C.L. Cazzullo D. Caputo A. Ghezzi
M. Zaffaroni (Eds.)

Virology and Immunology in Multiple Sclerosis: Rationale for Therapy

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Prof. Dr. Carlo L. Cazzullo
Dr. Domenico Caputo
Dr. Angelo Ghezzi
Dr. Mauro Zaffaroni
Centro Studi Sclerosi Multipla
Universita' Studi di Milano
Ospedale "S. Antonio Abate"
Via Pastori 4
21013 Gallarate, Italy

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Immunology, Virology, and Immunogenetics of Multiple Sclerosis

Immunocyte Abnormalities in Multiple Sclerosis

B. G. W. Arnason

Department of Neurology and the Brain Research Institute University of Chicago, Chicago, USA

The cause of multiple sclerosis is unknown, but there is reason to believe that the immune system has a role in its pathogenesis. Several lines of evidence support this postulate.

It is now established that certain gene products are overrepresented in multiple sclerosis. Included here are the major histocompatibility complex (MHC) allele known as DR2 on chromosome 6, the immunoglobulin allotype allele known as GM 1, 17:21 on chromosome 14, and possibly an allele of the third component of complement on chromosome 19 (see [1] for review). These three alleles segregate independently, suggesting that inheritance of susceptibility to multiple sclerosis is polygenic.

Some 60-odd associations of MHC alleles with disease are known. In general, such associations are found with diseases that are chronic or recrudescent as is multiple sclerosis, exhibit an inflammatory component to greater or lesser degree as does multiple sclerosis, and show a modest tendency to be inherited but in a fashion that does not obey classical Mendelian rules, a situation that is known to be the case in multiple sclerosis. When a cause has been assignable to diseases that show an MHC allelic association the vast majority have proven to be autoallergic or infectious processes. Arguing by analogy, multiple sclerosis, based on its MHC allelic association, is likely also to be an autoallergic or infectious disease.

Studies of identical twins support the postulate that inheritance of susceptibility to multiple sclerosis is polygenic. If one of a pair of identical twins has multiple sclerosis the chance that the other twin will exhibit clinical or laboratory evidence of multiple sclerosis (e.g., abnormal spinal fluid or white matter lesions on magnetic resonance scans) is of the order of 50% [2]. Concordance for multiple sclerosis among dizygotic twins or between siblings is much lower, approximating 1% or 2%. High concordance for multiple sclerosis in identical twins bespeaks a powerful genetic component in multiple sclerosis, and the rarity of concordance in dizygotic twins and non-twin siblings indicates that several genes must be in play simultaneously. While DR2 is overrepresented in multiple sclerosis not all patients carry this allele. Thus it may be that no single gene is critical for multiple sclerosis and that if, say, six genes are implicated, then possession of any three or four among them suffices to augment susceptibility markedly.

The fact that concordance for multiple sclerosis is not absolute for identical twins, and that severity of disease between them varies markedly, argues that an environmental factor is also likely to be in play. Assiduous epidemiologic search has failed to uncover any environmental factor relevant to multiple sclerosis, suggesting that

the factor is either extremely elusive or too obvious. I wish, however, to signal a caveat. It is not impossible that a "strategic hit" of multiple sclerosis within the central nervous system could determine subsequent course, in which case disease might remain subthreshold in one of a pair of identical twins even in the absence of any environmental trigger (see later). At the same time it is difficult to envisage an intrinsic trigger, as opposed to an environmental one, that could be responsible for acute attacks.

The lesions of multiple sclerosis contain B cells, T cells, and macrophages. Both T-helper and T-suppressor-cell lineages are found in and about multiple sclerosis plaques. The B cells within the brain are activated, and continuously so, as witnessed by the brisk production within brain of immunoglobulin by them even at times when disease is inactive by clinical criteria. The T cells in spinal fluid have also been shown to be activated in multiple sclerosis, even in cases judged on clinical grounds to be quiescent [3]. In the spinal fluid activation appears to be restricted to T cells of the T-helper subset [4]. These findings are consistent with an ongoing and persistent abnormality in immune regulation in multiple sclerosis.

Experimental allergic encephalomyelitis is a T-cell-mediated autoimmune disease. The antigen is myelin basic protein. Chronic and recrudescent forms of experimental allergic encephalomyelitis exhibit pathologic, electrophysiologic, and clinical features highly reminiscent of those seen in multiple sclerosis. Unfortunately, sensitivity to myelin basic protein is not detectable in multiple sclerosis although it is observed in postinfectious and rabies vaccination-induced encephalomyelitis. Nonetheless, study of experimental allergic encephalomyelitis has established that an autoimmune process can mimic multiple sclerosis.

Study of blood lymphocytes has also revealed aberrancies in multiple sclerosis. The literature on this topic is vast and has been reviewed by us recently elsewhere [5]. Here we will focus on alterations in T-suppressor cells and in their function as measured in vitro. Some years ago we showed that T-suppressor-cell function is grossly defective during flares of multiple sclerosis and that it recovers with remission [6, 7]. More recently we have been studying multiple sclerosis cases with rapidly progressive disease course, defined as a loss of 1 point or greater on the Kurtzke scale over the preceding 12 months. In our rapidly progressive group T-suppressor function remains grossly defective month after month with no overlap to date with values obtained in controls. Some patients have been studied serially for up to 2 years, and the defect has persisted throughout. Half the patients in this cohort have been entered into a trial of cyclosporine A treatment, the other half receiving placebo. Disappointingly, cyclosporine A treatment does not correct the T-suppressor-cell defect.

T-suppressor cells carry the phenotypic marker called T8 which can be detected using the monoclonal antibody known as OKT8. The number of T8-positive cells in the circulation is reduced to a modest extent when multiple sclerosis is active (reviewed in [5]) and the density of T8 on T8-positive cells is reduced as well [8]. The latter finding points to a modulation of lymphocyte surface markers in multiple sclerosis about which more will be said later. Deliberate stripping of T8 from lymphocytes compromises T-suppressor function to some extent [9], but this maneuver fails to duplicate the profound loss in T-suppressor function observed in rapidly progressive multiple sclerosis.

T8-positive cells have two known functions. In addition to the suppressor function already mentioned they also function as cytotoxic cells. Cytotoxic function, as measured by an in vitro assay, is normal in multiple sclerosis, even in rapidly progressive cases [10]. Thus, one function of T8-positive cells is compromised while another is preserved. Interestingly, cytotoxic function is abrogated by treatment with cyclosporine A and restored by provision of IL2. Thus, the drug fails to correct that which is abnormal (T-suppressor function) and makes that which is normal (cytotoxic function) deficient.

There is a monoclonal antibody known as 9.3 that can be used to separate T8-positive cytotoxic cells from T8-positive suppressors . The cytotoxic cells bind 9.3; the suppressor cells do not. Using this antibody we have been able to show (A. Reder and B. Arnason, unpublished studies) that T-suppressor cell/cytotoxic cell ratios in the circulation are normal in progressive multiple sclerosis, i.e., suppressor cells are there, but they do not work.

We have also generated T8-positive cells lines from multiple sclerosis patients and from controls. Freshly obtained cells are stimulated with the monoclonal antibody OKT3, which drives T cells to proliferate. OKT3 treatment is followed by interleukin 2. Large populations of cells can be generated by sequential pulsings with these two reagents. After 2–3 weeks, and numerous cycles of cell division, T8-positive cells from controls continue to exhibit suppressor activity, but cells from progressive multiple sclerosis patients remain defective. This need not imply that the defect, or change, whatever it may ultimately prove to be, is uncorrectable. For example, chicken hepatocytes pulsed with estrogen exhibit altered protein metabolism that persists through 10–15 cycles of cell division before reverting to normal [9].

The basis for abnormal T-suppressor-cell function in multiple sclerosis is not known, but there are some clues. All T cells bear a surface marker called T11 which is perhaps better known as the sheep red blood cell (SRBC) receptor. In fact, binding of three of more SRBCs has for many years been accepted as a criterion for defining a lymphocyte as a T cell. Some T cells bind many more SRBCs than this; we have defined cells binding ten or more SRBCs as avid T cells. Avid T cells comprise some 20%–30% of total T cells in controls. Avid T-cell percentages are decreased in multiple sclerosis overall [12] and are essentially absent in rapidly progressive cases (A. Reder, unpublished data). The finding would seem to indicate that something is different about T11 in multiple sclerosis. T11 is, however, present in normal amount on T cells of multiple sclerosis patients. T11 can be readily detected by monoclonal antibodies directed against it and quantitated by use of an FACS. We find normal amounts of T11 on T cells in multiple sclerosis. Thus, the alteration in T11, as reflected by the failure to form avid SRBC rosettes, would appear to be one of T11 structure rather than of number. T8-positive cells preferentially exhibit avidity since they normally express greater numbers of SRBC receptors than other T cells, and we suspect that the defect in avid T cells reflects an abnormality largely if not totally confined to suppressor cells. It should be pointed out that certain combinations of monoclonal antibodies directed against T11 will drive T cells to proliferate, suggesting that binding of some at present unknown ligand or lymphokine to T11 can lead to T-cell activation. It follows that a problem with T11 could tie to the failed suppressor cell activation observed in multiple sclerosis.

We have begun to study sympathetic nervous system function in patients with progressive multiple sclerosis. We find that galvanic current-induced sweat responses in the feet (invariably present in controls) are absent in 50% of progressive multiple sclerosis patients [13]. This difference between multiple sclerosis patients and controls is highly significant. Abnormal sympathetic function has been detected in multiple sclerosis by others using different techniques as well [14].

Might interruption of sympathetic outflow have consequences for immune regulation in multiple sclerosis. Might a "strategic hit" of disease lessen normal sympathetic tone, reset the immunoregulatory rheostat, and be responsible for a subsequently progressive course? The sympathetic nervous system is known to modulate immune function. This can be shown in animals in which the sympathetic nervous system has been ablated by treatment with the drug 6-hydroxydopamine. Immune responses are altered following this treatment. Antibody responses to some but not all antigens are increased [15], and the severity of experimentally induced myasthenia gravis and experimental allergic encephalomyelitis (a model, as already indicated, for multiple sclerosis) is augmented (E. Chelmicka-Schorr and M. Agius, personal communication). In addition, peripheral lymphocytes upregulate their β-adrenergic receptors following sympathetic ablation, i.e., they exhibit denervation hypersensitivity [16]. Given these considerations we reasoned that were sympathetic outflow defective in multiple sclerosis then β-adrenergic receptors might be upregulated. Accordingly, we measured β-adrenergic receptors on peripheral blood lymphocytes from multiple sclerosis patients and controls. Progressive cases of multiple sclerosis have a two- to three fold, on average, increase in the number of lymphocyte β-adrenergic receptors. T8-positive, 9.3-negative, lymphocytes (i.e., T-suppressor cells) normally bear more β-adrenergic receptors than other T cells [17], and we find a highly significant upregulation of β-receptors on T8-positive cells in progressive multiple sclerosis. We posit that T-suppressor-cell function fails when sympathetic innervation fails, and that this provides the basis for the increased severity of autoimmune disease that is observed in animals after sympathetic ablation. Whether our basic hypothesis has merit or not, and whether it is relevant to the upregulation of β-adrenergic receptors oberserved on T cells in multiple sclerosis or not, the fact that β-adrenergic receptors are upregulated in progressive multiple sclerosis (regardless of cause) offers the opportunity perhaps to modulate T-suppressor function directly using β-adrenergic agonists and antagonists.

The data discussed above point to several abnormalities in the surface phenotype of T-suppressor cells in multiple sclerosis. T8 is decreased, β-adrenergic receptors are increased, and the T11 molecule, based on admittedly indirect evidence, is modified. The challenge for the future is to tie these abnormalities one to the other, to T-suppressor-cell function, and hopefully ultimately to a role for T-suppressor cells in the pathogenesis of multiple sclerosis.

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Different T-Cell Response to Myelin Basic Protein in Multiple Sclerosis Patients and in Healthy Subjects

M. A. Bach, and *E. Tournier-Lasserve*

Unité de Pathologie de l'Immunité, Institut Pasteur, Paris, France

Introduction

Multiple sclerosis (MS) is a demyelinating disease, which shares clinical and pathological features with some models of experimental allergic encephalomyelitis (EAE) [1]. Thus, an autoimmune response directed against the myelin basic protein (BP) might be involved in the demyelination process as it is in EAE. Attempts to produce evidence of T-cell sensitization to BP in MS patients have led to controversial results [2] due to the poor reproducibility and/or sensitivity of the assays. We present here some data suggesting that T-cell response to BP is quantitatively and qualitatively different in MS patients from that seen in healthy subjects [3, 4].

Material and Methods

Subjects

Patients suffering from definite MS (as defined by Schumacher's criteria) were followed in Hospital de la Salpetrière (by Profs. F. Lhermitte, P. Brunet, and O. Lyoncaen) and in Hospital Saint-Antoine (by Prof. R. Marteau and Dr. E. Rollet), in Paris. Healthy subjects were blood bank donors or staff members.

Lymphocytes Transformation Test (LTT)

Peripheral blood mononuclear cells (PBMCs) were isolated over a Ficoll-Hypaque gradient and suspended in culture medium (RPMI 1640, Gibco) supplemented with 10% human AB serum; 10^5 PBMCs were cultured in triplicate with 100 μg/ml human BP (prepared by Dr G. Hashim, St Luke's Roosevelt Hospital Center, New York), or without antigen in wells of microculture plates (96 wells, Corning), containing 200 μl culture medium. Half of the cultures were harvested after 6 days. Other cultures were supplemented with 50 Units (Biological responise modifiers program) of interleukin 2 (IL2, lymphocult-TLF, Biotest, FRG) and harvested 2 days later. In all cultures, 1 μCi tritiated thymidine was introduced 18 h before harvesting on filter paper. Radioactivity of samples was measured in a liquid scintillation counter.

Basic Protein-Triggered T-Cell Lines and Clones

Peripheral blood mononuclear cells ($1–2 \times 10^6$) were cultured in 2 ml culture medium in 24 well trays (Corning), with 50 μg/ml human BP and restimulated 6 n 10 days later with the same BP concentration. IL2 was introduced 2 to 4 days after secondary BP stimulation, at concentrations varying from 5 to 50 BRMP units/ml, according to the degree of cell growth. Growing cultures were further propagated as T-cell lines by weekly restimulation with human BP plus irradiated autologous PBMCs (stored in liquid nitrogen) used as antigen-presenting cells (APCs), and IL2 supplementation (50 BRMP units/ml, three times a week).
Clones were obtained from the lines by limiting dilution. Growing clones were expanded as described for the lines.

Proliferation Assays of Line and Clones

Ten thousand washed T-cells were cultured for 48 days in 200 μl culture medium in round-bottomed microwells (Corning), with 5×10^4 iradiated autologous PBMCs and the antigen to be tested. Human BP as well as BP from other species (given by G. Hashim) was used at a concentration of 50 μg/liter. Thymidine incorporation was measured as described above after an 18 h pulse.

Results

Increased Proliferative Response to Human BP of PBMC from MS patients in Primary Cultures (Table 1)

In the absence of IL2, a significant proliferative response (stimulation index ≥ 2) was observed in 2 out of 10 healthy subjects only, but in 8 out 14 MS patients. When

Table 1. Proliferative response to human BP after primary stimulation[a]

Subjects	Δcpm[b]		Stimulation index[c]	
	−IL2	+IL2	−IL2	+IL2
MS patients	2548[a] ±1009	13033 ±5961	2.95 ±0.61 (8/14)	10.2 ±5.14 (9/11)
Healthy subjects	913 ±675	1751 ±1252	1.84 ±0.59 (2/10)	2.77 ±1.35 (5/7)

[a] Proliferative response to BP was assayed either after 6 days of culture without IL2, or after an additional incubation of 2 days in the presence of IL2
[b] Δcpm=cpm with BP − cpm without BP
[c] Stimulation index= $\frac{\text{cpm with BP}}{\text{cpm without BP}}$
In parenthesis, number of subjects with stimulation index ≧2/number of subjects tested

IL2 was added at the end of the culture, the BP-specific proliferative response increased in both groups: up to 5 out of 7 healthy subjects and 9 out of 11 MS patients did show a significant reactivity to BP, but the average response to BP was found higher in the latter group ($P < 0.05$).

Human BP-Triggered T-Cell Lines from Healthy Subjects and MS Patients Respond Equally Well to Human BP

Basic protein-triggered T-cell lines were derived from PBMCs of eight MS patients and five healthy subjects. T-cell lines of MS patients grew faster than those of healthy subjects. However, once the lines were established, their proliferative responses to human BP, as assayed in the presence of autologous APCs, was found of similar magnitude in both groups (Table 2).

T-Cell Lines of MS Patients Differ from Healthy Subject Lines by Their Proliferative Response to Heterologous BP (Table 3)

When the proliferative response to several heterologous BPs was compared with that triggered by human BP in the same experiment, healthy subject lines did respond to all BP tested, in most cases equally well or even better than to human BP. Conversely, several MS patient lines failed to react to either rat or monkey BP, or both. This occurred in patients with active disease as well as in patients in remission. A low response to bovine BP was also observed, but only in patients suffering relapse or progressive aggravation.

Table 2. Proliferative response to human BP of human BP-triggered T-cell lines from healthy subjects and MS patients

Subjects	Antigen added		
	None	Human BP	Stimulation index
Healthy subjects (N=5)	1075[a]	12656	25.3
	±600	±1635	±26.1
MS patients (N=8)	3395[b]	17553[b]	12.6[b]
	±4575	±11407	±11.4

[a] Mean±SD. Stimulation index $= \frac{\text{cpm with antigen}}{\text{cpm without antigen}}$

[b] $P > 0.1$ compared with healthy subjects

Table 3. Proliferative response of T-cell lines to heterologous BP as compared with human BP in MS patients and healthy subjects

	BP source		
	Monkey	Rat	Beef
Healthy subjects	−20±31[a] N=5	−18±86 N=5	+29±64 N=5
MS patients: all cases	−67±34 N=8	−67±37 N=7	−2±69 N=8
With active disease only	−57±25 N=5	−49±36 N=5	−46±35 N=5

[a] Percentage variation of the response as compared with that obtained with human BP within the same experiment (mean±SD). Significant values ($P < 0.05$) are underlined

Discussion

An important point of the present study was the detection of circulating T-cells recognizing human BP in a large percentage of healthy subjects. This observation confirms a previous work of Burns et al. [5] and suggests that T-cell autoreactivity to human BP is a physiological phenomenon.

Peripheral blood mononuclear cells from MS patients develop in primary cultures higher responses to human BP than healthy subjects. On the other hand, BP-specific T-cell lines from these patients did not proliferate better to BP than lines from healthy subjects. This suggests that MS patients differ from healthy subjects in having a higher number of BP-reactive T-cells, which may result from a previous sensitization, rather than having a better efficiency of antigen presentation or stimulation.

Another characteristic of MS patient lines was their failure to respond to one or several heterologous BPs, at variance with healthy subjects, whose specificity pattern suggests a preferential recognition of a conserved peptide sequence. Strain variations of the T-cell repertoire to BP have been described in rat and mouse [6, 7]. It must be stressed, however, that specificity studies at the level of T-cell lines, i. e., BP reactive-bulk T-cells, likely provides an incomplete picture of the T-cell repertoire to BP.

The relevance of these quantitative and qualitative abnormalities of the T-cell response to BP to the pathophysiology of MS remains unclear. T-cells that do react to BP in vitro are not necessarily encephalitogenic in vivo [7, 8] and the release of BP products, as a consequence of demyelination, could have expanded a pool of T-cell clones reacting to BP epitopes not normally exposed. Alternatively, some MS patients perhaps display a T-cell repertoire to human BP that includes the recognition of potentially encephalitogenic peptides and favors the development of the disease.

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Glial Cells and Products of Activated Inflammatory Cells: Implications for Pathogenesis and Treatment of Multiple Sclerosis

R. P. Lisak

Departments of Neurology and Immunology and Microbiology, Wayne State University School of Medicine, Detroit MI, and University of Pennsylvania School of Medicine, Phialdelphia, PA, USA

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system of unknown etiology, but a disease in which immunopathologic events are likely important [1]. It is a chronic disease, frequently with an clinically relapsing course [2], but the pathologic processes may actually be continuous, although accentuated at times [3]. It also has a restricted age distribution, with the peak onset between 20 and 40 years of age. In addition to the inflammation, which is predominantly made up of monocyte-macrophage-microglia and lymphocytes and/or their progeny, and demyelination, the other characteristic pathologic feature is gliosis [4]. This gliosis relates to the astrocytes, although there is some controversy as to whether these cells become more prominent, increase in number, or both.

There has been considerable interest in the possibility that MS is an autoimmune immunopathologic disease with a component of myelin/oligodendrocyte, the target for an antibody and/or cellular response. To date, the evidence is indirect [5, 6] and the antigen has certainly not been unambiguously identified [7]. More recently, there has been increasing interest in other interactions between the immune and nervous systems. This has been made possible by several scientific advances, including the ability to identify, purify, and study the function of subsets of cells of both the immune and nervous system as well as to purify and characterize secretory products of cells of both systems. In addition, genetic engineering has allowed the production of single products of inflammatory cells, and the use of monoclonal antibodies allows the unequivocal identification of epitopes of important surface and cytoplasmic components of cells of the nervous system on the immune system. Much of this recent activity has centered on interactions between products of activated inflammatory cells (cytokines=lymphokines+monokines) and glial cells [oligodendrocytes, astrocytes, macrophages, microglia (brain phagocytic cells)] and brain vascular endothelial cells [8, 9]. These cytokines are clearly present in the nervous system and CSF of patients with MS [10]. An extension of this line of investigation is the study of glial cells serving functions traditionally associated with cells of the immune system such as presentation of antigen [11], production of monokines [12, 13], phagocytosis [14], and production of enzymes such as proteases which could contribute to myelin breakdown and even act as stimulators of B-cell proliferation and differentiation [15]. Although much has been learned, there are many unanswered questions of considerable neurobiologic, immunologic, and pathologic importance.

Which products of activated inflammatory cells are mitogenic for which types and subtypes of glial cells?

The work of several groups [8, 9, 16, 17] makes it clear that unfractionated supernatants from activated inflammatory cells induce proliferation of astrocytes and CNS origin fibroblasts in vitro. As noted earlier, there is clearly gliosis associated with long-standing lesions of patients with MS, with the suggestion that astrocytes may undergo swelling, show an increase in intermediate filaments, and perhaps proliferate. In acute experimental allergic encephalomyelitis (EAE) as well as in the chronic and relapsing forms of the disease, astrocytes are also similarly affected. We do not know whether all astrocytes are affected equally [18]. Type II astrocytes, which in rats seem to arise from the same precursor glioblasts as oligodendrocytes [19, 20], are more numerous in white matter and some may be intimitely related to the paranodal region of the myelinated CNS axon [21]. Thus, it is conceivable that changes in this subclass of astrocyte, including proliferation, changes in phenotypic markers and functions, swelling, and failure to perform normal astrocytic functions, could lead to changes in CNS nerve conduction and result in symptoms. If the changes do not lead to demyelination or gliosis, it is possible that there might be rapid improvement in symptomatology. Could this be one of the mechanisms responsible for the rapid and often clinically complete recovery seen after exacerbations, especially during early phases of the disease?

Fontana's group [8, 16, 22, 23] performed preliminary studies on activated supernatants and identified a factor which seemed to induce astrocyte proliferation. There has been no further published work characterizing the factor further, which seemed to be a product of T cells, nor do we know is it has any effect on oligodendrocytes, microglia, or Schwann cells. Is it the only mitogen for astrocytes produced by T cells? It most likely is not the only mitogen produced by inflammatory cells since it has been shown that interleukin-1 (IL-1) (produced by macrophages and perhaps microglia and astrocytes themselves) induces astrocyte proliferation [17, 24]. It is of some interest that Schwann cells, at least cells from neonatal rat sciatic nerves, do not proliferate in direct response to IL-1 in vitro [25].

It has been reported that T-cell products stimulate oligodendrocytges to proliferate in vitro [26]. A 30000 mol. wt. protein is said to induce proliferation of oligodendrocytes and no other CNS, PNS, or non-NS cells [27]. Based on the mol. wt. of 30000, as well as its reported very restricted selectivity as a mitogen, it seems unlikely to be IL-1, interleukin-2 (IL-2), or Fontana's glial proliferative factor. We have been unable to demonstrate that unfractioned activated supernatants, IL-2, or γ-interferon (γ-IF) induce proliferation of oligodendrocytes [28]. Others have reported that IL-2 is mitogenic for oligodendrocytes [29], but at a very high concentration. Although cloned IL-2 has been used by both our group as well as those who detect a proliferative effect, the results differ. The need for very high concentrations to induce proliferation suggests either an effect of some carrier material or that oligodendrocytes have either very low density of IL-2 receptors or low-avidity receptros. Neonatal Schwann cells do not proliferate in vitro in response to IL-2, although they proliferate in response to the unfractionated supernatants of activated inflammatory cells [30]. It is not clear why these differences in results occur, but species and in vivo age of animals as well as in vitro age of cultures may explain some but not all of the conflicts. The question of the effect of age may be

important in consideration of which products of activated inflammatory mononuclear cells are capable of induction of major histocompatibility complex (MHC) antigens and which glial cells are susceptible to MHC antigen induction. Since the onset of MS shows age restriction and the pattern of clinical disease may differ in part related to the patient's age, the possible effect of age and the response to cytokines may be important.

Supernatants obtained from activated mononuclear cells have been reported to induce MHC type I antigens (HLA-A, B, C in man) on oligodendrocytes, astrocytes, and microglia-macrophages [28, 31–35]. The demonstration that type I antigens can be induced on a cell type which ordinarily does not bear such antigens is not trivial since it has been demonstrated that T-cell-mediated antigen-specific cytotoxic reactions against cellular antigens, including viral antigens in such cells, can only occur if the cells bear MHC type I antigens [36]. If glial cells serve as targets for such antigen-specific cytotoxic reactions in MS and other diseases, it would be required that type I MHC antigens be induced by a lymphokine, such as γ-IF, or by a viral infection [37]. There is much written about the search for type II (Ia) MHC antigens on glial cells in lesions of patients with MS but little about type I antigens on such glial cells. Parenthetically, such studies are likely forthcoming and we will need to remember that in a disease like MS that has periods of varying activity and a chronic course we may expect variable reports from different groups. The may be studying different lesions in different patients, or indeed different lesions in the same patient [38].

There is also interest in the question of which cells in the CNS naturally bear or can be induced to bear MHC type II (Ia) antigens (DR, DQ in man). It has been reported that activated antigen-specific T-cell lines, which would be capable of production and secretion of lymphokines including γ-IF, and γ-IF itself can induce Ia on astrocytes [8, 9, 11) which are ordinarily la negative [39]. These astrocytes can then present myelin basic protein (MBP) to the T-cells. It is a "requirement" for antigen-presenting cells to have type II on their surface. Recently, it has been reported that oligodendrocytes can have an accessory function on in vitro T-cell mitogenesis [40], but this is a different phenomenon from specific antigen presentation and antigen-specific proliferation. There is little evidence, if any, that oligodendrocytes become la positive in vitro or in vivo [39, 41, 42]. It has been reported, however, that astrocytes in MS and EAE lesions are type II MHC positive [10, 43]. Based on the earlier described in vitro evidence, it has been postulated that antigen presentation by astrocytes may contribute to propagation of inflammatory lesions in the CNS. Astrocyte lysis by MBP-specific T-cells has also been described [44].

The situation regarding astrocytes is not as straight forward as it had seemed. In EAE lesions in rats, macrophages become strongly positive before a relatively small percentage of astrocytes become Ia positive [45, 46]. We and others have not found induction of Ia on most astrocytes in vitro [28, 35]. Microglia-macrophages are the predominant Ia-positive cells in normal cultures [35,39] and after induction [28, 35]. MHC antigens are glycoproteins and may be found on cells adjacent to the cell that is actually producing la; i.e., it may be very difficult to localize la [35]. Disparity between immunofluorescence and immunoperoxidase techniques, and light and electron microscopic localization of Ia has been reported in other organs [47]. Cells

which are passively Ia positive likely do not function as antigen-presenting cells. Therefore, it will be important to determine, both in vitro and in vivo, if astrocytes or even oligodendrocytes actually can be induced to become la positive and which cytokines induce the antigens. It will likely require molecular biologic techniques, such as in situ hybridization combined with immunohistology, ultimately to settle the question.

What is the role of vascular endothelial cells in the initiation of and self-propagation of immunopathologic reactions with the CNS?

There is growing evidence that the endothelial cells of cerebral blood vessels may play an active rather than passive role in immunopathologic reactions within the CNS [9]. There have been reports of DR (Ia) on CNS endothelial cells in the CNS of animals with EAE [45, 46]. However, this has not been a universal finding and, more recently, it has been suggested that it is actually perivascular dendritic (microglia) that are the positive cells in the CNS vessels [49]. It has been reported that similar Ia localization is seen in MS lesions [50, 51], but, again, the exact nature of the positive cells in uncertain. It would not be surprising if a macrophage-microglial cell among the endothelial cells were induced to become Ia positive and interact with circulating T-cells, even presenting antigen [52]. It should be noted that how T-cells circulating at a high flow rate interact with endothelial or dendritic cells is still not clear. Changes in the endothelium could also allow passage of nonspecific inflammatory cells or serum proteins which could induce demyelination [53–55]. On the other hand, some of the changes reported in CNS vasculature in MS are seen in classic passive transfer delayed hypersensitivity reactions. Further comparative studies of passive and active EAE are clearly of importance here as are in vitro studies of endothelial cell-cytokine interactions and lymphocyte-endothelial cell interactions. It must also be remembered that the frequency of CNS antigen-specific T-cells is probably very different in animals with actively induced EAE, passively transferred EAE, and EAE passively transferred with T-cell lines or clones. Moreover, T-cell lines and clones, especially after reactivation or restimulation, may differ quantitatively and qualitatively from "normal" T-cells in many in vitro and in vivo characteristics.

For the most part, I have emphasized the potential pathogenic importance of the interactions of cytokines and glial cells, and I belive we are just beginning to understand this important area. Clearly, there are tremendous therapeutic implications. It has been traditional to consider how drugs and modifiers of biologic reactions would interact with the classic cells of immunopathologic reactions (T-cells, B-cells, monocyte-macrophages, and, more recently, K and NK-cells). The reports of some [56–59] but not all groups [60–62] of a defect of NK-cell function associated with (or perhaps caused by) a decreased γ-IF production in patients with MS lead to the suggestion that γ-IF might be of benefit in MS. Recently, a therapeutic study demonstrated that γ-IF was associated with an increase in exacerbations [63]. This might relate to activation of circulating cells of the immune system, but it is also possible that immunologic cells within the nervous system or glial cells were affected directly by γ-IF crossing a damaged blood-brain barrier [64]. Since glial cells have been postulated to produce IL-1 [8, 12] and proteases (which could interact with B-cells) [15], activation of glial cells by systemically administered lymphokines could also have a deleterious effect in an indirect fashion. Rather than

looking for biologic modifiers for treatment of MS, we may need antibodies directed against the modifier or its receptor.

There are other possible cytokine-glial cell interactions that have therapeutic implications. α- and β-IF inhibit cellular proliferation, as does γ-IF, under certain circumstances [65]. If the astrocytic response in MS is deleterious, then an inhibitory effect by one of the interferons or other biologic modifier might have a long-term beneficial effect in MS. However, if oligodendrocyte or oligodendrocyte precursor proliferation were inhibited by an exogenously administered agent, that might prove to be harmful. It has been reported that IL-2 induces synthesis of myelin-specific constituents, such as MBP, by oligodendrocytes [29]. Thus, inhibition of IL-2 production or blocking of IL-2 receptors, if present, on glial cells would be potentially harmful, even if inhibition of the effect of IL-2 on T or B-cells might be potentially helpful. Until we know more about the in vivo and in vitro effects of the many cytokines, as well as the effects of inhibition of the cytokines and their receptors, we are guessing in planning therapeutic studies.

Although there is no substitute for eventually performing well-controlled studies in patients, we need to learn much more about in vitro and in vivo effects of various potential biologic agents on glial cells before embarking on more and more treatment studies in patients.

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Intrathecal IgG Synthesis in Multiple Sclerosis: Correlation with Clinical Parameters

P. Livrea, I. L. Simone, M. Trojano, L. Pisicchio, G. Logroscino, and *A. Rosato*

Institute of Neurology, University of Bari, Bari Italy

Introduction

A variable amount of intra-blood-brain barrier (BBB) synthesis of oligoclonal IgG, accompanied by moderate pleocytosis and/or by a slight increase in BBB permeability, characterizes the CSF profile of multiple sclerosis (MS), patients. Correlations between clinical and CSF parameters have been attempted by several authors (review in [41]), but the high number of interdependent and ill-defined clinical variables involved in the disease likely account for the discrepant results, which have prevented definite conclusions from being reached.

Mononuclear pleocytosis usually below 50/μl [36] occurs in about 66% of patients, with no [38] or slight [22, 41] increase during exacerbations, and with a decline due to disease duration [23, 41]. Intrathecal synthesis of oligoclonal IgG is detectable in 50%–100% of patients (review in [17, 41, 42]): its frequency largely depends on the sensitivity of separation methods (review in [41]), and the lack of this abnormality has also been related to short-lasting [5, 42] or benign [21, 30, 31] clinical course of the disease. The amount of IgG synthesis, evaluated by different empirical formulae (review in [42]), has appeared to be independent from clinical variables [4, 8, 27, 40], but in other studies a high synthesis rate has characterized patients with clinically definite, disabling [36, 41], or active [37] disease. In some series, the amount of intra-barrier IgG synthesis is correlated with CSF pleocytosis [33, 37, 38, 41] and with the presence [30, 31] or number [40] of oligoclonal bands. The increased permeability of BBB to serum albumin is usually slight and confined to a small number of patients, without any clear correlation with clinical parameters [36, 37]; nevertheless, barrier opening to small [6] as well as large [32] serum molecules is found in higher percentages during acute or chronic progressive phases, respectively, of the disease.

In this paper a further investigation has been attempted to detect separate correlations among clinical variables and the frequency and/or the amount of CSF abnormalities. The results indicate that the evaluation of the CSF mononuclear reaction, of the BBB permeselectivity, and of the intrathecal IgG amount as well as isoelectric spectrum can be useful for the assessment of the disease activity.

Material and Methods

Patients

One hundred MS patients were classified according to McDonald and Halliday [19]: the disease was clinically definite in 32, early probable or latent in 33, progressive probable in 16, progressive possible in 10, and suspected in 9. Patients were grouped according to the phase of the disease [29]. Fifty-two patients were in acute phase from 1 to 60 days, 13 patients were stationary from 12 to 48 months, and 35 patients had slow progression of symptoms from 12 to 360 months. The severity of symptoms at the time of the lumbar puncture was scored by the Disability Status Scale [10]. Fifty-six patients were never treated by corticosteroids, and 28 of them had an acute exacerbation of symptoms at the time of the lumbar puncture. Twenty patients in acute phase were receiving dexamethasone (Decadron) 8 mg daily from 1 to 60 days. Twenty-four patients were free from previous corticosteroid treatments from 1 to 36 months. The average age, disease duration, number of bouts, and disability score in groups subdivided according to the clinical form and the phase of the disease is reported in Table 1.

Methods

The following tests were performed on every patient: CSF mononuclear cell count; assay of intrathecal IgG synthesis by the CSF IgG index [13] and by formulae estimating the de novo CNS IgG synthesis [37] or the pathological CSF IgG fractions [25]; search for IgG fractions, exclusively present in CSF or more intense in CSF

Table 1. Clinical parameters of MS patients

	n	Age (years)	Disease duration (months)	No. of bouts	Disability (Disability Status Scale)
Clinically definite	32	34±9	104±72	3.7±1.5	3.1±1.1
Probable initial latent	33	27±10	15±27	1.9±1.3	2.8±1.3
Progressive probable	16	43±14	109±101	0	4±1.9
Progressive possible	10	43±13	65±73	0	2±0.7
Acute phase	52	28±10	37±62	2.6±1.8	2.9±1.3
Stationary phase	13	35±10	60±63	3±3	2.2±0.6
Progressive	35	41±13	99±92	0.6±1.2	3.7±1.7

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than in serum (oligoclonal IgG) [38]; and assessment of BBB permselectivity by the CSF/serum albumin ratio (× 1000) [13] and by the CSF/serum alpha-2-macroglobulin-albumin ratio [25]. A significant correlation was found between the CSF IgG index and de novo CNS IgG synthesis (r=0.79), between the CSF IgG index and pathological CSF IgG fractions (r=0.72), and between pathological CSF IgG fractions and de novo CNS IgG synthesis ($r = 0.87$). Thus the CSF IgG index was used to indicate the amount of intrathecally synthesized IgG.

Cerebrospinal fluid mononuclear cells were counted using the Fuchs-Rosenthal chamber and stained by Test Simplets slides (Boehringer); albumin, IgG, and alpha-2-macroglobulin were assayed in CSF and serum by Laser Nephelometry (Behring) [18]; Isoelectric Focusing (IF) in a thin layer of polyacrylamide gel, pH range 3.5–9.5 (LKB), stained by Coomassie Brillant Blue [17] and, if necessary, scanned by quantitative Laser Densitometry (LKB) [35], was used for the detection of the oligoclonal CSF IgG bands. Thirty-one neurological patients aged 20–60 years, affected by disorders presumably not associated with any CSF cellular reaction, any blood-CSF barrier damage, any CSF flux damage, or any intrathecal IgG synthesis, were considered as controls. Mean+2SD values of the CSF/serum albumin ratio (× 1000), CSF/serum alpha-2-macroglobulin/albumin ratio, CSF IgG index, and CSF cells per microliter (Table 2) were considered the upper normal limits.

Student's t-test, chi-square, and multiple regression among nine variables (age, disease duration, disability, number of bouts, number of mononuclear CSF cells, CSF IgG index, CSF/serum albumin ratio, CSF/serum alpha-2-macroglobulin/albu-

Table 2. Mononuclear cells, total amount of intrathecal IgG synthesis, number of abnormal CSF IgG fractions, and BBB permselectivity in MS patients

	Controls (n, 31) (mean+SD)	MS patients (n, 100) (mean+SD	(frequency of abnormal values)
Mononuclear cells / µl	1±1.5	4.7±2.4[a]	50%[b]
CSF IgG index	0.45±0.08	0.94±0.54[a]	72%[b]
De novo IgG synthesis mg/day	0.76±2.8	11.4±13.6[a]	72%[b]
Pathological CSF IgG fraction mg/dl	< 0	1.7±3.3[b]	60%[b]
Number of CSF IgG abnormal bands	0	10.6±7.2[b]	100%[c]
CSF/serum albumin × 1000	3.7±1.4	4.7±2.4[a]	13%[b]
alpha 2 m/albumin CSF/serum	0.21±0.07	0.29±0.15[a]	22%[b]

[a] T-test, $P < 0.01$ vs. controls
[b] Chi-square, $P < 0.01$ vs. controls
[c] Chi-square, $P < 0.01$ vs. quantitative formulae

min ratio, number of abnormal CSF IgG fractions) were employed for statistical analysis. Supposing a combined influence of some variables on the same parameter, the multiple regression was used to detect the separate interdependence between variables, all the other variables being held constant. Multiple regression was separately tested in the following groups: clinically definite (*N*, 32), probable initial or latent (*N*, 33), progressive probable (*N*, 16), progressive possible (*N*, 10), suspected (*N*, 9), progressive primary (*N*, 26), progressive primary and secondary (*N*, 35), acute phase of exacerbation [52], clinically definite in acute phase (*N*, 18), probable initial or latent in acute phase (*N*, 28), acute phase never treated (*N*, 28), acute phase treated by corticosteroids (*N*, 20), first bout never treated (*N*, 14), and stationary disease [13]. Significant partial correlations between variables were determined by *t* values of respective partial regression coefficients. Only significant *t* values are reported.

Results

Mean values and percentages of abnormal CSF parameters found in MS patients subdivided according to the phase of the disease are reported in Table 2. The CSF mononuclear cell number and the mean CSF/serum albumin as well as the CSF/serum alpha-2-macroglobulin/albumin ratios were significantly higher in MS than in controls. The most frequent CSF abnormality was the presence of oligoclonal CSF IgG fractions. In Table 3, mean values are reported for patients subdivided according to the phase of the disease. CSF mononuclear cells were significantly lower in patients with progressive course than in patients with acute or stationary phase; during the progressive disease, the CSF/serum albumin ratio was higher than in the remaining groups; the number of abnormal CSF IgG fractions was lower in patients with stationary than in patients witch acute or progressive phase.

Table 3. Cerebrospinal fluid parameters during the course of MS

	Acute phase (n, 52)	Stationary phase (n, 13)	Progressive (n, 35)
Mononuclear cells /μl	10.7±17	8.9±16	3.9±3.8[a]
CSF IgG index	0.93±0.49	0.76±0.48	0.94±0.52
Number of abnormal CSF IgG fractions	10.1±7.9	5.2±2.6[b]	10.3±6.4
CSF/serum albumin × 1000	4.52±2.3	4.31±1.2	5.1±2.8[c]
Alpha 2 m/albumin CSF serum ratio	0.27±0.15	0.3±0.2	0.29±0.15

[a] Chi-square, $P < 0.001$; vs. acute phase, t-test, $P < 0.001$
[b] Chi-square, $P < 0.001$
[c] Chi-square, $P < 0.001$

Table 4. Correlations[a] among clinical parameters in MS patients

		Significant t-values of partial regressions				
		Duration vs. age	Duration vs. disability	Duration vs. bouts	Bouts vs. disability	Bouts vs. age
All cases	100	3.25[b]	3.14[b]			
Clinically definite	32	2.51[c]				
Probable initial latent	33				−2.45[c]	−2.53[c]
Progressive (probable + possible)	26	3.16[d]	2.52[e]			
Acute phase (all cases)	52			5.33[b]		
Progressive (all cases)	35		2.49[c]			

[a] Only significant values of partial regression t coefficients and only groups with significant values (see "Material and Methods") are reported, [b] $P < 0.005$, [c] $P < 0.01$, [d] $P < 0.025$, [e] $P < 0.05$

Several clinical parameters were correlated with each other (Table 4). Age and duration had a significant partial regression in the overall series, mainly for the regression found in the groups with clinically definite and primary progressive disease. Also duration and disability were correlated, mainly in progressive forms. The number of bouts was highly correlated with the disease duration in patients with relapsing remitting course and suffering an acute exacerbation. Both age and disability were negatively correlated with the number of bouts in probable initial or latent disease.

A significant partial regression between the number of abnormal CSF IgG fractions and CSF IgG index was found in all the groups. During the acute phase, the CSF IgG index was significantly correlated with the number of mononuclear cells, whereas this latter was negatively correlated with the number of abnormal CSF IgG fractions (Table 5).

During the acute phase, the number of previous bouts had a positive correlation with the number of abnormal CSF IgG fractions, but a negative correlation with the values of the CSF IgG index. In patients with progressive course of the disease, the number of CSF abnormal IgG fractions significantly correlated with the disease duration. The CSF IgG index was influenced in an opposite way by the disease duration in patients with acute exacerbations (positive regression) or chronic progressive course (negative regression) (Table 6).

The CSF/serum albumin ratio had a positive regression with age in patients with clinically definite disease and acute phase. Number of previous bouts seemed to influence the value of CSF/serum albumin ratio during both acute and stationary phases of the relapsing remitting disease, mainly in probable initial and latent forms.

Table 5. Correlations[a] among CSF IgG index, CSF mononuclear cells, and number of abnormal CSF IgG fractions in MS patients

		Significant t values of partial regressions		
		CSF IgG index vs. abnormal IgG CSF fractions	CSF IgG index vs. mononuclear cells	Mononuclear cells vs. abnormal IgG fractions
Acute phase (all cases)	52	7.58[b]		
Acute phase (without steroids)	28	5.38[b]	2.58[d]	
Acute phase (clinically definite)	18	4.93[b]		
Acute phase (probable initial latent)	28	6.53[b]	3.15[d]	−2.33[c]
Progressive (all cases)	35	5.81[b]		
Progressive (probable + possible)	26	5.94[b]		
Stationary phase	13	2.27[c]		
Clinically definite	32	3.97[b]		−2.21[c]
Probable initial latent	33	6.65[b]	2.9[e]	−2.07[e]

[a] See Table 4, [b] $P < 0.001$, [c] $P < 0.05$, [d] $P < 0.01$, [e] $P < 0.02$

In clinically definite patients the CSF/serum albumin ratio was negatively correlated with disease duration. The CSF/serum alpha-2-macroglobulin/albumin ratio was significantly correlated with the number of mononuclear cells in clinically definite patients, mainly during acute phase (Table 7).

Discussion

The CSF profile found in this series of MS patients is consistent with many previous reports (review in [22, 23, 36, 38, 41, 42]), the most frequent abnormality being the presence of intrathecal synthesis of oligoclonal IgG. IF of CSF and serum IgG is more sensitive for detecting this abnormality than formulae combining immunochemical assay of albumin and IgG in CSF and serum [15, 33]. The presence

Table 6. Correlations[a] between CSF IgG index, abnormal CSF IgG fractions, number of bouts, and disease duration in MS patients

		Significant t values of partial regressions			
		No. of bouts vs. abnormal IgG CSF fractions	No. of bouts vs. CSF IgG index	Duration vs. abnormal IgG CSF fractions	Duration vs. CSF IgG index
Acute phase (all cases)	52	2.48[b]	–2.52[b]		
Acute phase (clinically definite)	18		2.45[b]		2.94[b]
Acute phase (probable initial latent)	28				2.18[e]
Progressive (all cases)	35			3.19[c]	–2.26[e]
Progressive (probable + possible)	26			3.14[d]	–2.22[e]

[a] See Table 4, [b] $P < 0.02$, [c] $P < 0.005$, [d] $P < 0.01$, [e] $P < 0.05$

Table 7. Correlations[a] among BBB permselectivity, CSF mononuclear cells, number of bouts, age, and disease duration in MS patients

		Significant t values of partial regressions			
		CSF/serum albumin ×1000 vs. age	CSF/serum albumin ×1000 vs. No. of bouts	CSF/serum albumin ×1000 vs. duration	Alpha 2 m./ albumin CSF/serum ratio vs. CSF mononuclear cells
Acute phase (all cases)	52	2.1[b]	2.1[b]		
Acute phase (clinically definite)	18	2.12[b]			2.12[b]
Stationary phase	11		2.66[b]		
Clinically definite	32			–2.77[c]	2.16[b]
Probable initial latent	33		2.07[b]		

[a] See Table 4, [b] $P < 0.05$, [c] $P < 0.01$

and entity of CSF anomalies which are associated with the intrathecal IgG synthesis in different percentages has appeared to be influenced by many clinical variables of the disease.

In this series of MS patients, some groups differed with regard to age, disease duration, number of bouts, and disability (Table 1). Because of the interdependence of some of these variables (Table 4), the multiple regression analysis seemed to be more valuable than the total regression or the comparison of the mean values (Table 3) in order to detect the correlations between clinical and CSF parameters.

Decrease in MS CSF pleocytosis has been reported to be dependent on disease duration [23, 41] in no group did disease duration seem to be related with CSF mononuclear cells, whereas the low cell number characterized the progressive course of the disease. According to previous reports, mononuclear CSF pleocytosis [38] and the amount of intrathecally synthesized IgG [4, 8, 27, 40] did not differ in acute and stationary phases. Complex interdependence appeared in acute phase among the CSF mononuclear cell number, the amount of intrathecally synthesized IgG and the number of CSF IgG fractions; in turn, these parameters were correlated in different ways with the number of bouts and the duration of the disease.

As obtained by the enumeration of immunocompetent cells in CNS [24], CSF pleocytosis is representative of parenchymal infiltrates [2]; only a fraction of these cells [7] are capable of secreting unbound IgG in CSF [26] or in plaque tissue [20]; further, the IgGs secreted in vitro share electrophoretic features corresponding to the pattern found in CSF [26]. In agreement with other reports [33, 37, 38, 41] as well as with the measures of the both total and B-lymphocytes within active plaques [34], CSF pleocytosis and the overall amount of the intra-BBB IgG synthesis were correlated in acute phases. In this series, all (or the main part of) the intrathecal IgG synthesis appeared to be oligoclonal, as demonstrated in every group by the significant regression between the number of abnormal CSF IgG fractions and the values of the IgG index. Nevertheless, the complexity of the intrathecal IgG isoelectric spectrotypes had a negative correlation with the overall CSF mononuclear pleocytosis.

The molecular basis and the functional significance of the serum and CSF IgG isoelectric spectrum in health and in disease are unknown [43]. Monoclonal IgG secreted in serum by multiple myeloma results in multiple band spectrum, due to intracellular as well as extracellular postsynthetic charge modifications [1]. The presence of both kappa and lambda light chain antigenic determinants [14] in the same CSF IgG abnormal band separated by IF, and the isoelectric heterogeneity of monoclonal heavy chains compared with the homogeneity of light chains in multiple myeloma [12] or in subacute sclerosing panencephalitis (SSPE) CSF IgG [43], both indicate that IgG isoelectric spectra are not a reliable method of inferring the number of clones involved in their production. However, a faint correlation cannot be ruled out, while a relationship may exist between CSF IgG pattern complexity and the number of plaques within the CNS in MS. Monoclonal IgG are resolved by IF in a finite number of bands (from 3 to 15) [39, 45], and the IgG ageing which contributes to the heterogeneity in serum could not occur in CSF, whereby IgGs are quickly cleared [16]. In SSPE, where IgGs with the same heterogeneity are eluted from different CNS lesions [20], high-resolution two-dimensional electophoresis showed only one type of light chain in CSF [43]; on the contrary, in MS, where

different IgG isoelectric spectra are eluted from different plaques, light chains show a remarkable heterogeneity in both CSF [43] and brain [44].

If IgG isoelectric spectra are related to the number of B-cell clones and/or to the number of plaques present in CNS, the progression of the disease accompanied by the recruitment of new clones and/or by the formation of new lesions seems to induce an increase of CSF IgG spectrotype and a decrease of mononuclear CSF cells. The positive regression of the isoelectric spectrum of the CSF IgG abnormal fractions with the number of previous bouts or with the disease duration in patients with progressive course also supports this assumption. Accordingly, a low number of abnormal CSF IgG fractions occurs during the inactivity of the disease. Moreover, during the progression of the disease, as indicated by the number of acute phases or by the steady deterioration of symptoms, the overall intrathecally synthesized IgGs seem to decline. This could be consistent with the relatively high density of suppressor T cells at the edge of old plaques [34] and suggests that, besides the increasing number of CSF IgG fractions, the disease progression is accompanied by a decreased amount of each individual fraction.

A high CSF IgG index, high mononuclear cell count, and low number of prominent abnormal IgG fractions in CSF appear to be the hallmarks of less active or short-lasting disease. On the contrary, a low CSF IgG index accompanied by low mononuclear cell number and by numerous, faint abnormal bands, seems to characterize a long-lasting course with steady progression.

Blood-brain barrier data may also be useful for the assessment of the disease clinical activity. Histology in experimental models of MS [9], ultrastructural studies in autoptic material [3] and CT scan imaging [6] concur to indicate that BBB opening is linked to plaque formation and activity [28]. This is confirmed by the high values of albumin permeability coefficient found in primary progressive disease and by the positive regression with the number of exacerbations found in relapsing remitting forms. BBB opening to large serum molecules has already been reported [32]. BBB damage only for alpha-2-macroglobulin was found in 21/87 patients with normal permeability to albumin, and in only 1/13 patients with increased permeability to albumin. The distribution was not significant, but it seems to indicate that two kinds of BBB damage are likely to occur in MS. The leakage of large molecules is correlated with the transendothelial leukocyte immigration through the opening of tight junctions [11], whereas the increased number of endothelial pynocitotic vesicles with normal size detected in lesions [3] accounts for increased albumin permeability [17]. Further anaylsis in a larger series can clarify whether these events occur separately during the course of the disease.

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Loss of Reliability and Sensitivity of Agarose Isoelectric Focusing and Quantitative Formulae in the Detection of Intrathecally Synthesized IgG in the Presence of Blood-Brain Barrier Damage

P. Gallo,[1] *F. Bracco,*[1] *C. Andrian,*[2] *E. Toffanin,*[1] *G. Ongaro,*[2] and *B. Tavolato*[1]

1 Department of Neurology, University of Padova, Padova Italy
2 Immunohaematology and Transfusional Center, Padova Hospital, Padova Italy

Introduction

One of the unsolved problems in CSF IgG analysis is the reliable quantitation of intrathecally synthesized IgG in the case of blood-brain-barrier (BBB) damage [1–3].

In this report we conclude that the demonstration of IgG oligoclonal band patterns is the most sensitive and reliable method for the demonstration of pathological IgG synthesized inside the CNS, and that in the case of BBB damage there is currently no valid procedure to quantitate the intrathecal production of IgG.

Materials and Methods

The following patients were included in the study: 120 patients with clinically definite multiple sclerosis (MS) [4];, 30 normal subjects (patients with tension headache, uncharacteristic dizziness, or mild psychoneurotic disorders); and, 30 patients with noninflammatory BBB damage (glioma, neurinoma, meningioma, brain metastasis of microcitoma).

To demonstrate IgG oligoclonal bands (OBs) in unconcentrated CSF a very sensitive method was used [5, 6]. Briefly, agarose isoelectric focusing (AIEF) was followed by blotting of proteins to nitrocellulose membrane, double immunofixation, avidin-biotin amplification, and peroxidase staining. For each sample 225 ng IgG was loaded on the gel. Two or more bands found in CSF at pH values higher than 6.5, in addition to those in the corresponding serum, were considered to represent IgG OBs.

To estimate the intrathecal synthesis of IgG from a quantitative point of view two formulae were used:

1. Intra-BBB IgG syn Rate [7]:

$$\left[\left(\text{IgG CSF} - \frac{\text{IgG serum}}{369}\right) - \left(\text{Alb CSF} - \frac{\text{Alb serum}}{230}\right) \times \left(\frac{\text{IgG serum}}{\text{Alb serum}}\right) \cdot 0.43\right] \times 5$$

This formula is based on the assumption that there exists a linear correlation between the CSF/serum ratio for albumin and IgG and their CSF concentration, and

that the increase in CSF IgG, in the case of BBB damage, is directly proportional to the increase in

$$\text{CSF Alb} \times \frac{S\ \text{IgG}}{S\ \text{ALb}} \times K$$

(where K is always assumed to be 1.0). Normal values were considered those below 3.3 mg/liter.

2. A new formula proposed by Reiber and Felgenhauer [8]:

$$\text{IgG(loc)} = \left[\text{Q(IgG)} - \frac{a}{b} \sqrt{Q(\text{Alb})^2 + b^2 + c} \right] \cdot \text{IgG (Ser)}$$

This formula is, on the contrary, based on the assumption that the IgG CSF/serum quotient increases continuously but not linearly with increasing BBB permeability. In this formula a unique hyperbolic function describes the upper borderline of the group of values for discrimination between the IgG fraction which originates from blood and the one which is intrathecally synthesized. This formula was considered normal for values ≤ O.

The BBB function was evaluated by calculating the Alb CSF/Alb serum $\times 10^{-3}$ ratio [9]. This index was considered normal for values lower that 5.5.

Results and Discussion

The data concerning the MS CSF are summarized in Table 1 while the data on the CSF with noninflammatory BBB damage are summarized in Table 2.

Table 1. IgG hyperbolic function, intra-BBB IgG synthesis rate, and IgG oligoclonal bands in 120 patients with MS CSF. The samples are divided into three groups on the basis of the CSF/serum Alb ratio

$\frac{\text{Alb}^{CSF}}{S} \times 10^{-3}$		IgG hyperbolic function > 0	Intra-BBB IgG synthesis rate > 3.3 mg/liter	IgG OB +
	≤ 5.5=98 (81.66%)	75 (76.53%)	62 (62.26%)	97 (98.97%)
5.5 [9] *	≤ 7.5=16 (13.33%)	13 (81.25%)	14 (87.50%)	14 (87.50%)
	> 7.5= 6 (5%)	4 (66.66%)	6 (100%)	2 (33.33%)
		92 (76.66%)	82 (68.33%)	113 (94.16%)
	120			

Table 2. Positivity of IgG hyperbolic function and intra-BBB IgG synthesis rate in 30 patients with noninflammatory BBB damage

IgG hyperbolic function	Intra-BBB IgG synthesis rate
> 0=9 (30%)	> 3.3=22 (73.3%)
≤ 0=21 (70%)	≤ 3.3= 8 (26.6%)

The IgG OBs were found to be the most efficient marker of intrathecal IgG production in the presence of normal BBB function (98.97%). However, when severe BBB damage was present, the detection of IgG OBs was reduced to 33.33%. This effect can be explained with the dilution of the intrathecally synthesized IgG by the ingress of polyclonal IgG from serum to CSF through the damaged BBB.

The intra-BBB IgG synthesis rate showed peculiar behavior: its percentage of positivity increased with increasing BBB damage. Moreover, this index was often elevated in the CSF with noninflammatory BBB damage (Table 2).

The IgG hyperbolic function showed more homogeneous behavior: it was positive in 76.53% of MS CSF with normal BBB damage, in 81.25% of CSF MS with mild BBB damage, and in 66.66% of CSF MS with severe BBB damage. This formula was increased in nine patients with noninflammatory BBB damage. Considering all the 120 MS CSF, the detection of IgG OBs showed the highest percentage of positivity (94.16%). followed by the IgG hyperbolic function (76.66%) and by the intra-BBB IgG synthesis rate (68.33%).

Summarizing, even if all methods descriminated well between normal and pathological CSF, all of them were clearly influenced by the presence of BBB damage. Tourtellotte's formula seemed inadequate to correct the intrathecally synthesized IgG from serum-derived IgG and therefore should not be recommended for diagnostic clinical purposes. The IgG hyperbolic function appeared more reliable and, even if this formula also does not perfectly correct the effect of BBB damage, it can be used more safely in the evaluation of clinical trials, when the quantitation of local IgG is needed.

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Lymphocyte Subpopulations as Markers of Disease Activity in Multiple Sclerosis*

M. Zaffaroni, D. Caputo, A. Ghezzi, S. Marforio, and *C.L. Cazzullo*

Centro Studi Sclerosi Multipla, Università di Milano, Ospedale di Gallarate, Gallarate Italy

Introduction

For many years in our laboratory we have focussed our attention on the study of immunoregulatory mechanisms in patients with multiple sclerosis (MS).

Several data from the literature report the presence of different aberrations within the cell-mediated branch of the immune system. Among these, the most frequently reported findings are a defect of mitogen-driven T-suppressor activity [1], a reduced number of circulating suppressor T-lymphocytes as detected by different cell markers [2–4], in increased number of activated T-cells [5, 6] and reduced natural killer activity [7]. Since the advent of monoclonal antibodies several groups have been able to enumerate more reliably T-lymphocyte subpopulations. In spite of the discordant literature reports [8, 9], all investigators report some abnormality of immunoregulation with respect to normal populations. The most consistent finding in a large series of studies is a reduced number of circulating T-cells bearing the CD8 surface receptor during the active phases of the disease, also expressed as an increased CD4/CD8 ratio [10–14].

In a previous paper [15] we have pointed out that the loss of CD8-positive cells in the peripheral blood is paralleled by an analogue loss in the cerebrospinal fluid during clinical exacerbations. Moreover, we found an inverse correlation between the number of CSF CD8-positive cells and the amount of intrathecal synthesis of IgG, suggesting a possible influence of the lymphocytic setup on intracerebral Ig synthesis. This finding, however, does not explain the restricted heterogeneity of IgG produced in the central nervous system, which is the effect of the expansion of a few activated lymphocyte clones. Besides the pathogenetic significance, not yet clarified, of lymphocyte subpopulation imbalance in MS, we found it of interest to verify the utility of serial T-subset determinations in monitoring disease evolution.

Here we report the conclusive results of an 18-month-follow-up of 46 MS patients whose preliminary data are the subject of a recent publication [14].

Subjects and Methods

We followed-up 46 patients with definite MS according to the criteria of Poser et al. [16] for 18 months. They were 24 females and 22 males, aged between 18 and 48

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years (mean, 32.6 years), with disease duration ranging from 0.1 to 21 years (mean, 5.4 years). Patients were recalled monthly to our observation for neurological examination and clinical history updating: a clinical relapse was considered when the appearance of new clinical signs or symptoms lasted more than 24 h. A short course of dexamethasone was started in the case of clinical exacerbation.

At the same time of clinical examination, patients were venipuctured; to avoid circadian variations [17, 18] blood samples were always taken between 9.00 and 10.00 a. m. T-cell subsets were evaluated on whole blood as previously described [14] by direct immunofluorescence using an automated cytofluorometer (Ortho Spectrum III). Monoclonal antibodies against CD3, CD4, and CD8 surface antigens were used (Ortho-Clone OKT3, OKT4, OKT8 antibodies).

Statistical analyses were performed by *t*-test, X^2*t*-test, or analysis of variance where appropriate.

Results

Patients were classified into three groups according to their clinical evolution during the period of observation: chronic-progressive ($n=11$), relapsing ($n=15$), and clinically stable ($n=19$). The mean percentages of each lymphocyte subset are reported in Table 1 in relation to the three MS categories. Although not significant at analysis of variance among groups, the differences of T4 and T8 percentages between chronic-progressive and relapsing or stable patients are highly significant at *t*-test. Chronic-progressive patients show lower percentages of T8-positive cells (putative suppressor/cytotoxic T-lymphocytes) and higher percentages of T4-positive cells (putative helper/inducer T-lymphocytes).

In a previous study [14] we found that the T4/T8 ratio is the most reliable index of perturbation among lymphocyte subpopulations: by means of this parameter the different clinical courses of MS are better defined and distinguishable (Table 2). Chronic-progressive patients show significantly higher ratios with respect to other MS groups, with the widest fluctuations with time ($\triangle$ T4/T8). MS patients as a whole have significantly higher T4/T8 and $\triangle$ T4/T8 ratios with respect to controls ($P<0.001$ at *t*-test).

Figure 1 shows the distribution of the average T4/T8 ratio of each patient. We can note that the majority of chronic-progressive patients have very high ratio values. The ratios of relapsing patients overlap to a great extent those of stable ones, but we

Table 1. Mean T-cell subset percentages (±SDs) in the three MS classes

	%T3	%T4	%T8
Chronic-progressive (n=11)	77±4.8	53.7±5.4	25.6±4.9
Relapsing (n=16)	75.2±4.5	46.3±7	33.5±6.6
Stable (n=19)	73.8±7.3	46.7±7.7	31.7±7.5
ANOVA	F=1.026889 (NS)	F=4.097772 P<0.025	F=4.497422 P<0.025

Table 2. Mean T4/T8 and ΔT4/T8 ratios (±SDS) in MS classes and controls

Clinical course		Mean $T4/_{T8}$	Mean $\Delta T4/_{T8}$
Chronic-progressive	(n=11)	2.3±0.3	0.7±0.7
With relapses	(n=16)	1.5±0.4	0.5±0.3
Clinically stable	(n=19)	1.6±0.5	0.3±0.2
Controls		1.5±0.2	0.2±0.1
Statistical significance ANOVA		P<0.001 F=8.4	NS F=2.3

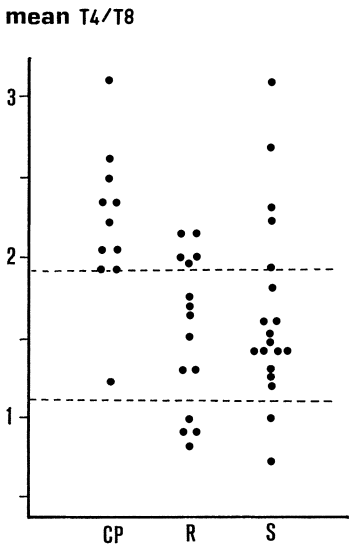

Fig. 1. Distribution of mean T4/T8 ratio in all MS patients, classified as chronic-progressive (*CP*), relapsing (*R*), and stable (*S*)

must remember that our data are the results of a longitudinal study: if we considered T4/T8 ratios recorded during relapses only, we would find relapsing patients behaving like chronic-progressive ones. In fact in an 18-month-follow-up we recorded a total of 44 clinical relapses, 41% of which were correlated with a high T4/T8 ratio. This figure would be higher if we also consider ed significant increases of the T4/T8 ratio over preceding values but still within the normal range (see, for example, the second relapse in Fig. 3).

Figs. 2–4 illustrate the sequential profiles of T-lymphocyte subpopulations of three typical chronic-progressive, relapsing and clinically stable MS patients: high T4/T8 ratios are clearly recognizable during progressive and active phases of the disease. It is noteworthy that while high ratios during relapses are due to a decrease of T8-positive cell percentages (Fig. 3), high ratios of chronic-progressive course are mainly due to high T4 percentages (Fig. 2).

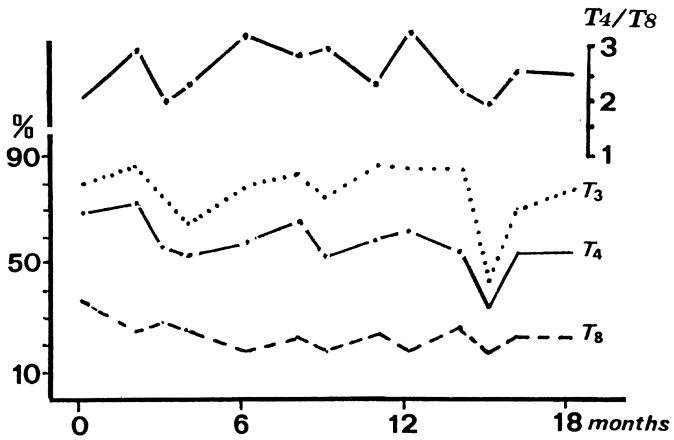

Fig. 2. Serial profiles of T-cell subsets and T4/T8 ratio in a typical chronic-progressive patient

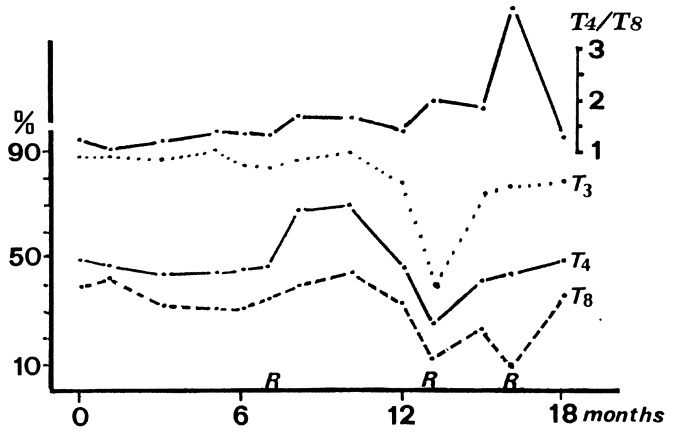

Fig. 3. Serial profiles of T-cell subsets and T4/T8 ratio in a typical relapsing patient. *R*, relapse

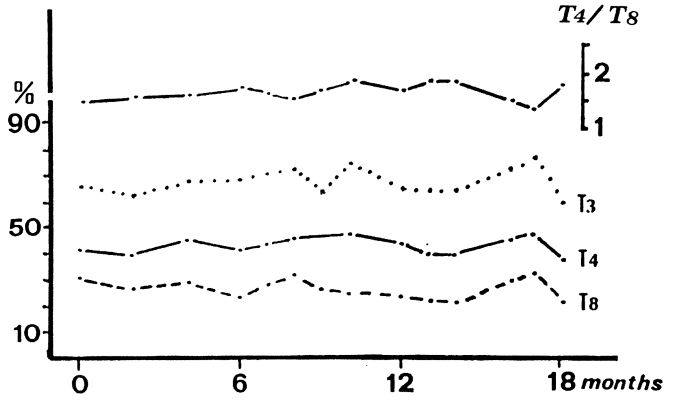

Fig. 4. Serial profiles of T-cell subsets and T4/T8 ratio in a typical stable patient

Eight patients (three stable, four relapsing, and one chronic-progressive) showed steadily reversed ratios (< 1), mainly due to high T8 percentages: during exacerbations, a further decrease of T8-positive cells and a consequent relative increase of T4/T8 ratio was observed (Fig. 5).

In order to verify the utility of lymphocyte subset enumeration in defining the different clinical courses of MS, we classified patients according to the percentage of abnormal (> 1.9) T4/T8 ratios recorded during the follow-up (Fig. 6). In this way we

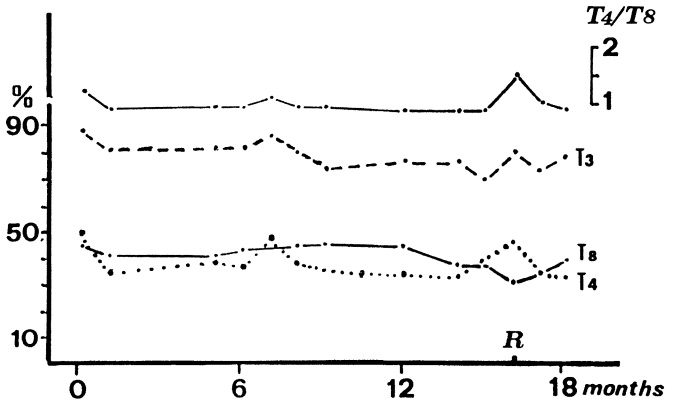

Fig. 5. Serial profiles of T-cell subsets and T4/T8 ratio in a relapsing patient with constantly reversed T4/T8 ratio. *R*, relapse

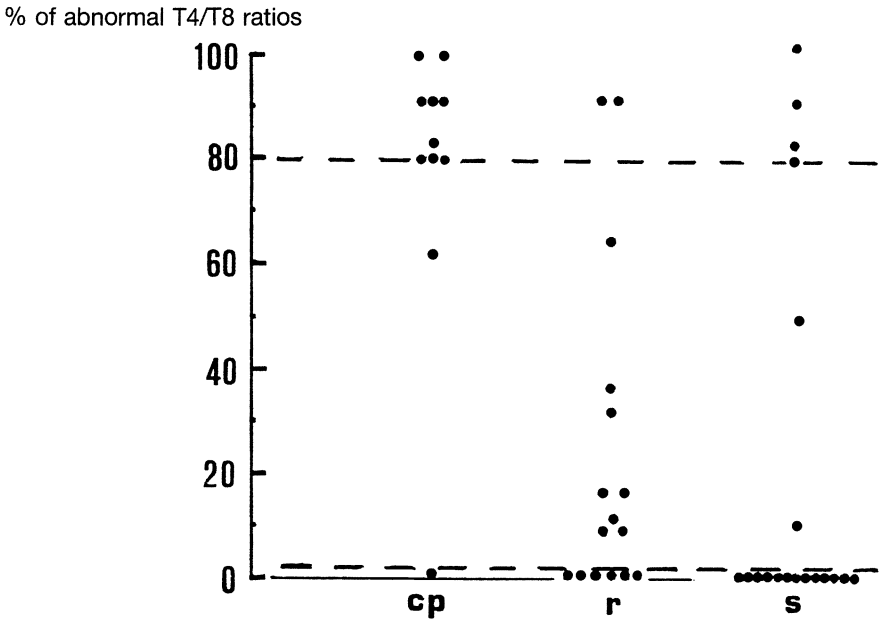

Fig. 6. Distribution of abnormal T4/T8 ratios in all MS patients, classified as chronic-progressive (*CP*), relapsing (*R*), and stable (*S*)

Table 3. Correlation of clinical course with mean T4/T8 ratio

Clinical course	T4/T8 ratio		
	Steadily high	Fluctuating	Normal
Chronic progressive (n=11)	9	1	1
With relapses (n=16)	2	8	6
Clinically stable (n=19)	4	2	13
Statistical significance	$\chi^2=20.5$ $P<0.0005$		

grouped patients into three classes: steadily high ratios (i.e., more than 80% abnormal ratios), fluctuating ratios (i.e., abnormal ratios less than 80%), and steadily normal ratios. The correlation of these groups with the three courses of MS yielded a significant result on X^2 analysis ($P<0.0005$, Table 3).

Discussion

The conclusive results of our 18-month-follow-up of 46 MS patients confirmed the findings of our preliminary [14] and other literature reports [10–13]. The sequential analysis of T-cell subpopulations for an adequate period yields useful data, in addition to clinical data, for an objective definition of MS course from an immunological viewpoint.

Lymphocyte subset analysis detected only 41% of clinical relapses and thus single determinations cannot be used as a reliable marker of disease activity. Nevertheless, serial determinations could define immunological patterns somehow related to clinical courses (Table 3). In particular, the chronic-progressive course is characterized by steadily high T4/T8 ratios with wide fluctuations; the relapsing course by ratios fluctuating from normal to high values more or less related to clinical relapses; finally, the stable course is characterized by steadily normal ratios, similarly to controls. There is no-clear-cut distinctions among subgroups and a more prolonged clinical follow-up will clarify, for example, the meaning of steadily high T4/T8 ratios in four stable subjects from a prognostic viewpoint.

The apparently low figure of clinical relapses detected by high T4/T8 ratios is probably determined by using 2 SD above mean normal values in defining abnormal ratios. It is also conceivable that smaller fluctuations of T4/T8 ratios above baseline values are of relevance in detecting clinical relapses. On the other hand, as stressed in a previous paper [19], the occurrence of apparently unexplained fluctuations of the T4/T8 ratio, paralleled by the finding of an abnormal cerebral evoked response, suggests that subclinical relapses may occur.

An important point in our study is the finding of different mechanisms causing raised T4/T8 ratios in chronic-progressive and relapsing patients. In the former we found mainly high T4 percentages, in the latter mainly low T8 percentages. This fact suggests two different immunological mechanisms in the progression of preexisting demyelinating lesions and in the appearance of new inflammatory foci.

Besides these speculations, the actual pathogenetic role of T-cell subset imbalance in MS is still unknown. Recently, a selective loss of the "inducer of suppressor" T-cell subset [20, 21] has been suggested as cause of the reduced T-suppressor activity in chronic-progressive MS [22, 23].

An underrepresentation of T8-positive cells in the peripheral blood of Ms patients during relapses of the progressive phase of the disease is not universally accepted [24–26]. Some groups [27] suggest that low percentages or T8+ cells may be apparently found because of a low density of CD8 receptors expressed by lymphocytes from MS patients. In addition, it was reported that cells that express a low surface density of CD8 molecules have natural killer function and lack CD3 (pan T-cell marker) [28]. Discrepancies may also be due to different techniques such as microscopic or cytofluorometric analysis as well as to different study designs. Kastrukoff et al. [29], for example, studied only 12 MS patients for 6 months and found no difference with respect to controls. Others [25, 26] performed only cross-sectional transversal studies. It is noteworthy that all groups that studied an MS population longitudinally found as we did a correlation between T-cell fluctuations and disease activity.

To conclude, the analysis of T-cell subpopulations can provide useful information in defining the clinical evolution of MS. Further investigations on the immune system in MS are needed to elucidate the relationships between T-cell perturbations and the pathogenesis of demyelination. These studies could possibly provide new and reliable markers of disease activity.

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Serological Studies of Patients with Multiple Sclerosis, controls, and Patients with Other Neurological Diseases: Antibodies to HTLV-I, HTLV-II, HIV, and STLV-III

D. L. Madden,[1] *F. K. Mundon,*[2] *N. R. Tzan,*[1] *D. A. Fuccillo,*[3] *M. C. Dalakas,*[1] *V. Calabrese,*[4] *T. S. Elizan,*[5] *G. C. Roman,*[6] and *J. L. Sever*[1]

1 National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health Bethesda, MD, USA
2 Electronucleonics, Columbia, MD, USA
3 Microbiological Associates, Bethesda, MD, USA
4 Medical College of Virginia, Richmond, VA, USA
5 Mount Sinai Medical Center, NY, USA
6 Texas Tech University Health Sciences Center School of Medicine, Lubbock, TX, USA

Introduction

Retroviruses have been associated with neurological disease in man. Recently, Kaprowski et al. [1] reported that outbreaks of mutliple sclerosis (MS) in Key West, Florida, and in Sweden seem to be associated with increased antibody for human retroviruses. In addition, homology studies under nonstringent conditions using CSF cells derived from MS patients reacted with human T-cell leukemia virus (HTLV)-I antigen. During the past 10 years more than ten different possible agents have been suggested as causes of MS [2]. These agents include a number of recognized viruses such as measles, canine distemper, scrapie agent, coronaviruses, and agents of unclear classification such as the MSAA (multiple sclerosis associated agent), bone marrow agent, and chimpanzee agent. We report here our studies of retrovirus antibody in a large number of sera and CSF collected from MS patients, matched controls, and patients with other neurological diseases (OND) prior to AIDS becoming a serious disease.

Review of the Literature

Retroviruses have been associated with a number of diseases in man and animals. In general these infections have been associated with leukemias, sarcomas, and immunosuppression. Neurological manifestations occur with some diseases. These agents have been listed as oncoviruses. In addition, retroviruses have been associated with slow virus diseases such as visna and progressive pneumonia (maedia). More recently immunosuppressive diseases caused by retroviruses have been recognized. These retroviruses are classified as nononcogenic or lentiviruses.

Retroviruses are enveloped viruses whose genome contain copies of high molecular weight, single-stranded RNA similar to messenger RNA. The virion contains various enzymes including reverse transcriptase (RNA to DNA). The retroviruses are classified into four groups: type A, B, C, and D, based upon dependency of

Table 1. Retroviruses of humans and non-human primates

Human virus	Disease
HTLV-I	Adult T-cell leukemia, tropical spastic parapresis
HTLV-II	Hairy cell leukemia
HIV/HTLV-III/LAV	AIDS
HTLV-IV/LAV-II	Unknown
Non-human-virus	
STLV-I/HTLV-I	Lymphoma in captured macaques, antibody present in many wild monkeys in Asia and Africa
SIV/STLV-III	Immunosuppressive disease of monkeys
SRV-I	SAIDS in monkeys
SRV-II	Retroperitoneal fibromatosis
Mason-Pfizer monkey virus (MPMV)	Causes SAIDS-like disease in infant monkeys
SSV-I	Woolly monkey sarcoma virus or simian sarcoma virus
GALV	Gibbon ape leukemia virus or lymphosarcoma or myelogenous leukemia virus
MAC-I	Macaque endogenous virus, nonpathogenic
Squirrel monkey	Squirrel monkey endogenous virus, nonpathogenic
PO-I-Lu	Langur monkey endogenous virus, nonpathogenic

reverse transcriptase upon Mm^{2+} or Mg^{2+}, the characteristic of the nuclear capsules, and the maturation of the intact virus from the cells.

A list of retroviruses currently identified from human and nonhuman primates is presented in Table 1. The first human virus (HTLV-I) was isolated in 1980. Several other viruses have been isolated. All of these agents belong within the type C group. Most of the viruses cause disease in the host from which they are isolated. HTLV-I has been isolated from humans with adult T-cell leukemia and recently has been associated with tropical spastic parapresis (TSP). HTLV-II was isolated from an individual with hairy cell leukemia. It is serologically related to HLTV-I. HIV/HTLV-III/LAV is the most pathogenic, now being recognized as the etiological agent of AIDS. Primary subacute encephalopathy and neuropathy are also recognized as important signs of infection. HTLV-IV has recently been isolated from normal individuals in Africa. It is serologically closely related to HIV/HTLV-III.

Many simian retroviruses have been identified; however, their disease spectrum is less well defined. STLV-I/HTLV-I are serologically and genetically similar. Recently this agent was isolated from three species of macaque with lymphoma. Many wild caught animals in Asia and Africa have HTLV-I antibody. An STLV-II/HTLV-II agent has not been identified. SIV/STLV-III has been isolated from monkeys at the New England Regional Primate Center. It has a one-way cross with HIV/HTLV-III, i.e., serum that reacts with HTLV-III antigen reacts with STLV-III antigen but monkey serum that reacts with STLV-III antigen may not react with HTLV-III antigen. This agent in monkeys produces an immunosuppressive disease similar to AIDS in man (infects the T4 cells) and at least one strain causes a severe

neurological disease. SRV-I is associated with the monkey SAIDS. This agent causes a generalized disease which in the acute form produces a leukopenia and anemia, with death occurring in 1–3 months. In the subacute disease the animals usually die of opportunistic infections in 3–12 months. Some antibody-positive animals may survive for years. No neurological disease has been associated with this infection. This virus is not serologically related to the other retroviruses. SRV-2 is associated with retroperitoneal fibromatosis. It also predisposes animals to opportunistic infections. It is not closely serologically related to the other retroviruses. Mason Pfizer monkey virus was isolated in 1970 from monkey breast cancer samples. When inoculated into infant animals it produced death. Its pathogenecity has not been well characterized. SSV-I was isolated from a woolly monkey with a sarcoma, and the GALV was isolated from a gibbon ape and causes lymphosarcoma or myelogenous leukemia. Three endogenous viruses have been isolated from monkeys. They are: MAC-1 virus, which was isolated from macaques, the PO-1-Lu virus, which was isolated from the Langer monkey, and the squirrel monkey virus, which was isolated from the squirrel monkey. These three are reported to be nonpathogenic for monkeys.

Several retroviruses have been associated with neurological disease in animals and man. The first recognized was that of visna and progressive pneumonia (maedia) of sheep. This disease syndrome has been recognized since the early 1930s and the virus identified in 1949. Recently, three diseases of man have been recognized. Tropical spastic parapresis (TSP) has been associated with HTLV-I virus and a subacute encephalopathy and neuropathy has been associated with HIV. An acute neurological disease in primates associated with SIV/STLV-III has been reported in rhesus monkeys. A summary of the information available about these three diseases is presented.

TSP has recently been associated with HTLV-I by antibody studies [3, 4]. The disease is prevalent in the Pacific lowlands of Columbia, Jamaica, Martinique, Seychelles, South Africa, India, other tropical countries, and Japan. It characteristically does not occur in those temperate zones in which MS has been reported. Clinically, TSP presents with a chronic spastic myelopathy of slow onset with minimal sensory deficits. TSP patients have a progressive involvement of the pyramidal tracts, bilaterally and symmetrically affecting only the lower extremities. This is manifested by difficulty in walking, spasticity, and hyperflexia of the legs. Spastic bladder, severe constipation, and impotence in males are common. TSP does not have the typical relapsing course or visual abnormalities common in MS. The CNS and spinal cord lesions of TSP on postmortem examination are significantly different from those observed in MS. Presence of oligoclonal bands in TSP have been reported. There are several contradictory reports on the effect of steroid treatment of TSP. Kogoshima in South Japan reports that the disease symptoms are diminished by steroids while Zaninovic reported that in Columbia steroids had no effect on the course of the disease.

Subacute encephalopathy and neuropathy have been described in HIV antibody positive patients [5, 6]. This virus has been isolated from brain tissue, CSF, spinal cord, and peripheral nerves. As many as 10% of the HIV antibody positive patients may present, with the first signs of disease being a neurological disorder. This initial neurological disorder usually occurs as a change in personality and abnormal

behavior; definable neurological signs may be present. In most of these patients other signs of AIDS developed later. In another 30%–40% of the AIDS patients, neurological disease – not due to opportunistic infections – occurs. The clinical syndrome includes dementia, gait disturbance, spastic paraparesis, and bilateral pyramidal tract signs. The pathological lesions observed consist primarily of a vacuolar myeloneuropathy associated with a subacute encephalitis. By in situ hybridization and immunohistochemical staining, virus-containing cells appear to be primarily large monocytic (macrophages) cells and multinucleated cells. These cells are not associated with the neuronal type of cells and are found primarily in frontal, temporal, and parietal regions. Oligoclonal bands have been found in a few cases in our laboratory. Dr. Ceroni of Pavia, Italy, while in our laboratory, has demonstrated the specificity of these bands for HIV.

Information about the epizootiology, pathogenesis, and pathology of STLV-III infection in monkeys is limited. Virus has been isolated from the African green monkey STLV-III^{AGM} and the rhesus monkey STLV-III^{MAC}. The incubation period following inoculation until clinical signs develop is 3–9 months or longer. Passage through tissue culture systems attenuates the pathogencity of the virus. The neurological disease seems to be associated with certain isolates of STLV-III while other isolates produce disease without neurological involvement. Serial animal-to-animal passage of isolates which produce neurological disease appears to increase the frequene and severity of the neurological signs. The neurological disease is associated with an acute encephalitis. Death usually ocurs within a few weeks after neurological signs appear. Virus has been found in macrophages and multinucleated cells in the brain parenchyma.

Material and Methods

We have several collections of sera from MS patients and matched controls and patients with other neurological diseases collected prior to 1980. These sera and CSF are listed. Sera from 62 MS patients and 62 controls collected from Milwaukee, Wisconsin, prior to 1979 were available. Sera from 45 MS patients, 53 patients with optical neuritis, along with CSF from 24 MS and 31 optical neuritis patients were collected by V.C. at Richmond, VA. Sera from 27 patients with postencephalitis Parkinson's, 26 age-matched controls, and 50 idiopathic Parkinson's were collected by T.E. Sera from 117 patients with other neuromuscular diseases were collected by M.D. All except those from postpoliomyelitis were collected prior to 1980. Sera from 25 AIDS patients and 54 homosexual controls were collected in collaboration with R.D. in Los Angeles in 1982–1983. Sera from 20 cases of TSP and 16 control subjects were collected by G.C.R. in the Seychelles in 1985. All sera from Milwaukee were tested for HTLV-I and HTLV-II, HIV, and STLV-III antibody using an indirect immunofluorescence test. All sera from AIDS and TSP patients were also tested for HTLV-I and HIV using the IFA. All serum and CSF except for AIDS was tested for HTLV-I, HIV, and STLV-III antibody using an ELISA test. Sera from AIDS patients and controls were tested by ELISA for HTLV-III only.

All ELISA tests were performed using commercially available kits. For the HTLV-I ELISA a commercial kit was purchased from Dupont Co., Billary, MA

(manufactured by Biotech Research Lab Inc.). ELISA tests for HTLV-III were performed using a commercially available kit purchased from Organon Teknika Corp, Oklahoma City (Bionetics Lab Products, Charleston, SC). All positives were confirmed by immunofluorescence antibody (IFA). The IFA tests were performed utilizing HTLV-I in Hut 102 cells and HTLV-II and HTLV-III in H9 cells. These antigens were prepared by Electronucleonics Lab Inc., Columbia, MD. The STLV-III IFA antigen was prepared in our laboratory. Positive and negative serum was available to determine the specificity of the reactions.

Results

The results of testing patients with AIDS, TSP, MS, and controls is presented in Table 2. All 25 of the AIDS patients had HTLV-III antibody. In addition, 21 of the 25 patients reacted with the STLV-III antigen. Antibody to HTLV-I was not present in these individuals. None of the controls had antibody to any of the three antigens tested. Antibody to HTLV-I was found in 17 of the 20 individuals with clinical symptoms compatible with TSP. Two of the seven patients with other neurological disease had antibody. One of these patients was diagnosed as having transverse myelitis and one with clinically probable MS. None of the normal controls had HTLV-I antibody. None of the MS patients or control patients from Milwaukee, WI, had antibody to any of the four retrovirus antigens tested.

All 25 AIDS patients had HIV ELISA optical density (OD) readings above the cutoff of 0.39, ranging from 0.85 to 2.0, and were antibody positive. Two of the 54 control samples had an ELISA reading above 0.39 (5.5 and 8.5). Neither of these samples could be confirmed as being positive by IFA or Western blot. Thus, all 54 samples were negative. None of the TSP patients, patients with optic neuritis diseases, or controls had ELISA OD readings above 0.39; thus all were negative for HIV antibody.

The results of the ELISA HIV tests on the samples from MS patients, patients with optic neuritis, controls, and patients with OND are presented in Fig. 1. None of

Table 2. Retrovirus immunofluorescent activity in serum from AIDS patients, TSP patients, and multiple sclerosis patients and controls

	No. of patients tested	HTLV-I	HTLV-II	HIV	STLV-III
AIDS patients	25	0/25	NT	25/25	21[a]/25
Controls	54	0/54	NT	0/54	0/54
TSP patients	20	7/20	NT	0/20	0/20
OND[b]	7	2/7	NT	0/7	0/7
Controls	9	0/9	NT	0/9	0/9
MS patients	62	0/62	0/62	0/62	0/62
Controls	62	0/62	0/62	0/62	0/62

[a] All positive of HIV
[b] PN 1 negative, TM 1 positive, GS 1 negative, PGP 3 negative, and MS 1 positive

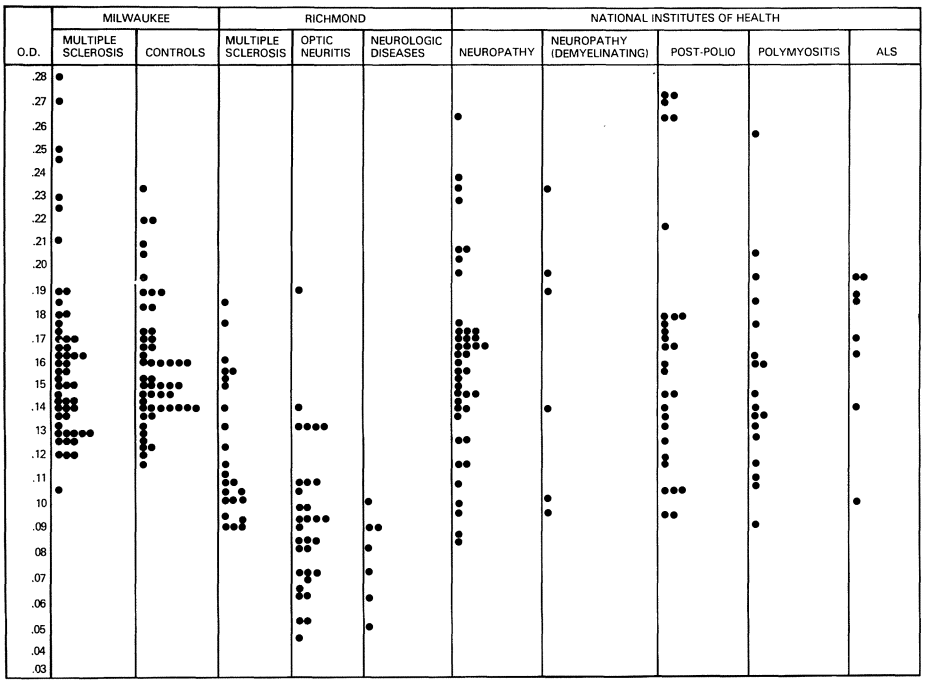

Fig. 1. HIV ELISA reactivity recorded as optical density (OD) on sera from MS patients, matched pal controls, and patients with other neurological diseases. The negative control cutoff was 0.39 and all of these samples were considerably below this level. The results obtained from the patients with Parkinson's disease and aged-matched controls were similar (data not shown). The OD readings on the CSF from multiple sclerosis, optic neuritis, and other neurological disease patients were lower but the distribution among the groups was similar (data not shown)

the OD readings were above the cutoff of 0.39. Analysis of the OD readings obtained did not indicate that the readings were higher in the MS patients as compared with the controls.

Seventeen of the 20 TSP patients had HTLV-I ELISA OD readings above the cut off of 0.36; 2 of the patients with OND had a reading above the cutoff. Three of the patients with clinical TSP, five patients with OND, and the nine controls had ELISA OD readings below the 0.36 cutoff and were considered negative.

The results of the ELISA HTLV-I tests on the samples from MS patients, patients with optical neuritis, controls, and patients with OND are presented in Fig. 2.

None of the OD readings were above the cutoff of 0.36. Analysis of the OD readings obtained did not indicate that the readings were higher in the MS patients as compared with the controls.

Discussion

Retroviruses have been associated with several neurological diseases. HTLV-I has been associated with TSP, which occurs in the lower latitudes and Japan. This

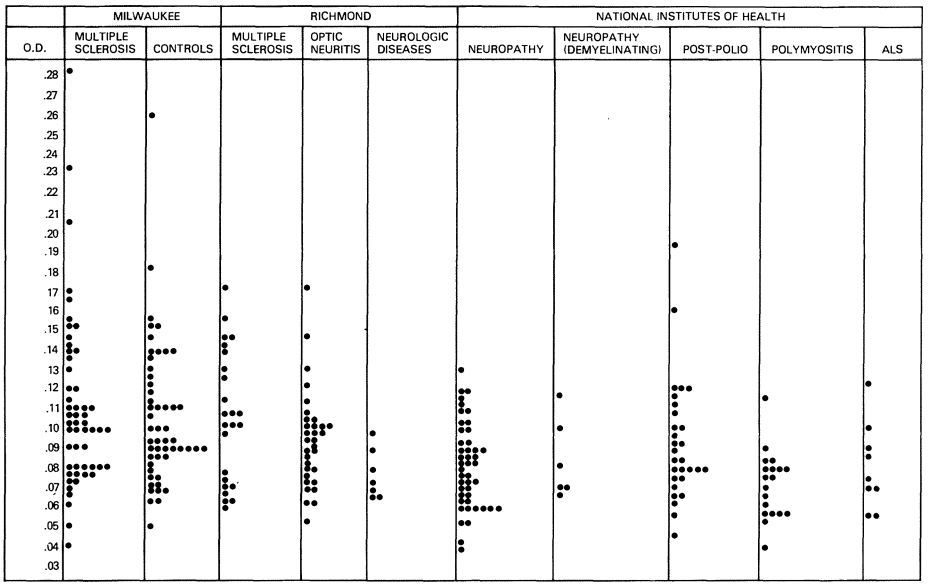

Fig. 2. HTLV-I ELISA reactivity recorded as optical density (OD) on sera from MS patients, matched pal controls, and patients with other neurological diseases. The negative control cutoff was 0.36 and all of these samples were considerably below this level. The results obtained from the patients with Parkinson's disease and aged-matched controls were similar (data not shown). The OD readings on the CSF from multiple sclerosis, optic neuritis, and other neurological disease patients were lower but the distribution among the groups was similar (data not shown)

association needs to be further confirmed by additional serological studies and virus isolation studies. The clinical signs and symptoms of disease resembles progressive multiple sclerosis but the pathological lesions are significantly different. HIV causes a neurological syndrome. This clinical syndrome as well as the pathology differs from MS. In our study on serum samples collected before HIV infection became prevalent we could not demonstrate a serological relationship of the human retrovirus as well as the simian retrovirus STLV-III with MS.

Several explanations for these differences and those reported by Kaprowski et al.[1] are evident. It is possible that some patients might have HTLV-I antibody since Key West is a tropical island on the edge of the TSP belt and have TSP, or an early stage of adult T-cell leukemia. Further, with modern transportation so easy, individuals who have lived or traveled to these HTLV-I regions may develop antibody and may return to the northern latitudes and develop MS independent of their HTLV-I status. Further, it should be expected that MS patients have life-styles that are similar to the general population. Some will develop HIV antibody due to homosexual activity, heterosexual transfer, drug usage, and transfusion. Careful questioning may identify these individuals. Thus, our findings in samples collected prior to 1980 are in agreement with the results reported by Hauser et al. [7] and Karpas et al. [8] and currently demonstrate that the recognized human related retroviruses are not etiologically related to MS.

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Correlation of Oligoclonal IgG Bands and Viral Antibodies in Twins with Multiple Sclerosis

J. Woyciechowska,[1] *J. Dambrozia,*[2] *A. Chu,*[1] *P. Leinikki,*[3] *C. Shekarchi,*[4] *D. Madden,*[1] and *J.L. Sever*[1]

1 The National Institutes of Health, Infectious Diseases Branch, IRP, NINCDS, Bethesda, MD, USA
2 The National Institutes of Health, Office of Biometry and Field Studies, IRP, NINCDS, Bethesda, MD, USA
3 University of Tampere, Department of Biomedical Science, Tampere, Finland
4 Microbiological Associates, Inc., Bethesda, MD, USA

Multiple sclerosis (MS) has been known for several immunological epiphenomena [1]. One of these characteristics of the CSF of MS patients is the presence of oligoclonal IgG bands (OCBs) in 90%–95% of specimens (Link 1981). Only a small percentage of OCBs have viral activity. The specificity of the majority of antibody activity is unknown [1–3].

Twins with MS can provide the most rigorous matching case-control group study and have been extensively investigated from a clinical and immunological point of view [4–6]. Recently, we have analyzed viral antibodies in the same twins [7]. In this study using specimens and data obtained at the time of original admission of these twins, OCBs were examined in unconcentrated CSF by the micromethod [8.] Results were compared with viral antibody titers an viral CSF/serum ratios corrected for blood brain *barrier* (BBB) permeability as previously described [7,9]. OCBs were also compared with serverity and duration of the disease and presence of DW_2 histocompability antigens [4].

Oligoclonal IgG bands were observed in the CSF of 47 individuals (Table 1). CSF was not available for one person. Among the discordant twins, the distribution of the number of bands for the affected twins ranged from two to six, whereas the unaffected twins had only two or three bands. Furthermore, among individuals with either two or three bands, the proportion of those with three bands was significantly greater for the MS (0.61) compared with the non-MS twins (0.44). Among the affected twins, those with positive DW_2 antigen had more IgG bands ($P = 0.002$).

Table 1. Number of CSF oligoclonal IgG bands in twin pairs

Discordant twins			Concordant twins		
	MZ	DZ		MZ	DZ
MS	3 4 5 3 5 3	2 5 5 2 3 3 3 4 2 5	MS	3 4 4 5 3 5	23
Non-MS	2 2 3 2 2 3	2 2 3 2 2 3 2 N 3 2	MS	3 4 6 6 2 2	34

N, no specimen available

Table 2. Oligoclonal IgG bands and mean titers of viral antibodies in CSF: CSF and CSF-serum viral antibodies ratio

	Measles				Rubella			
	Titers		Ratios		Titers		Ratios	
Bands	MS	Non-MS	MS	Non-MS	MS	Non-MS	MS	Non-MS
2	1.16	0.94	0.06	0.06	1.24	0.41	0.22	0.16
3	1.50	0.80	0.33	0.06	1.27	0.32	0.40	0.21
4	1.98		0.76		1.45		0.13	
5	1.78		0.35		1.17		0.27	
6	2.40		0.62		2.05		0.13	

A possible linear regression relationship between the number of bands and the duration of MS or the severity of the disease was not supported by the data ($P = 0.07$ and $P = 0.22$, respectively).

Comparison of CSF viral antibody titers with the number of OCBs is shown in Table 2. Among MS twins with either two or three bands, mean CSF viral antibody titers for rubella, vaccinia, and measles were higher than those of non-MS twins. Positive linear relationships between viral antibody titers an the number of bands were observed for measles ($P = 0.002$), rubella ($P = 0.006$), and mumps ($P = 0.044$) in all MZ and DZ concordant and discordant twins. Support for a positive linear relationship of vaccinia antibody levels and bands was not significant ($P = 0.092$), while corona titer levels and bands gave a weak negative relationship. Similar analysis with regard to oligoclonal IgG bands was performed on viral antibody CSF serum ratios. Linear relationships between these data were not found. All the above P values were based on the usual F-test for regression.

Positive correlation of the highest rubella and vaccinia antibody CSF mean titers with the highest number of the oligoclonal IgG bands in MS twins was found only when CSF mean titers were analyzed and disappeared with correction for BBB permeability and age. This is in agreement with Albrecht et al. [10], who emphasized the importance of including BBB permeability. In a longitudinal study on rubella, measles, and respiratory syncytial virus antibodies in serial serum and CSF specimens from MS patients, poor correlation between the fluctuation of the intrathecal IgG and the antibody production was found [11]. According to authors of that study, most of the antibodies may not be relevant to the disease process. We agree that synthesis of oligoclonal IgG and viral antibodies within the CNS may not be due to viral stimulation. Cross-reacting antigens or nonspecific stimuli may also be responsible for triggering immunological derangements in MS.

On the basis of our study, when data corrected for BBB permeability were used, CSF viral antibody ratio linear relationships to the oligoclonal IgG bands could not be established. This finding is consistent with other studies indicating that the majority of CSF oligoclonal IgG is different from viral activity [1–3].

The analysis of OCBs showed that more than three bands were seen only in affected twins. With the micromethod, the presene of two or more bands in the gamma globulin area is considered abnormal and ‹10% of the normal population has

Table 2. Continued

Corona				Vaccinia				Mumps			
Titers		Ratios		Titers		Ratios		Titers		Ratios	
MS	Non-MS	MS	Non-MS	MS	Non-MS	MS	Non-MS	MS	Non-MS	MS	Non-MS
1.20	1.06	0.08	0.26	0.90	0.49	0.33	0.05	0.20	0.16	0.23	0.14
1.60	1.65	0.27	0.20	1.16	0.85	0.14	0.07	0.38	0.22	0.05	0.06
0.95		0.10		1.06		0.25		0.65		0.26	
1.22		0.21		1.11		0.10		0.47		0.51	
0.50		0.07		1.05		0.06		0.60		0.05	

more than one band [12]. The unusual finding of bands in all of the non-MS twins suggests the possibility of subclinical disease in those twins or abnormalities in immune responses which are similar to those which occur in clinically overt MS. This was a possibility suggested in an original study of multiple sclerosis on the same twins by Williams et al. [4] based on the presence of CSF bands seen in 8 of 16 clinically normal twins using the macromethod. A similar impression has given by Xu, who recently evaluated OCBs in the same MS twins among other patients [5]. Xu was using the isoelectrofocusing method and subsequently identified OCBs by immunoperoxidase staining and silver staining of western transblotting. His results, obtained with very sensitive methods, confirm previous suspicions of Williams [4] and Woyciechowska [7] that the presence of immunological abnormalities can precede clinical diagnosis of MS. Longitudinal studies of MS twins and their families have revealed the presence of subclinical forms of MS in some cases [6].

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Detection of HLA Class II Restriction Fragment Length Polymorphisms in Multiple Sclerosis Using Pooled DNA Samples

R. N. S. Heard,[1,2] *I. A. Dodi,*[1] *A. W. Downie,*[3] *D. A. Francis,*[2] *J. E. C. Hern,*[3] *J. R. Batchelor,*[1] *W. I. McDonald,*[2] and *R. I. Lechler*[1]

1 Department of Immunology, Royal Postgraduate Medical School, Hammersmith Hospital, London, UK
2 Institute of Neurology, Queen Square, London, UK
3 Departments of Medicine and Neurology, Aberdeen Royal Infirmary, Foresterhill, Aberdeen, UK

Introduction

There is by now strong evidence that susceptibility to multiple sclerosis (MS) is influenced by one or more genetic factors [1]. The familial aggregation of MS, the higher concordance rate between monozygotic than dizygotic twins and the association with serologically typed alleles encoded within the major histocompatibility complex (MHC) all provide convincing data in support of this hypothesis. HLA typing of Causasian populations of northern European ancestry with high levels of MS, in many parts of the world, has consistently shown a higher than expected prevalence of the extended haplotype HLA A3, B7, DR2, the association with DR2 being stronger than with the class I gene products. HLA DR2 can be further divided by means of cellular proliferation assays into five distinct HLA-D clusters [2] and it can be demonstrated that MS is associated particularly with the most common subtype, Dw 2 [3]. It is not yet clear whether Dw subtypes of the serologically defined DR specificities represent separate loci within the MHC or are defined by the interaction of, for instance, DR and DQ specificities [4]. In a recent study in the Grampian region of northeast Scotland, Francis et al. [5] found that DQw1 was more strongly associated with MS than was DR2, although the frequency with which it was found in normal families was also increased.

It has been suggested on the basis of these population studies that there exists within the MHC a gene coding for a disease-specific susceptibility allele [6]. Indeed it can even be demonstrated theoretically that such a gene, if it exists, would exhibit dominant inheritance and have a high frequency and a very low penetrance [7]. Since the Ninth International Histocompatibility Workshop (1984), three DQ specificities have been officially recognized and are serologically defined by both alloantisera and murine monoclonal antibodies. However, it has become apparent from both nucleotide sequencing and T-cell clone reactivity studies that both DQα and DQβ genes exhibit an extremely high degree of polymorphism, and that current serological typing of the DQ region remains grossly inadequate. Since this region is in strong linkage disequilibrium with the DR region it is possible that a susceptibility gene might lie within it and yet not be detectable by current serological or cellular typing methods.

The analysis of restriction fragment lenght polymorphism (RFLP; "DNA typing") has enabled genotype polymorphisms to be detected without the need for sequencing and thus the technique lends itself readily to population and family studies. A polymorphism will be detected if it results in the loss or gain of a cutting site recognized by a given restriction endonuclease. Following electrophoretic separation of genomic DNA digested by this enzyme, immobilization on a nylon membrane, and hybridization with a suitable gene probe, a polymorphism is revealed as a differing pattern of bands on an autoradiograph. These RFLPs are inherited in classical Mendelian fashion and can therefore serve as markers for genes with which they are in linkage disequilibrium. The technique has been widely used in identifying disease-associated genes with some notable successes such as adult polycystic disease [8] and Huntington's chorea [9], and it has also been applied to various autoimmune diseases that are associated with the HLA class II antigens. In order to detect a discriminatory polymorphism it may be necessary to screen a large number of DNA samples with a wide range of restriction enzyme/gene probe combinations. The recently described technique of pooling DNA samples from disease and control individuals provides a rapid and economical method for this kind of study [10]. The results of RFLP analysis using pooled DNA will potentially differ from studies in which DNA samples are digested individually in that the hybridization intensity of a fragment will depend upon the frequency with which it is present in each sample contained in the pool. Clearly when searching for a disease marker only polymorphisms that are represented at high frequency are of substantial interest. A theoretical analysis of the method suggests that, given a reasonable level of disequilibrium between a marker locus and the disease-related locus, the hybridization intensity of the marker fragment will be at least three times greater than that in the control pool. Using this approach we have identified a number of RFLPs located at or near both the DQα and DQβ genes which may discriminate between "MS" and "control" pools both with and without HLA DR2.

Methods

Thirty-three MS patients from the Grampian region of northeast Scotland werde studied together with 58 healthy subjects drawn from the same population. Twenty-four patients belonged to families in which two or more first-degree relatives were affected Only patients with clinically definite MS according to the criteria of Poser et al. [11] were included. Both patients and controls have been previously HLA typed in our laboratory [5]. Genomic DNA was prepared from frozen blood samples by conventional methods [12] and stored at 4°C. Four DNA pools (MS DR2+, MS DR2-, control DR2+ and control DR2-) were constructed by mixing equal amounts of each individual DNA.

A panel of 14 restriction endonucleases was selected (*Tag*I, *Bgl*II, *Bgl*I, *Pvu*II, *Pst*I, *Eco*RI, *Eco*RV, *Sac*I, *Hinf*I, *Sma*I, *Ms*PI, *Bam*HI, *Sau*96I, and KpnI). In order to maximize the chances of including the enzymes which were most likely to detect polymorphisms within or immediately adjacent to the coding regions of the genes, computer-generated restriction maps of several class II α and β cDNA gene clones were studied and those enzymes which recognized only a small number of

cutting sites, and particularly within the hypervariable regions of the genes, were preferred. Samples of restriction enzyme cleaved DNA from each pool were electrophoresed in 0.7% agarose gels (Bio Rad) for 1200 V in 89 m*M* Tris-borate buffer, pH 8.3, containing 2.5 m*M* EDTA. Gels were depurinated and Southern transfer onto Gene Screen Plus filters was carried out overnight with 10 × SSC (1.5 *M* sodium chloride, 0.15 *M* sodium citrate). DNA was denatured in 0.4 *M* NaOH and the filters dried. Filters were prehybridized for 4 h at 42°C according to the manufacturer's instructions. Full-length cDNA clones of DQ*a*, DQ*ß*, and DR*ß* [13, 15] were radiolabeled by random hexamer priming to a specific activity of 10^8 cpm/*ug*. Hybridization was carried out overnight at 42°C in the presence of denatured sperm DNA. Filters were washed twice in 2×SSC at room temperature, twice in 2×SSC with 1.0% SDS at 65°C for 30 min, and once in 0.1×SSC at room temperature. Autoradiographs were exposed für 24–72 h at -70°C.

Results

Creation and Composition of the DNA Pools

As mentioned above all the individuals included in this study have previously been HLA typed in this laboratory. The numbers in each pool were as follows: MS DR2+ 24, MS DR2− 9, control DR2+ 29, control DR2− 29. It can be seen from Table 1 that the proportions of class II alleles other than DR2, while not exactly matched, are nevertheless broadly similar within each of the pools. We consider the chance of bias in the distribution of alleles in the pools resulting in detectable polymorphisms to be very small.

DQα Polymorphism Between MS and Control Pools

Using the DQα probe polymorphisms were detected with only one of the 14 enzymes used. Following digestion with *Ms*PI a 5.6-kb fragment was present in both MS pools but not in the control pools. Additionally a 6.1-kb fragment was also present in the MS DR2− pool. This is the only one of the 42 enzyme/probe combinations examined in which we have identified polymorphisms which discrimi-

Table 1. Composition of the pools in terms of class II alleles (%)

	MS DR2+	MS DR2−	Control DR2+	Control DR2−
DR1	2	28	0	5
DR2	71	–	65	–
DR3	10	39	10	26
DR4	13	0	12	22
DR5	0	0	3	12
DR6	0	0	0	10
DR7	4	11	10	15

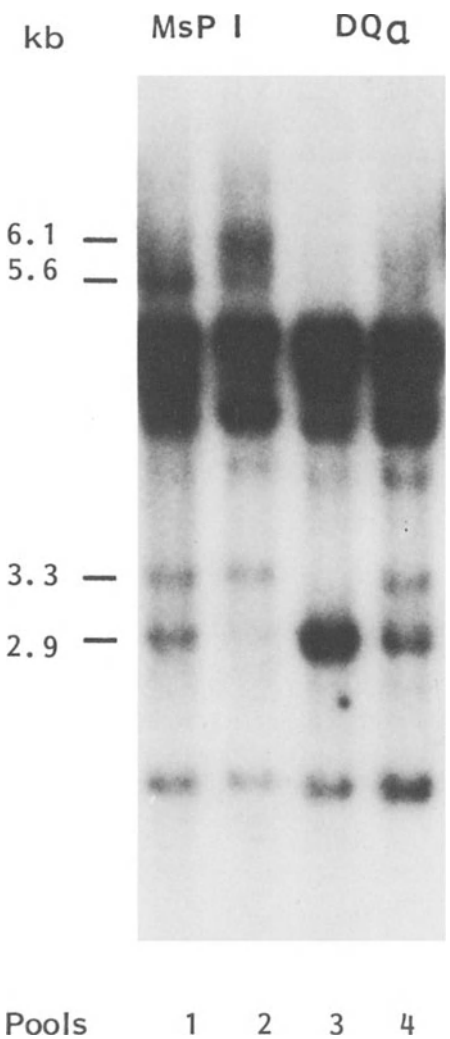

Fig. 1. Hybridization of a DQα chain probe to pooled DNA digested with *MsP1* (*Pool 1*, MS DR2+ve; *Pool 2*, MS DR2-ve; *Pool 3*, control DR2+ve; *Pool 4*, control DR2-ve)

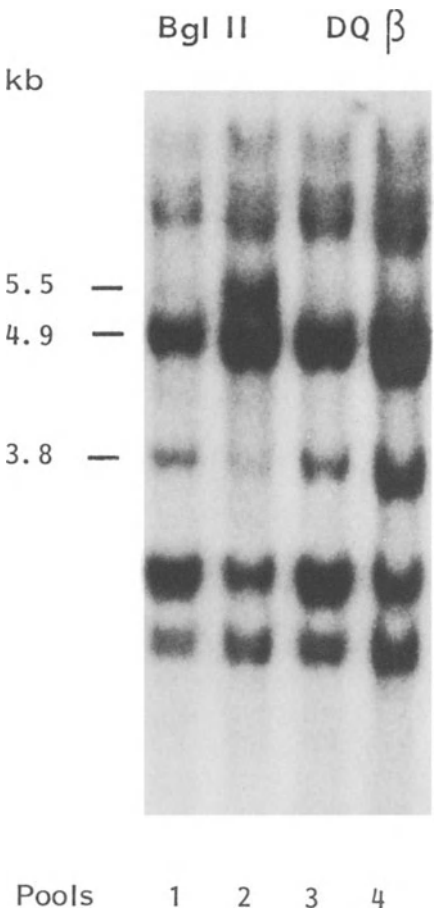

Fig. 2. Hybridization of a DQβ chain probe to pooled DNA digested with *Bgl*II

nate between the disease and control pools and which are unrelated to the presence of DR2.

DQB Polymorphism Between MS DR2 and Control DR2 Pools

Once again the majority of hybridizing restriction fragments were invariant between the four pools. However, in three instances polymorphic fragments were identified.

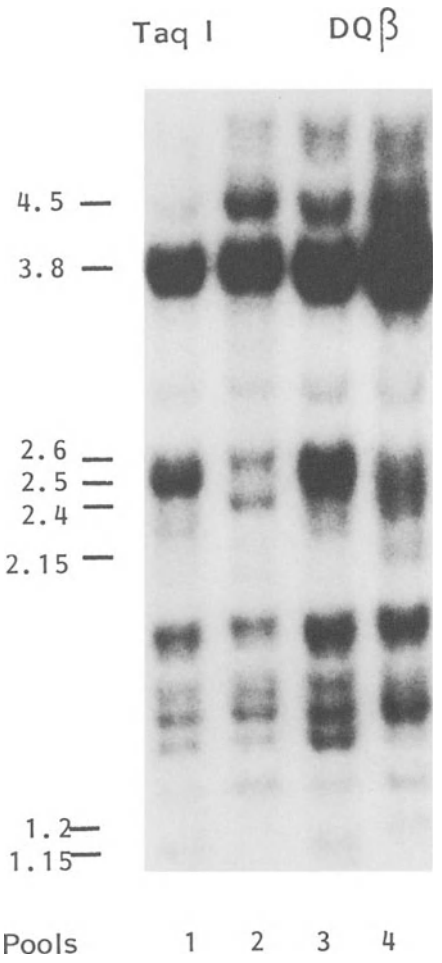

Fig. 3. Hybridization of a DQβ chain probe to pooled DNA digested with Taq_x

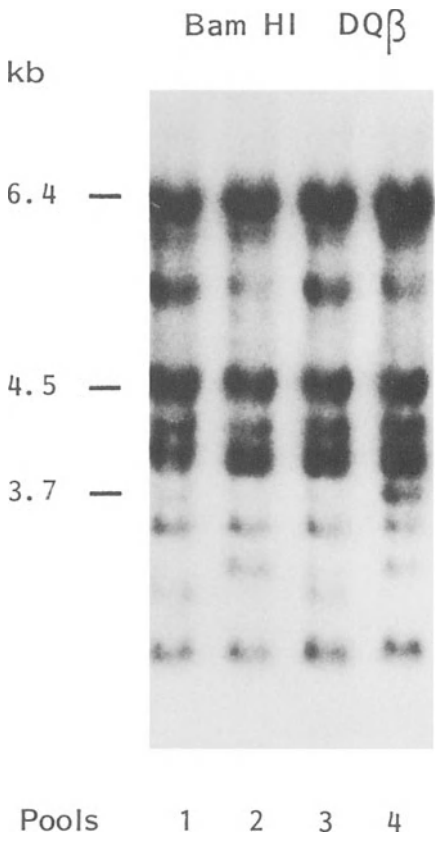

Fig. 4. Hybridization of a DQβ chain probe to pooled DNA digested with *Bam*HI

Following restriction endonuclease cleavage with *BgI*II, a 5.5-kb fragment hybridized strongly with DQβ in the MS DR2− pool but was only weakly present in the control DR2− pool. In contrast the situation was reversed for a 3.8-kb fragment. While it is possible that this difference reflects bias in the distribution of other class II alleles between the two DR2− pools it must be pointed out that no differences were seen with the DQα or DRβ probes. Thus these *BgI*II/DQ polymorphisms do appear to reflect polymorphism in the DQδ genes themselves and not cross-hybridizing genes.

Following digestion with *Taq*I a number of RFLPs were detected with DQβ. A 4.5-kb fragment was only weakly present in the MS DR2+ pool in contrast to the other there pools and a 1.2-kb fragment was unique to the control DR2− pool. A cluster of fragments sized between 2.6 and 2.15-kb appeared to differentiate

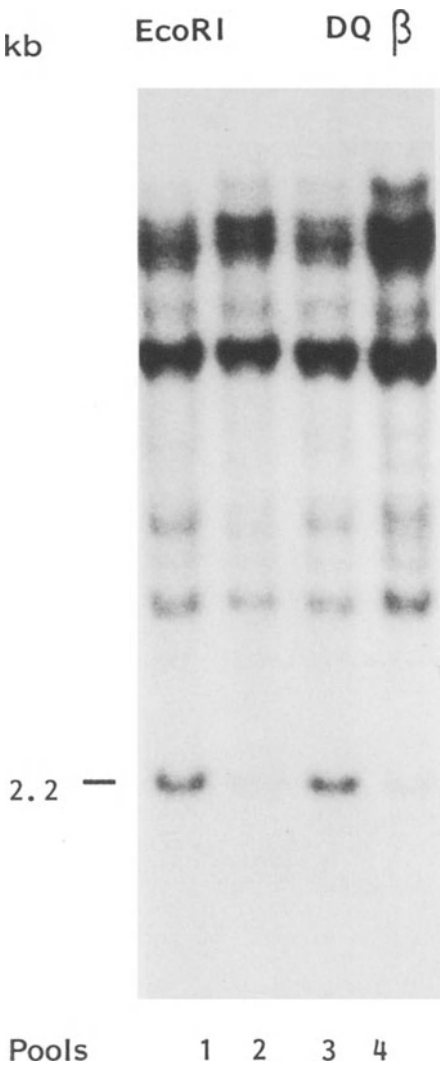

Fig. 5. Hybridization of a DQβ chain probe to pooled DNA digested with *Eco*RI

between the DR2− pools. The only other polymorphism detected using the DQβ probe was following *Bam*HI digestion when a 3.7-kb fragment also differentiated between the DR2− pools.

No polymorphic fragments discriminating between MS and control pools were seen using DRβ (data not shown). However, in many instances, and using each of the probes, fragments closely associated with the presence or absence of DR2 could be identified. An example is the 2.2-kb *Eco*RI/DQβ fragment, which has been described previously as defining DR2 Dw2 [16].

Discussion

In a well-characterized, HLA-typed population from a single geographical area we have defined several HLA-DQ RFLP differences between MS and control groups. Three of these were between DR2-negative disease and control groups using a DQβ probe and one, between disease and control groups, irrespective of whether DR2 was present, was detected using DQα probe. No differences were seen using a DRβ probe. Analysis with DRα, DPα and DPβ has yet to be completed. These results were obtained using a DNA pooling technique as described by Arnhein et al. [10]. Our experience seems to confirm the value of this approach for rapid screening of a disease group using a large number of different enzyme/probe combinations. If each of our patients and controls had been tested individually with each enzyme and each probe, 250 Southern blots would have been required.

Assuming our observations are repeated when the samples of DNA making up the pools are subjected to endonuclease digestion individually, a number of explanations can be put forward to account for our results. The first is that polymorphisms are the result of unequal distribution of DR and DQ alleles in our disease and control pools. However, it has been shown that the proportions of DR alleles other than DR2 in each of the four pools are broadly similar and since DQ genes are in linkage disequilibrium with DR it is unlikely that these results can be accounted for by a random unequal distribution of alleles between the pools.

Secondly it is possible that we are detecting differences due to a different distribution of Dw subtypes of DR2 between the MS and control DR2+ pools. DR2 possesses at least three distinct Dw subtypes which most probably represent different alleles of DQα and β, and which can be readily distinguished by their restriction patterns. Segall et al. [16] have recently analyzed the restriction patterns obtained from a panel of homozygous typing cells (HTCs) using various restriction enzymes and full-length DRβ and DQβ probes, when a number of bands specific to particular subtypes could be identified. One of these bands hybridizing to DQβ (a 2.2-kb *Eco*RI fragment) was present in all seven DR2 and Dw2 HTCs but not in the DR2 Dw12 or DR2 LD-5a HTCs. The same fragment was present equally in both our MS and control DR2+ pools (Fig. 5) and thus Dw subtype differences alone cannot account for our findings. Furthermore, the intensity of this 2.2-kb *Eco*RI/DQ fragment in our study confirms the previous finding that DR2 Dw2 is the dominant haplotype in MS and corroborates a recent finding that the relative prevalence of Dw2, Dw12, and Dw AZH as detected by RFLP is unchanged in MS patients as compared with healthy unrelated controls. It can now be demonstrated that a polymorphism previously thought to be in disequilibrium with an MS-associated locus was a fragment specific for Dw2 [17]. It was found that the 2.2-kb *Eco*RI fragment detected with a DQβ probe was present in 17/24 MS patients asopposed to only 6/22 random controls and only 1/27 patients with insulin-dependent diabetes, a disease negatively associated with DR2. However, the difference between the MS patients and controls arose because of bias in the controls due to an increased representation of the uncommon subtype Dw AZH. It has recently become apparent that many of the RFLP markers which have been described as being associated with various autoimmune diseases are in fact Dw subtype specific (C. Lock, personal communication).

Thirdly, these differences could be accounted for by the presence of a disease-specific susceptibility allele(s) in the disease individuals, which cannot be detected by current serological typing methods, but which is revealed by DNA-typing. However, the application of a recently developed technique for isolating genes [18] has allowed rapid sequencing of large numbers of class II genes in a number of HLA-associated diseases. Very recently, α and β genes of DQ and DR in five MS patients have been sequenced and shown to be unremarkable (J. Bell, personal communication). Thus, though the number of MS haplotypes sequenced at present is small, the possibility that a disease-specific allele will be found now appears to be increasingly unlikely.

We consider that the polymorphisms we have detected reflect sequence variation in noncoding regions of the DQ genes. Whether or not these polymorphisms might have functional significance has yet to be explored. However, the extremely interesting possibility exists that these polymorphisms may affect regulatory sequences upstream from the 5' ends of the coding regions of these genes. Important *cis*-acting positive and negative regulatory elements have been defined in a number of systems in the 5' flanking regions and are at present being dissected by means of sequence deletion studies [19, 20]. Some of these elements control the regulation of class II expression by γ-interferon. In view of the demonstrable importance of quantitative variation in the per cell level of MHC class II molecule expression, polymorphism in these regions could have profound effects on immune responses [21]. Abnormal regulation of α and β gene transcription may also result in the expression of a class II dimeric molecule which is qualitatively abnormal and the possibility that this aberrant expression might contribute to abnormal T-cell activation in autoimmune disease is currently being investigated.

Clearly the present data are preliminary and need to be confirmed by the analysis of individual samples of DNA. Should our findings be confirmed, the localization and functional significance of these polymorphisms will be explored. It is likely that RFLP studies, possibly using oligonucleotide sequences as probes, will continue to be a powerful tool in identifying genetic factors which are associated with susceptibility to MS, even should further sequence data fail to show abnormalities within the coding regions of the class II genes.

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HLA Studies in the Multiple Sclerosis Population

G. Veneroni,[1] *S. Marforio,*[2] *L. Rizzolo,*[1] and *M. Di Falco*[2]

1 Servizio Immuno-Trasfusionale, Ospedale Fatebenefratelli, Milano
2 Centro Studi Sclerosi Multipla, Gallarate, Italy

In recent years investigators have been oriented to consider multiple sclerosis (MS) as an immune or autoimmune disease. Occasional familial incidence strongly suggests the possible role of a genetic factor, and epidemiological data related to the incidence of MS within the kinship of the patients strongly supports MS heredity. Indeed [7] the incidence of MS is 7 times higher in first cousins, 12 times higher in parents, 22 times higher in brothers, 259 times higher in heterozygotes, and 535 times higher in monozygote twins. According to racial criteria, Caucasians are more affected than colored races [1–4].

For these reasons in recent years emphasis has been placed upon the importance of the HLA system in MS patients. Several studies performed on Scandinavian populations have shown two kinds of association between HLA und MS; one weak but statistically significant association between HLA-A3 and HLA-B7 and the disease and the very strong association between HLA-DR2 and MS; but such findings [3, 7] have not been found among Middle East and Japanese people. Since the published results are still inconclusive and conflicting, we thought that further investigations were needed to confirm the above data. Our research has the following aims:

1. Contribution to other studies based on the same pathogenetic assumption
2. Search for preferential alleles of the HLA system in Italians affected with MS (such findings could be of diagnostic value)

Material and Methods

Two hundred and forty-one unrelated patients were carefully followed for MS at the MS Center of Gallarate (VA) for a period of 2 years (1984–1985) and submitted to all the clinical and laboratory tests required for MS diagnosis according to the international standards of Schumacher. Selection of patients was limited to those living in north Italy and born from northern ancestors with the exclusion of those from Ferrara and Rovigo provinces and the southern part of the Venice province because of the high rate of Mediterranean anemia, which is evidence of a different ethnic group. The disease was classified according to the McDonald and Halliday criteria [8].

The patients were considered a unique group or divided into several subgroups according to the age of onset, severity of the disease [9], prevalence of the typical

syndrome, or prevalence of spinal or stem cerebellar symptoms. For this reason the number of patients in the different subgroups exceeded their total number.

Tissue typing of the A, B, C, [241], and DR [233] antigens was performed using the NIH lymphocytotoxicity test with 120 commercial sera (Biotest or Behringwerk) or local set sera defining 44 specificities. Control values were obtained from a large group of north Italian blood donors (452 for ABC and 247 for DR). A chi-square goodness of fit was performed to ascertain whether the observed frequencies were compatible with the expected frequencies. The *P*, value was corrected for the number of specificities tested. The strength of association between HLA and disease was measured by the Woolf's "relative risk" formula.

Results

There was no statistical difference between MS patients and controls for the alleles at the A and C locus while for the alleles at the B locus we found a significant decrease of the B12 antigen (8.7% vs. 18.1% in controls; *Pc*, 0.05) (Table 1). For the DR antigens we found an increase of DR2 (40% vs. 23.1% in controls; *Pc*, 0.005) (Table 2). We made the following observations on the different subgroups of patients (Table 3):

1. In the group of "typical" MS (143 patients) the difference observed in the whole group remained; however, the decrease of B12 was expressed more strongly (6.3% vs. 18.1% in controls; *Pc*,0.05).

Table 1. HLA-B typing of the total MS population and controls. The results of HLA-A and HLA-C typing has been omitted since there is no difference between patients and controls

Locus B	MS (N=241) (%)	Controls (N=452) (%)
B 5	25.31	22.49
B 7	16.18	10.51
B 8	16.60	13.10
B 12	8.71[a]	18.1
B 13	3.73	6.80
B 14	8.71	6.18
B 15	12.86	9.09
B 16	7.05	10.64
B 17	6.63	7.76
B 18	13.28	15.21
B 21	9.13	9.06
B 22	1.66	4.74
B 27	4.15	4.00
B 35	32.78	28.36
B 38	4.02	5.60
B 40	2.90	5.48
B 44	7.85	16.32
B 51	21.01	18.24

[a] X^2, 11.04; PC < 0.05; RR, 0.43

Table 3. HLA typing in MS patients according to type and severity of the disease

	Typical (N=143)		Spinal (N=65)		Stem-cerebellar (N=26)		Early onset (N=49)		Late onset (N=28)		Benign (N=17)		Severe (N=10)	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
B12	9	6.29[a]	7	10.77	2	7.69	4	8.16	3	10.71	2	11.76	1	10
	137		65		24		47		28		17		9	
DR2	54	39.42[b]	32	49.23[c]	7	29.17	14	29.79	13	46.40	8	47.06	5	55.56

[a] X^2, 11.77; PC < 0.05; RR, 0.30
[b] X^2, 11.44; PC < 0.05; RR, 2.16
[c] X^2, 17.26; PC < 0.005; RR, 3.23

Table 2. HLA-DR typing of the total MS population and controls

Locus DR	MS (N=233) (%)	Controls (N=247) (%)
DR1	9.87	16.31
DR2	40.77[a]	23.1
DR3	24.89	22.60
DR4	12.02	12.30
DR5	39.48	35.70
DRw6	4.72	6.90
DR7	21.46	24.40

[a] X^2, 17.35; PC < 0.005; RR, 2.29

2. In the "spinal" subgroup (65 patients) we observed a very strong positive association with DR2 (49.2% vs. 23.1% in controls; *Pc*, 0.005).
3. A progressive increase of the DR2 frequency was related to the increase of the age of onset of the disease (Table 4).
4. The frequency of DR2 in the "severe" group was higher than in the benign subgroup.

Table 4. HLA typing in MS patients according to age of onset

	Early onset (< 20 years) (N=49)		Onset (> 20 < 40 years) (N=157)		Late onset (> 40 years) N=28)	
	N	%	N	%	N	%
B12	4	8.16	11	7.01	3	10.71
DR2	14	29.79	66	43.71	13	46.40

Discussion

The frequency of the alleles at the A locus we did not report since we did not find the distortion of A3 or A1 antigens quite often published by several authors [7, 10]. From our studies we can confirm the well-known association between MS and DR2. Indeed the increase of DR2 is significant (*Pc*, 0.05) in the entire group and in the "typical" subgroup and highly significant (*Pc*, 0.005) in the "spinal" subgroup (Table 3). Such findings again confirm that DR2-positive patients experience a more rapid progression of the disease. This is also confirmed by the gradual increase of DR2 in patients with early onset of the disease where the impairment is mild, allowing self-sufficiency for a long time, compared with those with late onset (Table 4). Further evidence also comes from the frequency of DR2 in the "severe" group, which is higher than that in the benign subgroup (Table 5). We can therefore infer that the

Table 5. HLA typing in MS patients according to disease severity

	Benign (N=17)		Not benign Not severe (N=207)		Severe N=10)	
	N	%	N	%	N	%
B12	2	11.76	15	7.25	1	10
DR2	8	47.06	80	40	5	55.56

increase of DR2 is probably related more with the age of onset and the degree of severity than with a particular clinical feature.

As far as B12 is concerned this allele is constantly decreased in the different subgroups as well as in the "entire" groups. While our results disagree with those reported in Parsi and Indian people they correlate perfectely with those reported [10, 12, 13] on French and North European people. Some authors consider the decrease of B12 antigen a protective factor in the supposed susceptibility gene of MS. Unfortunately our results do not allow us to share such an idea. If the B12 were a protector we should expect a higher frequency in the benign group or in the early onset group. Therefore a question arises. Does it represent a difference limited to sex or age of onset of the disease as has been shown [11] in myasthenia gravis, where B8 is increased only in females with the onset of the disease before the age of 35? In this study we did not consider the sex factor in order to avoid an excess of subgroups with a very small number of patients. On the other hand, in a preliminary analysis performed on the whole group, where the sex factor was taken into account, we found no difference. We can therefore conclude that our research:

1. Confirms the well-known association between MS and DR2. The increase of DR2 is related to the severity and age of onset of the disease.
2. Does not confirm the increase of A3 and B7.
3. Shows that the B12 correlates in north Italian people with the decreased frequency reported in French and North Europeans. We underline such a result since it has never been reported among Italians.
4. Shows that statistical analysis among the different subgroups has a meaningful value only when the number of patients exceeds 50.

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Multiple Sclerosis: Phenotypic Immunogenetic Markers and Nucleic Acid Biochemistry

H. Zander

Laboratory of Immunogenetics, Kinderpoliklinik, University of Munich, Munich, FRG

Introduction

Twelve years ago, in 1974, the earliest use of the term "DNA probe" was made by Murphy and Attardi [19]. Since then, probes have rapidly propagated into every field of biology and medicine. In current practice, probes make it easier to determine genotype than phenotype. The approach to multiple sclerosis (MS) as proposed herein, although tremendously laborious, should provide the means of moving from linked marker genes to the very true genes that confer susceptibility to MS. The next step should be to sequence the susceptibility genes, to delineate their gene products – i.e., to identify the molecules that are involved in the pathogenesis – to study their function, and – ultimately – to provide a molecular basis for therapy.

Multiple sclerosis is widely considered to be a multifactorial disease. With regard to genetic factors contributing to the clinical manifestation of MS, there has been a century's equivocal discussion. The final report of the International Symposium on MS at Göteborg, 1972, concluded that there was "a trend for MS to accumulate among near relatives", without reliable knowledge, however, on whether this could be "due to either a genetic or an exogenous factor" [6].

In the same year, Naito et al. [20] found an association of MS with the immunogenetic trait HLA-A3, coded for by an allele within the major histocompatibility complex (MHC) on the short arm of chromosome 6.

Summing up a tremendous amount of work devoted to this issue by many investigators, the 7th International Histocompatibility Workshop at Oxford in 1977 definitely established an association at the population level between MS and HLA-A3, B7, Dw2 in Caucasians [3]. This association strongly suggested a genetic linkage between HLA marker genes and MS susceptibility genes. But early multiple case family studies failed to reveal a simple pattern of inheritance [2, 5, 13, 23, 36, 37]. In 1980, at the 8th International Histocompatibility Workshop at Los Angeles, 53 multiple case pedigrees with MS were analyzed. Genetic linkage of at least one major determinant for susceptibility to MS with HLA was accepted with a maximum lod score of 3.93, assuming a dominant action with low penetrance [33]. From the mathematical analyses, additional factors not linked with HLA were postulated but so far they could not be identified. No HLA associations with MS could be found in some other ethnic groups, e.g., Israeli Jews and Japanese. In Arabs, MS was reported to be associated with HLA-DR4 (for reviews, see refs. [33, 34]). These are important observations because they support the hypothesis that the HLA alleles or

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their products are only markers for the disease gene(s), and do not *per se* confer MS susceptibility. The linkage disequilibria between marker alleles and disease genes may differ from one ethnic group to another, which may result in different associations.

In January 1984, at a small conference in London organized by J. R. Batchelor and W. I. McDonald, the participants felt that one or more susceptibility genes for MS were now clearly ripe for being focused on by DNA biochemistry [18].

One major problem in molecular disease research is the interaction between clinics, which have access to patients, and laboratories, which have research facilities. Interdisciplinary cooperation is required, but a rapid advance is hampered by the limited number of informative patients. In another context, Roses [29] has stated that "the more glamorous recombinant DNA experiments have outstripped their clinical resources." With regard to MS, overcoming this lack of informative patients necessarily requires extensive fieldwork by clinical neurologists.

This lecture briefly presents clinical material that has been investigated in the Federal Republic of Germany by the author. It consists of 33 sib-pair double case families with MS that have been neurologically reexamined and reclassified. In addition, data from nine monozygotic twinpairs discordant for MS have been collected. In cooperation with research laboratories, patients and relatives have been analyzed for HLA-A,B,C,DR, for the polymorphism of glyoxalase I (GLO), for the complement factor polymorphisms of C2, Bf, C4A, and C4B located on the same chromosome, for the complement factor polymorphisms of C3 and C6, for quantitative serum levels of the complement factors C2, C4, and C3, for the IgG heavy chain markers Gm and the closely linked alpha l-antitrypsin allotypes, for the spontaneous and inducible natural killing activity of peripheral blood lymphocytes *in vitro*, and for their capability to produce interferons *in vitro* in response to viral and nonviral stimuli. Thus, these families are a unique clinical and immunogenetically well defined basis for forthcoming studies on the DNA level [35].

Clinical Features

Our study was originally designed to use the sib-pair double case approach of Penrose [25] to analyze associations and linkage between MS and immunogenetic markers [36, 37]. To investigate families having at least 2 siblings affected with MS, questionnaires were sent out to 15 000 patient members of the MS Society and to 4000 neurologists in the Federal Republic of Germany. Patients of the Göttingen MS pool (H.J. Bauer) and of the specialized MS center at Langscheid am Sorpesee (P. Evers) have been included.

All patients who could be motivated to participate in our study were visited at home, neurologically reexamined by the author, and clinically documented. All available hospital and consultant records were reevaluated. The revised Schumacher criteria as published by Rose et al. [28] were adopted for grading the diagnoses into clinically definite, probable, and possible MS. Some patients' diagnoses were upgraded from probable to definite MS after evoked potentials and brain imaging by nuclear magnetic resonance had been arranged for. Disability was scored according to Kurtzke [16]. Thus, 33 sib-pair double case families were available for study, with

28 families having 2 siblings with clinically definite disease. In five families, one sib had definite MS and the other one probable or possible MS. With regard to clinical features, the patients of this study did not differ from nonfamilial cases. In the 28 sib pairs with both siblings having definite MS, the male: female ratio was 24:32. The course was remittant in 12 patients, remittant and progressive in 41 patients, and primarily progressive in 3 patients. The mean age at onset was 29.2 years (SD ± 7.7 years, range 16–47 years). The mean disease duration at the time of study was 21.3 years (SD ± 9.3 years, range 5–48 years).

In the twin sample, monozygosity was established by typing for ABO, rhesus, Kell, MNSs, Duffy, Lewis, Kidd, Pl, and HLA-A,B,C,DR. Thus, nine monozygotic twin pairs discordant for MS were available for study, seven of them with clinically definite MS. The healthy twin had also been neurologically reexamined [1, 14, 36, 37, 39].

In MS, a pitfall of family studies is the occurrence of spinocerebellar ataxias, spastic parapareses, and other syndromes very similar to MS but known to be classically inherited disorders. A series of families which, upon careful clinical reexamination, were reclassified to suffer from inherited ataxias or spastic parapareses rather than from MS has been excluded from the MS sample.

To examine cellular markers and functions, heparinized blood from patients and relatives was taken by the author and sent to the laboratories by express mail or air cargo. Serum was centrifuged and frozen in liquid nitrogen within 90 min after bleeding.

Markers of Chromosome 6

HLA – A, B, C, DR, and GLO

The first 13 families of our sample – i.e., 26 patients all having clinically definite MS and 37 healthy siblings and parents – were incorporated as the largest single contribution into the joint analysis of the 8th International Histocompatibility Workshop, Los Angeles, 1980 [33, 38].

Twenty additional families were typed thereafter. In our entire sampling of 33 families, MS was again seen to be most closely associated with HLA-DR2. Typing for GLO was not informative but helpful for assembling the HLA haplotypes. Phenotypically, HLA-DR2 occurred in 24 out of 33 propositi with MS (with the family's first patient in birth order taken as the propositus) – i.e., in 72.7% vs. 25.2% in healthy Caucasian controls. Genotypically, HLA-DR2 was present in 28 out of the 66 propositus haplotypes, which corresponds to a gene frequency for HLA-DR2 of 0.424 vs. 0.133 in healthy Caucasian controls. When sharing of HLA haplotypes was analyzed, 5 affected sib pairs shared 2 haplotypes and 23 affected sib pairs shared 1 haplotype. Thus, there is a high number of five exceptions from joint segregation of HLA and MS. Essentially the same observation – i.e., high association of MS with HLA-DR2 on the one side but distorted segregation of MS with HLA on the other – has also been reported in large family studies by Haile et al. [11] and Ebers et al. [9], in the Workshop's joint analysis of 1980 [33], and in many smaller studies recently reviewed by Tiwari and Terasaki [34]. Deviations from an

expected joint segregation of MS with HLA may be explained by

1. difficulties in the clinical diagnosis of MS,
2. genetic recombination between the marker and the disease loci,
3. homozygosity for MS susceptibility in one parent linked with different HLA alleles on either haplotype,
4. MS susceptibility in both parents linked with different HLA alleles, or
5. the occurrence of extramarital haplotypes. The MS conundrum in HLA has been mathematically discussed in detail by Tiwari et al. [33]. In addition to the explanations offered above the data point to at least one additional factor not linked with HLA or to disease heterogeneity.

Complement Factors C2, Bf, C4

Our first 13 families we contributed to the Workshop of 1980 were further analyzed for the complement factor polymorphisms of C2, Bf, C4A, C4B, C3, and C6, and for quantitative serum levels of the complement factors C2, C4, and C3. The factors C2, Bf, C4A, and C4B map within the HLA complex between HLA-DR and HLA-B. Fifty parental haplotypes were derived from phenotype determinations (the missing two parental haplotypes did not occur in the offspring with the parents being deceased). A significant increase was seen for the C4 haplotype A4,B2 (gene frequency for A4=0.16 vs. 0.051 in normal controls, for B2=0.16 vs. 0.085 in normal controls). When a comparison was made within families between the patients and their healthy siblings, the data suggest that MS is even more closely associated with C4 A4,B2 (P=0.035) than with HLA-DR2 (P=0.33). The BfF allele was found in 5/50 parental haplotypes, which is not a significant deviation. Phenotyping for C2 polymorphisms was not informative. Our data on complement factor polymorphisms have been presented and discussed in detail by Schröder et al. [32]. Serum levels were found to be decreased for the factor C2 in patients and healthy relatives but the decrease correlated more closely with the zygosity of HLA-DR2 than with MS [22].

Other Markers

Phentotyping for the non-MHC-encoded complement components C3 and C6 was not informative due to the poor polymorphism of these markers [32].

The sera of our first 13 Workshop families were in addition typed for the IgG heavy chain markers Gm and for the alpha 1-antritrypsin allotypes. The genes for these markers are located on chromosome 14 in close linkage to each other. We saw no deviations from random distributions (unpublished data). Thus we cannot confirm the association between MS and Gm markers in serum that has been described in the literature [24, 26].

A possible deficiency of the natural killer (NK)/interferon (IFN) system has been attracting attention in the field of MS research [21, 4, 30, 12]. The results have been controversial, probably due to methodological difficulties and to genetic heterogeneity between patients and controls. Further, conventional patient-control

comparisons cannot provide information on whether a dysfunction is determined by genetic factors or whether it is acquired by disease. To overcome this problem we have compared three groups of individuals:
1. the MS patients,
2. their healthy HLA-identical siblings or – when possible – their healthy monozygotic twins, and
3. an unrelated healthy population matched for sex and age, but not for HLA.

So far seven HLA-identical sib-pairs and nine monozygotic twin-pairs discordant for MS have been studied. Three parameters of the NK/IFN system were investigated *in vitro* using nonadherent lymphocytes from peripheral blood:
1. spontaneous and
2. IFN β-inducible NK activity, and
3. production of IFN α and IFN γ in response to viral and nonviral stimuli.

Our observation was that the healthy monozygotic twins and healthy HLA-identical siblings of patients with MS displayed no functional deviations of all parameters tested when they were compared with unrelated healthy controls. This result argues against a preexisting genetic deficiency of the NK/IFN system in MS [1, 14, 39, 40]. Spontaneous NK activity was lower in some patients but the differences did not reach the level of significance when the patient sample was analyzed as a whole. Inducible NK activity and production of IFNs did not show any significant differences between patients and controls.

Conclusions and Perspectives

The HLA-D/DR loci on the short arm of chromosome 6 are the only marker loci so far that have been unequivocally established to include or to neighbor at least one gene that must be involved in the pathogenesis of MS. Our suggestion of an even closer association of MS with the complement factor allotype C4 A4, B2 than with HLA-DR2 in the German population remains to be confirmed by other investigators for other populations. Nevertheless, the C4A and B loci map within the HLA complex between HLA-D/DR and HLA-B, telomeric from HLA-D/DR. There is one report of an even closer association of MS with HLA-DQw1 than with HLA-DR2 in the Grampian population of northeast Scotland [10]. HLA-DQ maps very close to HLA-DR but centromeric from it. Preliminary results of Martell et al. [17] suggest that a germ line Ti alpha gene related polymorphism, as defined by restriction fragment length polymorphisms, may contribute to genetic susceptibility to MS.

Further progress, however, is not to be expected from phenotypic or functional studies alone. HLA-DR2 is a serologically well defined specificity but which is biochemically heterogeneous. This has been shown by analyzing its amino acid sequences [15] as well as by DNA hybridization technology [8].

DNA and HLA constitute an extremely rapidly developing field. So far, as many as 162 cloned human DNA sequences for HLA have been published. They were listed by Schmidtke and Cooper in October 1986 [31].

The DNA probes listed for the complement factor genes within the MHC, for the steroid C21-hydroxylase genes, etc., add up with the HLA probes to at least 200 human DNA probes covering the MHC. This number is still growing exponentially. Thus, DNA biochemistry offers a real chance to narrow more and more the chromosomal area within and around the marker loci for susceptibility to MS. An initial approach by Cohen et al. [7] has yielded promising results. The aim is to truly identify the disease gene, to sequence it, to delineate its product, to study the product's function and its role in the pathogenesis, and – therapeutically – to manipulate its function. Multiple case families are the clue here. Family studies may, in addition, answer the question of whether susceptibility to MS is conferred by autochthonous human DNA also present in healthy relatives – especially in a healthy monozygotic twin – or by additional – e. g., viral – or modified DNA not present at homologous loci in healthy individuals. The clinical material that has already been gathered in Germany, immunogenetically well defined, provides a unique starting point for forthcoming DNA studies [35]. An additional effort will be made to collect and to preserve genomic material from the families presented here provided that a neurological clinic will cooperate by doing the patient-related part of the work.

The DNA approach toward a molecular understanding of enigmatic diseases such as MS is ambitious as well as laborious but it has a good chance of being successful. The future looks bright indeed.

Any careful neurologist studying multiple case families with MS will meet with inherited ataxias and spastic parapareses. Our multiple case families suffering from ataxias and spastic parapareses may be available for DNA studies, too, although the marker situation is poor for these disorders. In any case, genomic material must be preserved from these families now, as long as the affected family members are still alive. Otherwise, with further delay, most valuable sources of information will be lost for genetic counseling as well as for clinical research. There is considerable clinical and genetic heterogeneity of these disorders and, consequently, considerable disagreement and confusion about their classification. Refsum and Skre [27] said "that there are as many proposed classifications as there are authors on the subject." The ultimate classification, however, will be based on DNA biochemistry, with beneficial consequences for the genetic counseling of affected families and – with realistic hope – for the therapy of the affected individual.

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Genetics in Multiple Sclerosis – with Special Emphasis on Twin Studies

A. Heltberg

Department of Neurology, Roskilde Amtssygehus, Roskilde, Denmark

Introduction

The etiology of multiple sclerosis (MS) is largely unknown. Genetic as well as environmental factors are probably involved, and therefore it seems relevant to include genetic aspects in this symposium. An increased risk for the disease in relatives to MS patients has been demonstrated in several studies. However, the prevalence in relatives is well below 25%, which is the lowest value predicted by ordinary Mendelian inheritance – so a simple Mendelian mode of inheritance is ruled out. The discovery of an association of MS with the genetic HLA system suggests the importance of genetic factors for the development of MS. This association suggests that MS may occur only in persons with a susceptible genotype.

The Twin Method

The classical method to evaluate whether genetic factors are involved in the etiology of a given disease, and if they are to what extent, is "The twin method". By this method it is evaluated how much of the variability observed between different individuals is due to hereditary differences between them, and how much is due to differences in the environment. However, to make genetical conclusions from twin studies it is of fundamental importance that the material is unselected, unbiased, and population based.

The twin method when applied for demonstrating a genetic contribution (variation) in the phenotype is based on the comparison of monozygotic (MZ) and dizygotic (DZ) concordance rates. If the concordance rate for a given disease is significantly higher for monozygotic than for dizygotic twin pairs and intrapair differences of environmental factors can be assumed to be equal for monozygotic and dizygotic twins, this can be taken as evidence that genetic factors are of importance for development of the disease. The concordance rate can be calculated in two ways: as pairwise concordance rate, which is the proportion of concordant pairs out of all pairs, and as proband concordance rate, which is essentially the risk or probability that a member of the twin pair will be affected if the cotwin is affected.

The heritability (h_2) can be defined as the proportion of the total phenotypic variants (genetic and nongenetic) which are due to additive genetic variants. The heritability can be estimated from twin data.

However, it should be noted that in the absence of environmental similarities concordance rates in monozygotic twins will not be expected to be high unless the heritability and the population frequencies are high. So, despite a high heritability the concordance may be low if the population frequency is low. It should also be noted that heritability is not a fixed attribute of a trait, but may vary in different populations and with time in the same population.

In MS the concordance in monozygotic twins in all studies is much less than 100% and this is clear evidence for the importance of nongenetic factors. What is inherited is susceptibility, and other (possibly environmental) effects determine whether a susceptible individual becomes affected.

A basic point in twin studies is the establishment of the zygosity diagnosis. The most reliable method for establishing the zygosity is based on extensive use of blood, serum, and enzyme group determinations. Another method which has been shown to be very valuable with a misclassification below 5%. A limiting factor for twin studies is the relative rarity of twins. In Europe about 1% of all births are twin births, and in many studies only like-sexed pairs are included. Other limiting factors are the biases that occur in the selection of twin samples and the difficulties which arise because of the interaction between genotype and environment.

The twin method offers recognition of the hereditary basis for phenotypic differences, but is not an analysis of the genotype responsible. The method does not answer the question of which kind of genes are responsible or what kind of environmental factors are involved.

As stated previously a fundamental point in twin research applied for genetical evaluation is the ascertainment method of the twin sample. Many of the prior twin studies on MS have been characterized by ascertainment difficulties.

In order to make genetic conclusions from a twin study the twin material must be unselected and unbiased population-based material. The twins should preferably be derived from whole populations or well-defined parts of populations, and the registration of twins within this area should be complete or nearly complete.

The correct ascertainment can only be applied in few countries because important requirements must be fulfilled before the results can be considered to be of more general value. A much-used method for collecting twin pairs for studies including genetical studies is the use of public appeal. It is, however, well documented, that by this method the twin material will be biased as this method attracts more monozygotic twin pairs and more concordant pairs. Also a tendency to obtain more female pairs by this method has been demonstrated. Some of the more important twin studies on MS will now be mentioned.

Twin Studies in MS

Thums from Germany in 1936 [1] collected a large material based on serial collection over several years. The distribution of sex was as in the normal population and the zygosity almost satisfactory. However, the zygositiy classification was not well documented. The 1936 material consisted of 22 twin pairs (7 monozygotic and 15 dizygotic); only 1 dizygotic pair was found to be possible concordant for MS. The final material (published in 1951) consisted of 43 twin pairs: 13 MZ and 30 DZ pairs.

Of these 1 MZ pair was found concordant for MS and 1 DZ pair was found of doubtful concordance. The conclusion was that genetic factors were of minor or no importance for the development of MS.

Bammer and Schaltenbrand in 1960 published a smaller series also collected over several years in the Würzburg area in the Federal Republik of Germany [2]. Thirteen pairs were ascertained, 6 monozygotic and 7 dizygotic pairs. As can be seen from these figures the material was biased with relatively too many monozygotic pairs. One monozygotic pair and one dizygotic pair were found to be concordant and the conclusion was that genetic factors were of minor importance for development of MS.

In the well-known study from United States by MacKay and Myrianthopoulos [3] the material was large, but was biased because of the mode of selection by public appeal. The bias was demonstrated by the ratio of monozygotic/dizygotic pairs, which was too high. Fifty-four twin pairs were ascertained, 29 monozygotic and 25 dizygotic. On diagnostic criteria two evaluations were made. By the "conservative" evaluation 2/29 monozygotic pairs were found concordant=a pairwise concordance rate of 5.9%, und 1/25 dizygotic was found concordant=a pairwise concordance rate of 3.8%. By a "liberal" evaluation 7/29 monozygotic paris were found concordant=a pariwise concordance rate of 20.6%, and 4/25 dizygotic pairs were found concordant=a pairwise concordance rate of 15.3%. The conclusion was that genetic factors were of some umportance for the development of MS.

In 1966 the authors published a follow-up on the prior twin series and also extensive family data were included [17]. The twin material was still biased, but the selection of relatives to the twin pairs was unbiased and valuable for genetic conclusions.

Sixty-eight pairs had now been ascertained, 39 monozygotic and 29 dizygotic, with an overemphasis of female twin pairs. With a "conservative" evaluation 6/39 monozygotic pairs were found concordant for MS=a pairwise concordance rate of 15.5%, and 3/29 dizygotic pairs were found concordant=a pairwise concordance rate of 10.3%. With a "liberal" evaluation 9/39 monozygotic pairs were found concordant=a pairwise concordance rate of 23.1%, and 6/29 dizygotic were concordant=a pairwise concordance rate of 20.7%. From the study on relatives it was concluded that the risk for MS in relatives to MS twin pairs was about 20 times higher than the risk in the normal population. It was also shown that the risk was highest for sibs to MS twins and becoming lower for other relatives. The authors concluded that MS might be caused partly by genetic factors, probably an autosomal recessive gene with a reduced penetrance about 43%.

In 1978 Bobowick et al. [4] published a paper, which primarily was a discordant analysis. The material was collected from twins who had been in military service. However, only about half of the sample could be included. Nine pairs, five monozygotic and four dizygotic pairs were ascertained, and this shows a bias concerning the zygosity. One of five monozygotic pairs was found concordant for MS and no dizygotic pairs were found concordant. However, as said before, this investigation was primarily a discordant analysis, and it was not possible from the material to make genetical conclusions.

In a later study by Williams et al. [5] 24 twin pairs were ascertained; 12 were monozygotic and 12 were dizygotic. Of the monozygotic pairs six were found

concordant, and of the dizygotic two were found concordant. However, this material was also biased and again ascertained by public appeal.

In later studies by Currier and Eldrige [6] and Eldridge and others [7] the materials were also biased because of the ascertainment method. These studies were combined and this revealed, of all the 51 twin pairs ascertained, 22 monozygotic pairs and 29 dizygotic pairs. The pairwise concordance rate for NZ pairs was found to be 36% and for DZ pairs 10%. Because of the biased selection it was not possible to make genetic conclusions from these investigations, but their results pointed to genetic factors being of some importance for the development of MS.

In a recent and very valuable Canadian study by Ebers et al. published in 1986 [8] the results of a large population-based study on Ms twins are reported. Ascertainment of twins for this study has been relatively unbiased and the patient cooperation nearly complete. The results are therefore valuable for genetic conclusions. Seventy twin pairs were included and the ratio of monozygotic to dizygotic pairs corresponds to expectation with 27 monozygotic and 43 dizygotic pairs. Of the 27 monozygotic pairs, 7 were found to be concordant for MS=a pairwise concordance rate of 25.9%, and of the 43 dizygotic pairs 1 was found to be concordant for MS=a pairwise concordance rate of 2.3%. Also included in the study were non-twin siblings of all patients attending the participating MS clinics in Canada. The concordance rate for non-twin siblings was found to be 1.9% and this number is of a similar magnitude to the concordance rate found for dizygotic twin pairs. The authors conclude that the study shows a major genetic component to MS susceptibility. By reviewing prior twin studies on MS they also point out that the method of ascertainment is a crucial factor for the validity of the evaluation of genetic factors in a twin study. Finally the authors showed the possibility for genetic heterogeneity in MS as they found a higher rate of familiar cases in concordant monozygotic pairs as compared with discordant monozygotic pairs. As part of the study magnetic resonance imaging (MRI) performed in the unaffected member of twin pairs is in progress. The authors have published the results on ten twin pairs (five monozygotic and five dizygotic), and two of the five monozygotic twins have shown MRI abnormalities consistent with MS. None of the five dizygotic unaffected twins were found to have abnormalities on MRI.

The same result has been observed by McFarland and his group [9] in Bethesda, Maryland – namely that the concordance rate was raised for monozygotic pairs, but not for dizygotic pairs when including MRI studies. These results indicate that genetic factors are more involved in the etiology of MS than previously demonstrated, but also that analysis of genetic influence on clinical grounds alone may be misleading.

The establishment of population-based registers as for example in Denmark has helped to facilitate adequate sample selection. In the Danish study the probands were primarily ascertained by a matching of The Danish Twin Register [11] and The Danish MS Register. In this way the material is relatively unbiased and derived from a twin population. The Danish Twin Register at The University Institute of Clinical Genetics in Odense was founded by Professor Hauge and coworkers and cover all like-sexed twin births in Denmark in the period 1870–1930. All twin pairs recorded are followed from birth until a given date or to death without paying attention to the presence or the absence of disease or to zygosity. The nationwide Danish MS

Register was founded by Hyllested in 1948. The majority of Danish MS cases (mostly reported from hospital departments) are registered here. The twin pairs in the Danish study were primarily ascertained by a comination of these two registers. The diagnosis of MS was based on the clinical diagnostic criteria of the Schumacher panel. In twins alive at the time of the investigation a personal neurological examination was performed, and in the remaining cases the diagnosis was based on case records and when available, autopsy reports. The zygosity diagnosis when possible was established by extensive blood, serum, and enzyme group determination performed by The Institute of Forensic Medicine, University of Copenhagen, and in the remaining pairs the zygosity diagnosis was based on the similarity method. HLA determination was performed by the tissue typing laboratory at Rigshospitalet University Hospital, Copenhagen.

Ascertainment procedures revealed 61 twins fulfilling the proband criteria. One twin had to be rejected due to lack of information about the cotwin. The proband material consists of 60 twins from 56 pairs. Examination of cotwins revealed no secondary cases. Twenty-one pairs were monozygotic=37.5%, and 35 pairs were dizygotic=62.5%. Thirty-three were female pairs=58.9%, and 23 were male pairs=41.1%. The age at onset varied from 14 to 49 years, with a median age at onset of 30.5 years. Three of the 21 monozygotic pairs and 1 of the 35 dizygotic pairs were concordant for MS. The pairwise concordance rate for monozygotic twins was 14.2%, and the proband concordance rate was 25.0%. The pairwise concordance rate for dizygotic twins was 2.8%, and the proband concordance rate was 5.6%. The heritability estimate is about 0.80. The proband concordance rate in dizygotic twins, which is comparable with the recurrence risk of ordinary sibs, is similar to risk figures of sibs. The high heritability estimate of about 0.80 suggests that genetic factors are of importance for the development of MS. HLA determination was established in 30 probands, of which 19 were HLA-DR2 positive=63.3%. Of the normal Danish population 25.4% are HLA-DR2 positive, and this gives a relative risk of above 4. Also other investigations were made on the twin material, but I will not go into further details with these.

Conclusion

The reported twin studies in MS at least indicate that if we assume etiological homogeneity there is no genotype which irrespective of environment always leads to MS. There may be certain genotypes which imply a higher susceptibility for developing MS, but nongenetic factors will be rather decisive in provoking or preventing the disease. MS could not be due to only one single gene; environmental factors also contribute to the development of the disease. Thus regular dominant, recessive, and sex-linked inheritance is ruled out.

Two types of genetic determination might be possible. One gene (or a pair of identical recessive genes) with reduced ability to penetrate and to dertemine the phenotype might be responsible, or the development may be determined by more than one gene (or a set of genes), which is polygenic inheritance. But also because the concordance even in monozygotic twins is not greater than around 30% despite

the similarity of the environment, it is possible that the susceptible genotype is an (absolute) requirement for developing MS.

The recessive mode of inheritance for MS is suggested by the study of MacKay and Myrianthopoulos [17] in which the frequency of MS in sibs of MS twins was greater than in parents and children and other relatives. However, other analyses support a dominant mode of inheritance, which is suggested by the distribution of HLA-DR2 genotypes in MS cases and by pedigree analyses incorporating HLA. It is consistent with the findings in these studies that the frequency of MS in sibs is only slightly greater than in other relatives. However, at the present time we must still conclude that the genetics of MS remains poorly understood. In the future it will be highly desirable that new twin series are collected and studied. The increased communication between the European countries – also within science and technology – will hopefully facilitate further research, including twin studies in MS.

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Risk Factors, Course, Prognosis, and Treatment of Multiple Sclerosis

Risk Factors, Course, and Prognosis of Multiple Sclerosis

J.F. Kurtzke

Neurology Service and Neuroepidemiology Research Program, Veterans Administration Medical Center and Departments of Neurology and Community and Family Medicine, Georgetown University Medical School, Washington, DC, USA

It has been said that to know multiple sclerosis (MS) is to know neurology – and vice versa. In the occident this is among young adults the most common disease with primary pathologic changes in the central nervous system. It has been characterized as a disease of unknown cause, inadequate treatment, and unpredictable course. It has also been the single neurologic disorder about which the most has been written, and in this regard epidemiology has contributed its share. There is then some information available for this disease as to risk factors, the course of the illness, and factors predictive of this course.

Risk Factors in MS

Risk

According to Fox et al.: "the basic premise of epidemiology is that disease does not occur randomly but in patterns which reflect the operation of the underlying causes... that knowledge of these patterns is not only of predictive value with respect to future disease occurrence, but also constitutes a major key to understanding causation..." [1]. The "patterns" mentioned are those which comprise the "risk factors" for a disease. To consider risk factors, we must first define risk [2].

Take a group or cohort of 1000 healthy people. Over a given interval they will incur a certain number of events, such as stroke. If ten strokes occur in 1 year in that cohort, its annual frequency is 1%. Thus the experience of a population cohort followed over time provides a measure of the cumulative frequency of the event over time, i. e., the chances or probability of an event. This is risk, and for this defined group it is the *absolute risk* of the event.

Risk is measured by the frequency of later events in a population defined at the start of the period of observation. Incidence and mortality rates are based on counts of events over time within the average population during the interval. In real life, the distinction is trivial, and an *annual absolute risk* of stroke is very well approximated by the *annual incidence rate* of stroke. Attributes which alter the expected absolute risk or probability of disease are referred to as risk factors.

If we know the absolute risk (or the incidence rate) in two population subgroups which differ as to the presence of a factor, then the ratio of the risk in those with the factor, versus that in those without, is a measure of the *relative risk* (RR) for that

factor. If the incidence rate of a disease is 8/100 000 for those with factor X and 4/100 000 for those without factor X, then for factor X the relative risk is 2. In situations where true incidence rates are unknown, prospective studies of comparable groups, one with and one without the factor, will also provide relative risk ratios. If proportionately twice as many with the factor develop the disease as do those without the factor, then again the relative risk is 2.

Relative risk therefore is the *ratio* of the rate in the "exposed" to the rate in the "standard" ("unexposed"). The higher this ratio, the more likely is this factor to be directly related to the cause or precipitation of the disease in question.

When prospective studies are not feasible, one can compare groups of the affected with appropriate concurrent controls for the presence or history of a risk factor. Such retrospective case control comparisons were previously considered to provide direct relative risk ratios. However, it has been concluded that this statement is valid only when the true risk ratio is 1 – and that is the instance of least interest. This comparison is now simply called the *odds ratio* (OR), and it is defined as the quotient of the product of a times d over the product of b times c in the following table:

Factor	Case	Control	Total
+	a	b	$a+b$
−	c	d	$c+d$
Total	$a+c$	$b+d$	

The product ad represents the "hits" for the factor: present in cases (a) and absent in controls (d), while the product (bc) reflects the "misses": present in controls (b) and absent in cases (c). The quotient (ad/bc) then gives the odds for (or against) a relationship between the factor and the disease. The higher the OR, the stronger the positive relationship.

The *excess* of the rate of occurrence of disease in those "exposed" to a factor beyond the rate in the "standard" not so exposed provides a measure of the amount of disease blamed on or attributed to this factor, and this excess is called the *attributable risk*. It is the quantitative amount of disease that one could hope to avoid by removal of the risk factor in question.

Geography

The geographic distribution of MS has been studied extensively, particularly in the last quarter century. But even in 1868, Charcot [3] had commented:

> *Après M. Cruveilhier [1835–1842], Carswell, dans l'article* Atrophy *de son Atlas (1838), a fait dessiner des lésions qui se rapportent à la sclérose en plaques. Mais cet auteur, qui a puisé surtout les matériaux de son ouvrage dans les hôpitaux de Paris, ne relate à ce propos aucun fait clinique. Même aujourd'hui, je ne crois pas que la sclérose en plaques soit connue en Angleterre."*

The first case report from Britain, indeed, was that of Moxon in 1873 [4], while for some years previously in both France and Germany the disorder had already seemed quite common.

Table 1. Multiple sclerosis: average annual age-adjusted (US 1950) death rates per 100 000 population for selected countries for 1951–1958 [5] and 1967–1973 [6]

Country	Rate per 100 000 1951–1958	1967–1973	Country	Rates per 100 000 1951–1958	1967–1973
Norway	1.5	1.1	Greece	0.3	0.4
Sweden	1.0	0.8	Italy	0.7	0.6
Finland	0.9	0.6	Portugal	1.2	1.1
Denmark	2.0	1.5	Israel	0.5	0.6
Scotland	3.0	2.1	Iceland	0.3	1.0
England-Wales	1.6	1.5	Canada	1.2	1.1
Northern Ireland	3.3	2.1	United States	0.9	0.8
Ireland	2.9	2.1	Mexico	0.2	0.1
FRG	2.1	1.5	Colombia	0.2	0.2
France	2.7	0.8	Uruguay	0.6	0.6
Belgium	2.0	1.4	Chile	0.3	0.2
Netherlands	2.0	1.5	Australia	0.7	0.6
Switzerland	2.2	1.8	New Zealand	1.2	1.1
Austria	1.9	1.4	Phillipines	0.0	0.1
Czechoslovakia	2.0	1.6	Japan	0.1	0.1

Death Rates

Multiple sclerosis death rates among countries, all age adjusted to the same (US 1950) standard, show notable variations (Table 1). In the 1950s [5], the rates ranged from well over 3/100 000 population to almost zero. The highest rates were those for Northern Ireland, Scotland, and the Republic of Ireland. Other western European countries had rates of 2/100 000, except for the northernmost lands of Norway, Sweden, and Finland and the Mediterranean countries of Greece, Italy, and Portugal. The rates for those two groupings were closer to 1/100 000, similar to those for Canada, Australia, and New Zealand, and for United States whites; nonwhites in the United States had half the rates of whites. Lowest by far were rates from Asia, Africa, and the Caribbean. Later data were provided by Massey and Schoenberg for the 1967–1973 interval [6]. The majority of the countries then reported lower rates, but the ranking was quite similar to that of the earlier series.

Age-adjusted death rates by state of residence in the United States revealed a north-south difference. All states below the 37th parallel of latitude were low, although in the east the dividing line seemed to be at 39°, with Virginia and Maryland below the mean. The same distribution was seen for whites alone and for crude death rates by place of birth [7].

Prevalence Rates

There are now nearly 300 population-based surveys available to define the frequency of MS about the world [8, 9]. Summary comments as to these data follow.

Europe

Prevalence rates per 100 000 population for Europe and the Mediterranean basin are plotted against geographic latitude in Fig. 1. The rates appear to separate into two zones or clusters, one with rates of 30 and over, considered high frequency, and the other with rate below 30 but above 4/100 000 population, classed as medium frequency. Using only those judged class A studies (those with good case ascertainment and comparable methodology and diagnostic criteria), the high prevalence band as defined extended from 44° to 64° north latitude.

The northernmost parts of Scandinavia and the Mediterranean basin comprised medium prevalence regions. Geoffrey Dean has contested the inclusion of Italy within the medium zone, first with his survey of Enna in Sicily, where a rate of 53/100 000 was recorded [12]. This has received considerable support, and there are now a number of other recently published surveys from Italy and its islands with rates in the high-frequency range [13]. The question now is whether this is a recent change in MS frequency or whether earlier Italian surveys were all markedly incomplete.

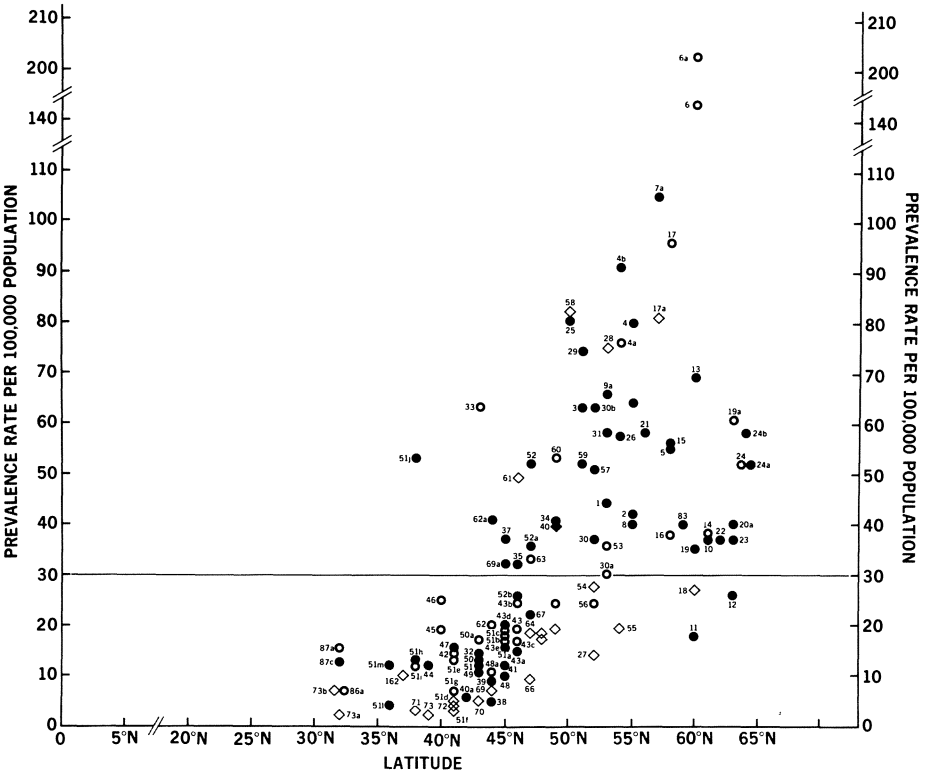

Fig. 1. Multiple sclerosis: prevalence rates per 100 000 population for survey sites plotted by latitude, Europe and Mediterranean area as of 1980. *Numbers* identify studies in Kurtzke (1975, 1980) [10, 11]. *Solid circles* are class A (best) surveys, *open circles* class B, *open diamonds* class C (inadequate), *solid diamonds* class E (estimates). Class C studies are listed only if no better quality survey is available for the site. From Kurtzke (1980) [11].

There is also evidence of clustering of MS within Norway, Denmark, Sweden, Switzerland, and northern Scotland. In Norway, Denmark, and Switzerland two generations of patients (and doctors) had been surveyed. The focal distributions were highly correlated betweeen each survey, but with evidence of diffusion over time [8]. The clustering, as well as the broader geographic distributions, would indicate that the occurrence of MS is intrinsically related to geography. If this is correct, then MS can be defined as an acquired, exogenous, environmental disease.

Americas

As to the distribution of MS in the Americas, there have been a moderate number of prevalence studies, and they appear to fall within the same three risk zones: high frequency 37°–52°, medium frequency 30°–33°, and low frequency (prevalence less than 5/100 000) 12°–19° and 63°–67° degrees north latitude [10, 11]. Our best information as to the nationwide distribution of MS in the United States, though, arose from a series of 5305 United States veterans of World War II or the Korean Conflict, who were adjudged by the Veterans Administration to be "service connected" for MS. Each of the MS patients was matched to a military peer on the basis of age, date of entry into and branch of service, and survival of the war. This provided an unbiased, pre-illness, case control series of nationwide composition and unprecedented size [14].

Figure 2 shows the distribution of MS expressed as case control ratio percentages for the white male veterans of World War II according to the state of residence at entry into military service. Based on estimates of the prevalence rates to which these ratios refer, this provided the same division as in Europe. States below the 37th parallel all fell in the medium-frequency zone, and the states (and northern

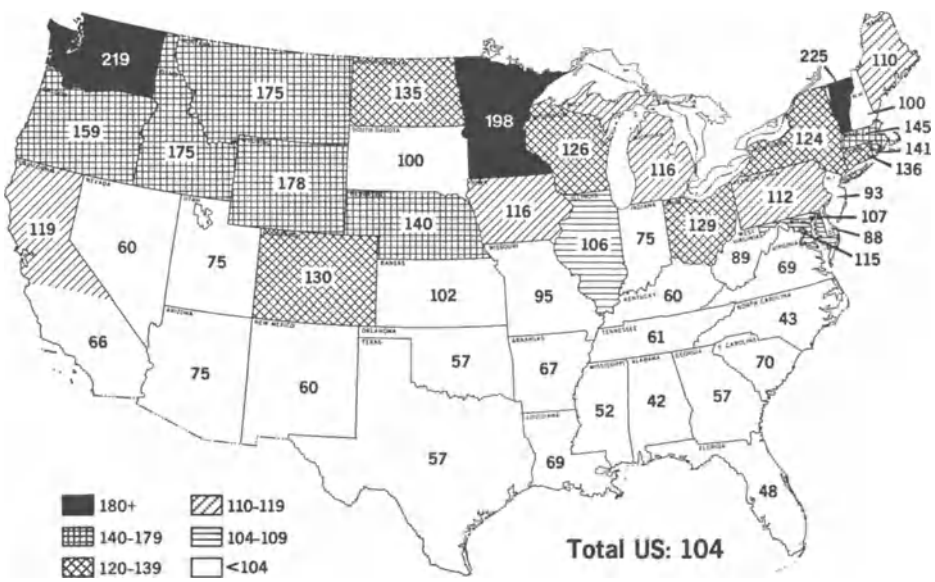

Fig. 2. Multiple sclerosis: case control ratio percentages for white male veterans of World War II by state of residence at entry into service. Modified from Kurtzke (1978) [15]

California) above the 37th parallel were in the high-frequency zone, except for Virginia (0.69 ratio) and Kentucky (0.60). In the east, then, the high- to-medium dividing line reached the 39th parallel. This distribution was therefore very similar to that for MS death rates.

Worldwide Distribution

Space precludes review of the prevalence rates from other regions, but they conformed in general with the threefold division noted. The distribution of MS about the world may then be described within these three zones of frequency or risk (Fig. 3).

The high-risk zone, with prevalence rates (as of 1980) of 30 and above /100 000 population, included northern and central Europe into the USSR, the northern United States and southern Canada, New Zealand, and southeastern Australia. These regions were bounded by areas of medium frequency, with prevalence rates between 5 and 29/100 000, consisting of the southern United States, southwestern Norway, and northernmost Scandinavia, and probably the USSR from the Ural mountains into Siberia as well as the Ukraine. Except for Italy with, now, high rates, the entire Mediterranean basin from Spain to Israel was also of medium prevalence. In this zone too fell most of Australia and perhaps Hawaii and the mid-portion of South America, plus one white group of South Africa. Low-frequency areas, with prevalence rates below 5/100 000, comprised all other known areas of Asia and Africa, Alaska and Greenland, and the Caribbean region to include Mexico and probably northern South America.

Migration

The fate of migrants who move into regions of differing risk for MS is critical to our understanding of geography in this disease. If the risk of MS is defined at or near birth, or the disease is innate, then migrants from low to high regions would demonstrate no increase in risk, and their MS frequency would be that specified by birthplace alone.

Figure 2 shows state of residence at entry into military service for the United States veteran case control series. These residences were allocated within three horizontal tiers for the coterminous United States: a northern tier of states above 41°–42° north latitude, a middle tier, and a southern tier below 37° including California from Fresno south. Migrants would be those born in one tier who entered service from another. The marginal totals of Table 2 provide the ratios for birthplace and for residence at service entry within the three tiers for white males of World War II service. The major diagonal (north-north, middle-middle, south-south) gives the case control ratios for nonmigrants, and cells off this diagonal define the ratios for the migrants [16].

All ratios decrease as we go from north to south. The nonmigrant ratios are 1.41 north, 1.04 middle, and 0.56 south. For the migrants, those born north and entering service from the middle tier have a ratio of 1.26; if they entered from the south their ratio is 0.70, only half that of the nonmigrants. Birth in the middle tier is marked by

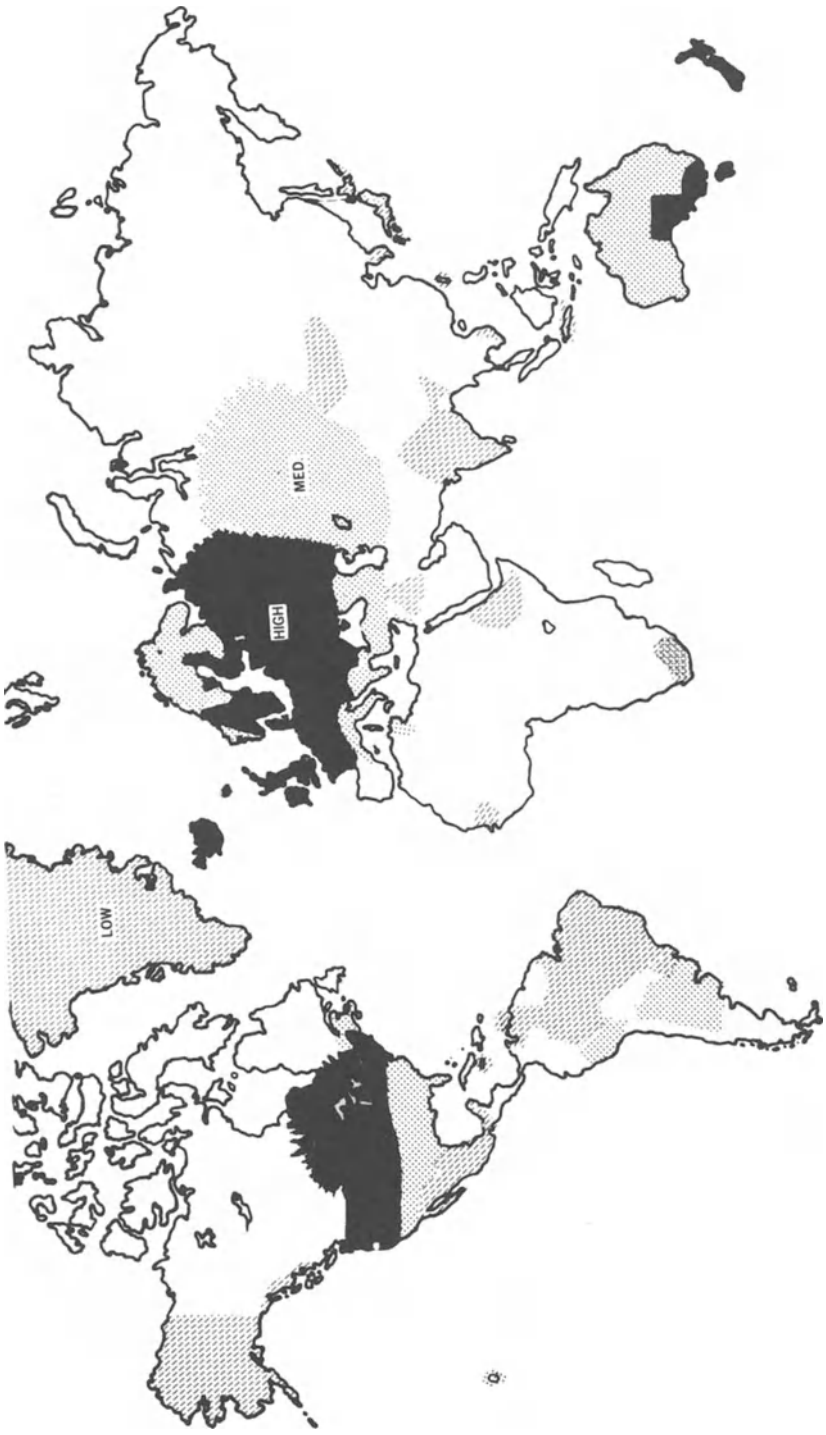

Fig. 3. Multiple sclerosis: worldwide distribution as of 1980 according to high (*black*), medium (*dots*), and low (*diagonal dashes*) zones of prevalence or frequency. *Open areas* are regions without data; South American frequencies are tentative. From Kurtzke (1980) [11]

Table 2. Multiple sclerosis: case control ratios for white males of World War II by tier of residence at birth and at entry into active duty (EAD)(, coterminous United States military-veteran series [16]

Birth tier	EAD tier			Birth
	North	Middle	South	Total
Case control ratios				
North	1.41	1.26	0.70	1.38
Middle	1.30	1.04	0.72	1.04
South	0.73	0.62	0.56	0.57
EAD total	1.39	1.04	0.58	1.04
Case/control/numbers				
North	1611/1140	112/89	32/46	1755/1275
Middle	125/96	1544/1482	68/94	1737/1672
South	16/22	42/68	439/788	497/878
EAD total	1752/1258	1698/1639	539/928	3989/3825

an increase in the MS/C ratio for northern entrants to 1.30 and a decrease to 0.72 for the southern ones. Migration after birth in the south seems to raise the ratios to 0.62 (middle) and 0.73 (north). The migrant risk ratios, therefore, are intermediate between those characteristic of their birthplace and their residence at service entry.

There are other prevalence studies for migrants from high- to low-risk areas which also suggest that the age of adolescence was critical for risk retention: those migrating beyond age 15 retained the MS risk of their birthplace; those migrating under 15 acquired the lower risk of their new residence. Several low-to-high studies also showed that those migrating in childhood or adolescence did in fact increase their risk of MS [8, 9, 16].

The veteran series provides our best evidence as to increasing MS risk by migration from low to high areas – and it also indicates that the time when such moves are critical is well before clinical onset. In an earlier US Army study, similar case control ratios were found for the same three tiers for residence at birth and at entry into service; but the gradient had totally disappeared for residences while in military service but before clinical onset [17]. Overall, the evidence suggests that for natives of high-risk areas the acquisition of MS occurs between approximately ages 10 and 15 years.

Multiple Sclerosis Epidemics

Thus MS is a place-related disorder, and one's risk is primarily dependent upon one's location, particularly during late childhood or adolescence. As to the place itself, the risk has been relatively fixed over time. However, we seem to have encountered two separate populations with epidemics of MS, which in fact may have common precipitants, and which have occurred in the ethnically similar lands of the Faroe Islands and Iceland.

Hyllested and I have been studying MS on the Faroes intensively since 1972. There was no MS among native resident Faroese before 1943, but in 32 patients clinical onset of MS occurred between 1943 and 1973, and comprised three separate epidemics of decreasing incidence. The first epidemic attained a maximal annual incidence rate of 10/100 000 population. We concluded that MS was introduced into the Faroes by asymptomatic British troops who had occupied the islands in 1940–1945. We believe that they brought an infectious agent which caused what we called the "primary MS affection" (PMSA), which years later produced clinical neurologic MS (CNMS) in only a small proportion of the affected. We feel that PMSA was later transmitted from and to cohorts of young asymptomatic Faroese, with again small proportions of the affected representing the clinically involved patients comprising the later epidemics. It was also concluded that, in this virgin (as to MS) but susceptible population, 2 years of exposure from at least age 11 years were required for acquisition of PMSA and that neurologic symptom onset, if it occurred, took place an average of 6 years after acquisition [18, 19].

In Iceland, there seemed also to be a postwar epidemic: the annual incidence rate doubled in the 1945–1954 period over previous experience, and declined thereafter [20]. Iceland too was heavily occupied by the military in World War II.

Age, Sex, and Race

Most of the more recent prevalence studies report higher rates among females than males, with a ratio of about 1.5:1. The sex difference is most marked at the younger ages, with little variation beyond age 50. Age-specific prevalence rates are maximal at about age 42, with most patients between 20 and 70 years of age.

For Denmark, average annual incidence rates were calculated from the incident cases 1939–1945. An annual rate of 3.4/100 000 was found: 3.0 male and 3.7 female. Age-specific rates rose rapidly from essentially zero in childhood to a peak at about age 27 of more than 9/100 000 for females and almost 7 for males (Fig. 4). Beyond age 40 there was little difference between the sexes, both of whose rates declined equally to zero by age 60.

Race

From the worldwide distribution of Fig. 3, it may be seen that all the high- and medium-risk areas were those of predominantly white populations. Death rates in the United States indicated that nonwhites had this disorder recorded as the cause of death only half as often as whites. Further, in the veteran series, blacks or Negroes had only half the risk of white males regardless of residence (Table 3). Note too that these young white females had nearly twice the risk of MS as did the white males. The same series indicated a paucity of Orientals, Filipinos, and Amerindians, though with small numbers; an apparent deficit of Latin Americans was explicable by geography.

Prevalence estimates among native Japanese have been uniformly low [8–11]. Detels et al. [23] have presented good evidence for low MS mortality and prevalence among Japanese and Japanese-Americans in California and Washington State,

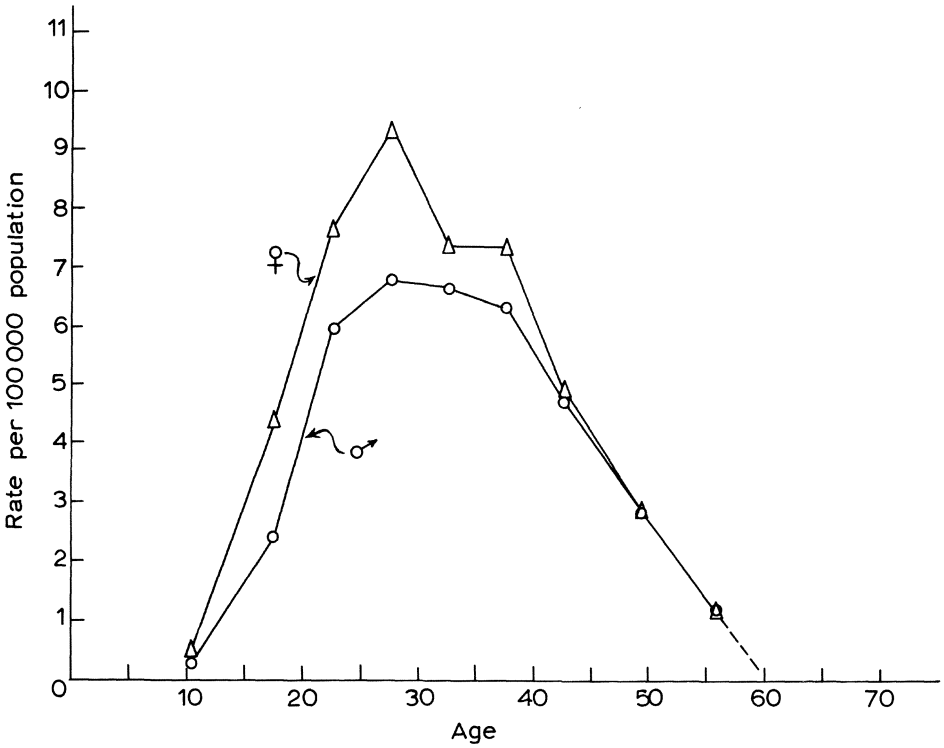

Fig. 4. Multiple sclerosis: average annual age-specific incidence rates per 100 000 population by sex, Denmark, 1939–1945 ; MS series of Hyllested (1956) [21]. From Kurtzke (1968) [22]

Table 3. Multiple sclerosis: case control ratios by tier of residence at entry into active duty (EAD) for the major sex and race groups, United States military-veteran series[a]

Sex and race	Tier of residence at EAD			
	North	Middle	South	Total[b]
Case control ratios				
White male	1.41	1.02	0.58	1.04
White female	2.77	1.71	0.80	1.86
Black male	0.61	0.59	0.31	0.45
Total series[c]	1.41	1.00	0.53	1.00
Case control numbers				
White males	2195/1544	2059/2022	688/1161	4922/4737
White females	97/35	65/38	20/25	182/98
Black males	28/46	88/150	61/194	177/390
Total series[c]	2323/1647	2213/2219	762/1425	5298/5291[b]

[a] Modified from Kurtzke et al. 1979 [14]
[b] Excludes 1 male case and 11 male controls inducted in foreign countries
[c] Includes black females and persons of other races

although these estimates for the American born were approximately twice those reported from Japan itself. Data from Hawaii [24] suggested low MS risk among Polynesians and Filipinos residing in the state.

Multiple sclerosis, then, is predominantly the white man's burden. However, the other racial groups still manifest the same geographic gradients as the whites, but at the lower levels characteristic for the group.

Genetic Factors

Many studies have shown that familial aggregation is excessive in MS [8, 9]. The disease reportedly occurs among siblings of affected patients at a rate perhaps six to eight times that expected. There may also be some excess among parents of MS patients.

Unbiased data on twins, which might clarify the role of genetics, are inconclusive. Most published twin studies on MS suffer from selection bias and retrospective family ascertainment. The only ones for which the *prospective* occurrence of multiple sclerosis within a defined cohort of twins has been assessed are those of Bobowick et al. [25] and Heltberg and Holm [26]. In neither instance was there a significant difference in MS concordance ratios between monozygous and same-sexed dizygous twins, but the numbers were small: 1/6 MZ vs. 0/8 DZ_1 were concordant in the former, and 4/19 MZ vs. 1/28 DZ_1 in the latter. Ebers et al. [27] have concluded, based on their retrospective, population-based series of MS cases, that there is a major genetic factor in MS; they reported 7/27 MZ (including some clinically "possible" cases) as concordant vs. 1/43 for *all* DZ sets, regardless of sex, a significant difference. It the concordant pair were in DZ_1 (same-sexed) pairs, which is the proper comparison, then this ratio of 1/20 would not have differed significantly from the MZ ratio.

The information that certain histocompatibility (HLA) antigens may be related to MS, at least in northern Europe and the United States, has broadened the controversy surrounding the role of genetics in this disorder. Space precludes elaboration, but essentially, in whites of northern European ancestry, there is generally an excess of HL-A3, B7, and especially DW2 (DR2) among MS cases. A large and contradictory literature has arisen as to shared genetic components, predominantly in the HLA series, in families of MS patients. Some studies indicated that the majority of affected siblings in multicase families shared a common haplotype, but different haplotypes had been noted in different families. Other investigations, however, have failed to demonstrate one parental haplotype segregating preferentially in affected siblings as compared with unaffected siblings [28].

Roberts et al. [29] studied family histories in cases and controls ascertained in the MS surveys of the Orkney Islands, which share with the Shetland Islands the highest rate of multiple sclerosis so far reported (numbers 6 and 6a in Fig. 1). They found that MS patients did not differ from controls in HLA patterns or in other blood groups, isoenzymes or serum proteins, nor were they more or less inbred than controls. The only possible single-gene inheritance the authors felt at all likely was an autosomal recessive with 12% penetrance. "Much more likely is a complex aetiology, the genetic contribution being polygenic and possibly subordinate to the environmental..." [29].

Other Risk Factors

Attempts to identify factors which would explain the unique geographic distribution of MS have been numerous but unsuccessful (see ref. [28] for details of the following). Dietary fat, trace elements, and heavy metals have been implicated, but these observations remain largely unsupported. Most retrospective case control comparisons by a number of workers have revealed no significant associations (other than the accepted geographic variations) with the exception of one finding that patients had had tonsillectomy in more instances than had either their spouses or their nearest siblings; but these results also have not been replicated. In studies in Israel there were possible inverse relationships with indices of poor sanitation and a direct relationship to some features of urbanization; however, the most impressive feature of answers to a detailed questionaire was the absence of strong correlations. Reports implicating dog exposure have been published, but to date remain unverified.

In a recent case-control study [30] conducted in Olmsted and Mower Counties, Minnesota, small family size was significantly associated with multiple sclerosis. Whether this reflects socioeconomic status (see below) is uncertain, but urban residence and high educational achievement did have relative risk estimates greater than one. However, for prior infections, trauma, animal exposure (including dogs), prior surgery, and other illnesses, no association could be demonstrated. In a small series, Bobowick et al. [25] reported a significant excess of prior "environmental events" among affected versus unaffected twins. These "events" included operations, trauma, and infections as the major groups, and differentiating frequencies were mostly within the 20 years before clinical onset rather than in early childhood.

Beebe et al. [17] compared MS patients with matched controls from the earlier United States Army series, utilizing data collected *before* multiple sclerosis was diagnosed. Among characteristics that significantly differentiated patients from controls was (as mentioned above) geographic location at birth or service entry but not during military service. There was a strong positive correlation with urbanization of residence, high socioeconomic status, and visual defects (refractive errors) at

Table 4. Multiple sclerosis control ratios by socioeconomic status (SES)[a] and urbanization classification of residence at entry into service, US Army WWII series [17]

Urbanization class	Socioeconomic status (SES)[a] scores (MS/C ratios)				Total	$(N)^b$
	000–069	070–109	110–149	150–200		
Metropolitan	2.00	3.67	1.70	3.50	2.40	(48)
Other urban	1.60	1.33	2.17	1.67	1.67	(40)
Mixed	0.63	0.88	1.21	0.98	0.98	(209)
Rural	0.62	0.36	1.09	0.63	0.63	(71)
Total	0.70	0.81	1.33	1.61	1.00	
$(N)^b$	(85)	(96)	(100)	(87)		(368)

[a] Sum of scores for pre-service occupational status (Bureau of Census codes) and codes for educational level, each ranging from 0 to 100; the higher the scores the higher the SES

[b] Number of MS cases

Table 5. United States veteran series: MS case and control (MS/C) pairs county of birth and latitude of birth[a]

	Latitude: case=control Paris with A			Factor A: case=control Pairs with latitude		
Factor A	MS>C	MS>C	P	>C	MS>C	P
Mean annual freeze-free period (F-F)	388	359	0.15	501	320	$<10^{-9}$
Annual solar radiation (SOL)	207	210	0.46	894	530	$<10^{-21}$
Mean annual hours of sunshine	504	487	0.30	552	307	$<10^{-16}$
Mean annual days temperature >90°F	394	431	0.10	333	222	$<10^{-5}$
Mean annual days temperature <31°F (Cold)	577	537	0.12	170	105	0.00006
Mean July relative humidity	489	486	0.47	616	338	$<10^{-18}$
Mean annual pan evaporation (PAN)	269	255	0.28	565	359	$<10^{-10}$
Mean annual days of precipitation (RAIN)	534	517	0.31	504	300	$<10^{-12}$
Mean annual days of forecasted air pollution	653	658	0.46	309	173	$<10^{-9}$
Ground H_2O minerals	408	392	0.30	821	508	$<10^{-17}$
Elevation, feet above sea level	553	509	0.09	552	286	$<10^{-19}$
COLD × RAIN	751	681	0.03	77	39	0.0003
(5-SOL) × (7-PAN)	376	389	0.33	452	280	$<10^{-9}$
(7-F-F) × RAIN)	669	646	0.27	187	139	0.005
COLD × (7-PAN)	609	606	0.48	128	81	0.007

[a] Modified from Norman et al. 1983 [31]

entry into service. Table 4 summarizes the first two of these factors, indicating each one to be an important variable. A lower risk of MS was also found among blacks – less than half the expected number, regardless of residence.

The large data set of the military-veteran series was analyzed for meteorologic and climatic correlates of county of birth of the some 4400 white male MS cases and their controls. When analyzed as single variables, each of the factors cited in Table 5 was highly significantly related to the risk of MS. However, when latitude was controlled, none of these factors showed a significant association (left-hand columns of Table 5), while within each of the factors, the relationship with latitude per se remained striking (right-hand columns).

Course and Prognosis in MS

Survival

In studies antedating World War II, and based on records of nonsurvivors in hospital series, the average duration of life after onset of MS was estimated at 10 years or

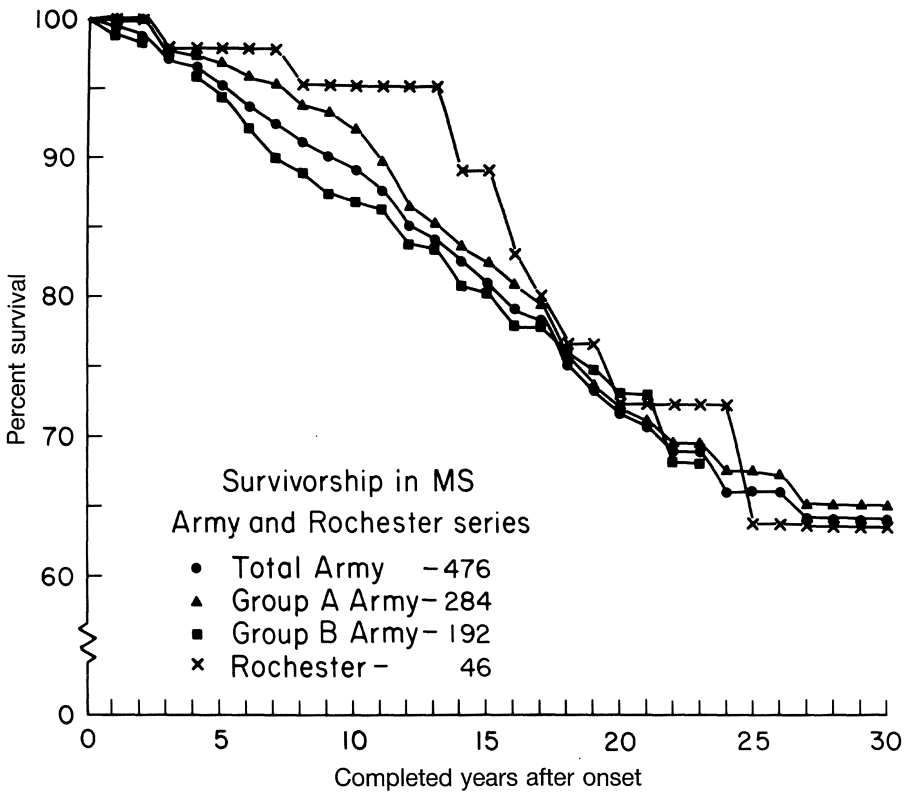

Fig. 5. Multiple sclerosis: percentage survival by years after onset, Rochester, MN residents and United States Army males (group A, those with bouts before Army diagnosis; group B, Army diagnosis at first bout). Modified from Kurtzke 1970 [34]

less. The prevalence studies of the early 1950s indicated that survival averaged at least 20 years after clinical onset. Survival rates were calculated by life-table methods for the patients resident in Rochester, Minnesota [32], and for United States Army male patients hospitalized in the period of World War II [33] (Fig. 5). Among both Army groups and the Rochester series, the average duration of illness or median survival was estimated to be at least 35 years from onset. About three-fourths of the patients had survived 20 years of illness.

These findings, considerably in excess of other estimates, probably reflect the more complete ascertainment (including benign cases) in Rochester, and enumeration of all cases (irrespective of severity) developing in a defined population (Army series), with both cohorts followed over a lengthy period of time. There is, though, additional support for these figures from two other population-based series which will be published. Both in Lower Saxony, Federal Republic of Germany (Sigrid Poser, unpublished data), and in the Faroe Islands (Kurtzke, unpublished data), median survival has been calculated also to be in the order of some 35–40 years from onset.

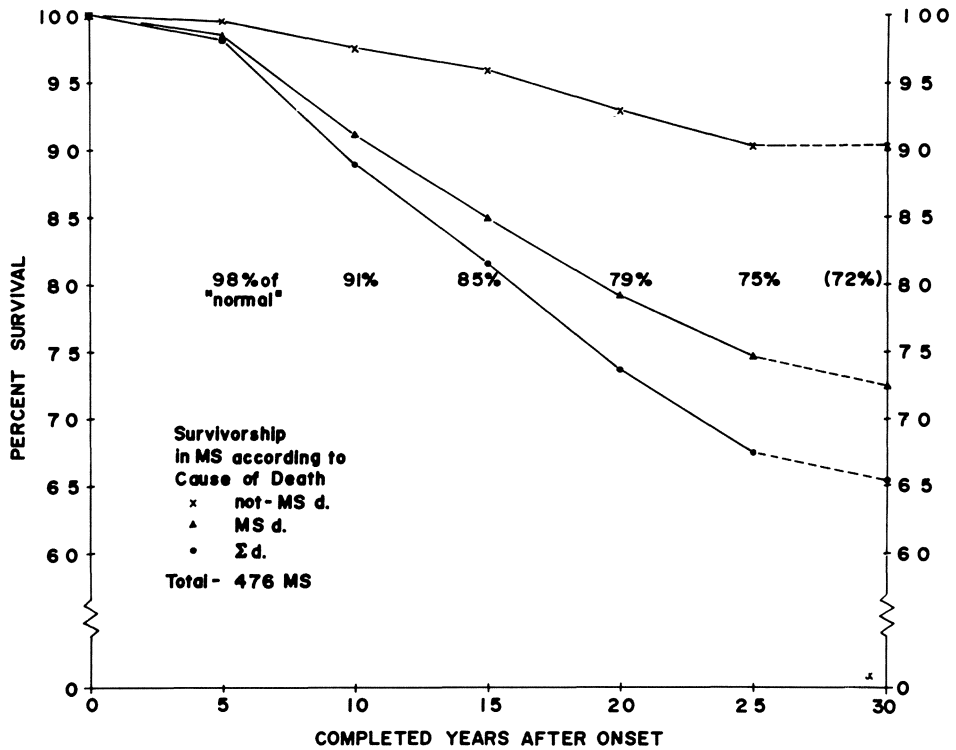

Fig. 6. Multiple sclerosis: percentage survival years by after onset according to cause of death (MS or not), United States Army total series of Fig. 5. Modified from Kurtzke et al. (1970) [33]

As more MS patients survive to older ages, a greater proportion of them can be expected to die of causes unrelated to multiple sclerosis and thus will not be coded as deaths from MS. This last point was supported by analysis of contributory causes of death in multiple sclerosis patients in Denmark and the United States [35]. The increasing proportion of other causes of death with increasing duration was also noted in the Army survivorship study (Fig. 6). Here causes were allocated by personal review of the records, and not taken from death certificates.

Figure 6 also demonstrated that the excess of deaths due to MS, although clear, is less than might be anticipated. As seen in Table 6 as well, in all Army subgroups and in the Mayo series, even after 25 years of illness MS patients had a survival ratio that was still three-fourths of normal (expected) survival.

Age was not a predictor of survival in the Army series. For each subgroup aged 20–24 through 35–39 years at time of diagnosis the overall survival was the same 75% 20 years later (Table 7).

Table 6. Survival in MS as percentage of normal survival a for Mayo Clinic series [32] and the US Army WWII series [33], the latter divided into those with MS bouts prior to Army diagnosis (group A) and those diagnosed in the Army at the onset bout (group B)

Years from onset	Percentages of normal survival[a] Mayo	Total Army	Army Gp A	Army Gp B
0	100	100	100	100
5	99	98	99	96
10	98	91	94	88
15	94	85	85	85
20	80	79	78	82
25	74	75	74	77

[a] Normal survival for United States whites age 30 in 1940 (Mayo) and for non-MS deaths among Army MS series

Clinical Course

The Army WWII series also afforded an opportunity to describe the clinical course of illness through the 15 years following Army diagnosis [36]. Patients were neurologically defined at all examinations in accordance with a Disability Status Scale (DSS), an overall scale of neurologic impairment with grades from 0 (normal) to 10 (death due to MS), as well as with subscales for neurologic functional systems (pyramidal, cerebellar, brain stem, sensory, bowel and bladder, visual, cerebral, other) [37]. Figure 7 shows the distribution of MS patients according to DSS scores at diagnosis, and at 5, 10, and 15 years after onset. In Fig. 8, involvement by functional system is specified according to each one's frequency within groups defined by overall DSS scores (not by time). It is clear that, as *groups* of MS patients worsen, they tend to worsen in all testable areas of neurologic impairment. Not shown is the fact that this correlation is not only for frequency but also for severity of involvement in each functional system.

Predictors of the Course of Illness

We have seen above that in the Army series there was no correlation of survival with age at diagnosis (Table 7); this held too for age at onset, and also for the course of illness was defined by DSS groupings. In addition, all demographic features (education, occupation, place of residence, urbanization) were similarly unrelated to the course of the disease.

Clinical and laboratory features of the onset bout and of the Army diagnostic bout were essentially unrelated to prognosis as well. Overall impairment as measured by DSS at Army diagnosis showed little predictive value (Table 8); this diagnostic point averaged some 2–3 years after onset.

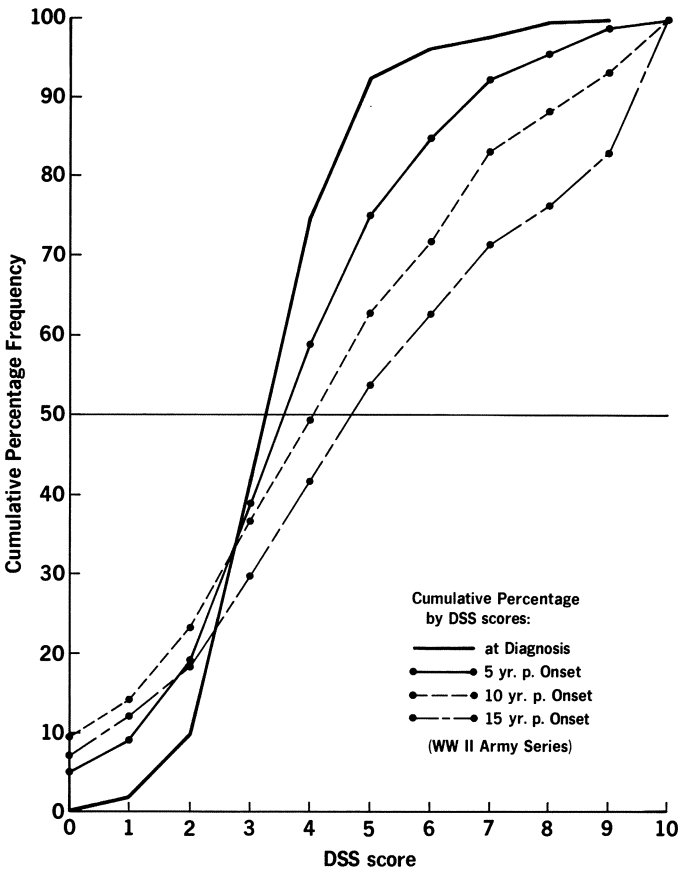

Fig. 7. Multiple sclerosis: cumulative frequency over time for neurologic impairment as measured by the Disability Status Scale (DSS) [37] in 527 definite and probable MS patients followed through 15 years after diagnosis, Army World War II series (data of Kurtzke et al. 1977 [36]); cited here are distributions at time of Army diagnosis (*bold line*) and at 5, 10, and 15 years after onset

Table 7. Survival in MS according to age at time of Army diagnosis, US Army WWII series [33]

years from diagnosis	Age at Army diagnosis (years) 20–24	25–29	30–34	35–39
0	100.0	100.0	100.0	100.0
5	96.8	97.9	96.3	94.0
10	91.9	89.4	89.0	90.0
15	87.0	84.4	77.1	82.0
20	75.4	76.2	73.4	75.2
(*N*)	(123)	(141)	(109)	(50)

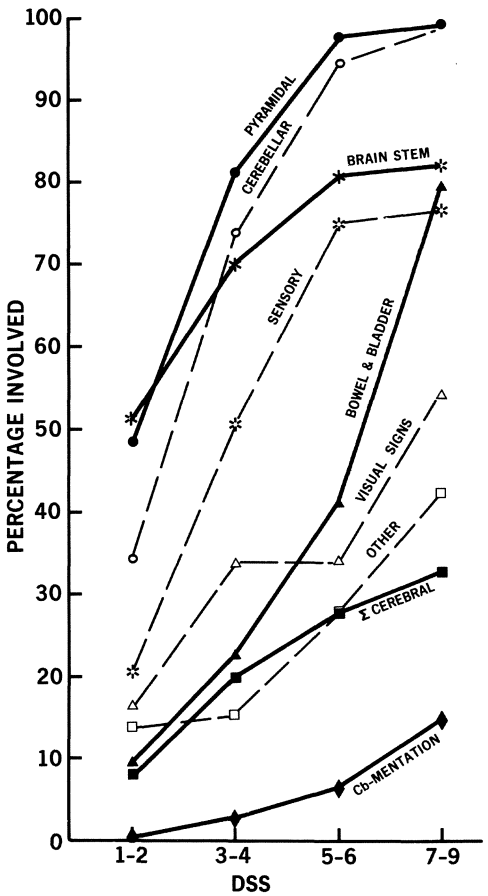

Fig. 8. Percentage frequency of involvement for each functional system according to DSS scores of the patients, based on some 2000 neurologic examinations among 527 men of the United States Army World War II series during a 20-year course of illness [38]

Table 8. Prognosis in MS: correlation of Disability Status Scale (DSS) score groups at Army diagnosis and at 10 and 15 years after diagnosis in US Army WWII series [36]

DSS at diagnosis	DSS at follow up 10 years after diagnosis Mild (0 2)	Severe (death)[a] (6+ (10))	15 years after diagnosis Mild (0–2)	Severe (death)[a] (6+ (10))
			(percentages)	
Mild (0–2)	13.5	40.5 (8.1)	6.7	56.6 (23.3)
Moderate (3–5)	21.4	43.4 (13.6)	21.2	48.2 (24.1)
Severe (6–9)	11.8	55.9 (23.5)	16.0	72.0 (40.0)
Total	19.9	44.2 (13.9)	19.5	50.8 (25.2)

[a] Percentages for deaths (DSS 10) included in severe (DSS 6+) class

Table 9. Prognosis in MS: correlation of frequency of bouts (exacerbations) during the first 5 years of illness with severity of illness, as measured by DSS score groups, at 15 years after diagnosis, US Army WWII series [36]

Number of bouts[a] in 5 years	(N)	DSS at 15 years after diagnosis Mild (0–2)	Severe (death)[b] (6+ (10))
		(percentages)	
1	(126)	22.2	40.5 (14.3)
2	(98)	15.3	54.1 (32.7)
3–4	(68)	17.6	60.3 (32.4)
5+	(17)	17.6	64.7 (29.4)
Total	(309)	18.8	50.5 (24.9)

[a] Includes onset bout
[b] Percentages for deaths (DSS 10) included in severe (DDS 6+) class

Table 10. Prognosis in MS: correlation of DSS score groups 5 years after onset ant at 10 and 15 years after diagnosis in US Army WWII series [36]

DSS at 5 years after onset	DSS at follow up 10 years after diagnosis Mild (0 2)	Severe (death)[a] (6+ (10))	15 years after diagnosis Mild (0–2)	Severe (death)[a] (6+ (10))
			(percentages)	
Mild 0–2	63.2	7.4 (1.5)	66.0	11.4 (3.8)
Moderate (3–5)	16.1	32.6 (7.3)	14.5	39.5 (15.1)
Severe (6–9)	2.2	92.2 (32.2)	1.3	98.7 (54.5)
Total	21.7	42.8 (12.5)	20.6	50.2 (23.8

[a] Percentages for deaths (DSS 10) included in severe (DSS 6+) class

Bout frequency early in the course of illness was also poorly related to the later severity of illness, either positively or negatively (Table 9). The distribution did not differ significantly from homogeneity, even at the 5% level, and even when the comparison was for 1 vs. 2 vs. 3+ bouts. Results were similar at the 10-year follow-up.

However, the results were markedly different when the neurologic status was assessed, not at diagnosis, but rather at approximately 5 years after onset (Table 10). There was then a very strong correlation between neurologic severity of illness as measured by DSS and the later course. For those with mild impairment (DSS 0–2) at 5 years, 2/3 were still mild at 15 years after diagnosis and only 1/9 were severely involved or (4%) dead. A similar finding is seen if we consider the two principal types of neurologic deficit, pyramidal and cerebellar functional systems (Table 11). In multiple linear regressions these two FS at 5 years were the only ones with significant independent contributions to the DSS scores at follow-up [36]. An

Table 11. Prognosis in MS: correlation of pyramidal and cerebellar function system scores at 5 years after onset with the DSS score groups at 10 and 15 years after diagnosis in US Army WWII series [36]

FS and FS grades at 5 years postonset	DSS at follow-up 10 years after diagnosis Mild (0 2)	Severe (death)[a] (6+ (10))	15 years after diagnosis Mild (0–2)	Severe (death)[a] (6+ (10))
			(percentages)	
Pyramidal				
0	50.7	5.8 (1.5)	50.9	11.9 (1.7)
1–2	25.6	27.8 (8.9)	23.9	34.3 (14.9)
3+	7.3	65.7 (19.1)	4.9	74.3 (38.2)
Total	21.1	43.3 (12.8)	19.6	50.7 (24.4)
Cerebellar				
0	48.3	9.5 (2.4)	52.1	9.9 (4.2)
1–2	22.5	32.4 (9.0)	17.5	46.3 (17.5)
3+	2.5	73.7 (24.6)	1.0	82.2 (45.5)
Total	22.0	41.9 (13.1)	20.6	50.4 (25.0)

[a] Percentages for deaths (DSS 10) included in severe (DSS 6+) class

Table 12. Prognosis in MS: pyramidal (P) and cerebellar (Cll) signs at 5 years after onset as predictors of DSS score groups at 15 years after diagnosis, compared with DSS scores at 5 years as similar predictors, US Army WWII series [36]

Predictors at 5 years postonset	DSS at 15 years 0–2	6+
	(proportions)	
P + Cll		
Both none (0 and 0)	0.72	0.05
Both severe (3+ and 3+)	0.00	0.88
DSS		
Mild (0–2)	0.66	0.11
Severe (6–9)	0.01	0.99

overview of their combined contribution to prognosis is defined in Table 12. Essentially, if groups of patients are minimally involved at 5 years after onset, the chances of still being minimally involved almost 20 years later are high: between 2/3 and 3/4 such will still be minimally affected; and their chances of being severely involved are much lower, at 1/10 or 1/20. Conversely, for groups severely affected at 5 years, virtually none will be mild at follow-up and almost all will then be severely involved – or dead. Thus, the strongest predictor of the later course of illness in this series was the behavior of the groups at 5 years after onset – but not earlier.

Summary

Risk factors for MS include geography, age, sex, and race. Annual age-specific incidence rates are maximal at age 27 and 0 below 10 and beyond 60. Geographic distributions from prevalence surveys indicate that all high and medium MS risk areas are in Europe or its colonies like Canada, United States, New Zealand, and Australia, while risk in the tropics and in all of Asia is low. In Europe there is evidence of both a focal distribution and its spread over time. Migration studies suggest acquisition of MS at adolescence for high to low migrants, and an increased risk for children or young adults migrating from low- to high-risk areas. The epidemics of MS on the Faroe Islands have led to the thesis that the primary MS affection is ordinarily a specific, widespread, transmissible infection of adolescence that only rarely leads years later to clinical neurologic MS.

From a massive case control series of 5300 MS among United States veterans of World War II and Korea, it was found that the risk in white women was twice that of white men, while that for black men was half that of whites, and Orientals and American Indians were also low. Sex and race differences persisted regardless of geography. The MS risk was altered by change of residence between birth and service entry: moves from high- to low-risk areas decreased the risk while moves from low- to high-risk areas increased it.

A separate study of United States Army men hospitalized in World War II indicated that high socioeconomic status and urbanization of residence were also risk factors independent of geography. Meteorologic correlates of birthplace for the large veteran series were all MS risk factors when assessed independently, but none showed any significant association when latitude was controlled.

Course and prognosis were studied in the Army WWII series. As groups of patients worsen, they worsen in all testable aspects of neurologic dysfunction. Survival was some 65% after 30 years of illness, which represents nearly three-fourths of normal expectancy. Age at diagnosis did not differentiate survival through the next 20 years of illness. Survivorship was the same as in the Army series for residents of Rochester, Minnesota, and probably for residents of the Faroe Islands and of southwestern Saxony, Germany. In the Army series no features of the early course of illness were of prognostic value, but the severity of neurologic impairment at 5 years after onset was highly predictive of the degree of impairment 15 years after diagnosis. Of the group with mild disability at 5 years, 2/3 were still mild and 1/9 severe at the latter point, and of the group with severe disability at 5 years 99% were still severe (or dead) by that point.

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Epidemiological Studies of Multiple Sclerosis in Western Norway: Possible Factors in Etiology and Prognosis

J. P. Larsen,[1] *T. Riise,*[2] and *H. Nyland*[1]

1 Department of Neurology, University of Bergen, Bergen, Norway,
2 Section of Medical Statistics, University of Bergen, Bergen, Norway

In spite of our ignorance to many of the key questions in multiple sclerosis (MS), most agree on the hypothesis that MS is a disease induced by an environmental agent in genetically susceptible individuals. The susceptibility gene is believed to be located on the short arm of the 6th chromosome and its action is through a modification of the immune responsiveness.

The exogenous factor or factors are on the other hand still open to any possible guesses. This paper will try to bring some light to these factors by describing some epidemiological features of MS in Norway.

Previous studies of MS in Norway have shown great variations in prevalence. The occurrence of MS in Scandinavia and Norway is highest in the inland of central and eastern parts of Norway and with a low frequency of the disease along the coast in western Norway.

This distribution of MS in Norway is based on an incidence study by Swank, several prevalence studies, and studies of registrations of death certificates and cases of disability assistance. From these different sources it seemed established that the frequency of MS formed a rather unique pattern, with a low frequency of the disease in coastal western and northern Norway, while other parts of the country had a high frequency of the disease. This distribution of MS in Norway has been thought to have etiological implications. Swank suggested nutritional differences to be important, while Kurtzke has proposed a Fenno-Scandinavian focus of MS. The studies establishing these interesting figures of MS in Norway were all based on MS cases with onset before 1960.

We have recently performed an updated epidemiological study of multiple sclerosis in the county of Hordaland in western Norway. The population in this county on 1 January 1963 was 350 000 and on 1 January 1983 nearly 400 000. The main city of Bergen has above one-half of the total population in the county. The Norwegian population is generally stable and the Public Health Service System has been well developed and stable for many decades.

The following sources for MS patients were examined; hospitals files, regional health officers, nursing homes, and neurologists in private practise. Furthermore, the files of possible MS patients not in contact with the Department of Neurology in Bergen in a prevalence study by Presthus in 1960 were available. His sources were mainly the same as those explored by us.

The diagnosis of MS was based on the criteria presented by MacAlpine. The patients were classified in definite, probable, and possible groups.

Our total MS material comprised 426 patients. In the prevalence study we found that the prevalence on 1 January 1963 was 20.1/100 000 for definite and probable MS. When including possible MS the prevalence figure was calculated to be 25.0/100 000, which is in good agreement with the previous prevalence calculations by Presthus around 1960.

On 1 January 1983 the prevalence showed a threefold increase compared with the 1963 figures to 59.8 and 75.5/100 000, without and with possible MS included, respectively.

Several factors able to explain a change in prevalence figures have been evaluated. Migration, case ascertainment, and survival have not been found to have any major influence on the figures in our study. A change in time interval from onset to year of diagnosis was, however, found to be important, but could only partly explain the change in prevalence.

In a subsequent study we have therefore examined the incidence of the disease. The annual number of new MS patients in Hordaland in the period 1943–1982 according to year of onset showed an increase from about 1960. The annual number of MS patients was five to eight from 1943 to about 1960. This number slowly increased to about 15–20 new patients each year in the period 1970–1980. The incidence figures in 5-year periods from 1953 to 1982 show for all MS patients an increase from about 2/100 000 per year in 1953–1962 to about 4/100 000 in the period 1968–1977. The increase can also be seen for definite and probable MS alone. In the 5-year period 1978–1982, the incidence was lower. The data for this period must be evaluated with some caution because of the long time interval from onset to diagnosis for some patients. This may also bear some relevance for the period 1973–1977. Later on we have found nearly 100 new MS patients in 1983, 1984, and 1985. Most of these patients had onset of disease from 1978 to 1982 and further strengthened the rise in incidence.

The increase was found both for females and males, but surprisingly for the past 10 years a consistent increase was found in females while a decrease was observed in males. In addition, patients with age at onset at 40 years or more did not show any increase. The increase was found exclusively in the younger patients.

Even though we thought that the earliest part of the 1953–1982 period was consistent with the later years with regard to collection of all MS patients, there was a need for a control disease. We have therefore studied the incidence of another demyelinating disease, acute Guillain-Barré syndrome (GBS). We did not observe any change in incidence of GBS in the period 1957–1982.

Furthermore we have studied the incidence and prevalence of MS in the county of Vestfold in eastern Norway. Identical methods of case collection and diagnostic criteria, as in the Hordaland study, were used. We found in this high-risk area for MS a consistent high prevalence in 1963 and 1983, and no change in incidence in the period 1953–1982. The period with the highest incidence was 1953–1962.

We have therefore considerable evidence for the hypothesis that the observed change in MS occurrence in the county of Hordaland is due to a real biological change in the population. This shows that MS is not a stable endemic disease, but is influenced by exogenous factors that can change in exposure to the population over time.

We have tried to examine this increasing occurrence of MS in Hordaland in more detail.

We found significantly lower prevalence and average annual incidence rates in the coastal area compared with the inland area. Especially in the earliest part of the study period, 1953–1982, the incidence was low in the coastal area. In the past 10 years the incidence on the coast has equaled the inland incidence and thus the increase for the whole period has been most dramatic in the coastal area.

The comparison of the occurrence of MS in urban (city of Bergen) and rural (Coastal and inland area) districts showed small variations. The increase in incidence seemed, however, to come first in the urban area and then about 10 years later in the rural area.

When studying each administrative unit in the county, the municipality, there were no great variations. To come closer to the question of clustering, we therefore performed a formal mathematical clustering analysis in both time and space. No significant space/time clustering was found. Although the methods available for such studies are insufficient, we conclude that the epidemiological pattern of MS is not compatible with known viral infections, like infectious hepatitis.

Furthermore, we have compared the epidemiology of the most suspected viral agent to cause MS, the measles virus, with the frequency of MS in our area. Data on measles infections in Hordaland are available for several decades. Measles has been stable for several years until about 1972, when a vaccination program was started. Thereafter the infection has decreased dramatically in frequency. The incidence of MS shows no correlation with the measles infection whether one postulates an incubation time of a few years or of up to 20 years. The epidemiological data thus show that measles at least cannot be the most important factor to cause MS.

Another postulated infectious agent to cause MS is canine distemper infections (CDV) in dogs. The latest epidemic in Hordaland occurred in the years 1958–1962 and no cases have been reported after 1963. Dogs have been vaccinated against CDV since 1950 and the epidemiological survey of diseases in animals is good. Furthermore, five of our MS patients were born after 1963. Thus a direct association between CDV and MS seems unlikely.

The more detailed evaluation of the increasing incidence of MS in western Norway also revealed another interesting finding. As you may remember, no increase in incidence was observed among patients with age at onset of 40 years or more. Because of this we wanted to study the epidemiology of chronic progressive MS known to have an older age at onset compared with the patients with a relapsing course of the disease with or without a later progressive phase. We used the criteria of Confavreux to divide the patients in to groups of remittent, remittent/progressive, and progressive MS. The remittent and remittent/progressive patients we called the remittent form of MS.

We found that the average annual number of cases of progressive MS was stable during the period 1953–1982. It varied from one to three new patients. However, the number of remittent MS patients per year increased during the same period from three to five patients in 1953–1960 to 10–14 patients in 1970–1982. The annual average age-adjusted incidence rates showed the same different trends in incidence for progressive and remittent MS.

Furthermore, these two forms of MS differed with regard to clinical findings as well as age at onset. Gait difficulties or motor weakness in one or more limbs were the presenting symptoms in nearly 90% of the patients with a chronic progressive

MS. In remitting MS paresthesias and motor weakness were the first symptoms in about one-third of the cases. Eye symptoms occurred in nearly 40% at onset of these patients.

The mean age at onset in progressive MS was 40.9 years and in remitting MS 30.2 years and the distribution by age is also quite different. For remittent MS the peak incidence is in the age groups 20–29 and 30–39 years. In chronic progressive MS the age at onset has no clear age peak, and most patients experience their first symptoms between 30 and 49 years of age.

Accordingly, we have found that remittent and chronic progressive MS have different epidemiology as well as different symptomatology and age at onset.

Aditionally a group in France has studied genetic markers in these two main forms of MS. In remittent MS they found an excess of HLA-B7 and -DR2. In progressive MS in contrast they found excess of -A1, -B8, and -DR3.

Together these data show that the two forms of MS may be caused by different etiological factors or even represent two different diseases.

In conclusion, the epidemiology of MS in the county of Hordaland has shown that multiple sclerosis is not a stable endemic disease, but more probably an acquired disease caused by an exogenous factor. The nature of this factor has to include a possibility to change over time in exposure to the population or more precisely the genetically suspectible part of the population. We also find it unlikely that measles or canine distemper can be this determining environmental factor.

The observed differences in epidemiological features of remitting and chronic progressive MS may represent true biological differences. These differences will then be important in terms of etiology and pathogenesis and may point to a different cause to these two forms of MS. Our results show that there is a need for detailed classification of subgroups of MS when trying to study both etiological factors and the pathogenesis of the disease.

Finally I will bring you some of our unpublished data on prognosis of MS evaluated by survival analysis. Our results will then be independent of prognostic evaluation based on disability rating.

We have in this study included all cases in the incidence studies in both Hordaland and Vestfold with onset after 1 January 1953. This material comprises 542 MS patients of whom 96 patients were dead on 1 January 1985.

To study the prognostic effect of different clinical variables simultaneously we applied Cox's proportional hazards regression model with incomplete data. The estimated 75% survival for the total MS population was 21 years.

Age at onset was the variable which had the strongest ability to predict the duration of disease. A poorer prognosis seemed to be linked to a high age at onset also when considering the natural shorter life expectancy of the old patients as in the normal population.

The symptom at onset of disease that predicted the best prognosis was motor weakness. This is in contrast to results from studies examining prognosis from disability rating where the ability to walk has a major negative influence on the rating. The symptom at onset predicting a poor outcome was vertigo.

I have already described epidemiological differences between remittent and chronic progressive MS. The survival analysis showed the poorest prognosis for progressive MS regardless of age at onset.

Furthermore, there was a tendency to a more unfavorable prognosis for men. The high- or low-prevalence area did not differ with regard to survival, and there was no evidence of improved survival over time in the studied 30-year period.

In summary, the combination of high age at onset of MS, a chronic progressive course of the disease, and vertigo as first symptom in a male MS patient, indicate an unfavorable prognosis. On the other hand, the combination of a female patient with low age at onset, remittent course of the disease, and motor weakness as the first symptom indicates a long survival.

Multiple Sclerosis: Stochastic Models of Prognosis

C. Confavreux,[1] and *C. Wolfson*[2]

1 Service de Neurologie, Hopital Neurologique, Lyon, France
2 Department of Epidemiology and Biostatistics, McGill University and Montreal Neurological Institute, Montreal, Quebec, Canada

Introduction

Prior research concerning the prognosis of multiple sclerosis (MS) has produced conflicting results. Several explanations can be given. First, it is possible that MS has a really unpredictable course. Second, comparison of the methodological approaches taken by several authors shows basic differences resulting in the impossibility of comparing these results. For example, there ist not even agreement about what constitutes a prognostic outcome. Some research relies on relapses. Other is based upon a progression score, the definition of which is not even uniform. Third, the methods used to date are still rather primitive. On one hand, the disease activity is expressed in a monoparametric way, for example relapse rate or progression index or interval to onset of disability. No presently published approach allows a multiparametric global "vision" of the disease. On the other hand, at the stage of testing potential prognostic variables, these are studied independently. When they are combined with each other, this usually results in too fine a stratification and a loss of power of the statistical tests. The interrelationship between variables may be complex and not simply additional. The univariate approach may be somewhat misleading. Lastly, quantitative variables such as the age at the onset of the disease are studied discontinuously, for example before 30 and after this age, and not on a continuous mode. It is unrealistic to suppose that something suddenly occurs at 30 years of age to alter the prognosis significantly.

This prompted us to follow a more adapted approach and to elaborate mathematical models of MS course. There are two kinds of mathematical models: deterministic and probabilistic. The first approach has already been used by Fog and Linneman in 1970 [1] and Patzold and Pocklington in 1982 [2], with very close results. In these studies, each patient was given, at each examination, a neurological score. The course of the disease was then reflected by plotting successive scores against time. Regression analysis was employed to choose the best-fitting curve from competitive regression curves, i. e., linear, parabolic, hyperbolic, and polynomial.

This approach gives a personalized description of the disease for each patient but no global view of the disease for a set of patients. It requires a substantial follow-up for each patient before ascertaining the "best" curve: to some extent, the predicted prognosis is an "a posteriori" one. The disease course is reflected by the neurological score as the only marker and this may be disputed.

Following our 1980 publication in *Brain* [3] about the course and prognosis of MS, we have had the opportunity to collaborate with Christina Wolfson from the Department of biostatistics and epidemiology, McGill University, Montreal. Two probabilistic or stochastic models of the course of MS have been elaborated [4, 5]. They rely upon probability calculations from our cohort of patients.

The Markov Model

The first model is of the Markov type and is the simpler [4]. The first assumption is that, at any point in time, a MS patient may be classified into one of several disease states. Throughout the course of the disease, the patient moves between these states. Each of these movements represents a "transition." The second assumption is for the sake of simplification and inherent to Markovian models: the probability of each state transition depends upon the present and the next state but is independent of the former states. This can be considered as an "amnestic model."

Informations given by the Markov approach are of two kinds but remain independent. The first is a list of the transition probabilities between states. This is presented in a transition matrix. This information is given without reference to time. The second is a list of transition time distributions. It reflects the time factor of the transitions.

Concretely, we have classified each MS patient in five different disease states as shown in Fig. 1: R=pure relapses, without sequelae; RS=relapses with sequelae; P=progression; D=deceased; C=censored, i.e., patients lost for the follow-up before death or the end of the survey. In MS, these transitions follow logical sequences only.

This model has been applied to our population of 278 definite and probable MS cases from Lyon. The first step was to calculate, for each kind of transition, the

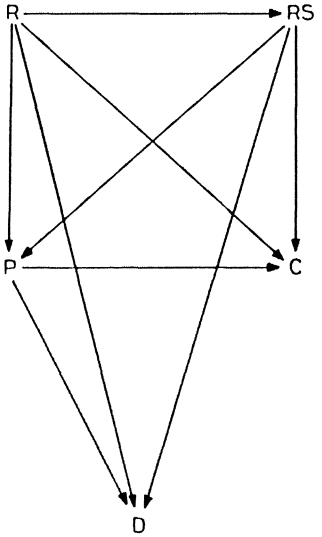

Fig. 1. State diagram

Table 1. Observed clinical course

Course type	n	%
R only	94	32
R to RS	36	13
R to P	56	20
RS only	25	9
RS to P	17	6
R to RS to P	11	4
Progressive from onset	39	14
Total	278	100

Table 2. Transition matrix for all 278 patients (693 transitions)

Source	Destination				
	R	RS	P	D	C
R	0.689 (298)	0.127 (55)	0.113 (49)	0 (0)	0.069 (30)
RS	–	0.636 (119)	0.176 (33)	0.005 (1)	0.182 (34)
P	–	–		0.149 (14)	0.811 (60)

Number of transitions in parentheses

observed number in the population. In Table 1, the patients were distributed according to their disease course. The total number of R to R transitions was calculated from the patients whose disease was of an R only, or R to RS, or R to P, or R to RS to P type. In a similar way, the number of R to RS transitions was calculated from the patients with an R to RS, or R to RS to P type.

The second step was to present these results in a table and to calculate, for each disease state – R, RS, P – the frequency of transition to the same state or to others (Table 2). This is a transition matrix. Let us look at the first row of the table. From state R, a total of 298+55+49+0+30=432 transitions have been observed. Conditional upon being in state R, the probability of making a transition to states R, RS, P, D, and C are 69%, 13%, 11%, 0% and 7%, respectively. From state RS, the probability of making a transition to states RS, P, D, and C is 64%, 18%, 0% and 18%, respectively.

The last column in the matrix displays the censoring probabilities. This provides no information concerning the natural history of MS but needs to be considered when drawing conclusions from the matrix. For example in row one, the probability of being censored following state R ist 7%. Assuming that these 7% of patients, followed longer, would have made transitions to R, RS, P, and D with a breakdown of 298, 55, 49 and 0, respectively, it is possible to recalculate the transition probabilities under this assumption. This is displayed in the transition matrix

Table 3. Transition matrix adjusted for censoring

Source	Destination			
	R	RS	P	D
R	0.741 (320)	0.136 (59)	0.123 (53)	0 (0)
RS	–	0.775 (146)	0.214 (40)	0.006 (1)
P	–	–	0.810 (60)	0.189 (14)

Number of transitions in parentheses

Table 4. Transition matrices for age at onset

Source	Destination			
	R	RS	P	D
a Age at onset less than 35 years (625 transitions)				
R	0.759 (306)	0.134 (54)	0.107 (43)	0 (0)
RS	–	0.791 (129)	0.202 (33)	0.006 (1)
P	–		0.779 (46)	0.220 (13)
b Age at onset 35 years and over (68 transitions)				
R	0.448 (13)	0.172 (5)	0.379 (11)	0 (0)
RS	–	0.667 (16	0.333 (8)	0 (0)
P	–	–	0.933 (14)	0.067 (1)

Number of transitions in parentheses

adjusted for censoring (Table 3). Conditional upon being in state R, the estimated transition probabilities to states R, RS, P, and D become 74%, 14%, 12%, and 0% respectively.

At this stage of the analysis, the effect of five potential prognostic variables on the transition probabilities was evaluated: age at onset, sex, optic neuritis versus other symptom at onset, mono-versus polysymptomatic onset and interval between the first two attacks or, if preferred, length of the first remission.

The age at onset and the sex were the only variables to show a significant effect on transition probabilities. Table 4 displays the transition matrices for patients with age at onset under 35 years and 35 years and over. Conditional upon being in state R, the probability of transition to state R is significantly higher and the probabilities of transition to states RS and P are significantly lower for the younger patients. Similarly, conditional upon being in state RS, the probability of transition to state RS is higher and the probability of transition to state P lower for younger patients. This argues for a favorable effect of younger age at onset on the outcome. Table 5 displays the results for males and females. Here males were found to be less likely to make an R to P transition (8% vs. 15%) but more likely to make a transition from P to D (33% vs. 11%) than females. These results are difficult to reconcile.

Table 5. Transition matrices for males and females

Source	Destination			
	R	RS	P	D
a Males (288 transitions)				
R	0.760 (132)	0.156 (27)	0.080 (14)	0.00 (0)
RS	–	0.750 (66)	0.250 (22)	0.000 (0)
P	–	–	0.667 (18)	0.333 (9)
b Females (405 transitions)				
R	0.726 (188)	0.123 (32)	0.150 (39)	0.000 (0)
RS	–	0.808 (80)	0.182 (18)	0.010 (1)
P	–	–	0.894 (42)	0.106 (5)

Number of transitions in parentheses

In a second stage, the time spent making transitions with the effect of potential prognostic variables was also studied. In this approach, indication of a more severe prognosis in patients with older age at onset was found again.

As can be seen, this model has several advantages: the first one is its simplicity. The transition matrix display of the results is suggestive and meaningful for the clinician. In addition, by mixing various disease states, it accounts for a global description of the disease. On the other hand, this model possesses some shortcomings. Information about transition probabilities and times spent in transition are given separately, in an uncoupled way. This is not very convenient. Prognostic variables must be considered independently by a univariate analysis with the drawbacks already mentioned. Quantitative variables must be studied discontinuously. This prompted us to generate a more appropriate model.

The Stochastic Survival Model

This model represents an extension of the survival analysis [5]. Concretely, for any given state at the onset of the disease and for each year of disease duration, the patients were displayed in the disease states described above. For each kind of transition, a curve of the observed transition frequency according to the disease duration was traced. For example, for patients in state R at the onset, there are four different curves, corresponding to the R to R, R to RS, R to P, and R to C transitions.

The following step was to use the stochastic survival formula. It is an original formula, typical of the model and quite complex. It comprises several parameters which must be adjusted to the observed data. We may consider it as a "black box." Let us see together what is there to put in it, what is there in it, and what can we get from it.

What is there to put in the "black box"? Consider, for example, the R to RS transition. A first factor, $h_{R.RS}$, is the underlying hazard. It is specific for the

transition under study and it is assumed to be the same for any individual. It is then combined in the hazard function, which takes into account the covariates, i.e., the potential prognostic variables:

$$h_{R.RS\,i}=h_{R.RS}\exp(\beta X_i)$$

βX_i represents the covariate factor for the patient *i*. β is constant, specific for the transition and the covariate under study. X_i is the actual value of the covariate for the patient *i*. As can be seen, $h_{R.RS\,i}$ is constant over time, inherently modeled by the observed values of the prognostic variables, and specifically adjusted for the patient *i*. Lastly, the probability of R to RS transition for a patient *i*, according to disease duration *t*, can be expressed as follows:

$$P_{R.RS\,i}(t)=\int_o^t [\exp\{-(h_{R.RS\,i}+h_{R.Pl\,i}+h_{R.C\,i})\,t'\}\,h_{R.RS\,i}]$$

$$[\times \exp\{-(h_{RS.Pi}+h_{RS.Ci})\,(t-t')\}\,dt'].$$

In the stochastic survival model, the probability of each kind of transition is dependent on the others.

What is there in the "black box"? The underlying hazards for the various transitions have been estimated (Table 6). The hazard functions were also estimated, following the analysis of the five potential prognostic variables already defined. Only three transitions were significantly affected by at least one of these variables. Results may be summarized by the following three equations:

$$h_{R.RS\,i} = 0.046 \exp\{-0.153\ (1\text{st rem.})\}$$
$$h_{R.P\,i} = 0.050 \exp\{-0.622\ (\text{pluri}\Sigma) - 0.169\ (1\text{st rem.})\}$$
$$h_{RS.P\,i} = 0.017 \exp\{1.03\ (\text{male}) + 0.862\ (\text{pluri}\Sigma) - 0.244\ (1\text{st rem.})\}$$

What can we get from the "black box"? Results of calculations based upon the three preceding equations are given in Tables 7–9. The R to RS transition probability is negatively affected by the length of the first remission. The longer the remission, the lower the probability of transition from R to RS. Table 7 displays results of calculations for various values of the covariate. From the second equation (Table 8), is follows that the R to P transition probability is negatively affected by the length of the first remission and a plurisymptomatic onset. Table 8 displays results of calculations for several values of these two covariates. Lastly, the RS to P

Table 6. Estimates of underlying hazards

Transition	Parameter	Estimate
R to RS	$h_{R.RS}$	0.046
R to P	$h_{R.P}$	0.050
R to C	$h_{R.C}$	0.023
RS to P	$h_{RS.P}$	0.017
P to D	$h_{P.D}$	0.009
P to C	$h_{P.C}$	0.066
RS to C	$h_{RS.C}$	0.052

Table 7. Numerical examples of changes in hazard (R to RS) due to length of first remission $h_{R.RSi}=0.046 \exp(-0.153$ (1st rem))

Length of first remission	Estimated hazard
2 months	0.045
6 months	0.043
1 year	0.039
2 years	0.033
3 years	0.029
5 years	0.021
10 years	0.010

Table 8. Numerical example of changes in hazard (R to P) due to prognostic variables $h_{R.Pi}=0.050 \exp(-0.169$ (rem.1)-0.622 (pluriΣ))

Length of first remission	Estimated hazard Monosymptomatic onset	polysymptomatic onset
2 months	0.048	0.026
6 months	0.046	0.024
1 year	0.042	0.023
2 years	0.036	0.019
3 years	0.030	0.016
5 years	0.021	0.011
10 years	0.009	0.003

Table 9. Numerical example of changes in hazard (RS to P) due to prognostic variables $h_{RS.Pi}=0.017$ exp (-0.244 (rem.1)$+0.862$ (pluriΣ)$+1.03$(male))

Length of first remission	Estimated hazard monosymptomatic females	polysymptomatic females	monosymptomatic males	polysymptomatic males
2 months	0.014	0.034	0.040	0.095
6 months	0.013	0.031	0.037	0.088
1 year	0.012	0.028	0.033	0.078
2 years	0.009	0.022	0.026	0.061
3 years	0.007	0.017	0.020	0.048
5 years	0.004	0.010	0.012	0.029
10 years	0.001	0.003	0.004	0.009

transition probability is affected negatively by the length of the first remission but positively by a plurisymptomatic onset and by male sex. Examples of results are shown in Table 9.

How may we summarize these results? The salient feature is that the length of the first remission significantly affected the R to RS, R to P, and RS to P transitions in a similar negative way. These three transitions define the movement of the patient to a worse state. It may be inferred that the longer the remission, the better the

prognosis. The effect of plurisymptomatic onset was somewhat confusing: negative for the R to P transition, but positive for the RS to P transition. In this approach, sex affected the RS to P transition only. The two other potential prognostic variables studied, i.e., age at onset and optic neuritis at onset, did not affect the transition probabilities.

In fact, the more informative way for presenting results of the stochastic survival model was to plot the changes in probabilities over a range of t (Fig. 2). The curves can be assessed for the total set of patients with a given state at onset. It may also be

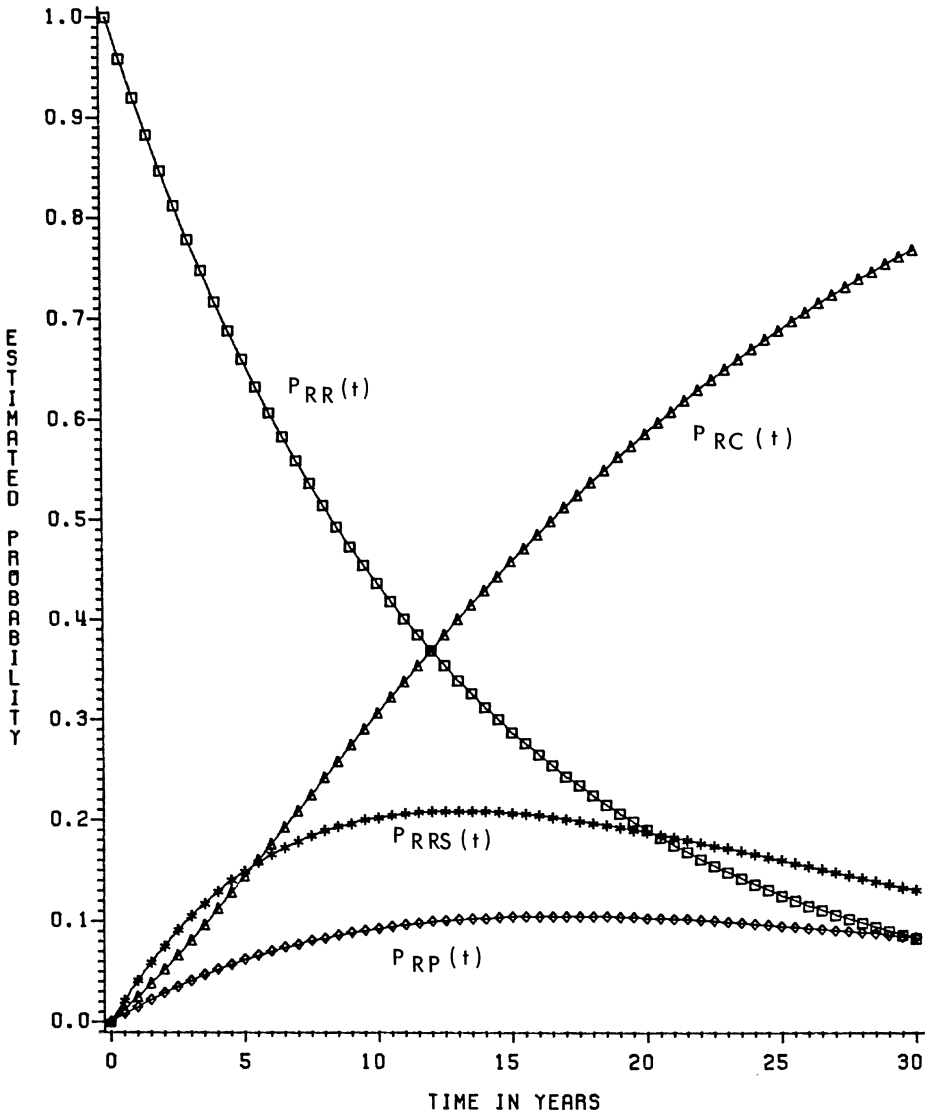

Fig. 2. Transition probabilities for patients in state R

assessed for any covariate value, i.e., males vs. females or mono-vs. plurisymptomatic onset, and, finally, be adjusted to any given patient.

The advantages of the stochastic survival model are numerous. Despite complex theoretical grounds, it provides explicit and convenient results, a synthetic multiparametric display of the disease and estimations for transition probabilities and times for transitions in a combined way. It incorporates the variables into the model altogether. The analysis can be multivariate. It is then possible, for each type of transition, to evaluate the effect of covariates, individually and in the presence of the others. Quantitative variables such as the age at onset and the length of the first remission are incorporated in the model as continuous variables, with their actual value. Lastly, and this is likely to be one of the major advantages of the method, estimated transition probabilities can be precisely adjusted to the observed parameters step after step in an individual patient.

Conclusion

Here is the present state of our stochastic approach of MS prognosis. The Markov model is simple but quite rigid. The survival model is more sophisticated but much more flexible, adjustable to any given patient. Note that results of these two approaches are not quite concordant. For example, the prognostic effect of age at onset is clear in the Markov analysis and absent in the survival model. The reverse is true for the length of the first remission. Such discrepancies are difficult to reconcile. Anyway, this demonstrates the methodology effect on the prognosis analysis.

What about the future? Our stochastic approach still needs refinements. First, it is probable that an alternative definition of the disease states may be informative. A distinction between none, moderate, and severe disability stages and death may be suggested. Second, an adjustment for censoring is to be found in our survival model. The increase in the proportion of patients lost to follow-up as disease is going on limits the pertinence of the presently designed curves. Third, new potential prognostic variables have to be tested and, eventually, incorporated in the survival model.

Our present results also need to be validated. This work relies upon a population of MS patients surveyed in 1976 in Lyon. A new survey is in progress for the same patients. The purpose is to compare the estimated prognosis and the actual outcome. The cohort of MS patients newly diagnosed in the Department since 1976 will also be used as control.

If these two conditions – refinement and validation – are satisfied, the concrete usefulness of this stochastic approach may be of prime importance. An universal tool for testing potential prognostic variables and treatments may become available. It is currently held that immunosuppressants, for being effective in MS, need to be administered as soon as possible after the onset of the disease and under an aggressive regimen. By providing a personalized prognosis for each patient, the survival model may help to appropriately select patients for trials.

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Studies of the Natural History of Multiple Sclerosis

B. Weinshenker, B. Bass, and *G. C. Ebers*

Dept. of Clinical Neurological Sciences, Division of Neurology, University Hospital, London (Ont.), Canada

Investigators conducting clinical therapeutic trials in patients with multiple sclerosis (MS) are often surprised at the favorable outcome of their control groups [1, 2]. Numerous ongoing trials address the treatment of patients with progressive MS. While progressive disease has been widely shown to herald a poor prognosis [3–5], precise data are not available on the expected rate of deterioration, once chronic progression has been indentified. Many lessons are to be learned by studying the natural history of MS, such as the decrease in attack rate with increasing duration of disease regardless of outcome (see below). This finding has great implications for selection of patients and endpoints considered in clinical trials. While concurrent controls are essential in therapeutic trials, these and other considerations demonstrate the need for natural history information.

Methodologic Considerations

The principal difficulties in defining the outcome of MS are the inability to define an "inception cohort" [6] (i. e., a group of patients followed from onset of disease) and the highly unpredictable and protracted course over which the disease evolves [7]. The obvious approach is a prospective study of an inception cohort over a prolonged period. However, patients often do not seek medical advice with their first symptoms, or referral is not made to centers where comprehensive longitudinal assessments are conducted. Of 1099 consecutive patients that were registered at the MS Clinic at University Hospital, London, Canada, between 1972 and 1984, only 197 (18%) were seen from this first unequivocal symptom. Furthermore, the most important outcome of MS, permanent neurological disability, evolves slowly over decades [7]. The few prospective studies [8–11] in existence have provided follow-up of less than 1 decade, and in some cases have used scores that show considerable fluctuation over time [8] and have an unclear relationship to fixed disability.

Retrospective studies have thus been necessary. Their value is highly dependent on their adherence to the methodologic requirements outlined in Table 1. As tertiary referral centers typically evaluate severe or unusual cases of MS, patients are thereby selected for referral based on factors which may influence their outcome. Clinic- and hospital-based studies are less reliable than geographically based studies in which all patients in a given area are included. Few centers have been capable of such studies which require a pattern of practice that permits such

Table 1. Studies of the natural history of MS – methodologic requirements

1. Complete, unbiased ascertainment
2. Adequate population size
3. Short interval from onset to ascertainment
4. Prolonged follow-up
5. Follow-up complete
Assure patients are not lost for reasons arising from their outcome (i.e. death, institutionalization)
6. Standardized assessment and recording of data
7. Clinically relevant endpoints with adequate sensitivity
8. Agents which potentially alter natural history should not be used

centralized evaluation and follow-up of MS patients. Poser [12] has documented the difference in outcome between a population-based and hospital-based practice, confirming the expected bias toward patients with rapid progression in hospital-based series.

The variability documented by virtually every investigator necessitates that large enough patient samples be studied over prolonged periods to permit significant statements regarding outcome. Often patients are lost to follow-up for reasons directly arising from the outcome of their illness, i. e., institutionalization or death. The lack of a specific treatment modality that requires ongoing monitoring has often resulted in patients becoming lost to follow-up after a definitive diagnosis has been reached.

Retrospective studies can be classified either as longitudinal, in which patients are followed over time by the investigator, or cross-sectional, in which the status of patients in a clinic, geographic area, or other identifiable group is determined at a given point of time and indexed to the onset of symptoms. The former approach is attractive, in that statements regarding outcome are not based on a single examination and generally convey permanent disability. The latter approach has the disadvantage of interpolating the clinical course from the time of onset to the point of assessment as a linear variable or "progression index" [3, 4]. The number of patients, however, tends to be higher in cross-sectional than in longitudinal studies (see Table 2).

Finally, natural history studies require a scale that provides significant sensitivity, yet avoids the fluctuation over time which can be explained by the fluctuation in the symptoms of Ms due to fatigue, fever, and other factors not reflecting permanent disability. Our impression has been that the Kurtzke disability status (KDS) scale [13] is the most useful index. We have demonstrated that the time to reach KDS 3 has weak predictive value as to the time to reach higher levels of disability (see below). This argues in favor of nonlinearity of the disease, if not of the Kurtzke scale itself. Thus, an additional advantage in longitudinal studies of MS is the recording of the status of patients at various points along their course, thereby avoiding the assumption that progression is linear.

Table 2. Existing studies of natural history in MS: methodologic considerations

Investigator	Location	Year	Population size	Population[a] based	Longitudinal follow-up	Duration of follow-up (years)	Duration of disease[b,c] (years)
Thompson [20]	Dublin	1986	290	—	—	—	11.4
Visscher, Clark, Detels [9–11]	Los Angeles County: Washington and Pierce Counties, United States	1982–84	834–941	+	+	10	10–20
Verjans [4]	Belgium	1983	200	—	—	—	2–48
Patzold [8]	Hannover, FRG	1982	102	—	+	3.5	
Poser [3]	Göttingen, FRG	1982	2058	—	+ (only 221 patients)		11
Broman [17]	Gothenburg, Sweden	1981	312	+	+		13–27
Confavreux [21]	Lyon, France	1980	349	+	+	9	
Kurtzke [7]	United States	1977	527	+ (Army discharges)	—	—	10–21
Percy [22]	Rochester, MN, United States	1971	67	—	—		
Gudmundsson [23]	Iceland	1971	104	+	+	14–23	24
Fog [16]	Copenhagen, Denmark	1970	73	—	+	10	20
Panelius [18]	Finland	1969	146	+	—	—	0–39
Leibowitz [24]	Israel	1964	266	+	—	—	11.5
McAlpine [25]	England	1961	241	—	+	>10	10–29
McAlpine [19]	England	1952	675	—	—	—	

a According to author, supported or unsupported, most cases in the study area were identified
b Mean duration or range is given, where this could be determined
c At the conclusion of follow-up

Of the available studies, that of Kurtzke [7] is of particular interest and value because of the following considerations: large population (n=527); prolonged tracing of patients (although not longitudinal follow-up in a strict sense as the patients were not followed by the investigators); relative completeness of follow-up (disability data are available on 62% of patients at 15 years from onset); nearly complete ascertainment of cases is likely because patients received compensation for their disease; and finally short onset to ascertainment interval. The major drawbacks to the study are the potential difficulties in using traditional medical records to extract information about permanent disability, and the unrepresentative nature of the cohort (World War II era veterans of the United States army).

A brief summary of methodologic aspects of previous studies of natural history is given in Table 2. For a summary and comparison of results from these series, the reader is referred to our recent review [14].

Survey of the London MS Clinic

With these considerations in mind, we undertook a study of our patients at the MS Clinic in London, Canada. We felt that our study was unique for the following reasons:

1. A high degree of ascertainment of cases in the community. A recent prevalence study of MS in Middlesex County has shown that 90% of cases of MS are registered with our clinic [15].
2. A large population of patients. London is the major referral center for cases of MS in Ontario south of Toronto.
3. Longitudinal follow-up. We traced those patients lost to follow-up so as to ascertain their status.
4. Carefully recorded details of disability at each outpatient visit, so that the time to reach successive levels of disability on the KDS scale was known. We, in fact, collected data with the intention of generating a data base appropriate for this study.

Our objectives were the following:

1. To describe the characteristics of a large clinic population of MS patients including age of onset, symptomatology, clinical course (replapsing remitting vs. progressive), and attack frequency, among others.
2. To provide precise data on the percentage of patients who had progressed beyond given degrees of disability according to the KDS scale indexed both from onset of disease and from identification of a progressive course.
3. To determine if the early tempo of the disease predicts the development of more advanced disability and the rate at which it is reached. The early course was assessed by the rate at which low levels of disability were reached, and by the attack frequency in the first 2 years.
4. To determine if various demographic and clinical characteristics such as age at onset, sex, initial symptomatology, and positive family history predict outcome.

We analyzed 1099 consecutive patients seen for variable durations of follow-up between 1972 and 1984. We identified 197 patients who were seen from the apparent

Table 3. London MS clinic – characteristics of patient subgroups

	Total population		Middlesex county		Seen at onset	
	N	(%)	N	(%)	N	(%)
Number of patients	1099		196		197	
Number of males	377	34.3	63	32.1	62	31.5
Number of females	722	65.7	133	67.9	135	68.5
Sex ratio (M/F)	0.52		0.47		0.46	
Diagnostic certainty						
Probable	916	83.3	176	89.8	162	82.2
Possible	183	16.4	20	10.2	35	17.7
Disease duration (years)						
Mean	11.91		12.96		4.09	
Median	10		12		3	

onset of their disease, and these were also separately analyzed as an "inception cohort." Furthermore, 196 patients were resident in Middlesex County at onset of their disease, and were analyzed separately as a population-based sample based on our high ascertainment of cases in this county. The key features of our patient groups are shown in Table 3.

The nature of the clinical course (relapsing remitting, relapsing progressive, chronically progressive, or combinations thereof) is shown in Table 4. Assuming that our Middlesex County population reflects MS patients at large, 35% of patients at any given time have purely relapsing remitting disease, whereas 53% are developing at least some degree of permanent sequelae. The distribution is quite similar for our total clinic population. The discrepancy between prospectively and retrospectively obtained information, noted by others [8, 16], is evident in the difference between the percentage of patients chronically progressive from onset between the "seen at onset" group and the other two groups. Patients with progressive disease may be less inclined to remember earlier attacks from which there was recovery, and, consequently, the number of patients felt to have chroni-

Table 4. London MS clinic – clinical course

	Total population		Middlesex county		Seen at onset	
	N	%	N	%	N	%
RR only	407	37.0	69	35.2	138	70.0
RR-RP	109	9.9	14	7.1	19	9.6
RR-CP	196	17.8	36	18.3	10	5.0
RP only	163	14.8	32	16.3	14	7.1
CP only	213	19.3	22	11.2	15	7.6
Unclassified	11	10.1	23	11.7	1	0.5
	1099		196		197	

RR, Relapsing remitting; RP, Relapsing progressive; CP, Chronically progressive

Table 5. London MS clinic – attack frequency in the first 2 years (mean±SE)

	Total population	Middlesex County	Seen at Onset
Year 1			
RR	1.57±0.05	1.57±0.11	1.80±0.10
RR-RP or RR-CP	1.28±0.05	1.41±0.12	2.08±0.22
RP	1.42±0.06	1.22±0.12	2.33±0.37
Year 2			
RR	0.35±0.04	0.44±0.32	0.55±0.09
RR-RP or RR-CP	0.40±0.06	0.45±0.40	1.08±0.22
RP	0.48±0.07	0.40±0.35	1.43±0.37

RR, Relapsing remitting; RP, Relapsing progressive; CP, Chronically progressive

cally progressive disease from onset is significantly smaller in the seen at onset group.

The attack frequency is shown in Table 5. We analyzed attack frequency in the first 2 years of disease. Previous studies which have averaged the number of attacks over longer durations fail to take into account the documented decrease in the number of attacks with increasing duration [8, 17–19], which is, apparently, independent of the rate at which disability develops [7, 8, 16]. The differences between prospective and retrospective data are again evident, the attack rate being significantly greater in patients followed from onset.

The extent of disability defined by the KDS scale indexed to duration of disease is shown in Table 6. The values shown here are for our entire patient population and the Middlesex County subgroup. Our data on disability at durations of disease greater than 12 years is potentially biased in favor of more benign cases, as patients who died or were institutionalized before our clinic was established were unlikely to

Table 6. London MS clinic – progression of disability indexed to duration from onset

Duration (years)	Patients Alive		Percentage of patients at or beyond					
			KDS 3		KDS 6		KDS 8	
	TP	MC	TP	MC	TP	MC	TP	MC
2	1032	193	18	16	6	3	0	0
5	863	165	33	29	14	12	1	1
10	569	115	45	45	24	22	3	2
15	350	76	51	57	32	36	4	1
20	203	38	57	66	37	45	5	5
25	113	19	66	68	40	47	4	1
30	49	8	69	50	41	38	8	13
40	10	2	80	100	50	50	0	0

KDS, Kurtzke disability status; TP, total population; MC, Middlesex county

come to our attention. Data from the seen at onset subgroup provide similar results, though follow-up is only available for 12 years from onset at most, and the mean duration of follow-up is 4 years.

We have studied the predictive value of the early rate at which disability develops in MS. We explored whether the time of reach KDS 3 predicts the time to progress beyond KDS 3 to KDS 6, KDS 8, and KDS 10. Using a regression analysis, the effect of time to KDS 3 in predicting the time to reach more advanced disability was small. This again demonstrates the unpredictability of this disease based on the early clinical course. Other predictive variables presently being explored include attack frequency, time from first to second attack, and clinical features that can be determined at diagnosis, including initial symptoms, age at onset, and sex. Ultimately, predictive models expressing the risk of reaching advanced disability, as well as the rate at which it is reached, may be generated. Any such model would of course have to be prospectively validated. To date, numerous claims have been made about the influence on outcome of such parameters at age of onset, sex, and initial presentation [14]. Widespread agreement is lacking about any prognostic indicator.

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Space-Time Aggregation in a Small Village in Italy: Buti (Pisa)*

D. Caputo,[1] *P. Ferrante,*[2] *F. Parenti,*[3] *L. Mendozzi,*[1] and *C. L. Cazzullo*[1]

1 Centro Studi Sclerosi Multipla Università degli Studi di Milano Ospedale di Gallarate, Gallarate, Italy
2 Istituto di Virologia Università degli Studi di Milano, Milano Italy
3 U.S.L. no. 16 Pontedera (PI), Italy

Introduction

Epidemiological studies have shown that there are 50 important factors in the consideration of multiple sclerosis (MS) as an exogenous disease dependent on environmental factors. The distribution of the disease is not homogenous everywhere in the world, but areas with high, middle, and low risk do exist. Furthermore, studies on emigrating populations have demonstrated that birthplace is more important than racial factors in producing the risk of being affected by MS. In fact, in both South Africa and Israel it has been observed that the course of MS begins during youth before the age of 15 years [1–3]. On the other hand, different aggregations in space and in time have been pointed out for patients affected by MS. In Table 1 the most important among these aggregations and the suggested correlations are listed. One investigation has hypothesized that one or two different

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Table 1. Main MS space-time aggregations in different areas of the world with proposed possible correlations

Localities	Prevalence (per 100 000)	Authors
Duxbury, Massachusetts	163	Deacon 1959 [4]
Mansfield, Massachusetts	309	Eastman 1973 [5][a]
Orkney, Islands	184	Poskanzer 1980 [6][b]
Shetland, Islands	141	Poskanzer 1980 [6]
Faroe, Islands	64	Kurtzke 1979 [7][b]
Iceland	–	Kurtzke 1981 [8][b]
Sitka, Alaska	–	Cook 1985 [9][b]
Hordaland	75.5	Larsen 1984 [10][c]
Macomer, Sardinia	–	Carreras 1985 [11][c]
Leszno, Poland	130.93	Wender 1985 [12][c]
Guiezno, Poland	122.83	Wender 1985 [12][c]

[a] The appearance of MS preceded 23 years previously by a typhoid fever epidemic
[b] The appearance of MS was preceded by distemper epidemics in dogs
[c] The appearance of MS was attributed to the change in population life habits because of industry installations

environmental factors may participate, at different times, in producing the risk of being affected by MS: one of these factors operates about 20 years before the onset of the disease and the same or another one operates the year before the outcome of the first disease symptom [13].

Materials and Methods

Throughout the time we have been involved in MS research, we have observed a group of subjects with MS from Buti, a small village in the district of Pisa, in Tuscany. Buti commune is subdivided into four communities: Buti itself and Cascine di Buti are the most important, and La Croce and Castel di Nocco are two tiny hamlets. Buti commune covers an area of 23.08 km^2 and it has a fairly constant number of inhabitants: in fact according to the 1951 census there were 4771 inhabitants, 5111 in 1961, 5168 in 1971, and 5253 in 1980. Buti commune is placed in a closed valley surrounded by the steep slopes of Monti Pisani, whose vegetation consists of chestnuts, olives, and pines. Buti itself is placed in the inner valley, whereas Cascine di Buti extends toward the plain. Buti is a very ancient village: the first historical information dating back to 960 A.C. Through the ages farming has been the main economy, namely olive oil and corn. For 20 years olive growing has been substituted by handicraft (Buti baskets). Figures 1 and 2 show the employment trends in Buti commune from 1948 to 1980 and the number of employed subjects.

The search for subjects suffering from MS was made easy by the contribution of the medical officers, and by the courtesy of the President of USL (Local Sanitary Unit) No. 16, of Prof. Fabiani, Head Physician of the Department of Neurology, and of the Director of Pontedera Hospital, who allowed us to use the archives of the hospital.

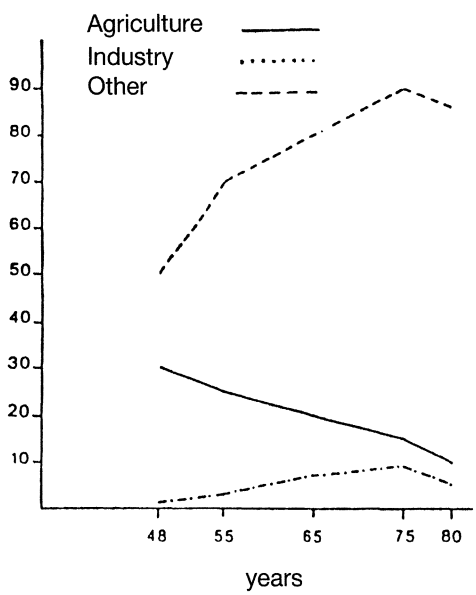

Fig. 1. Employment trends in Buti commune 1948–1980

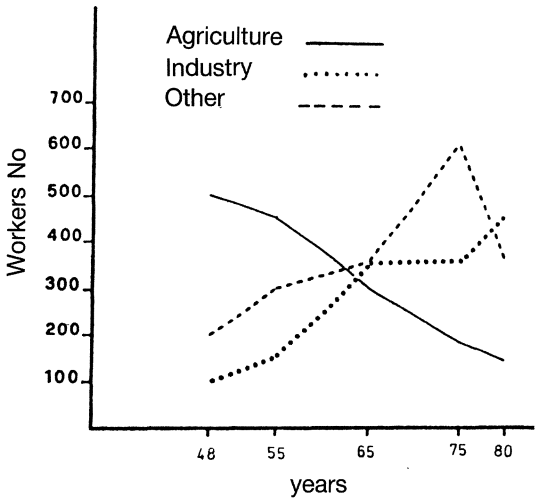

Fig. 2. Employment trends in But commune 1948–1980

We carried out a door-to-door investigation of all the villagers and also examined all neurological patients and subjects with suspected neurological pathology. Furthermore, thanks to the contribution of the municipality and of the parish we carried out the following:

1. Analysis of causes of death and Public Health registers during the period 1933/1960
2. Collection of information about military activities during World War II from local military authorities and from witnesses and news issued by specialized reviews
3. Examination of data about possible zoonoses from local veterinary surgeons and directly from breeders
4. Small case-control research to outline the presence and frequency of distemper cases

The MS cases were classified according to the McDonald and Halliday criteria [14].

General Data

1. Feeding: typical Mediterranean food with great use of olive oil. Meats mainly used are rabbit, chicken, pork, and beef. Great use is made of wine.
2. Most frequent pathologies: acute rheumatic fever, chronic and asthmatic bronchitis, liver diseases, diabetes, high blood pressure, and cardiovascular disorders.

Results

We observed four cases of definite MS (three males and one female) and one case of suspected MS (female). They all lived in Buti itself, whereas one case of definite MS

(female), who had immigrated to Buti when the disease had already manifested itself, was observed in the hamlets. One case of (ALS)* was found. Therefore we applied our attention to Buti itself and to the subjects affected by ascertained MS. Tables 2 and 3 show the clinical characteristics of the subjects. Table 4 illustrates the prevalence indexes in the years 1951, 1961, 1971, and 1980 in Buti itself and in other communities.

Table 2. Clinical aspects in observed cases

Pt. No.	Sex	Age (years)	Age of onset (years)	First symptom	Course of the disease	Diagnosis
1. B.L.	F	50	30	Amaurosis	Remitting	Definite MS
2. L.M.	M	47	27	Diplopia Weakness of right leg	Remitting	Definite MS
3. C.G.	M	46	26	Paresthesia	Progressive	Definite MS
4. B.E.	F	46	14	Weakness of lower extremities	Progressive	Possible MS
5. L.E.	M	45	25	Cerebellar ataxia	Remitting	Definite MS
6. M.M.	F	28	14	Weakness of right arm	Remitting	Definite MS
7. B.D.	M	33	32	Dysarthria Dysphagia	Progressive	ASL

Table 3. Other data on patients with definite and possible MS

Pt. No.	Date of birth	Place of birth	Residence	Year of onset
1. B.L.	20. 6. 30	Buti itself	In Buti from birth	1960
2. L.M.	18. 7. 33	Buti itself	In Buti from birth	1960
3. C.G.	15. 1. 34	Buti itself	In Buti from birth	1960
4. B.E.	19. 3. 34	Buti itself	In Buti from birth	1948
5. L.E.	9. 8. 35	Buti itself	In Buti from birth	1960
6. M.M.	30. 7. 53	Bientina	In Cascine since 1974	1966
7. B.D.	5. 4. 47	Capannori	In Buti since 1970	1980

Table 4. Multiple sclerosis prevalence index in Buti

Year	Buti Inhabitant prevalence		×10	Other communities Inhabitant prevalence		×10	Buti Inhabitant commune prevalence		×10
1951	1	3.454	28.95	0	1.317	0	1	4.771	20.96
1961	5	3.251	153.8	0	1.860	0	5	5.111	97.8
1971	5	3.102	161.2	0	2.064	0	5	5.166	96.8
1980	5	3.098	161.4	1	2.155	46.4	6	5.253	114.2

Specific Data (Public Health Registers)

1. High incidence of deaths from tuberculosis until 1949
2. High incidence of deaths from gastric carcinoma in 1936, 1942, 1948, 1958, and 1959
3. High incidence of deaths from respiratory diseases, not tuberculosis
4. High incidence of deaths from Parkinson's disease in 1942
5. Measles epidemics in 1938, 1948, 1973, and 1979
6. Frequent cases of brucellosis with an epidemic in 1952

Military Occupation

Between spring 1944 and spring 1945 first german troops and then Allied troops (American and English) encamped in Buti, which was next to the Gothic line. English troops (XII Army Corps) were five Commonwealth Divisions strong. Everybody in Buti remembers the war events but one particular fact stands out: all the subjects with ascertained MS had lived during their youth in the highest part of the village, named Panicale; first German troops and then Allied troops encamped just in this area. Two of the patients shared their house and rations with soldiers.

Familiarity

Two of the four subjects with ascertained MS are first cousins. We have not observed other relationship correlations until the fifth generation. The subject with ALS died in 1983. His father was affected by ALS and also a paternal first cousin fell ill with ALS in 1985.

Measles and Distemper

Table 5 shows the measles epidemics which have occurred in Buti, but they do not seem to have significantly affected the lives of the subjects with MS. Five hundred hunters lived in Buti in 1980, and 250 dogs were unregistered, with about stray dogs

Table 5. Buti measles epidemics

1938	Supposed measles cases according to the age of two patients affected by MS
1948	350 definite cases
1973	53 definite cases
1979	40 definite cases

Table 6 Retrospective study of canine distemper (CD) epidemiology in Buti

Official records	No cases of CD in Buti
Interviewed	5 MS patients 30 healthy, age-matched subjects
Results	No cases of CD reported from MS patients 5 sporadic cases of CD in different years reported from controls
Conclusions	CD was an endemic disease in Buti in the past years No epidemics of CD are evident

100. Official data deny distemper as being present, but our investigation has showed the presence of distemper in Buti in a sporadic form (Table 6).

Respiratory Diseases

Analysis of deaths caused by respiratory diseases other than Tuberculosis revealed very interesting data. It is well known that this kind of increase is extremely indicative of flu epidemics. An important finding stands out from this analysis: all MS cases were probably involved during their early childhood in the flu epidemics of the 1935–1936 and 1936–1937 winters (Fig. 3).

These epidemics were due to the virus which caused the 1918 pandemic. Furthermore, all cases of ascertained MS had their onset in spring/summer 1960. This coincides with a high mortality rate for respiratory diseases observed in Buti due to the flu pandemic caused by H_2N_2 virus, which struck more than 2 000 000 people (Fig. 3).

Discussion

We must take into consideration the following factors:

1. Before 1960 definite MS had not been recorded in Buti.
2. Italy is a high- and middle-risk area for this disease, even if recent investigations highly criticize this finding [15]
3. Disease almost contemporarily appears in four subjects whose age is very much alike
4. All these subjects have lived until 15 years of age in Panicale hamlet.

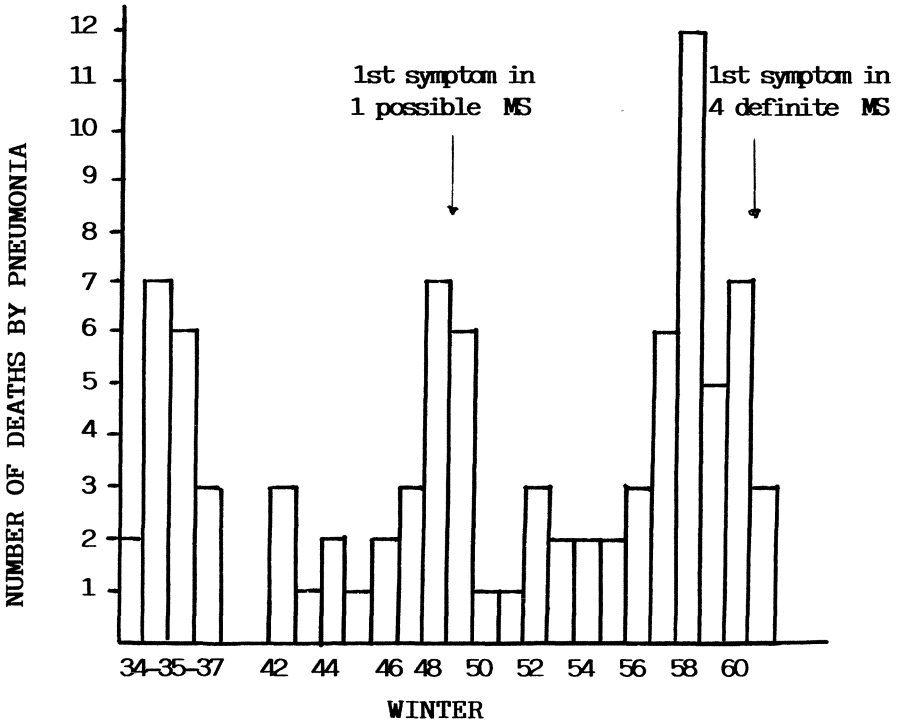

Fig. 3. Death for respiratory disease and as omset in Buti

Thus we can maintain that an MS space-time aggregation took place in 1960 in Buti. This confirms that the disease has an exogenous origin, even if we can consider the familiarity between two patients as a demonstration of the existence of a genetic background which makes it easy for environmental factors, to have an influence. It is not so easy to define these factors:

1. Contacts among the patients during their 15 years of life are expected.
2. German troops and Allied troops, coming from countries with MS risk, have encamped in Panicale.
3. The measles virus epidemics which occurred in Buti do not seem to be related to the appearance of MS in Buti. Presumably patients were infected by the 1938 measles virus.

As far as dog distemper virus is concerned, it is involved in MS etiology according to some recent investigations [16, 17]. Even if official data deny the presence of this virus, we have seen that distemper has been recorded in a sporadic form in control subject's dogs but not in patient's dogs.

The H_2N_2 role flu of the pandemic seems to have been very important in the appearance of MS in Buti. In our neurological experience we have observed how MS patients fear flu, not so much for the fever as for neurological consequences.

Our data fit very well with the Poskanzer hypothesis [13], which indicates the different points at which environmental factors may operate: the first about 20 years before the onset of the disease, the second in the year before the onset. Our research has indicated the second factor to be the H_2N_2 flu pandemics. It is more difficult to indicate the first environmental factor, which may be:

1. An environmental factor which was present in Panicale during the patient's youth
2. An environmental factor which occupation troops carried with them during World War II
3. Exposure during early childhood to the H_2N_2 flu pandemic during the 1935–1936, 1936–1937 winters

Antiviral antibody acquisition curves for different classes of ages (paramixovirus, herpes viruses, flu viruses) and HLA typing in affected subjects, their relatives, and control subjects will confirm what we have observed.

Acknowledgments. We thank for their courtesy and useful contribution the inhabitants of Buti; Buti commune; Dr. Volpe, President of Pontedera USL No. 16; Prof. Fabiani, Head Physician of the Department of Neurology of Pontedera Hospital; and Buti MS patients, particularly Mr. G. Cavani.

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Immunotherapy in Multiple Sclerosis: Arguments for Early Treatment

O. R. Hommes

Institute of Neurology, Catholic University, Nijmegen, The Netherlands

Introduction

Immunotherapies in multiple sclerosis (MS) are frequently discussed. A recent review by Mertin [1] indicates that in some instances these therapies have obvious effects, but that unequivocal proof is still required. Most treatment trials are inconclusive.

The difficulties of demonstrating effects of treatment are well known and are related to the erratic course of the disease, the absence of reliable parameters of disease activity, and a clear placebo effect of many treatments. But one of the main questions in treatments of MS has shifted to a more hopeful situation. Even now that hereditary and environmental backgrounds of MS are under discussion, there is little sign that these could be helpful for treatment. The shift of attention from causes of the disease toward immunological pathogenic phenomena has been of great importance in studies of MS treatment. It is here that in the past 20 years most of the studies on treatment have started.

Pathogenesis of MS

From EAE (Experimental Allergic Encephalitis) studies it seems likely that one of the first abnormalities in MS may be a change in the blood-brain barrier of postcapillary venules (Fig. 1 I). The abnormalities may be connected with local adherence of granulocytes to the vessel wall, and the presentation of free radicals that open the blood-brain barrier. Then a sequence of cellular infiltration of brain tissue occurs, with antigen-presenting cells, plasma cells, B-cells, T-helper cells, and T-suppressor cells (Fig. 1 II). The dynamics of these T-helper and T-suppressor cells are under study now in EAE [2, 3]. Cellular infiltrations may cause demyelination, but can also disappear without having caused demyelination. The demyelination may thus be a secondary process that once set in motion may continue itself by liberation of many myelin-associated antigens (e.g., myelin basic protein, proteolipid protein, galactocerebroside), and so run an exacerbating and remitting course depending on the amount of antigen presented (Fig. 1 III).

The presence of many antigen-presenting cells in so-called "normal white matter" of MS brains has directed attention to the possibility that the immunological process is not restricted to areas around post-capillary venules and to areas of demyelination

in spinal cord and brain, but that it really involves the whole of the brain white matter.

Recently it has been demonstrated that demyelination can be followed by remyelination of oligodendrocytes (Fig. 1 IV). The frequency and extent of this remyelination is unknown. In some instances remyelination by Schwann's cells in myelin has been found and it has been suggested that this in itself may be a source of pathogenic mechanisms [4]. The area of demyelination seems to be preferred by future reactivations of the inflammatory process, in this way creating the well-known perivascularly extending and increasing round or oval lesions. Finally, however, astrocytic proliferations will create the scar that give the disease its name (Fig. 1 V). At the moment of scar formation the inflammatory process is over.

However, in the scar demyelinated fibers will have difficulty surviving and a substantial number will degenerate. This may cause a loss of function superimposed on that caused by the inflammatory process. These two mechanisms of functional loss may throw light on the clinical course of the disease and may together determine its clinical picture. In the years of onset inflammatory activities may predominate, showing themselves as exacerbations and remissions, slowly superseded by scar formation, showing itself as the chronic progressive course of the disease.

Early Diagnosis

Diagnosis of MS has been revolutionized in recent years. The clinical criteria have been better described and extended, and it is now clear that pain, epileptic seizures, rapidly fluctuating functions, and tiredness are part of the clinical picture.

Spinal fluid changes have been demonstrated to have a high sensitivity for MS. With improving techniques of electrophoresis spinal fluid abnormalities can be demonstrated in more than 90% of MS patients. Evoked response studies have become of great value, not only in the diagnoses, but also in the follow-up of MS patients. They can demonstrate conduction changes in systems that are not implicated on clinical grounds, thus making the diagnostic process much quicker than it was previously.

Finally nuclear magnetic resonance (NMR)-scanning has been of great importance for the diagnosis of MS. The number and size of the lesions, even in the optic nerve, can be demonstrated. It made clear that the greater number of lesions cannot be correlated with clinical signs and symptoms, and that on clinical grounds and on the basis of spinal fluid and evoked response studies, the real mass of lesions shown by NMR scanning cannot be suspected.

Studies in monozygotic twins [5, 6] demonstrated NMR lesions suspected for MS in the asymptomic twin. It demonstrated again that abnormalities in the brain and spinal cord may be present for a long time without clinical symptomatology, and probably a long time before clinical symptoms start. Immunological abnormalities in the brain may precede clinical symptoms for years.

With the tools of clinical observation, spinal fluid and evoked response investigations, and NMR scanning, the diagnosis of definite MS can be made at a much earlier point in the course of the disease than a few years ago. Internationally accepted rules for the diagnosis of MS have been recently changed accordingly [7].

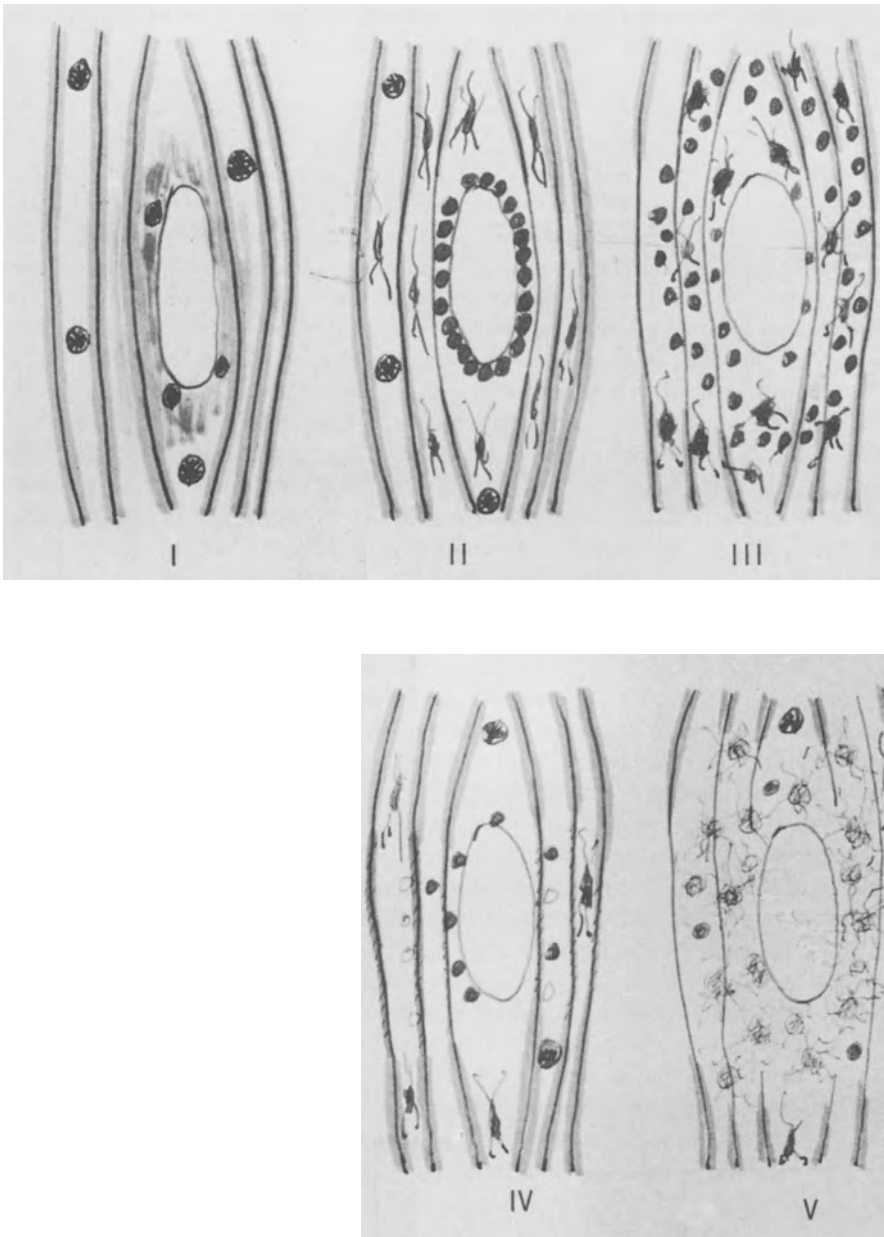

Fig. 1. *1*= Early toxic and degenerative phase, predemyelinating. Disturbance of blood-brain barrier. IgG deposits on myelin and oligodendrocytes. Presence of many plasma cells in the surrounding tissue. *2*= Early active demyelinating phase. Perivascular infiltrates of small lymphocytes. Ia-positive macrophages and microglia in the surrounding tissue. *3*= Chronic active demyelinating extending phase. Macrophages and suppressor cytotoxic T cells (CD8 cells) in the lesion and myelin phagocytosis at the lesions's edge. *4*= Massive remyelination at the lesion's edge, mainly by oligodendrocytes but also by Schwann's cells. Decrease in number of inflammatory cells. *5*= Gliotic scar formation: definite plaque. Loss of nerve fibers

Thus we are now confronted with a new generation of definite MS patients, young, with a very short disease duration, without much disability, even without any disability at all.

Treatment Trials

The diagnosis "definite MS" is required to include patients in treatment trials. Such a diagnosis could be made in the past only several years after onset of the disease, with a mean of 5 years [8, 9]. The category early, undisabled, young, definite MS patients hardly existed, because diagnostic criteria could not be fulfilled in the early phase of the disease. In some trials of immunosuppressive treatment beneficial effects were found in young patients with a short disease duration [10, 13], suggesting that early stages of the disease may react better on immunological therapies than later stages. These findings are logical because it is clear that immunological treatments will influence inflammatory reactions only. On the other hand many immunotherapy failures in MS may now be explained by inappropriate selection of patients. Patients in later stages of the disease, with mainly definite scars, are by their nature insensitive to this type of treatment.

One of the crucial questions in selection of MS patients for immunotherapy is therefore: how much active inflammation is present and how much clinical symptomatology depends on scar formation and its secondary fiber loss. And consequently one of the main questions in diagnosis is to differentiate between inflammatory lesions and scars. These questions have hardly been tackled as yet, but NMR studies show good prospects. With this background and with this knowledge a new approach to the problem of clinical trials of immunosuppressive treatment is possible.

If immunosuppressive drugs are effective, this would be in the inflammatory phase of the disease. It now seems likely that in the very early stage of the disease inflammatory reactions are most numerous and scar formation may be little or absent. The size of the area of inflammation may be small. In these early stages demyelination may be initial or even absent so that reduction or inhibition of inflammation may leave no destruction at all, or areas that can be easily and quickly remyelinated.

Let us consider some arguments is more detail, which could lead us to the selection of a group of early definite MS patients for treatment trials.

1. In very early stages of the disease the immunological abnormalities may be more sensitive and more easily modulated by immunosuppressive or immunomodulatory treatment. One of the examples here is the use of colchicine to block the initiating role of granulocytes in the early phase of an inflammatory reaction. Another possibility is the use of naturally occurring immunoregulating substances like "uromodulin" [12].
2. In early inflammatory lesions dysfunction of the blood-brain barrier may give easier access of the drug to involved areas, where it should deploy its effect.
3. In early stages of the disease the number of lesions is probably low. Most of the lesions then probably have inflammatory characteristics. The extent of the lesions and the "bystander" effects may be small and demyelination may be limited.

From EAE studies it is known that infiltration without demyelination occurs. Inhibition of immunological reactions before demyelination occurs is therefore a therapeutic possibility. Prevention of demyelination could be achieved by drugs that inhibit deposition of IgG on myelin, and that inhibit the production of T-helper cells, natural killer cells, and antigen-presenting cells.

4. In the early stages of the disease scar formation may be limited or absent. We still do not know what initiates scar formation, but some preliminary reports indicate that antimitotic substances show some preference for inhibition of astrocytic proliferation, while oligodendrocyte proliferation is activated [11]. On the other hand, activation of oligodendrocyte remyelination in itself may be the inhibitor of astrocytic scar formation. It is possible that Copolymer I may have such an effect.
5. Self-propagation of the lesions's edge may be prevented by treatment in the early stage of the disease. The mechanisms of plaque growth may depend on antigen-presenting cells swarming out from the active area into surrounding normal white matter [4]. Early treatment may reduce such a blood-borne population.
6. Remyelination of demyelinated areas may be complete if treatment inhibits active plaques at an early stage. The remyelination may be wholly dependent on oligodendrocytes, less on Schwann's cells. In these remyelinated areas early treatment may prevent a second wave of demyelination of the recently remyelinated fibers, and so again prevent growth of the plaque and progression of the lesion.
7. In early stages of the disease the patient usually presents in a good condition, without disability, without sources of secondary infections (bladder, lungs, bedsores), and generally in a better regenerative condition.

Early Treatment Trials

With a new set of diagnostic criteria a group of definite young, early, undisabled MS patients can be selected. In such a group of patients immunoregulatory and immunosuppressive treatments can be investigated, starting from a clinically more advantageous point, than when the disease has already led to extensive destruction. Recent population studies [8, 14] have demonstrated that only a minor portion (maximal 20%) of patients will run a benign course in the 3 or 5 years following the onset of the disease. If the sample size is large enough an answer to the main clinical questions can be found in the first years of the disease, e.g., frequency, duration, intensity, and remission of exacerbations, overall increase of disability, number of NMR lesions, changes in evoked responses, and spinal fluid abnormalities.

The aim of these trials should be to stop or greatly reduce clinical and paraclinical progression of the disease, and reduce or stop the exacerbations in all their aspects.

It should be discussed whether such studies should be blind and placebo controlled, blind and two armed, or completely unblind and two armed.

The effects on the course of the disease may be much clearer than in a population with wide variation in disease duration, active and inactive inflammation and scar formation, and large variation of general clinical condition and age.

Early treatment trials could be much more conclusive than those that we have executed in the last decennia.

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Selective Immunosuppression in Multiple Sclerosis

L. Massacesi, and *L. Amaducci*

Istituto delle Malattie Nervose e Mentali, Università di Firenze, Firenze, Italy

Introduction

Immunosuppressive agents are widely employed for clinical situations characterized by altered immunological reactivitiy and manifestation of autoimmunity. Because of the complex and poorly understood mechanism of these conditions, such use is not generally approved and remains largely investigational.

In multiple sclerosis (MS), the antigen target of the immune reaction is still unknown and the immunosuppressive therapies generally accepted for this disease are necessarily not specific and so far based on antimitotic-cytotoxic agents. The development of therapies with more selective activity is one of the aims of the research currently in progress in immunology and in MS. The recent advances in the knowledge of the immune system physiology now permit the development of tentative theories about the immunopathogenesis of MS.

Advances in the Immunopathogenesis of Demyelinating Diseases

Recent in vitro and in vivo experiments with experimental allergic encephalomyelitis (EAE), suggest that, in this experimental model of MS, probably the sensitized immune cells meet the target brain antigen on the blood-brain barrier [1], associated with class II major histocompatibility complex (MHC) antigens as for the T-helper (TH) lymphocytes [2], and probably associated with class I as for cytotoxic T-lymphocytes (CTLs) [3]. During this "presentation" of the antigen, CTLs express the receptor for interleukin 2, and TH lymphocytes release interleukin 2 and other lymphokines [4], expanding in this way a reaction initially involving only a few cells. This hypothesis is based on the crucial demonstration that cells other than monocytes, dendritic cells, and Langherans cells could, under particular conditions, act as antigen-presenting cells (APCs) expressing class II antigens (also known as DR antigens in humans and Ia antigens in rodents) [5]. The stimulus capable of inducing Ia antigen expression on these facultative APCs is the gamma-interferon (γ-IFN) released by sensitized T cells [6]. After the development of techniques for the culture of endothelial cells, McCarron et al. demonstrated that these cells could present myelin-basic protein (MBP) to sensitized T-lymphocytes if the cells were previously challenged with a conditioned medium containing γ-IFN [7]. A similar observation was carried out by Fontana et al. using astrocytes as facultative APCs

[8]. On the other hand, this group sustain the hypothesis that the antigen target of the immune reaction is probably presented to the T cells inside the CNS on the astrocytes carrying the class II antigens [9].

The critical role of γ-IFN seemed to be confirmed by the clinical trial recently carried out. The administration of this molecule induced a significant increase of the number of relapses in relapsing-remitting MS patients. DR-antigen expression in monocytes, as well as mitogen- and MBP-induced proliferation in lymphocytes, were also remarkably increased. These data, indirectly, seem also to confirm the role that MHC antigens probably play in the pathogenesis of MS [10].

Selective Immunosuppressive Strategies Currently in Progress

The classical strategies of immunosuppression in immunomediated diseases include antimitotic-cytotoxic drugs such as azathioprine and cyclophosphamide. A more specific approach has been tried in MS through specific desensitization with MBP and with the Copolymer 1 (Cop 1) of MBP. This molecule was shown to be completely safe and the clinical study on MS is in progress. Preliminary results indicate a good activity of the Cop 1 in reducing the number of relapses in relapsing-remitting patients [11].

Waiting for the definitive results, other strategies of therapy have been developed to obtain immunosuppression wihtout the side effects of antimitotic-cytotoxic drugs.

Mainly, the rational basis of these strategies is:

- Block of antigen presentation
- Suppression of lymphomonokine activitiy

Monoclonal antibodies (Mabs) and molecules with pharmacological activity have been proposed for these goals. A number of them are currently under study in MS:

- Anti-T-lymphocyte Mabs
- Anti $T4^+$-lymphocytes Mabs
- Cyclosporin

Other agents have been to be able to suppress EAE:

- Anti-Ia antigen Mabs
- Retinoic acid

The experiment by Steinman using anti-Ia antigen Mabs indicates that when these molecules are administered at the time of immunization, only a few animals developed the disease, suggesting that the T cells have not been sensitized because MBP has not been presented to them. When the anti-Ia Mabs were administered during the latent period of the disease, the majority of the mice developed the disease 6 days later, when the antibodies were probably catabolized or inactivated, indicating a probable interference with the Ia-restricted recognition of MBP on the target organ by the sensitized lymphocytes [12].

After the suppression of EAE with Mabs anti the mouse correspondent of the T4 antigen [13], a clinical trial with these Mabs has been developed. The trial has just begun and no data are available so far with respect to the therapeutic activity. On

the other hand, some data have been presented by Hafler et al. on the effects of administration of Mabs anti the T12 antigen of lymphocytes [14]. In this study, a marked decrease in the expression of the T12 antigens was observed. The decrease of positivity was not reflected by a significant loss of cells, meaning that the Mabs were not lymphocytolytic. The therapeutic activity after 10 days of administration was difficult to evaluate considering that most patients received prednisone, daily, to prevent allergic reactions, but a long-term evaluation did not seem to show improvement of the disease. The side effects of the antibody administration seem related to a sensitization to allogenetic proteins with the development of serum sickness and to the synthesis of antiidiotypic antibodies with annihilation of the activity and necessity for frequent administrations.

Even if the serum sickness could be overwhelmed by corticosteroid administration and by the development of chimeric human-mouse antibodies, the antiidiotypic reaction will probably be a more difficult problem to resolve. For these reasons our interest is currently focused on immunosuppressive molecules with a selective activity on the immune system.

Molecules with Selective Immunosuppressive Activity

Cyclosporin is an immunosuppressive agent which showed an intensive suppressive activity on EAE [15, 16]. Its action mechanisms is probably related to the inhibition of interleukin 1 and 2 activity [17] and a suppressing activity of this drug on Ia expression on endothelia has been observed [18]. The drug is currently under study in multicentric trials in England, Holland, the Federal Republic of Germany, and the United States. The results of these studies have not been published so far, but the side effects of the drug, mainly related to renal toxicity and hypertension, induced many patients to interrupt the treatment [19].

The critical role that γ-INF and MHC class II antigens may play in the described steps of the pathogenesis of the immunomediated demyelinating diseases have led our interest to molecules which can interfere in these steps, in order to obtain a selective immunosuppression avoiding the undesirable side effects of the antimitotic-cytotoxic drugs. Some of the most interesting molecules which may have such characteristics are the retinoids, a class of vitamin A derivatives [20]. These molecules, in particular retinoic acid (RA), are lysosomotropic agents [21, 22] and showed inhibiting activity on γ-IFN release and activity [23, 24] and on MHC class II antigen expression [25]. A similar activity on FC receptor expression on monocytes [26] has been described. Moreover, retinoids showed in vivo immunosuppressive activity on experimental models of cellular immunity [27] and of rheumathoid arthritis [28]. On the other hand, a reported adjuvant effect has been so far observed on humoral immunity [29] or in vitro on the function of isolated immune cell subpopulations [30, 31].

The following immunological activities of RA seem therefore to be the most relevant for experimental therapy in demyelinating diseases:

- Inhibition of γ-IFN synthesis and activity
- Inhibition of IFN-induced class II antigen expression on APCs
- Inhibition of FC receptor expression on monocytes

As lysosomotropic agent [21, 22], we believe also that it can interefere with the antigen handling and processing by APCs and therefore with the antigen presentation.

We would like here to emphasize that the authors of the abovedescribed γ-IFN study in MS patients concluded their preliminary report with the following sentence: "Further immunotherapeutic trials in MS should include agents which specifically inhibit production or activity of γ-IFN" [10].

Retinoic Acid Activity in Experimental Demyelinating Diseases

The effects of all-*trans* RA (300 mg/kg) and 13-*cis* RA (150 mg/kg) suspended in corn oil have been examined in our laboratory, with respect to the vehicle. The treatments were administered to matched groups of immunized animals and Freund's adjuvant injected controls (FACs), from day 9 to day 11 after immunization, in the 3 days before the expected onset of the disease. The treatment administration was carried out through gastric intubation, in a single dose for all-*trans* RA, daily, and 13-*cis* RA was administered in two divided doses.

No neurological symptom was observed in any group until day 11 or 12. At that time, immunized animals receiving corn oil developed EAE. No all-*trans* RA and 13-*cis* RA-treated animals showed any neurological symptom. Histological examination of the spinal cords did not show perivascular cuffs in the 13-*cis* RA-treated rats and only minimal cell infiltrates in two out of eight all-*trans* RA-treated rats. The clinical and pathological findings were reflected in the vehicle-administered animals by the highly significant increase in spinal cord DNA. In the RA-treated animals the absence of infiltrates was reflected by the complete suppression of such a DNA increase. Comparing therefore immunized animals of all-*trans* RA and 13-*cis* RA-treated groups versus the rats which developed EAE, significant differences in the spinal cord DNA concentration were observed.

Preliminary immunohistochemical studies have been carried out in 13-*cis* RA-treated and control spinal cords, using anti-Ia antigen Mabs. In rats which developed EAE, a high number of cells showed positivity for Ia antigens, some of them being observed on the capillary luminal surface. The characterization of the cellular type with specific monoclonal antibodies and double staining is in progress. On the other hand, in immunized rats with EAE suppressed with RA, the specific staining for Ia antigens was similar to the one observed in nonimmunized controls, suggesting that the treatment could interfere with the expression of these molecules.

These data show a powerful suppressive activity of RA on EAE. The time of administration of the drug is very important for a tentative hypothesis about its action mechanism. The drug administration schedule beginning from day 9 after the immunization, 3 days before the expected onset of the disease, indicates a RA activity on the efferent phase of the immune response that probably persists until the drug is washed out of the tissues. The immunohistochemical data suggest a possible interference of the molecule on the Ia expression at the level of CNS APCs. If this hypothesis is correct, there is indirect evidence of a possible interference of the drug with the self-antigen presentation to the sensitized T cells at the level of the CNS APCs, i.e., endothelia and astrocytes, that may involve MHC class II antigen

expression and/or lysosomal processing in the CNS APCs. Considering that RA so far, has not shown any antimitotic-cytiotoxic activity [20], it seems possible to hypothesize that EAE and other immunomediated demyelinating diseases may be suppressed through nonproteic molecules acting on antigen presentation.

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Use of Azathioprine in Multiple Sclerosis

R. A. C. Hughes

Dept. of Neurology, United Medical and Dental Schools, Guy's Hospital, London, UK

Introduction

Many arguments can be marshalled to support the use of immunosuppressive treatment in multiple sclerosis (MS)

1. Pathology – inflammatory
   – resembling chronic relapsing EAE
2. CSF – intrathecal Ig production
3. Blood – leucocyte alterations
4. Clinical immunological evidence of autoimmunity
5. Efficacy of steroids
6. Benefit from immunosuppression in non-randomised *open* trials
7. Benefit in *small* randomised open trials
8. Equivocal benefit in small randomised *"masked"* trials

The earliest histological lesion is a perivascular infiltrate of lymphocytes, which at least indicates inflammation and probably marks an active immunological reaction. The main evidence that this reaction is autoimmune comes from the close pathological resemblance between chronic relapsing experimental allergic encephalomyelitis (EAE) and MS. The CSF abnormalities, increased cellular content and intrathecal immunoglobulin production, are further signs of inflammation and indeed of an immune response occurring within the nervous system. The increased polymorphonuclear leucocyte count and decreased proportion of CD8+ to CD4+ lymphocytes in the peripheral blood which have been recorded in some, but not all, series of patients in relapse indicate that the immune response in MS is not confined to the nervous system. Unfortunately clinical immunological evidence of an autoimmune response in patients, which bears a consistent relationship to the presence and activity of the disease, the sort of evidence provided by the antibodies to the acetylcholine receptor protein in myasthenia gravis, continues to elude us. Until such evidence is provided the case for use of immunosuppression in MS must remain empirical. Limited support is provided by the results of available trials of steroids: both ACTH and probably intravenous methylprednisolone (see[1] and [2] for reviews) have a beneficial effect in acute relapses. However, this result might be the consequence of an anti-inflammatory rather than an immunosuppressive action.

Small open trials of powerful immunosuppressive regimes, such as high-dose intravenous cyclophosphamide [3], and also small masked trials of others such as plasma exchange [4], have appeared to benefit the treated group. Although each regime awaits independent validation so that none can be recommended for general use, the combined results encourage further attempts to investigate these and other forms of immunosuppressive treatment in MS.

Azathioprine

Azathioprine is one of the most commonly used and least toxic immunosuppressive agents in clinical practice. Its chemical formula is 6 – (l-methyl-4-nitroimidazol-5-yl-thio) purine. Thus it is a nitroimidazole-substituted form of 6-mercaptopurine into which it is rapidly converted in the body. Some pharmakokinetic properties are:

Readily absorbable orally Peak plasma level in 2 h Plasma half-life 5 h Rapidly converted into 6-mercaptopurine Cerebrospinal fluid concentrations 2% of plasma levels

Its properties of ready oral absorption, short plasma half-life and low CSF concentrations are noteworthy in relation to multiple sclerosis.

The sites at which azathioprine acts are multiple, complex and incompletely understood because 6-mercaptopurine is an analogue of hypoxanthine, which occupies a central position in purine and nucleic acid synthesis.

Mechanisms of action of azathioprine are: inhibition of purine synthesis inhibition of DNA and RNA synthesis inhibition of membrane glycoprotein synthesis alkylation of -SH groups

There are also suggestions that azathioprine may be more active than, or have different actions from, 6-mercaptopurine possibly because of additional effects from the nitroimidazole side chain.

The cellular effects of azathioprine are correspondingly complex and broad spectrum. Table 1 lists some of the major effects which have been demonstrated in vitro, which are predominantly on T-cell function, but also on the induction of antibody formation. Animal experiments illustrate the wide range of effects in vivo. The observation of a significant suppression of the non-specific inflammation induced by the subcutaneous injection of a non-specific irritant [5] is worth stressing because if an effect were observed in a disease such as MS it should not be deduced ipso facto that the disease is immunological in nature. In common with other

Table 1. Azathioprine: effects

1. Addition in vitro inhibits:
   Lymphocyte sheep red cell rosette formation
   Lymphocyte responsiveness to PHA
   Mixed lymphocyte reaction
   Induction of antibody response
2. Animal experiments show inhibition of:
   Inflammation by non-specific irritants
   Induction of primary antibody responses
   Induction of immunological memory
   Graft rejection
   Experimental autoimmune disease
   Adjuvant arthritis
   NZB/NZW mouse lupus-type nephritis
   Experimental allergic encephalomyelitis
3. Observations in man suggest inhibition of:
   Immunoglobulin synthesis
   Induction of delayed hypersensitivity
   Graft rejection
   Autoimmune disease, e. g.
   Myasthenia gravis
   Chronic inflammatory demyelinating polyradiculoneuropathy
   Systemic lupus erythematosus
   Polyarteritis nodosa
   Polymyositis

immunosuppressive agents azathioprine is much better at blocking early than established immune responses, and induction rather than production of antibody. Azathioprine is well suited to suppressing graft rejection, as first demonstrated by Calne and Murray in 1961 [6], when it can be started at the time of immunisation. If the widespread lesions seen on MRI scans in early MS indicate an established immune response it may be more difficult to discover a therapeutic effect with azathioprine even early in the course of the disease. The same stricture can be applied to the suppression of experimental autoimmune diseases, including EAE in rats [7], by treatment which is given at or soon after the time of immunisation. In man azathioprine has been shown to have inhibitory effects predominantly on cell-mediated functions including induction of delayed hypersensitivity by DNCB and graft rejection. The effects on humoral immunity are generally thought to be less: immunoglobulin levels and pre-existing antibody levels usually remain unchanged although IgG and IgM synthesis has been shown to be reduced. Specifically in MS, Oger et al. [7a] have shown that pokeweed mitogen-induced IgG secretion is reduced in azathioprine-treated patients. Beneficial effects have been claimed in a variety of conditions thought to have an autoimmune aetiology, including myasthenia gravis [8], chronic inflammatory polyradiculoneuropathy [9], systemic lupus erythematosus, polyarteritis nodosa and polymyositis. In most of the studies the results have been suggestive but the sample size has been too small to provide a reasonable power or chance of detecting a significant effect. An overview of trials on

lupus nephritis showed a significantly lower rate of renal deterioration on azathioprine and steroids than on steroids alone [10].

Non-Randomised Studies of Azathioprine in Multiple Sclerosis

Because of its broad-spectrum immunosuppressive action, oral route of administration and tolerable side effects, azathioprine has been adopted for more clinical trials in MS than other agents. In an overview of early uncontrolled trials on about 200 patients with MS, Ellison and Myers [11] concluded that about 35% of patients worsened over about 6 – 12 months compared with a similar proportion in a group studied by Millar [12] in the control arm of their chronic ACTH study. Unfortunately these early reports do not determine whether azathioprine has any effect or the results merely reflect the natural history of the disease. Further experience with azathioprine has been summarised by Mertin [20] and the largest recent series are recorded in Tables 2 and 3. Table 2 describes the results in relapsing and remitting MS in which the two recent French series are notable since they are both large carefully collected retrospective series and their results conflict. The patients reported by Aimard and colleagues [16] had a much more favourable course than a group of historical "controls": for instance 46% of the treated patients had no more relapses compared with only 13% of the controls; and 10% entered a secondary progressive phase compared with 36% of the untreated patients. The patients treated with a similar regime for a similar period by Lhermitte and colleagues [17]

Table 2. Azathioprine: effect on relapsing-remitting MS: uncontrolled trials

Ref.	Daily dose (mg/kg)	Treatment (years)	Observation (years)	No.	Result
[14]	2–3	>2	5	66	Relapse rate reduced from 0.65 to 0.12 per year 52% stable or improved
[15]	2–3	2.5	>2.5	50	68% no more relapses 75% improved
[16]	2–3	4	10	128	46% (13%[a]) no more relapses 36% (35%[a]) relapsed but did not worsen 8% (17%[a]) relapsed and worsened 10% (36%[a]) secondary progression
[17]	2–5	6.3	10	97	21% no more relapses 42% relapsed but did not worsen 34% relapsed and worsened 14% secondary progression

a These percentages refer to 78 patients treated without azathioprine before 1977

Table 3. Azathioprine: effect on progressive MS (uncontrolled trials)

Ref.	Daily dose (mg/kg)	Duration (years)		n	
		Treatment	Observation		
15.	2.5	2.5	>2.5	51	30% (P)–36% (RP) stable
18.	Variable	10	10	(85)	2 of 23 became unable to walk without support
16.	2–3	3	3	31RP 16P	Progression slowed
17.	2.5	4.3	8.7	48	35% stable

P, progressive from onset; RP, relapsing then progressive

did not fare as well as the group treated by Aimard et al [16] in that only 21% had no more relapses: in Lhermitte's series [17] while only 14% entered a progressive phase, a larger proportion, 34%, continued to have relapses and worsened during the observation period.

The effect on the progressive phase of MS is just as difficult to assess (Table 3). In the first place it is not always clear whether the patients have had a purely progressive course or have entered a secondary progressive phase after a relapsing onset. Two groups have reported that about a third of their patients have stabilised [15, 17] and another [16] that the rate of progression is slowed compared with a historical control group or the course of the patients' own disease before the onset of treatment. Rosen [18] reported 23 patients who were still able to walk at the start of treatment; only two became unable to walk without support after10 years.

Controlled Trials of Azathioprine

Two small masked controlled trials of azathioprine, one of the drug alone [19] and one combined with prednisolone and antilymphocyte globulin [2], failed to show any significant benefit to the active treatment group. However the trial of combined treatment did show a trend in favour of the active drug with respect to relapse rate and differences in clinical deterioration measured with the Kurtzke score. In a randomised but open study 2 of 22 treated patients came to require wheelchairs after 6 years compared with 13 of 20 untreated patients [18]. This striking difference might be due to the more intensive regime used by the author which required adjustment of the dose to maintain a marginal leucopenia. However, the open study design does not eliminate either placebo effects or observer bias. The same strictures can be levelled at the largest controlled trial of azathioprine yet published in which 56 patients were randomised to receive a relatively low dose of azathioprine (2 mg/kg daily) and 51 patients to linoleic acid [21]. The randomisation procedure unfortunately allocated more severely affected patients to the azathioprine than the control group, which complicated the analysis. The final results showed no significant differences between the disease courses of the two groups taken as a whole. The

course of the disease was subclassified before entry into the trial into those with exacerbations and complete remissions, with exacerbations and incomplete remissions and chronic progressive. Only the middle stratum with an intermittent-progressive course, i.e. exacerbations and incomplete remissions, had a more benign course with a mean rate of deterioration of disease severity which was significantly slower in the azathioprine-treated group. Resort to analysis of subgroups in order to "dredge" a significant result weakens the confidence which can be placed on this finding. Furthermore the open study design and exclusion of patients who discontinued treatment provide additional difficulties of interpretation. Another controlled trial in which about 90 patients have been randomised to receive azathioprine and placebo, azathioprine and methylprednisolone or double placebo will shortly be published [22]. Dr. Ellison has advised me that there was a reduction in the relapse rate in the two groups which received azathioprine compared with the double placebo group but no change in the overall deterioration. Their conclusion was that a full trial is warranted and necessary to determine the efficacy of azathioprine.

British and Dutch Multicentre Controlled Trial of Azathioprine

This trial has randomised 354 patients in about 20 centres in the British Isles and Holland to receive either azathioprine 2.5 mg/kg daily or placebo for at least 3 years. Follow-up will be complete in October 1987 although for ethical reasons provision has been made for a clinical audit committee, unconnected with the care of the patients, to end the trial earlier if a highly significant result has been achieved. Patients are being monitored at 3-month intervals by observers with no access to laboratory results and no knowledge of the treatment allocation. The Kurtzke extended disability status scale [3] and ambulation index [23] are being applied at each visit and the number of relapses and patients' subjective opinions of wellbeing are also being recorded. We hope that the large sample size will make the trial sufficiently powerful to permit limited subgroup analysis and we have defined the subgroups in which we will be interested.

Side Effects of Azathioprine

Against the theoretical and possible real benefits of azathioprine we must set the well-recognised side effects [24]. In Table 4 the theoretical risks are listed with an indication of the incidence of side effects in two series, one from a series of patients with neuromuscular disease, who may have been treated with rather large doses [25], and one from the large French series [17]. Of particular concern is the theoretical risk of cancer which the worrying data of Lhermitte et al [17] suggest might be a real risk (Table 5). It should be noted however that the comparison was retrospective and not case controlled and the increased risk of cancer of the breast is not one which was theoretically expected or anticipated from the data from transplant recipients in which the increase in incidence of neoplasms is predominantly of non-Hodgkin's lymphoma. A prospective study (Table 6) of more than 1000 non-transplant patients treated with azathioprine did show an increased incidence of

Table 4. Azathioprine: side effects

		Percentage[a]	Percentage[b]
Haematological	Macrocytosis	20	
	Leucopenia	22	6[c]
	Anaemia		3[c]
	Thrombocytopenia		1.5[c]
Infection	Especially viral		5
Gastrointestinal	Nausea, vomiting, abdominal pain	12	8 1
	Hepatic dysfunction	9	
Hypersensitivity	Erythema nodosum, fever, arthralgia		2
Teratogenicity	Theoretical risk		
Cancer	Theoretical risk	0.7	

[a] Ref. [25] (n=64)
[b] Ref. [17] (n=211)
[c] Plus 3% who had a combination of haematological abnormalities

Table 5. Azathioprine: cancer risk. [17]

131 MS patients Azathioprine 10-year follow-up		131 MS patients No azathioprine 10-year follow-up
Breast	5	
Ovary	1	
Skin	1	
Cervix	1	
Stomach	1	
Colon	1	
Total	10	Total 4

Table 6. Prospective study of incidence of neoplasms in non-transplant patients after immunosuppresive treatment. [26]

Type of cancer	Azathioprine		Cyclophosphamide		Chlorambucil	
	Observed	Expected	Observed	Expected	Observed	Expected
Non-Hodgkin's lymphoma	5	0.38	1	0.16	0	0.01
All skin	4	2.58	1	1.00	0	0.06
Squamous cell	2	0.42	1	0.17	0	0.01
Basal cell	2	1.89	0	0.73	0	0.05
Bladder	1	1.14	5	0.47	0	0.02
Others	30	24.51	16	9.33	2	0.62
Total	40	28.61	23	10.96	2	0.71

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non-Hodgkin's lymphoma, but not other types of neoplasm [26]. Some of this increase could be accounted for by a higher incidence of non-Hodgkin's lymphoma in patients with rheumatoid arthritis who formed the majority of these subjects. Nevertheless the risk of neoplasm with immunosuppressive treatment in MS must be monitored during and after treatment trials and must be balanced against the likely protection from becoming seriously disabled.

Conclusions

Azathioprine is a convenient oral immunosuppressive drug with a broad spectrum of action. It causes gastric intolerance and hypersensitivity, usually early in a course of treatment, in only a small proportion of patients and is otherwise well tolerated. There is a theoretical and possible small actual increased risk of developing neoplasia, especially non-Hodgkin's lymphoma. Uncontrolled trials suggest but do not establish that the drug reduces relapse rate and slows progression. The controlled trials conducted to date show a trend in favour of azathioprine but have not been large enough to have an adequate power to detect a small effect. The results of the current large controlled trials should clarify the place of azathioprine in the treatment of multiple sclerosis.

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Immunosuppression with Azathioprine in Multiple Sclerosis*

A. Ghezzi, M. Zaffaroni, D. Caputo, S. Bianchi, S. Marforio, and *C. L. Cazzullo*

Centro Studi Sclerosi Multipla, Ospedale di Gallarate, Università di Milano, Gallarate, Italy

Introduction

Although the etiology of multiple sclerosis (MS) is still unknown, there is growing evidence that immunological factors play an important role: for this reason many therapeutic attemtps have been made with immunoactive drugs [1–3]. Among these, azathioprine (AZA) and cyclophosphamide have been evaluated in several clinical trials, with somewhat inconclusive results (for a review see ref. [4]). This fact largely depends on objective difficulties of conducting therapeutic trials in MS including the variability of symptoms and course, the occurrence of spontaneous remissions, and the lack of a reliable marker of disease activity.

In the present study clinical and laboratory results of AZA therapy on different series MS patients are reported.

Part 1: Retrospective Clinical Study of 86 Patients

A retrospective study was carried out on a series of 86 definite MS patients collected in our department from 1982 to 1983. They were submitted to AZA therapy for at least 18 months and received corticosteroids during relapses: charactoristics of the patients are reported in Table 1. Clinical status was scored by Kurtzke scales [5].

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Table 1. Patient characteristics

Eighty-six definite MS patients: 39 females, 47 males
Mean age: 30.7 years, range 18–47 years
Disease duration: 5.9 years, range 1–20 years
Follow-up: 29.2 months, range 18–48 months
Dose: 2.5 mg/kg/day
In eight patients the treatment was stopped because of adverse effects

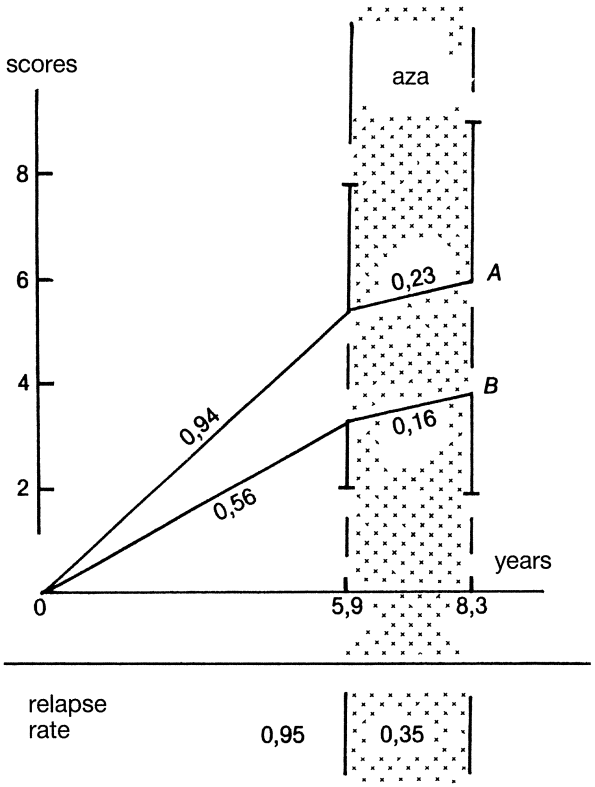

Fig. 1. Disease progression and relapse rate in relation to AZA therapy

Disease progression (disability status/disease duration) and relapse rate before and after the treatment were considered.

Results

A slower disease progression and a lower relapse rate were observed after AZA-therapy (Fig. 1). Data were analyzed with respect to disease duration, clinical course, and age at onset. Evolution was defined as "unchanged" when the final value of disease progression was similar to the initial one. When it was lower, the evolution was called "better"; when it was higher it was termed "worse". Results were not statistically significant (Table 2).

Part 2: Randomized Controlled Study of 39 Patients

In order to obtain more reliable data on disease progression, a controlled randomized study has recently been carried out. A series of 39 definite MS patients [6] (20 females, 19 males) were included in the study according to the criteria shown in

Table 2. Clinical evolution with respect to disease duration, age at onset, and course

Disease duration	Evolution		
	Better	Worse	Unchanged
< 3 years (n. 20)	12 (60.0%)	6 (70.0%)	2 (10.0%)
3–5 years (n. 22)	14 (63.6%)	7 (31.8%)	1 (4.6%)
> 5 years (n. 36)	14 (38.7%)	15 (41.7%)	7 (19.4%)
Age at onset			
≤ 20 years (n. 20)	13 (65.0%)	6 (30.0%)	1 (5.0%)
21–30 years (n. 30)	18 (45.0%)	16 (40.0%)	6 (15.0%)
≥ 31 years (n. 18)	9 (50.0%)	6 (33.0%)	3 (16.7%)
Course			
Remittent (n. 31)	20 (64.6%)	8 (25.8%)	3 (9.6%)
Progressive (n. 47)	20 (42.6%)	20 (42.6%)	7 (14.8%)

Table 3. Criteria of selection of MS patients

1. Definite MS, in the relapsing progressive form
2. No steroids/immunosuppressants within 6 months
3. Disease duration: 2–12 years
4. Age: 18–48 years
5. Disability (DS): 2–5

Table 3. Patients gave their informed consent to take part in the trial and were randomly allocated into two groups: one received AZA 2.5 mg/kg per day (20 cases); the other served as controls (19 cases). All cases received dexamethasone in the case of relapse, starting from 4 mg/day tapered over 3–4 weeks. The follow-up lasted 18 months. Patients were evaluated monthly with Kurtzke functional systems (FS) and disability status (DS) scales [5] by two independent blinded neurologists. On the same day the patients were submitted to determination of blood OKT3-, OKT4-, and OKT8-positive cells by automated cytofluorography (for details see ref. [7]. Before and after the trial patients were submitted to recording of visual (VEP) brain-stem auditory (BAEP), and somatosensory (SEP) evoked potentials, with the methods extensively described elsewhere [8]. Before and after the trial patients were also submitted to lumbar puncture, to determine the IgG index [9].

Five patients of the AZA-treated group did not conclude the follow-up: one because of family problems, four because of side effects: transient severe anemia (one case), recurrent infections (one case) gastric complications (two cases). Two patients among the controls did not conclude the follow-up because of family problems.

The characteristics of the two groups are summarized in Table 4.

Table 4. Clinical characteristics of MS patients

	AZA treated	Untreated
Males/females	8/7	7/10
Age (years)	27.8±8.1	33.1±9.1
Range	18–46	19–49
Disease duration (years)	5.3±2.8	5.6±2.8
Range	2–10	2–12
Disability status	3.3±1.2	2.5±0.8
Functional system	6.0±3.0	4.6±2.0

Results

In spite of randomization, the AZA-treated group presented a lower age, a lower disease duration, and a higher disability with respect to the control group, although the differences were not statistically significant. Nevertheless clinical results, as well as neurophysiological ones, were evaluated statistically by covariance analysis to avoid the influence of different starting points.

The initial disease progression (disability status/disease duration) was 0.82 in the AZA-treated group and 0.69 in the control group. At the end of the trial it was 0.24 and 0.20 respectively (Fig. 2): results were corrected by covariance analysis and did not differ significantly in the two groups. Clinical changes in the single patients are reported in Fig. 3: it shows a similar evolution of the two groups in relation to therapy. Relapse rate before the study was quite similar in the AZA-treated and untreated patients(0.64 and 0.54, respectively) and did not change significantly at the end of the trial (0.61 and 0.58, respectively).

Evoked potentials were recorded before and at the end of the study. The following measures were considered: VEP P 100 latency, BAEP I-V interpeak

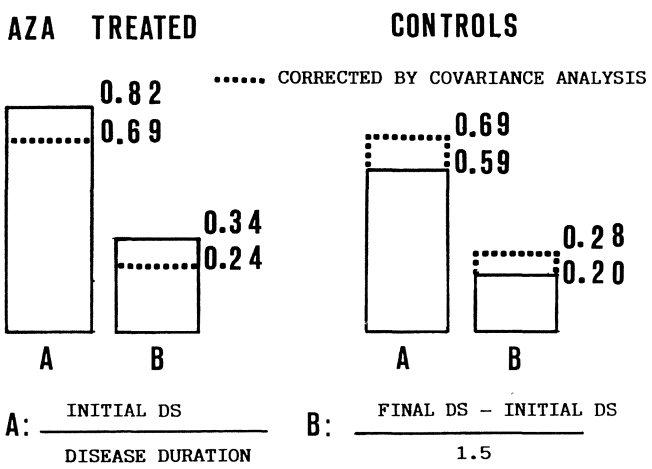

Fig. 2. Disease progression before and after the controlled trial

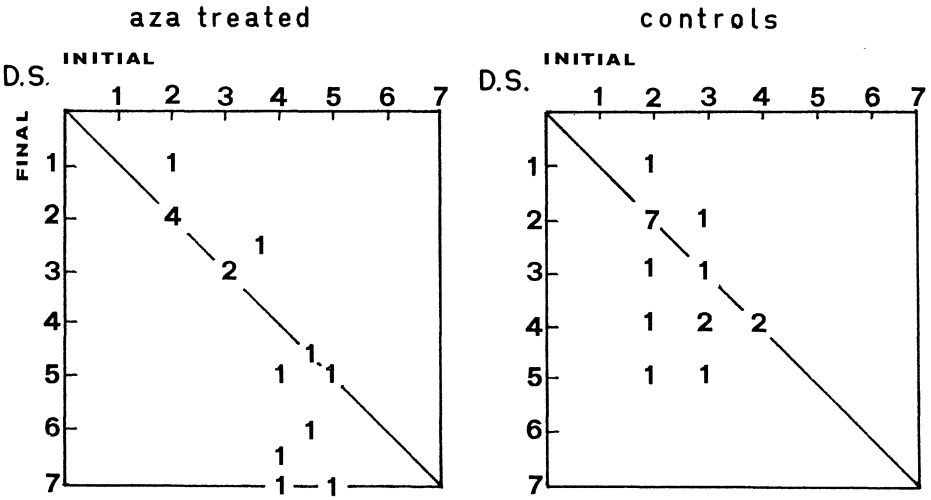

Fig. 3. Changes of final vs. initial disability status scores (DS) in AZA-treated and control patients

latency (IPL), and SEP N9-N20 latency. The initial values of VEP P100 and of BAEP I-V IPL did not differ significantly at t-test in the two groups, whereas SEP N9-N20 latency was significantly more delayed in the immunosuppressed group (Table 5).

The final mean latencies appeared slightly increased in both groups, but not to a significant degree.

The IgG index decreased from 1.12 to 0.96 in the AZA-treated group (P=0.07 at t-test on paired data), whereas the difference between the initial and final IgG index did not change in controls (P=0.76 Table 6). This interesting finding has been

Table 5. Neurophysiological results before and after the controlled trial. In parentheses, normal values and S.D. Values expressed in ms

	AZA treated		Controls	Analysis of Covariance
VEP P 100	I 128.6 ± 23.0	N.S.	124.8 ± 25.6	
(99.6±4.5)	F 135.6 ± 30.6		129.8 ± 25.1	N.S.
	+5.4%		+4.2%	
BAEP I–V IPL	I 4.84 ± 1.08		4.66 ± 0.99	
(3.94±0.17)	F 5.27 ± 1.25	N.S.	4.81 ± 1.08	N.S.
	+8.8%		+2.6%	
SEP N9–N20	I 18.3 ± 9.3	P< 0.05	11.6 ± 3.7	
(9.3±0.6)	F 21.4 ± 10.0		14.3 ± 8.0	N.S.
	+16.7%		+22.6%	

I=initial; F=final

Table 6. IgG index before and after the controlled trial

	AZA treated	Controls
IgG index		
Initial	1.12 ± 0.49	1.13 ± 0.47
Final	0.96 ± 0.54	1.09 ± 0.42
	−14.0%	−3.8%
P	0.07	0.76

Table 7. Mean T4/T8 and Δ T4/T8 ratios in normal subjects, immunosuppressed patients and control patients

	Normal subjects	AZA-treated	Controls	t-test
T4/T8	1.5 ± 0.2	1.76 ± 0.60	1.67 ± 0.56	N.S.
ΔT4/T8	0.2 ± 0.1	0.40 ± 0.22	0.28 ± 0.14	N.S.

successively verified in a larger series of patients (see "Part 3"). The mean OKT4/OKT8 ratio was calculated in each patient and then in the two groups. Fluctuations among successive subset determinations, defined by the △ OKT4/OKT8 parameter (Table 7), did not differ significantly at t-test in the two groups.

Part 3: CSF Study

The immunological hallmark of MS ist the intrathecal synthesis of IgG. Although the pathogenetic relevance of this finding is not completely understood, it seems reasonable that one of the goals of an effective immunosuppressive treatment in MS may be the reduction of intrathecal IgG synthesis.

The IgG index [9] before and after AZA therapy was studied in a larger and different series of 66 definite MS patients. Forty patients received AZA 2.5 kg/mg/per day plus a course of dexamethazone during clinical relapses [23 males/17 females, mean age 31.4 years, mean disease duration 5.4 years, mean disability (DS)2.8]. The remaining 26 subjects received only dexamethazone during relapses (13 males/13 females, mean age 33.4 years, mean disease duration 5.8 years, mean disability 2.8)

The whole series was submitted to lumbar puncture to calculate the IgG index before and at the end of the follow-up, which lasted 18–24 months.

Results

Comparative results in the two groups of patients are reported in Table 8. The distribution of IgG indices in AZA-treated patients (Fig. 4) shows that only four subjects had normal pretreatment values (< 0.7) while 17 presented an index higher than 1.5. In contrast, after AZA therapy, only four subjects had an index higher

Table 8. Comparative results in patients treated with AZA + DEXA and DEXA

	No.	IgG index Pretreatment	Posttreatment	Mean difference	P
AZA+DEXA	40	1.64 ± 1.28	1.04 ± 0.54	−0.6	0.001
DEXA only	26	1.00 ± 0.57	0.86 ± 0.35	−0.14	0.104

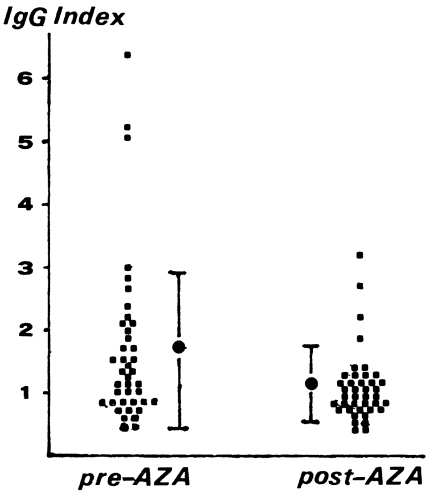

Fig. 4. Distribution of the IgG index pre- and post-treatment with AZA [25]. Reprinted, with permission, from Caputo et al. 1987 (Acta Neurol. Scand.)

than 1.5: it is noteworthy that these patients had started from the highest pretreatment values. Only nine patients showed an increase of IgG index after AZA therapy, which ranged from +0.1 to +0.7 (mean +0.3). Linear regression analysis showed a high correlation coefficient ($R > 0.9$) between the starting values of the IgG index and its reduction after AZA treatment (Fig. 5). As for CSF oligoclonal bands, they were present in 39 out of 40 patients treated with AZA (97%) and in 16 out of 26 patients treated with DEXA (61%). In one case (a young woman treated with AZA) oligoclonal bands were recognizable only in the second CSF specimen. The results concerning subgroups of AZA-treated patients, defined by sex, disease duration, disability status, and starting IgG index, are reported in Table 9.

Conclusions

Data in the literature about the effectiveness of AZA in the treatment of MS disagree: a beneficial effect in preventing disease progression [10–13] and in reducing relapse rate [10, 12, 14] have been found, but these positive results have not been confirmed in other studies [15–17]. Patzold et al [18] and Lhermitte et al [19] found a beneficial effect in the remittent, but not in the progressive form of MS.

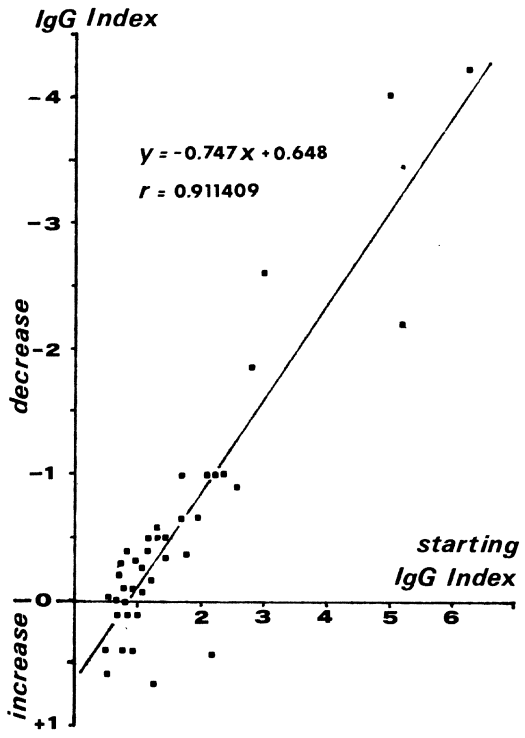

Fig. 5. Correlation between the starting values of the IgG index (*abscissa*) and their modification after treatment with AZA (*ordinate*) [25]. Reprinted, with permission, from Caputo et al. 1987 (Acta Neurol. Scand.)

Table 9. Results in subgroups of patients treated with AZA + DEXA

		No.	IgG index Pretreatment	Posttreatment	Mean difference	P
Men		23	1.5 ± 1.4	1.05 ± 0.6	−0.47	0.034
Women		17	1.8 ± 1.1	1.03 ± 0.5	−0.70	0.017
Duration	≤ 3	20	2.1 ± 1.6	1.2 ± 0.6	−0.88	0.008
	> 3	20	1.2 ± 0.6	0.9 ± 0.3	−0.83	0.049
Disability	≤ 3	25	1.8 ± 1.5	1.0 ± 0.6	−0.76	0.008
	> 3	15	1.4 ± 0.6	1.0 ± 0.5	−0.34	0.013

Differences in the size of the series, in the length of the follow-up, and in disease severity, course, and duration make the results difficult to compare. An additional limitation is caused by a lack of a control group in almost all studies, with few exceptions [18, 20].

In the randomized double-blind study by Mertin et al [20] antilymphocyte globulin and prednisone were administered in addition to AZA: a beneficial effect was found on the overall relapse rate and clinical progression in immunosuppressed patients, but not to a significant degree.

In our clinical uncontrolled study of 86 MS patients a reduced disease progression and relapse rate were observed after AZA treatment. However, when clinical results after immunosuppression were compared with those of a control group in a different series of patients, differences did not appear statistically significant. This phenomenon can be explained in different ways: it should be a consequence of the natural course of the disease or the effect of corticosteroids given during relapses. The placebo effect of frequent medical contacts may also be an explanation. Results were analyzed by covariance analysis to correct the different starting index of disease progression and did not differ significantly in the two groups at the end of the follow-up. In other words, our results do not enable us to conclude strongly and convincingly that the positive evolution of MS was caused by AZA therapy.

Neurophysiological measures were also employed in the controlled study as objective indices of neurological damage. A slight increase of the final mean latencies was found in both the AZA-treated and -untreated group without a statistically significant difference, in agreement with clinical results.

The immunosuppressive action of AZA is not completely understood: AZA seems to affect both humoral and cellular immune reactions [21]. B cells appear more sensitive than T cells [22]. Among these, T-suppressors seem poorly sensitive and T-helper/inducers quite resistant [23]. Trotter et al [24] reported that OKT8-positive lymphocytes are significantly reduced in MS patients treated with AZA. In our study the mean values of OKT 4/OKT8 and of $\triangle$ OKT4/OKT8 ratios (related to serial subset fluctuations [7]) did not differ significantly in relation to therapy: these results suggest that AZA does not affect the balance among monoclonal antibody-defined T-lymphocyte subsets. Conversely, a role in humoral immunity is suggested by CSF study in two different series of our study: a decrease of the IgG index was observed after AZA therapy. An effect by AZA synthesis has also been found by Oger et al [26], consisting of a reduced IgG secretion in vitro by cultured lymphocytes of AZA-treated MS patients.

Since the pathogenetic role of CSF IgG is undefined, the finding of a reduced intrathecal IgG synthesis after AZA therapy does not suggest its use per se in MS patients. The clinical study of more extended groups will give more conclusive results.

The problem of the potential risks of immunosuppression must also be carefully considered. In our series pharmacological side effects occurred in 10%–20% of cases: they were reversible but discouraged us from continuing the treatment. Long-term risks, particularly the occurrence of malignancy, are also reported [3, 4, 19]: a long-term survey seems necessary to better evaluate both clinical results and risks.

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Multiple Sclerosis: Preliminary Reports of a Therapeutical Trial and Magnetic Resonance Imaging Correlates

G. Tilia,[1] *R. Floris*[2], *M. Spadaro*[1], and *V. Floris*[1]

1 Istituto di Clinica delle Malattie Nervose e Mentali, Università degli Studi di Roma "La Sapienza", Rome, Italy
2 Cattedra di Clinica Neurologica, II Università degli Studi di Roma "Tor Vergata", Rome, Italy

All the therapeutic trials in the field of MS are very difficult to carry out and particularly complex to evaluate. First of all, though we have formulated some pathogenetic hypotheses, we still do not know what the etiological agent of the disease is: thus a concrete theoretical basis for therapeutic purposes is lacking; for these reasons, any kind of therapeutic approach can easily be criticized. In addition, data interpretation is further complicated by the variability and irregularity of the clinical course of the disease.

On the other hand, the results of many therapeutic trials employing steroids or ACTH and cytostatic immunosuppressive drugs support the hypothesis that these substances are effective in the treatment of the disease [1–6] providing a basis for their more frequent utilization in MS therapy.

A fairly important problem related to the use of such drugs, particularly in chronic administration, concerns the appearance of relevant toxic effects due to both substances. The steroids, in fact, in addition to their antiinflammatory and immunosuppressive action, are able to provoke, in a prolonged administration, serious side effects, such as osteoporosis, peptic ulcer, diabetes, hypertension, and Cushing's syndrome. Azathioprine, which is one of the most used cytostatic immunosuppressive drugs, expresses its therapeutic activity by interrupting DNA and RNA synthesis and by inhibiting antibody production; on the other hand, in a chronic treatment, it can cause hypoaplastic or megaloblastic anemia, hepatopathies, and neoplasms, particularly lymphomas.

These are the main reasons why the treatment with cytostatic immunosuppressive drugs is reserved for chronic progressive (CP) forms, in a last attempt to stop the disease progression, and is therefore justified by the risk/benefit ratio, while the acute bouts of relapsing-remitting forms (RR) are usually treated with high-dosage steroids for short periods. Separately, we can consider those clinical forms in which acute phases are followed by an incomplete recovery of the symptomatology, with a trend to worsening after each relapse, and others that show a very slow progression of symptoms, aggravated by acute bouts. These forms, which we define relapsing-progressive (PR), suggest the opportunity for administrating both an acute and a long-term therapy, in order to prevent the relapses and halt the disease progression.

Such therapeutic needs, very difficult to satisfy at the same time, together with the necessity of utilizing substances with high toxic potentiality, suggest that an associa-

tion between steroids and cytostatic immunosuppressants in alternate administration should balance the inhibition of the bone-marrow functionality induced by immunosuppressive drugs, so tath lower dosages of both substances in a more diluted administration could be utilized.

Steroids in fact increase the quantity of hemoglobin and the amount of blood red cells: they are also able to stimulate a normoblastic response in patients with megaloblastic anemia [7], inducing in their turn an irrelevant and short-lasting lymphopenia. For these reasons, they seem to be able to balance, at least in part, some of the bone-marrow abnormalities caused by cytostatic immunosuppressants that, in reverse, allow a reduction of steroid dosages. This is clearly shown by the utilization of such associations in specific immunosuppressive posttransplantation therapies, and from the current administration of this therapeutic schedule in rheumatology, for the treatment of some collagenophaties [7].

For the practical application of this theoretical basis we chose, in our therapeutic trial, a long-acting synthetic corticosteroid (triamcinolone), in association with one of the less toxic and more convenient immunosuppressive drugs, azathioprine. The therapeutic protocol included the alternate administration of azathioprine 2 mg/kg per day for 15 days followed by triamcinolone 40 mg i.m. in a single dose during the following 15 days.

Thirty-four outpatients with definite MS according to McAlpine [8] were selected, excluding all the subjects with severe intellectual deterioration and associated diseases (diabetes, hypertension, peptic ulcer, bleeding diathesis, infections, etc) and pregnant women. The group was composed of 24 females and 10 males, with an age range from 25 to 55 years (mean, 37.5 years). Seven had a relapsing-remitting course with a relapse rate >2/year; 15 showed a relapsing-progressive course with a mean relapse frequency of 1.8/year; 12 had a chronic-progressive course lasting at least 8 months-1 year. Disease duration ranged from 3 to 16 years (mean, 7.3 years) (see Table 1). Clinical evaluation before and after treatment was carried out by means of Kurtzke's Expanded Disability Status Scale (EDSS) [9].

Table 1. Outpatients with clinically definite MS

N°. subjects	34
Sex	24F, 10 M
Age	24–56 (mean, 35.7)
Disease duration (years)	3–16 (mean, 7.3)
Disability scores[a]	0–5.5 (mean, 3.3)
Treatment duration (months)	8–42 (mean, 19.7)
Course	Relapsing – remitting Relapsing – progressive Chronic – progressive

[a] Kurtzke's Expanded Disability Status Scale

Table 2. Results

	Relapsing-remitting	Relapsing-progressive	Chronic-progressive
N°. subjects	7	15	12
Sex	5F–2M	10F–5M	9F–3M
Age	25–45 (mean, 29.8)	24–47 (mean, 34.5)	30–55 (mean, 43)
Disease years duration (x)	3–8 (mean, 5)	4–12 (mean, 6.8)	3–16 (mean, 9.9)
Treatment duration (months)	11–42 (mean, 18.4)	9–41 (mean, 22.6)	8–27 (mean, 17.08)
EDSS			
Pre	0–2 (mean, 1.1)	2.5–4.5 (mean, 3.4)	3.5–5.5)mean, 4.6)
Post	0–2 (mean, 1.2)	2.5–4 (mean, 3.4)	3.5–6.5 (mean, 4.9)
Relapse rate (years)			
Pre	1.8–3 (mean, 2.2)	0.6–2.6 (mean, 1.8)	
Post	0.6–1.9 (mean, 0.8)	0–2.1 (mean, 0.75)	

All the information regarding the conduction of the study and the clinical evaluation of the results have been previously communicated at the International Symposium for Multiple Sclerosis (Rome, October 1986).

A summary of our results is shown in Table 2. It can be seen that in relapsing forms (relapsing-remitting and relapsing-progressive) a reduction of relapse rate has occurred, while mean EDSS scores remained unchanged; in chronic-progressive forms as well disability scores did not vary (Table 2).

Despite all the limitations due to the lack of control cases and by the impossibility of performing a statistical evaluation because of the low number of patients, our data allow some preliminary conclusions. This treatment appears particularly indicated in relapsing-progressive forms, while it does not seem to affect at all chronic-progressive forms; its effectivenesas is uncertain in relapsing-remitting forms, as the interpretation of the data is, in such cases, extremely difficult. Overall, our results are very similar to those obtained by Myers et al (1983) in an analogous study on an MS population.

The efficacy of the therapeutic association between steroids and cytostatic immunosuppressive drugs in alternate administration can thus be overlapped with that of the two drugs administered separately, with the additional advantage of reducing the risks related to the chronic administration of the single substances.

Checking and monitoring the effectiveness of the therapy provides a further problem to face, looking for clinical, immunological, and electrophysiological parameters, and techniques that could let us have more detailed information on the state and number of the lesions. A new diagnostic tool that seems particularly useful for this purpose is magnetic resonance imaging (MRI).

Magnetic resonance imaging has in fact proved to be a very sensitive method for detecting white matter lesions and is considered an important tool for the diagnosis

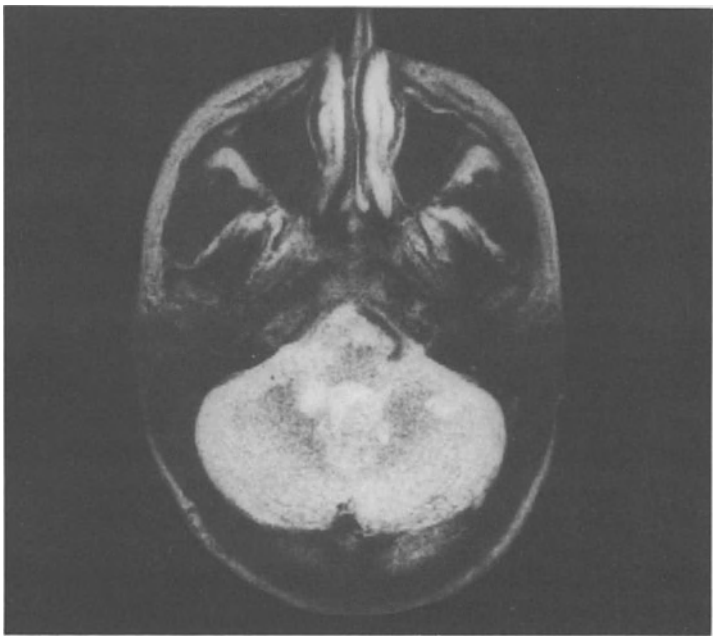

Fig. 1. Case 1 – S.E. 1800/100. Lesions with right middle cerebellar peduncle and in the left cerebellar hemisphere (acute phase)

and study of MS [10, 11]; of great interest as well are its possible applications in the therapeutic field.

We have performed MRI follow-up studies over a 3- to 6-month period in three cases of definite R-R MS, before and after treatment. The first MRI was carried out in the acute phase of the disease, while the second and the third were performed under treatment, respectively after 3 and 6 months from the beginning of the therapy.

In the first patient (No. 1) in the acute phase MRI showed several high-signal areas on T_2-weighted images in the right middle cerebellar peduncle, in the left cerebellar hemisphere, and in the periventricular white matter (Fig. 1). The patient was subsequently admitted to the therapeutic trial described above, being administered azathioprine and corticosteroids according to the protocol. Follow-up examination 3 months later was unchanged, while MRI scan after 6 months (the patient was still receiving the therapy) showed the lesions in the middle cerebellar peduncle and in the left cerebellar hemisphere to be small, er; the other lesions were unchanged (Fig. 2).

The other two patients (Nos. 2, 3) underwent MRI study of the cervical spinal cord, as clinical symptoms and signs suggested a prevalent involvement of the spinal cord at this level; MRI performed during the acute phase revealed an enlargement of the spinal cord in both patients on T_1-weighted images with a high signal area on T_2-weighted images in the corresponding location (Fig. 3). Treatment with corticos-

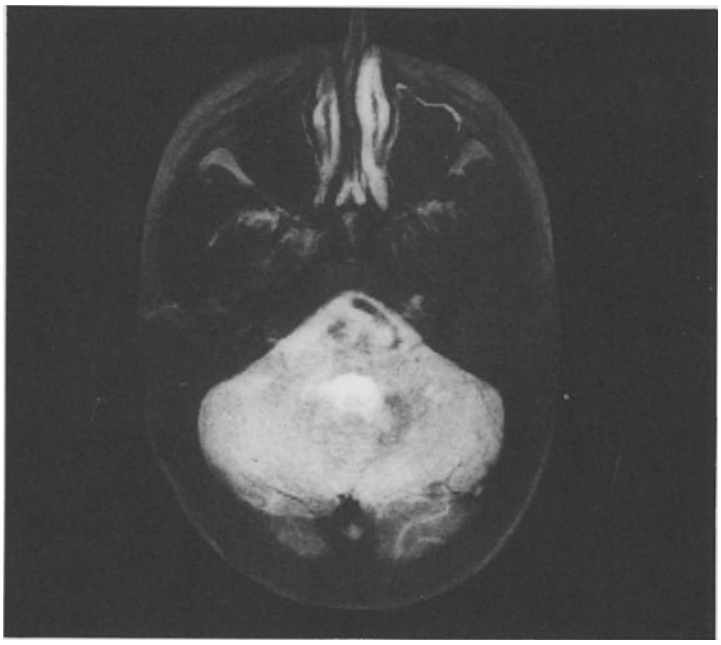

Fig. 2. Case 1 – Six months follow-up study. S.E. 1800/100. Reduction in size of the cerebellar lesions, more evident in the right middle cerebellar peduncle

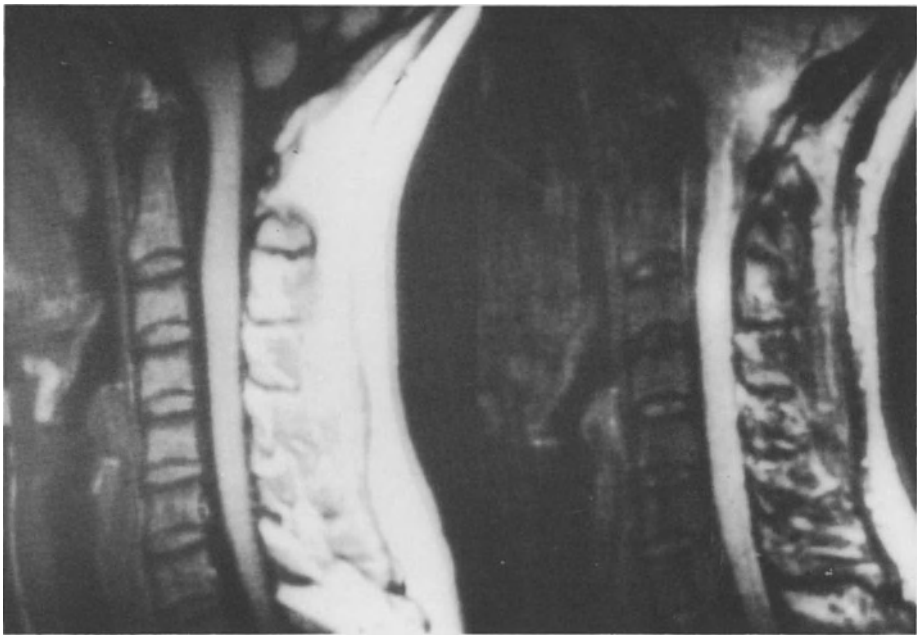

Fig. 3 Case 2 – P.S. 600/25 and S.E. 2000/40 – MRI shows an enlargement of the cervical spinal cord at C2 – C3 level with a long T2 lesion at the same level (acute phase)

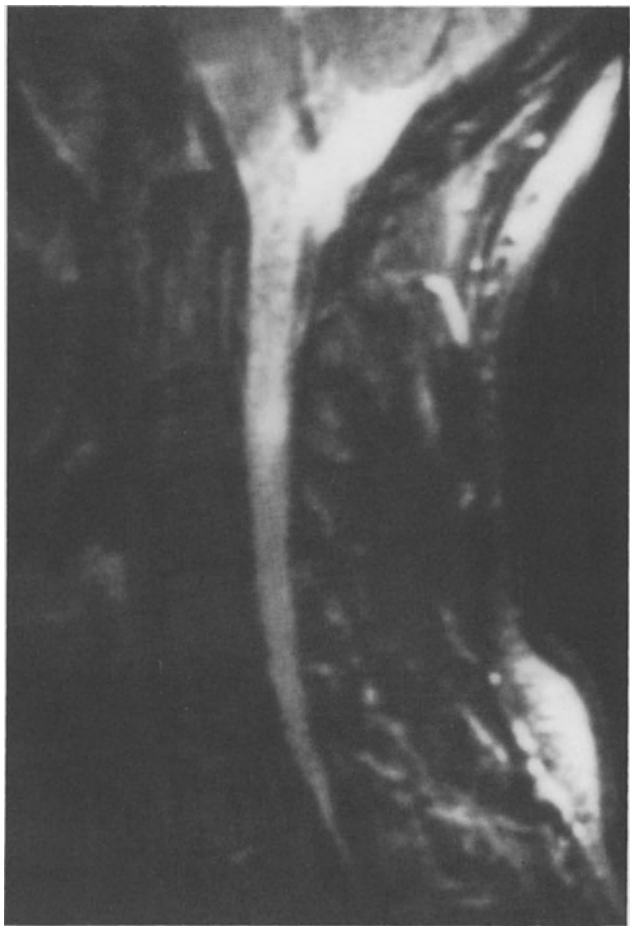

Fig. 4. Case 2 – Six months follow-up study. S.E. 2000/40. MRI shows a normalization of spinal cord diameter and a smaller intramedullary lesion at C2 level

teroids (periodic administration of ACTH) was begun and follow-up examinations 3 months later were unchanged. The third follow-up MRI scan, performed 6 months after the beginning of therapy, demonstrated a normalization of spinal cord diameter and showed the intramedullary lesion to be smaller (Fig. 4).

The results of this small follow-up study do not prove, of course, that the reduction in the extension of the lesions is directly or certainly related to the effectiveness of the treatment; they are only meant to give a suggestion for the assessment of case-control MRI studies in MS patients, in order to compare clinical course and MRI picture of the disease in subjects who receive treatment, with the same parameters in patients who have not been administered any therapy.

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New Trends for Treatment in Multiple Sclerosis

R. E. Gonsette

Belgian National Center for Multiple Sclerosis, Melsbroek, Belgium

Introduction

The 1980s will probably remain the decade of a worldwide concern in multiple sclerosis (MS) therapy. Indeed a better understanding of immune mechanisms associated with the development of CNS lesions have rekindled the interest for basic treatments capable of influencing the progression of the disease.

More than 25 different techniques of immunotherapy have been applied in MS in 20 years, but it is still difficult to define their respective interest and possible efficacy.

An important message from recent well-conducted multicenter, controlled, and/or blinded clinical studies is that, in spite of some encouraging preliminary reports, there is no definite evidence that various immune treatments such as thymectomy, lymphocytapheresis, plasmapheresis, levamisole, myelin basic protein, transfer factor, and hyperbaric oxygen are of any therapeutic value in MS. On the contrary, enough information is available today to prevent further clinical trials with these treatments.

The rationale for immunosuppression was based at first on the correction of hyperimmune reactions observed in MS patients. Nonselective and quite empirical techniques were used since the aim was to eliminate lymphocyte cells presumably involved in the development of local CNS immune events.

At that time, our knowledge of detailed immune mechanisms was still limited and, in addition, no practical parameters were available for the clinician to appreciate the effects of immunosuppressive agents.

The recent introduction of monoclonal antibody (Mab) techniques as a routine in clinical practice has yielded interesting information about lymphocyte subsets in nontreated MS patients, as well as in patients submitted to various immunotherapies; and, in this review, we would like to compare the clinical results with their potential effects on lymphocyte subsets.

Immune and Clinical Effects of Currently Used Immunosuppressive Agents

Among the immunosuppressors used for many years, *chlorambucil* should be mentioned, because it has been reported by Lees et al [1] in a recent clinical study that this substance does not correct the CD4/CD8 imbalance in MS patients and

does not influence the progression of the disease. The carcinogenicity of chlorambucil appears unquestionable and has been recently underlined by Aimard et al [2]. Its use therefore in MS patients should be advised against.

In spite of the fact that *azathioprine* (AZA) has been used in several well-conducted clinical studies, it is still difficult to really appreciate its potential benefit. It appears from the literature that AZA does not exert any major effect on the progression either of the remittent course or of the chronic progressive form. A mild beneficial effect has been reported in patients with the remittent-progressive form treated for years [3].

As far as the influence of AZA on lymphocytes is concerned, according to our experience as well as recently published studies [4], it appears that this substance does not exert any definite effect on lymphocyte subpopulations.

Cyclophosphamide (CY) is a potent immunosuppressive agent and has been used for many years in Europe [5, 6], and more recently in the United States [7]. In both European and American studies, intensive short-term CY was found effective in halting the progression of MS. This beneficial effect is observed in 60%–70% of patients, but is transient. Indeed, as a rule, a reactivation of the disease is observed 2 or 3 years after an acute, intensive CY immunosuppression. In our experience [8], this stabilization period can be prolonged for 5 years and over in patients submitted to a subsequent chronic oral CY immunotherapy.

A higher incidence of cancers has not been observed in our chronically CY-treated patients, even with a follow-up of 350 patient-years. The risk of adverse reactions, however, must not be underestimated and a new protocol, with intermittent bolus infusions, has been recently designed to reduce the annual cumulative dose of CY.

Where effects of CY on lymphocyte subsets are concerned, a mild but significant reduction of CD4 cells and a concurrent marked increase of CD8 cell percentages were observed in our chronically CY-treated patients, and, as a result, the CD4/CD8 ratio returned to normal values or even lower. These findings confirm the previous observations of Brinkman et al [9] in CY-treated patients with a progressive form.

Among the new generation of immunosuppressive agents, *cyclosporine A* (CSA) specifically inhibits interleukine production and, in opposition to other immunosuppressive agents, has no antimitotic or cytostatic effects. Two blinded studies with CSA are currently under way in Europe: one versus placebo in the United Kingdom and one versus AZA in the Federal Republic of Germany. In addition, a large clinical trial will enrol over 500 patients in the United States.

The results of the German trial have been recently published [4] and no differences concerning disease progression and relapse rate were found between patients treated with CSA or AZA. CSA appears therefore of little benefit, if any, in MS.

In the same way, neither AZA nor CSA produced lymphocyte changes in this blinded study. Of note is that functional studies recently reported by Bania et al [10] have demonstrated that chronic administration of CSA in MS patients reduces the cytolytic function of CD8 cells, but does not correct the aberrant suppressor function observed in most MS patients.

The double-blinded controlled clinical trial with *Copolymer I* (COP I) by Bornstein et al [11] appears promising. In 25 MS patients treated for 3 years, a significant

decrease of the exacerbation rate was observed in the COP I versus the placebo group. In the same way, a clinically important and statistically demonstrable slowing of disease progression was noted in the COP I-treated group. In spite of the fact that it has been suggested that the beneficial effect of COP I is partly due to lymphocyte subsets changes, no lymphocyte typings were performed in this study.

Isoprinosine(ISO) has been used for many years as a tentative treatment of subacute sclerosing panencephalitis with conflicting results. Three clinical trials in MS patients have been recently published in France and Italy. In two blinded studies [1, 12] ISO appears definitely more effective in reducing the annual relapse rate and in slowing the disease progression than placebo or chlorambucil. The same results were observed in the Italian open study [13]. According to Pompidou et al [12] and to Lees et al]1], ISO appears capable of restoring a normal CD4/CD8 ratio in MS patients. To confirm these observations, an open study is currently under way in our department, with serial lymphocyte determinations in MS patients treated with ISO for 1 year.

In a blinded study recently reported by Cook et al [14] about *total lymphoid irradiation* (TLI) in MS, a stabilization of the disease was observed more frequently in irradiated patients than in the sham group, but this benefit was transient. A lymphopenia below 1000/ml was noted in most patients for 1 year. Lymphocyte subset determinations were not done in this study, but in a preliminary report by Kolar et al [15] the failure of TLI to correct the CD4/CD8 ratio was associated with a therapeutic ineffectiveness in a particular case.

More specific immunological interventions include treatments with *interferons* (IF), but little is known about the exact immune effects of these substances. From a clinical point of view, intrathecal IF bèta possibly exerts a favorable effect on the exacerbation rate but seems poorly effective on the progression of MS [16]. The same holds for i.v. IF alpha, as recently reported by Camenga et al [17]. According to Panitch [18], a prolonged beneficial effect was observed 2.3 years after cessation of treatment with IF alpha. Of great interest is that in patients treated with IF gamma [19] the exacerbation rate was significantly increased. This negative effect of IF gamma is not totally unexpected from animal immunological studies since IF gamma increases expression of major histocompatibility complex geneantigens, what is probably happening in MS patients all along. However, this possibly opens new therapeutic avenues, for instance by developing anti-IF gamma monoclonal anti-bodies.

Poly ICLC is an IF inducer already used in postinfectious demyelinating encephalomyelitis. According to Bever et al. [20], a stabilization was observed in 18 treated MS patients, but secondary hyperthermia often provokes a transient neurological deterioration.

New Avenues in MS Immunotherapy

Preliminary results with substances less toxic (COP I, ISO) than the usual cytostatics are an incentive for further research with new agents. If we accept that experimental allergic encephalomyelitis(EAE) is a good animal model for the screening of potential therapeutic agents in MS, *mitoxantrone* could be a good candidate.

Indeed, it was found able to prevent EAE in rats [21], to suppress EAE even when administered after the onset of clinical signs, and to prevent further relapses in the remitting form [22]. As far as we know, lymphocyte changes have not been monitored during these experiments.

According to Bicker et al. [23], *ciamexone* should exert a selective action on the CD4/CD8 balance without any cytotoxic effect. Ciamexone would specifically increase suppressor cells and this effect could be favorable in MS patients with a low percentage of this lymphocyte subpopulation. In our laboratory, however, no suppressive effect of ciamexone on EAE in guinea pigs was found in preliminary experiments.

Monoclonal antibodies against CD4 + cells have been extensively used in EAE. According to Sedgwick and Mason [24], prevention of EAE seems to result rather from an interference with the disease-inducing capacity of CD4+ cells than from a clonal deletion. These Mabs should block the interaction mechanisms between activated cytotoxic effector cells and their putative target in the CNS. Preliminary trials in MS patients with anti-Pan T-cell antibodies or with anti CD4+ antibodies resulted in a transient elimination of the target cells, without perceptible clinical side effects [25]. In this short-term open study, patients were treated concurrently with prednisone and no conclusions about the therapeutic value of Mabs in MS can be drawn at this moment.

It appears from Sedgwick and Mason studies that inactivation of CD4+ cells seems feasible without cell lysis. In addition, more specific Mabs would spare possibly existing CD4+ suppressor cells, partially responsible for the natural self-tolerance. Of note is that, according to Rose et al. [26], a two-color FACS analysis has demonstrated a selective loss of a "CD4+ subset", maybe CD4 suppressor cells, in active MS patients.

In the same way, a selective loss of a CD4 lymphocyte subpopulation inducing suppressor T-lymphocytes has been observed by Morimoto et al. [27] in MS patients with the progressive form.

Lymphocytotoxic antibodies have been observed recently by Van Lambalgen [28] in sera from MS patients. This cytotoxic activity preferentially directed against CD8+ cells is more pronounced during exacerbations, and correlates with a concurrent increased CD4/CD8 ratio resulting from a decrease in CD8 cell percentage. If the existence of these specific lymphocytotoxic anbibodies is confirmed, and if they play a role in disease activity, their monitoring in MS patients and their removal form the sera could be a new therapeutic approach.

Other immunological interventions are still to be investigated. We were particularly interested in the publication by Bowern et al. [29] about the suppression of EAE in rats with *desferioxamine* (DFOM), an iron-chelating agent, and we were able to confirm this protective effect, albeit less pronounced, in guinea pigs. The exact mechanisms of action of DFOM are still to be elucidated, but from preliminary experiments, it appears that immune effects of this substance are associated with lymphocyte cells changes.

During this meeting, Arnason suggested a possible relationship between the sympathetic nervous system and immune regulatory mechanisms. Of note is that *prazosin*, an alpha 1 adrenergic receptor antagonist, has proved effective in suppressing EAE in rats [30].

These observations demonstrate that existing, nontoxic substances are able to influence CNS immune reactions and there is a reasonable hope that such substances could be of therapeutic value in MS.

The latest developments in molecular biology suggest that the definite treatment of MS could be based on molecular interventions. Indeed, Fujinami and Oldstone [31] have shown, with the aid of computerassisted analysis, that amino acid sequence homology exists between viral membrane and myelin basic protein. Rabbits immunized with homologous synthetic viral peptide develop antibody to MBP, mononuclear cells responding to MBP and CNS perivascular as well as meningeal infiltrates.

Since the major histocompatibility complex expressed on T cells determines the locus recognizing the homologous sequence of viral membrane, it has been postulated by Steineman [32] that a possible therapeutic approach for MS would consist of the development of Mab directed against this active part of the HLA system. Up until now, however, no differences concerning HLA DR and DQ sequences have been found between MS patients and healthy controls.

Conclusions

Our concepts of the basic therapy of MS have changed considerably in 20 years. Many clinical trials with different immunological techniques have been published. Most of them were found of no therapeutic value and they are no longer in common use. As we do not know yet the cause of MS and that no perfect, reproducible animal model is so far available, numerous tentative treatments, most of them unsuccessful, are the only way to explore new potential therapeutic avenues. A recent and important step forward has been made with a better understanding of immune abnormalities associated with MS evolution, as well as with the development of new immunological techniques in clinical routine. These techniques allow the follow-up of immunological changes produced by immunosuppressive treatments in MS patients, as well as correlations between immune effects and clinical results. It is striking to observe that treatments with a negligible influence on the disease, if any, do not correct the CD4/CD8 imbalance resulting from a loss of CD8 cells, which is the most frequently observed immune abnormality in MS patients. On the contrary, CY, a potent immunosuppressive agent, appears capable both of halting the disease transiently and of restoring a normal CD4/CD8 ratio by increasing the percentage of CD8 cells. Unfortunately the risk of life-threatening adverse reactions after a prolonged administration of CY is obvious and there is an urgent need for new agents with the same beneficial effects and devoid of long-term toxicity.

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Short Communications

Limitations of Peroxidase Staining in the Detection of CSF IgA Separated by Agarose Isoelectric Focusing

P. Gallo, S. Morara, M. Piccinno, and *B. Tavolato*

Department of Neurology, University of Padova, Padova, Italy

Introduction

Cerebrospinal fluid immunoglobulin electrophoretic abnormalities are characteristic of multiple sclerosis (MS). Intrathecal synthesis of IgG has been intensively investigated, while little attention has been directed to CSF IgA and IgM. The detection of intrathecally synthesized IgA oligoclonal bands (OBs) in MS CSF has been reported by Arnason's group [1]: IgA oligoclonal bands were found in 16 of 20 MS CSFs studied by agarose isoelectric focusing (AIEF), transfer of protein to nitrocellulose membrane, specific immunofixation, and peroxidase staining. However, there are several reasons to question the possibility of a reliable detection of IgA OBs by AIEF of native as well as concentrated CSF. We investigated the occurrence of IgA OBs in CSF from MS and normal subjects to verify the previously reported data.

Materials and Methods

The routine detection of albumin and IgG in serum and CSF was performed by immunoprecipitation nephelometry, while the detection of IgA was performed by laser nephelometry. The IgA level was measured in 63 MS CSFs and 30 normal CSFs. The intrathecal synthesis of IgA was evaluated by calculating the IgA index (i.e.; CSF IgA / serum IgA: CSF Alb/serum Alb): a value higher than 0.5 was taken as index of intrathecal synthesis of IgA. AIEF was carried out as previously described [2] with some minor modifications. After nitrocellulose blotting, the detection of IgG was achieved by the following methods:

1. Single immunofixation and peroxidase staining [1]
2. Double immunofixation and peroxidase staining [3]
3. Double immunofixation, avidin-biotin amplification, and peroxidase staining [4]

From 100 to more than 600 ng IgA was loaded on the gel.

Results

Figures 1 and 2 summarize the quantitative data about CSF IgA. In MS CSF the IgA levels ranged between 0.7 and 17.7 mg/liter (average value, 3.38 mg/liter) while in normal CSF the range was between 0.7 and 4.5 mg/liter (average value, 2.2 mg/

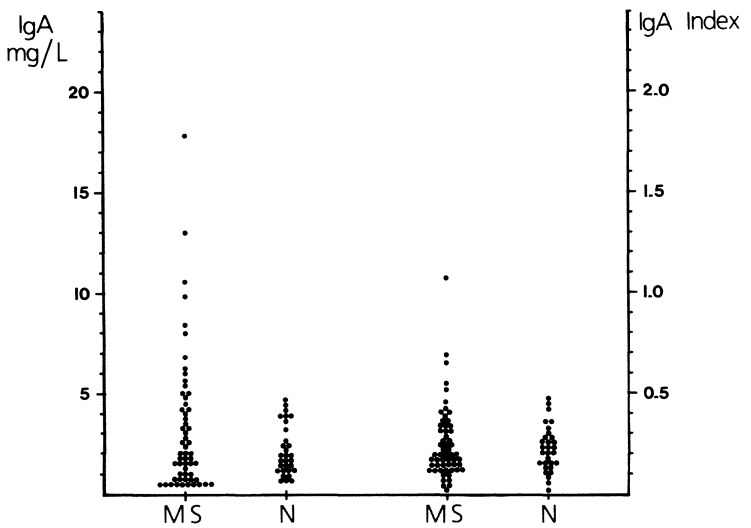

Fig. 1. IgA levels and IgA index values in 63 MS CSFs and 30 normal CSFs

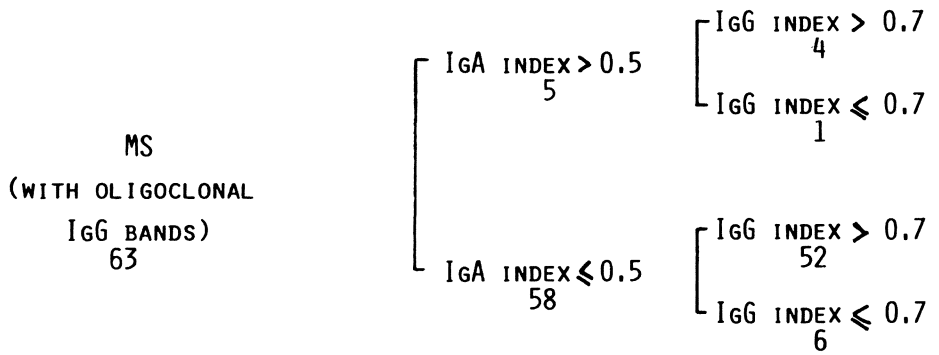

Fig. 2. Correlation between the IgA index and IgG index in 63 MS CSFs with IgG oligoclonal bands

liter). The IgA index was always ≤ 0.5 in normal CSF. Five MS CSFs showed an increased IgA index (>0.5). Only one sample had an increased IgA index and a normal IgG index (≤ 0.7).

In spite of the different amplification techniques, unspecific stainings were always obtained from AIEF followed by specific IgA immunofixation. When IgG and IgA were studied in parallel on the same gel (Fig. 3), it was possible to demonstrate that all the IgA OBs detected corresponded to IgG OBs.

Even when single immunofixation and peroxidase staining was used, the background due to IgG was high enough to make the detection of IgA unreliable (Fig. 3). The highest background was obtained with avidin-biotin-peroxidase complex staining. Sometimes, some additional CSF bands were found around pH 6.0

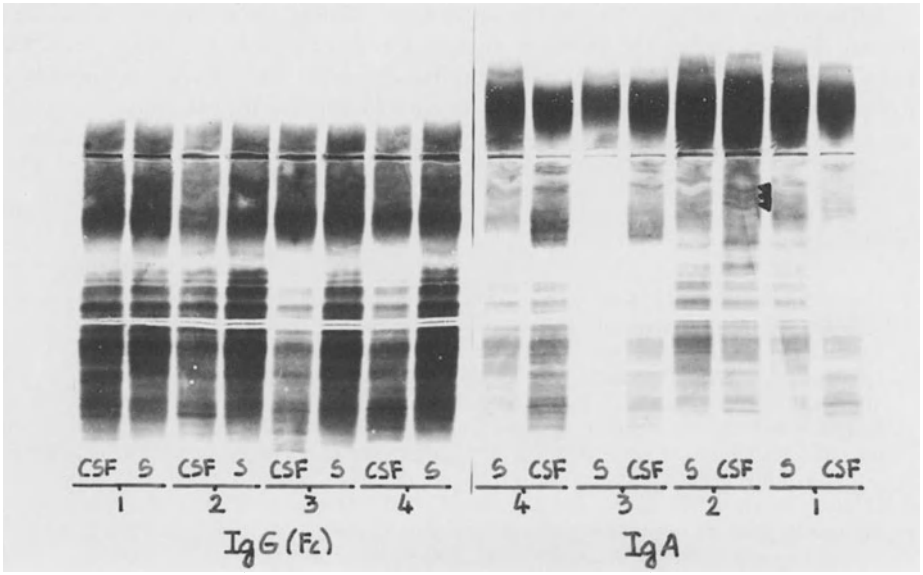

Fig. 3. Nitrocellulose membrane stained by single immunofixation and peroxidase staining. Four MS CSFs and serum-paired samples were studied in parallel on the same AIEF gel and developed with anti-IgG (Fc fragment) (*left*) and anti-alpha (*right*) antisera. The IgG OB patterns were also stained by the anti-alpha antiserum. In *CSF 2* some additional IgA bands, around pH 6.0, are indicated by *arrowheads* (see text for explanation)

which were stained by the anti-alpha antiserum and not by the anti-IgG (Fc) antiserum (Fig. 3, arrows-heads). In these cases the bands were interpreted as IgA by comparison with the IgG pattern.

As expected, IgA OBs were never found in CSF from normal individuals.

Discussion

The routine determination of IgA in MS CSF and the calculation of the IgA index was found to be useless in the diagnosis of the disease. In fact, only five CSFs, among 63 specimens studied by laser nephelometry, has an increased IgA index. Only one sample had an increased IgA index and a normal IgG index: however, this sample was IgG OB positive.

From a qualitative point of view, we stress the importance of a correct interpretation of the data achieved by AIEF followed by transfer to nitrocellulose and peroxidase staining. In all instances, the IgA immunofixation revealed a pattern similar to that of oligoclonal IgG, although fainter. These results were further corroborated by experiments performed on serum specimens from myeloma IgA and IgG. Summarizing these data, it is clear that all amplification systems used in the present study are not suitable for the analysis of oligoclonal IgA which might be present in MS CSF.

These limitations seem due to the peroxidase staining since they are a common feature of all methods. The avidin-biotin complex increases, as previously described for CSF IgM [5], the intensity of unspecific stainings. Therefore, one should be extremely careful in interpreting IEF immunoglobulin patterns when using the above-described methods.

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Failure to Detect Anti-HTLV-III Antibodies in Serum and CSF of 84 Neurological Patients

E. Merelli,[1] *P. Sola,*[1] and *G. Montagnani*[2]

1 Department of Neurology of the University of Modena, Modena, Italy
2 Immunohematology, Center of the University of Modena, Modena, Italy

Introduction

A unique virus or group of viruses has not been unequivocally linked to multiple sclerosis (MS), in spite of intensive investigations; however, a recent virological study [1] once again raises the question of a specific viral cause of MS. In particular human T-cell lymphotropic retroviruses (HTLV), because of their biological and physicochemical properties, have been postulated to play a role in the pathogenesis of MS. We examined CSF and serum of neurological patients including MS, in order to detect antibodies against HTLV, type III.

Material and Methods

Eighty-four paired sera and CSF from neurological patients coming from the Emilia-Romagna region of Italy were examined. Fifty-two patients were affected by MS, classified according to Poser et al. [2], while 32 patients presented other neurological diseases (OND) CSF and serum immunoglobulin concentration was determined with a Beckman Auto ICS Nephelometer. Using LKB equipment, 6% polyacrylamide gel isoelectric focusing (IEF), pH range 3.5–10, was carried out. CSF and serum oligoclonal IgG bands were identified by direct immunofixation with monospecific anti-human IgG (Dako). Link's IgG index, Tourtellotte's IgG synthesis/day, and CSF/serum albumin ratio were calculated. The presence of anti-HTLV-III antibodies was determined by ELISA, using polystyrene microplates coated with purified HTLV, type III (Organon, Teknika). All the sera were diluted with phosphate-buffered saline (PBS)/20% normal goat serum and CSF adjusted to a final IgG concentration of 4 g/liter and incubated at 37°C for 30 min. Each well was washed four times with PBS and subsequently 100 µl HRP-labelled goat anti-human IgG was added to each well and incubated at 37°C for 30 min. After extensive washing, 100 µl OPD (*O*-phenylenediamine-dihydrochloride) was added to each well end kept in the dark at 25°C for 30 min. The reaction was stopped by adding 100 µl 4*N*sulphuric acid. The plates were read at 492 nm using a multichannel photometer. Absorbance values above the cut-off level of $0.5(\overline{N}+\overline{WP})$ were considered positive.

Table 1. Data of MS patients

	No.	M	F	Mean age	CSF/S albumin 0.0055	>5.5	IgG index >0.7		IgG syn./day >3.3		Oligoclonal IgG bands3		Pleocytosis Cells/mm^3		Anti-HTLV III
Definite MS (CDMS A1–A2, LSDMS B1-B2-B3)	30	10	20	37	4	13%	25	83%	24	80%	30	100%	17	57%	0
Probable MS (CPMS C1-C2-C3, LSPMS D1)	22	9	13	31	2	9%	14	64%	12	54%	20	91%	11	50%	0
Total	52	19	33	34	6	12%	39	75%	36	69%	50	96%	28	54%	0

Table 2. Data of OND patients

	No.	M	F	Mean age	CSF/Serum albumin 0.0055	>5.5	IgG index >0.7		IgG syn./day >3.3		Oligoclonal IgG bands 3		Pleocytosis Cells/mm^3		Anti-HTLV III
Polyneuropathies	11	5	6	55	9	82%	1	9%	0	0%	3	27%	4	36%	0
Spinal cord disease	5	3	2	46	3	60%	0	0%	0	0%	0	0%	0	0%	0
CNS inflammatory diseases	8	3	5	36	8	100%	3	37%	2	25%	3	37%	8	100%	0
Headache and dizziness	8	3	5	39	0	0%	0	0%	0	0%	0	0%	0	0%	0
Total	32	14	18	44	20	62%	4	12%	2	6%	6	18%	12	37%	0

Results

Data of MS patients (Table 1) show clear blood-brain barrier (BBB) damage in 12% and pleocytosis in 54% of cases, thus indicating the presence of disease activity. IgG index and IgG synthesis/day were above the normal range in 75% and 69% of patients, respectively. CSF oligoclonal IgG bands were present in all the definite MS and in 20 out 22 cases of probable MS. Table 2 shows that in the OND patient group intrathecal synthesis of IgG with IEF oligoclonal bands takes place only in Guillain-Barré polineuropathy and in CNS inflammatory diseases. All CSF and sera samples from MS as well as from OND patients did not contain anti-HTLV-III antibodies at detectable levels. Since in a few cases the sample may have contained such a low level of anti-HTLV-III that the test result was repeatedly just below the cutoff value of 0.5 ($\overline{N}+\overline{P}$), the so-called "gray zone," we also considered a lower cutoff level, e.g., 0.2 ($4\overline{N}+\overline{WP}$). In spite of this adjustment all the results were still negative.

Discussion

HTLV retroviruses have been supposed to be involved in the pathogenesis of MS because of their properties, which include: a potential neurotropism (i.e., AIDS encephalopathy), a reverse transcriptase, evident tropism for OKT4 lymphocytes, and the capacity to inhibit T-cell function, to kill, or to transform infected T cells [3]. Koprowski et al. [1] found antibodies against HTLV-I and HTLV-III in serum and CSF of MS patients from Sweden and Key West, Florida. However, our results are at variance. No positivity was found in CSF and serum of the OND patients as well as the MS group for HTLV-III antibodies. These data agree with Madden et al. [4], who reported the absence of HTLV-I, -II, -III antibodies in MS and with De Rossi et al. [5], who failed to detect antibodies against HTLV-I and -III in MS patients from the Venetian region, Italy. This study needs to be completed with HTLV-I antibody search both with ELISA and Western Blot analysis. Fluctuations in the antibody titers were observed, due to the disease activity; nevertheless it should be noted that most of our patients were in the acute phase of MS.

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Absence of Serum HTLV-I and -III Antibodies in Multiple Sclerosis Patients

E. Salerni,[1] *R. Barnabei,*[2] *C. D'Aurizio,*[1] *F. D'Andrea,*[1] *D. Adorno,*[3] *P. Pellegrini,*[3] *A. M. Berghella,*[3] *C. Maschio,*[2] and *M. Prencipe*[1]

1 Clinica Neurologica, Universitá de L'Aquila, L'Aquila, Italy
2 Laboratorio Analisi, Ospedale S. Salvatore L'Aquila, L'Aquila, Italy
3 Istituto CNR Tipizzazione Tissutale e Problemi della Dialisi L'Aquila, L'Aquila, Italy

Introduction

Multiple Sclerosis (MS) is often considered to be an autoimmune disease initiated by infection, probably sustained by many enveloped viruses acting together or individually rather than by one virus. Koprowski et al. [2] observed high titers of antibodies reactive with antigenes of human T-lymphotropic viruses HTLV-I, -II, -III in sera and CSF of some MS patients. More recently this finding was not confirmed by Spadaro et al. [4], who did not detect anti-HTLV-I antibodies in sera of seven MS patients and by De Rossi et al. [1], who failed to detect anti-HTLV I-III antibodies in sera and CSF in a group of 32 MS patients.

In the present study we submitted 26 MS patients to viral and retroviral antibody evaluation.

Patients and Methods

Twenty-six MS patients (11 male, 15 female), aged between 18 and 61 years (mean age, 34.6±1.7 years), all meeting established criteria for definite relapsing-remitting MS [3] were studied. Disease duration ranged from 0.5 to 24 years (mean, 9.4 years). Control serum samples were concurrently obtained from 30 healthy blood donors. Sera analysis to investigate the presence of antibodies reactive with antigens of the HTLV-I and -III viruses was performed during the remitting phase in all MS patients. In ten of them the analysis was repeated during the relapsing phase. Serum samples were assayed for IgG antibody against the major core proteins of HTLV-I and -III viruses by enzime-linked-immunosorbent assay (ELISA) (Biotech Research Lab, Dupont). In this group of ten patients, antibody activity against measles, rubella, and Epstein-Barr virus (EBV) viruses was examined during the two different clinical phases of the disease. All patients were also submitted to T-lymphocyte subset analysis. No patient had received any therapy 1 month before sampling. Statistical analysis was performed by means of Student's t-test.

Results

Multiple sclerosis patients and blood donor sera did not show significant levels of IgG antibodies against HTLV-I and -III major viral core proteins. All patients studied during relapse showed a significant decrease of T8+ cells.

Anti-measles, anti-rubella, and anti-EBV IgG antibody titers were found higher than normal values in all MS patients studied either in relapse or in the remitting phase. We found IgM antibody anti-measles in 70% (7/10) of patients during the relapse and in 60% (6/10) of those on remission. Anti-rubella IgM antibody titers were higher than normal values (1/40) in 80% (8/10) of patients on relapse and in 60% (6/10) of those on remission. Anti-EBV IgM antibody titers were higher than normal values (1/20) in 70% (7/10) of patients on relapse and in 80% (8/10) of those on remission. No statistically significant difference was found between antibody titers observed in the relapsing phase and those collected in the remitting phase.

Discussion

Our data indicate the absence of antibodies reacting with HTLV-I and -III antigens in a group of Italian MS patients. These findings contrast with previous literature reports [2] and might be explained by different hypotheses: 1. only a proportion of MS patients might respond with the production of antibodies that cross-react with HTLV-I and -III antigens, but in none of our 26 patients did we detect anti-HTLV-I and -III antibodies; 2. antibody levels could fluctuate during the disease, but our patients have a wide disease duration range and were examined in both remitting and relapsing phase; or 3. the distribution might be strictly geographically dependent. It may be supposed that in our country MS patients are not at risk for HTLV-I or -III infection.

On the other hand, anti-measles, anti-rubella, and anti-EBV activity was present in almost all MS patients, but it was not correlated with the different clinical phases. In addition we found higher rubella- and EBV-specific IgM antibody titers and the presence of IgM antibodies against measles in MS patients. This finding was not previously reported by others [5].

These data confirm that MS patients show a non specific immunological response to undefined stimuli.

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Antigen Histocompatibility (HLA) in Multiple Sclerosis Patients from Gorski Kotar, Yugoslavia

J. Sepčić,[1] *L. Antonelli,*[1] *J. Rudez*[1] and *D. Rukavina*[2]

1 Department of Neurology University of Rijeka, Rijeka, Yugoslavia
2 Department of Physiology, University of Rijeka, Rijeka, Yugoslavia

Introduction

Epidemiologic investigations indicate Gorski Kotar, a mountainous region in northwest Yugoslavia, to be an area with a high prevalence rate of multiple sclerosis (MS) – 201.9/100 000 inhabitants and distinct familial aggregation of the disease, 64.7/100 000 inhabitants [3]. This paper analyzes the distribution of tissue compatibility antigen in MS patients from this region and ascertains a possible association of certain HLA loci with infections transmitted by neurotropic viruses.

Subjects and Methods

HLA-A, -B, -C, and -D antigen typification was performed by the micro-lymphocytotoxic method according to Terasaky [5] in 21 clinically definite MS patients (Bauer's diagnostic criteria) and 39 controls. The ethnic groups of the examined patients were determined by studying the historic anthropography of Gorski Kotar, the population of which displays heterogeneous origins: Slavic, Saxon, Germanic, Czech, and Italian. The level of antiviral antibodies in the serum was examined by a complement fixation test (CF).

Statistics

HLA antigen distribution was examined by a relative risk determination (RR).

Results

The distribution of HLA antigens in the examined groups is shown in Table 1. It is evident that multiple sclerosis in Gorski Kotar is associated with HLA-All, HLA-B35, and HLA-DR2 bearers. Serologic analysis of viruses in MS patients shows more frequent presence of antibodies to of measles, rubella, and herpes simplex relating to the other viral infections. MS patients with HLA-B35 antigen displayed more frequent presence of serum antibodies to measles and rubella ($P < 0.05$). On the other hand, a signifacantly higher frequency of positive antibodies to herpes simplex was expressed in subjects without HLA-A11 (Table 2).

Table 1. HLA antigens in MS patients from Gorski Kotar, Yugoslavia

MS patients (N=21)	%	Control group (N=39)	%	RR
A2	42.9		53.8	0.64
A3	23.8		23.1	1.04
A9	42.9		30.8	1.69
A11	19.05		7.7	2.82
B7	14.3		20.5	0.65
B13	4.8		7.7	0.6
B35	57.1		23.1	4.44
DR1	33.3		23.5	1.63
DR2	53.3		23.5	3.71
DR3	40.0		23.5	2.17
DR5	13.3		47.1	0.17

Table 2. Positive serum titers of virus antibodies and HLA antigens in MS patients from Gorski Kotar, Yugoslavia

HLA	antigens	Herpes simplex		Measles		Rubella	
	with	14.3%	28.6%	42,9%			
All			$P < 0.05$	NS		NS	
	without	85.7%	71.5%	57,1%			
	with	71.5%	83,3%	85,7%			
B35			NS	$P < 0.05$		$P < 0.05$	
	without	28.6%	16,7%	14,3%			

Discussion

Nowadays investigations concerning HLA antigen distribution of MS patients in Yugoslavia are very rare. Results of such examinations point to a varying distribution among different ethnic groups. Daskalova et al. [1] found an increased frequency of HLA-A3 and HLA-B15 antigens and an absence of HLA-Aw24, HLA-A32, and HLA-Bw16 antigens in Macedonian MS patients. Lević et al. [2] recorded an increased frequency of HLA-Al and HLA-B8 antigens in clinical casuistry (probably ethnically heterogeneous) in Belgrade. According to these authors HLA-B8 antigen is more frequently associated with malignant and HLA-A2 with a benign course of the disease in Serbia. Investigation of HLA antigen distribution among MS patients from the island-coastal part of the Rijeka region revealed a high relative risk toward the disease in the examined patients with HLA-All (*RR*, 6.38) and HLA-B13 (*RR*, 6.38). These values stand out from those cited in literature for MS patients in the Mediterranean basin.

No connection between hypocomplementemia and HLA-B18 determinant was found by Sepčić et al. in this population [4]. Tissue compatibility antigen research in Gorski Kotar confirms the well-known fact that multiple sclerosis is significantly

associated with the HLA-DR2 antigen in the areas with a high prevalence rate of the disease. The HLA-B35 antigen is a disposing rather than a protective disease marker in MS patients from Gorski Kotar, Yugoslavia.

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Diagnostic and Predictive Value of Nonclinical Tests in Retrobulbar Optic Neuritis*

A. Ghezzi, M. Zaffaroni, D. Caputo, C. Locatelli, S. Marforio, and *C.L. Cazzullo*

Centro Studi Sclerosi Multipla, Ospedale di Gallarate, Università di Milano, Gallarate, Italy

Introduction

Retrobulbar optic neuritis (RBON) is a frequent initial symptom of multiple sclerosis (MS). The progression of MS after an acute attack of RBON it differently evaluated in the literature, being from 13% [1] to 85% [2] of cases. The rate has been correlated to CSF pattern (intrathecal IgG synthesis) and HLA histocompatibility antigens [3]. A prognostic value has also been ascribed to tests for demonstration of multifocal CNS involvement evoked potentials (EPs) and magnetic resonance imaging (MRI) [4].
In our study we discuss the diagnostic and prognostic value of CSF examination and of multimodality evoked potentials in patients affected by RBON. Our preliminary data on MRI are also briefly discussed.

Subjects and Methods

We studied 49 patients affected by RBON; 32 were females, 17 were males. The attack was unilateral in 43 patients, bilateral in 6. The mean age was 29.0 years (range 20–40 years). In four cases a relative was affected by definite MS. Visual, brain stem auditory, and somatosensory evoked potentials were recorded according to the methods described elsewhere [5]. Responses were considered abnormal by a delay of visual evoked potential (VEP) P 100 wave, by a delayed I-III or I-V or III-V brain stem auditory evoked potential (BAEP) interpeak latency, or by a delayed N9-N13 or N13-N20 somatosensory evoked potential (SEP) latency (± 3 SD the normal mean value). Intrathecal IgG synthesis was demonstrated by the presenc of CSF oligoclonal banding [6] or by an IgG index > 0.7 [7]. MRI was performed in ten cases, according to the protocol described in a previous paper [8]. Forty-one cases were included in a follow-up study: excluding patients whose follow-up was < 12 months, there remained 37 cases (mean duration of follow-up: 4.0 ± 2.1 years). They were periodically examined by a neurologist every 4-6 months. The diagnosis of MS was made when a neurological symptom or sign was seen outside optic pathways. Eight patients whose CSF presented indices of intrathecal IgG synthesis were submitted to therapy with azathioprine (AZA) 2.5 mg/Kg per day for 18 months.

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Table 1. Results of CSF examination and evoked potential recording in patients with RBON

Intrathecal IgG synthesis	23/45	51.0%
Second site of CNS involvement at evoked potentials	7/39	17.9%
Contralaterally delayed VEPs	10/43	23.2%

Results

The percentage of abnormal findings at the tests is reported in Table 1: CSF was examined in 45 cases and indices of intrathecal IgG synthesis were found in 23 (51.0%) patients. SEPs and BAEPs could be recorded in 39 cases and demonstrated a CNS involvement outside optic pathways in 7/39 patients (17.9%). VEP results were analyzed in patients with unilateral RBON: a delayed response in the unaffected eye was recorded in 10/43 eyes (23.2%). MRI was available in ten cases and revealed lesions suggesting a demyelinating process in six cases: it was normal in one case with CSF oligoclonal bands. The number of patients who developed MS within 5 years is reported in Fig. 1 in relation to the length of the follow-up. Follow-up data are examined with respect to CSF pattern and AZA therapy in Table 2: results were not statistically significant. Grouping patients who developed MS and a further attack of RBON, P was between 0.10 and 0.05 (Table 2). The correlation of follow-up data with neurophysiological results is reported in Table 3: no relation was found with BAEP and SEP abnormalities; a higher risk of developing MS appeared related to the finding of a contralateral delayed VEP in unaffected eyes (42.8% VS. 29.6%, $P<0.05$).

Table 2. Follow-up results in relation to CSF pattern (A) and azathioprine therapy to which eight CSF + patients were submitted (B)

	Follow-up					
	MS		Recurrent RBON		Neg.	
A						
Intrathecal IgG synthesis +						
21 cases	6	(28.5%)	4	(18.0%)	11	(52.5%)
Follow-up 3.5±2.1 years						
Intrathechal IgG synthesis −						
15 cases	2	(13.3%)	0		13	(86.75%)
Follow-up 4.3±2.9 years						
B						
Azathioprine +						
8 cases						
Follow-up 3.7±2.5 years	4	(50.0%)	2	(25.0%)	2	(25.0%)
Azathioprine −						
13 cases						
Follow-up 3.4±1.8 years	2	(15.4%)	2	(15.4%)	9	(69.0%)

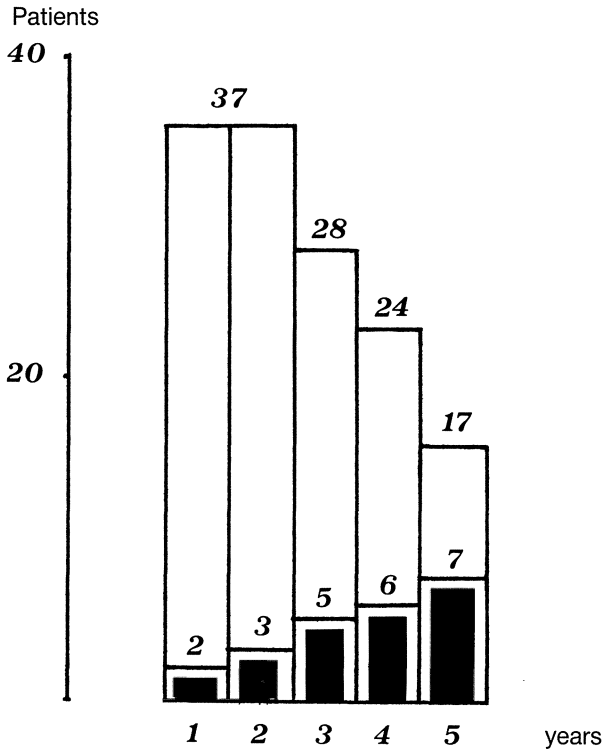

Fig. 1 Number of patients who developed MS (*black columns*) in relation to the length of follow-up

Table 3. Follow-up results in relation to neurophysiological results

	MS	Follow-up: Recurrent RBON	Neg.
BAEPs and/or SEPs			
Normal (n,27)	6 (22.2%)	4 (14.8%)	17 (63.0%)
Delayed (n,5)	1 (20.0%)	1 (20.0%)	3 (60.0%)
		P > 0.10	
Contralateral VEP			
Normal (n,27)	8 (29.6%)	2 (7.4%)	17 (63.0%)
Delayed (n,7)	3 (42.8%)	2 (28.6%)	2 (28.6%)
		P > 0.10	

Discussion

The demonstration of intrathecal IgG synthesis suggested a demyelinating pathogenesis in about 50% of our RBON patients; a similar finding was given by MRI, but in a more restricted series. In one patient MRI was normal but CSF showed oligoclonal bands: any other inflammatory autoimmune disease, namely a

collagen disease, was excluded. Evoked potentials revealed CNS involvement outside optic pathways in a smaller number of cases (10/43=23.2%).

Our preliminary data concerning the clinical follow-up showed that MS occurred in 41% of cases 5 years after RBON. Neurological complications were more frequent in CSF+cases (10/21) than in CSF-cases (2/15). AZA seemed ineffective at preventing the progression toward MS in high-risk (CSF+) cases.

A delayed VEP was recorded in the unaffected eye of seven cases with unilateral RBON: the percentage of patients who developed MS was higher than that of patients with normal responses. In our series an abnormal BAEP and/or SEP did not appear related to MS prognosis. To conclude, CSF study, multimodality evoked potentials, and, more recently, MRI appear important tests for disclosing the possible underlying demyelinating nature of so-called idiopathic uncomplicated RBON. In the absence of a resolutive therapy for MS, the contribution of these tests is actually restricted to their prognostic value: our results, although not significant, seem to suggest that CSF pattern is correlated with the risk of developing further neurological attacks, as demonstrated by other authors [3, 9]. A prognostic value is also suggested by the VEP pattern in the unaffected eyes.

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Contribution of Magnetic Resonance Imaging in the Assessment of Multiple Sclerosis*

D. Caputo,[1] *A. Ghezzi,*[1] *M. Zaffaroni,*[1] *S. Marforio,*[1] *G. Scotti,*[2] and *C. L. Cazzullo*[1]

1 Centro Studi Sclerosi Multipla, Università di Milano, Ospedale di Gallarate, Gallarate, Italy
2 Neuroradiologia, Ospedale di Niguarda, Milan, Italy

Introduction

The presence of intrathecal IgG production and of evoked potential (EP) abnormalities have been recently included as diagnostic indices of multiple sclerosis (MS) [1]. In recent years magnetic resonance imaging (MRI) has been demonstrated to be a sensitive tool in detecting demyelinating CNS lesions. In these studies [2–4], MRI was found capable of demonstrating abnormalities, suggesting MS in a higher proportion of cases with respect to CSF analysis and EP studies, even in early stages of the disease. Our study was carried out in order to assess the diagnostic efficacy of MRI in comparison with CSF and EP.

Subjects and Methods

Seventy-six MS patients were studied: 45 had definite, 17 probable, and 14 possible MS, according to McAlpine's criteria [5]. Ten patients affected by retrobulbar optic neuritis (RBON) were also studied. MRI was performed according to the method extensively described in a previous paper of our group [4]. Visual, brain stem auditory, and somatosensory EPs were recorded with standard techniques [3]: special attention was paid to the demonstration of an abnormal response in clinically uninvolved CNS pathways. Oligoclonal banding and IgG index were assessed according to the methods previously described[7].

Results

The results are analytically reported in Table 1. MRI showed lesions suggesting a demyelinating process in 95.3% of MS cases and in 60% of RBON cases. Intrathecal IgG synthesis was demonstrated in 62.2% and 60% of cases respectively; EPs revealed lesions in clinically unaffected pathways in 71.9% and 20% of cases

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Table 1. Percentages of abnormal findings in our series

		MRI+		IEF+		EP_S	
		No.	%	No.	%	No.	%
Definite MS	(n,45)	44	97.7	40	88.8	43	95.1
Probable MS	(n,17)	15	88.2	9	52.9	14	82.3
Possible MS	(n,14)	13	92.8	7	50	6	42.8
Total	(n,76)	72	94.7	56	73.7	63	82.9
RBON	(n,10)	6	60.0	6	60.0	2	20.0

Table 2. Distribution of abnormal findings in different groups

	Definite MS	Probable MS	Possible MS	Total	RBON
MRI, IEF, EP_S	34	7	5	46	1
MRI, IEF +	5	–	1	6	4
MRI, EP_S	4	5	1	10	1
IEF, EP_S	1	2	1	4	–
MRI +	1	3	6	10	–
IEF +	–	–	–	–	1
All negative	–	–	–	–	3

respectively. Comparative results of the three tests are reported in Table 2: at least one test was abnormal in all MS cases; MRI alone revealed lesions in ten cases but was normal in four cases with intrathecal IgG synthesis and/or delayed EPs. In RBON patients a demyelinating process was suggested by MRI in five cases.

Conclusions

The sensitivity of MRI in detecting demyelinating lesions has been stressed in several studies [2–4]. In our definite MS cases MRI showed abnormal findings in 97.7%. This percentage was slightly higher than that revealed by CSF and EP studies. In many probable and possible MS cases MRI was performed when CSF and EPs were normal: the low percentage of abnormal CSF and EP results can depend on this factor. Comparisons between the tests must obviously be considered with this limitation: however, MRI revealed lesions in a very large number of cases.

For the same reason the number of cases with a normal MRI and abnormal CSF and/or EPs cannot be exactly estimated. However, we can conclude that demyelinating lesions cannot be detected by MRI in some instances; probably it depends on their localization (optic nerve, spinal cord) or their size.

A limit of MRI in spite of its higher sensitivity is probably represented by its low specificity. Several other pathological processes involving the white matter can have a similar appearance: infarcts, metastases, infection processes. Abnormal findings have also been described in patients with epilepsy, psychosis and heredodegenerative disorders [2–4]. The requirement of a "no better explanation" must be always

considered as well as for the interpretation of CSF/EP abnormalities. To conclude, it seems advisable that the diagnosis of MS, in clinically suspected cases, is confirmed by the comparative results of several tests.

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Serial Evoked Potentials and Disease Progression in Multiple Sclerosis*

A. Ghezzi,[1] *E. Mazzalovo,*[2] *D. Caputo,*[1] *M. Zaffaroni,*[1] and *R. Montanini*[2]

1 Centro Studi Sclerosi Multipla, Ospedale di Gallarate, Università di Milano, Italy
2 Divisione Neurologica, Ospedale di Gallarate, Italy

Introduction

Cerebral evoked potentials (EPs) have been included among the tests for diagnosis of multiple sclerosis (MS) as methods for paraclinical demonstration of multifocal CNS involvement [1]. Their use has also been suggested in MS monitoring in order to evaluate disease progression and treatment efficacy objectively [2–4]. In this point the role of EPs is not well established and results are inconclusive [5–10].

In a previous paper [11] we evaluated the neurophysiological modifications of serial EP recordings in relation to disease evolution and to an immunological index (blood lymphocyte helper/suppressor ratio). Our results seemed to demonstrate that stable responses were observed more frequently in stable patients, but worsening responses in chronic progressive ones. In the present paper we describe in more detail neurophysiological modifications after a longer follow-up (18 months) in a larger series of patients (36 cases).

Subjects and Methods

Thirty-six patients affected by definite MS (according to Poser et al. [1]) took part in the study. They were 19 females and 17 males, mean age 32.1 years (range 17–44 years) mean duration of disease 7.9 years (range 2–20 years).

Clinical status, scored with the Kurtzke Functional Scale (FS), Disabilitiy Status (DS) Scale [12], and clinical history were assessed before each session. EPs were recorded every 3 months. The methods have been extensively described elsewhere [11]. Briefly, visual evoked potentials (VEPs) were recorded in response to a LED pattern reversal, brain stem auditory evoked potentials (BAEPs) in response to monoaural alternating clicks 70 dB SL, and somatosensory evoked potentials (SEP_S) in response to medial nerve stimulation. The following measures were considered: VEP-P100 latency, BAEP I–III, I–V, interpeak latency (IPL), and SEP N9–N20 latency (N13 was hardly recognizable in some recordings).

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The limits of test-to-test variability were respectively: 8.7, 0.25, 0.43, 2.3 ms and were adjusted for each patient proportionally to his or her own latency, according to the criterion described in a previous paper [11].

Results

Visual Evoked Potential Recordings

At the first examination VEPs were delayed in 29 out of 36 cases (80.5%). Changes occurred in the course of the study in 12 cases, as indicated in Fig. 1: in 5 cases they concerned the 2 eyes, in 7 cases only 1. Responses worsened in 13 eyes and improved in 2: a clinical correlation was observed in three and two cases respectively, in two patients responses worsened temporarily: in one the final latency was more delayed with respect to the initial value.

Brain Stem Auditory Evoked Potential Recordings

Abnormal responses at entry were found in 19 patients (52.8%). Modifications of I–V IPL occurred in 15 sides (12 patients): responses worsened in 14 recordings and improved in 1. A clinical correspondence was observed in three and one patients, respectively (Fig. 1). The increase of I–V IPL was associated with an increase of I–III IPL in five recordings (four cases). Changes of I–III IPL were detected in seven patients (nine sides). Responses worsened in eight cases, in one in relation to a relapse with a brain stem dysfunction. In one patient a temporary change was observed.

Somatosensory Evoked Potential Recordings

Somatosensory evoked potentials were above the normal limits in 24 out of 36 patients (66.7%) at the beginning of the study: in 10 recordings because of a delayed N9–N13 latency, in 18 because of a delayed N13–N20 latency. A clinically detectable defect of sensory pathways was present in 14 cases. During the study, changes occurred in 20 sides: in 9 sides N13 was recognizable with difficulty in at least one recording, so only N9 N20 latency was considered. Of the other 11 sides, in 10 the abnormality concerned the N13–N20 latency, in 1 the N9–N13 latency. Responses worsened in 18 recordings, in 6 (5 patients) coinciding with a relapse involving the sensory pathway. In two instances the latency increased temporarily, but in one the final latency was more delayed that the initial value (Fig. 1).

Clinical Electrophysiological Correlation

Cases were grouped into two categories according to the final DS and FS scores: in 15 cases a progression of disease was observed, with increased final FS and DS scores; 21 patients appeared stable in the course of the study. In this group cases 22

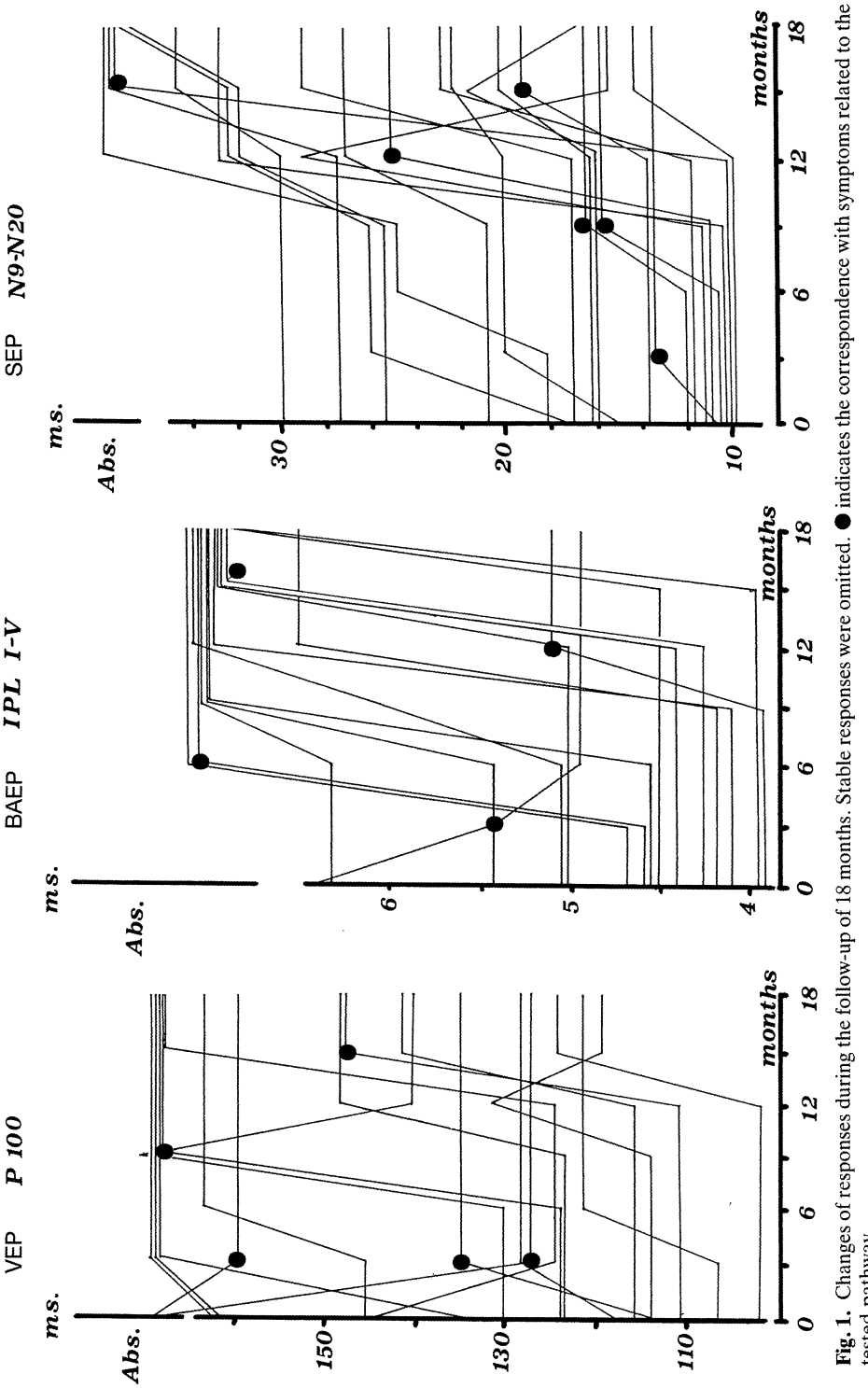

Fig. 1. Changes of responses during the follow-up of 18 months. Stable responses were omitted. ● indicates the correspondence with symptoms related to the tested pathway

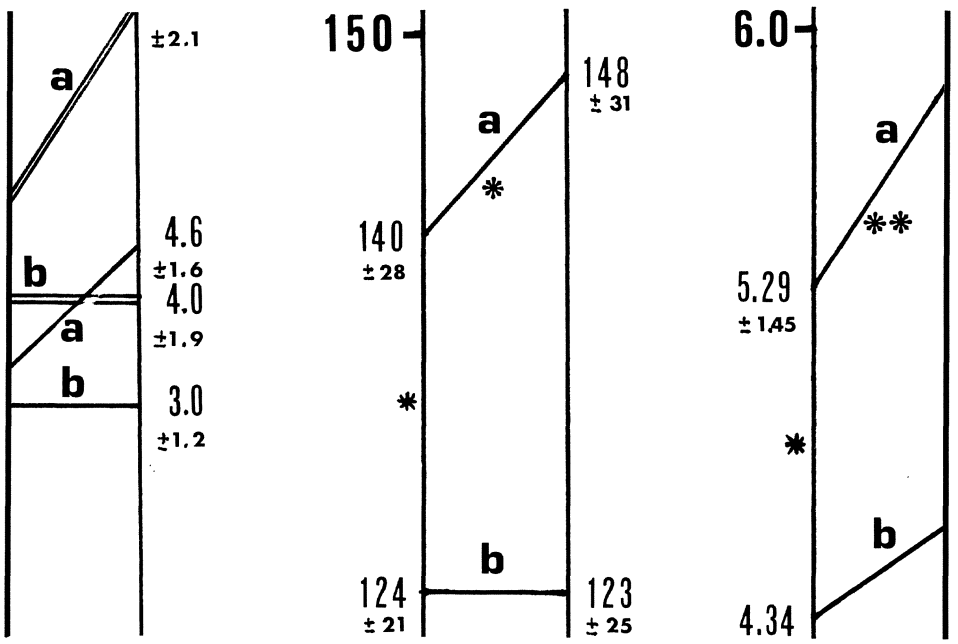

Fig. 2a, b. Changes of Kurtzke's scores (*FS*, functional system, *DS*, disability status) and of neurophysiological responses at the end of the follow-up. **a**, progressive patients; **b**, stable patients; * $P<0.05$; ** $P<0.01$

and 26 were also included: the former presented a partial recovery from an acute attack involving the optic nerve at entry; the latter was recovering from a relapse with vestibular-cerebellar symptoms. At the beginning of the study responses were significantly more delayed in progressive than in stable patients for the three types of EPs. Changes at the end were constantly and significantly more severe in progressive than in stable cases (Fig. 2). In patients whose responses were absent, we assigned the higher value observed in our laboratory, beyond that responses are unrecognizable. It was 200 ms for P100, 3.7 ms for I–III IPL, 6.7 ms for I · V IPL, and 35 ms for N9–N20 latency.

According to the final latency, responses were classified as stable, improved, or worsened, with respect to the limit of test-to-test variability, for each modality. Results indicate that the percentage of "worsened" responses was higher in progressive than in stable patients, the opposite for "stable" responses (Table 1).

Discussion

The progression of MS is often variable and unpredictable: for this reason the opportunity to dispose of an objective and quantifiable measure of neurological damage is commonly stressed. An important, but not well-defined role has been ascribed to EPs which have been used in numerous laboratories as possible indices

Table 1. Final responses at the three EP modalities in relation to MS evolution

	Multiple sclerosis			
	Stable		Progressive	
Responses	No.	%	No.	%
Improved	2	1.6	1	1.1
Stable	110	87.3	52	57.8
Worsened	14	11.1	37	41.1

$P < 0.001$

of neurological dysfunction of the respective pathways [2–12]. Two findings have been commonly observed: (a) a variability of responses in MS subjects, either in relapsing or in stable ones [6, 9–12] and (b) the rather frequent occurrence of changes in patients with relapses concerning other not tested pathways [3, 6, 11]. In agreement with previous observations, our results indicate that changes of responses can effectively occur with no correspondence with the status of the tested pathway, but, differently from other authors, only in rare cases were changes temporary, probably because of our more restrictive limits of test-to-test variability [11]. In these instances neurophysiological changes do not necessarily reflect pathological changes, but also a dysfunction of conduction because of immunological, chemical, or physical factors (such as the occurrence of blacking factors or the imbalance of electrolytes, pH, temperature [11]).

Most authors have concluded that EPs are a reliable test for neurological changes in MS patients, with more sensitivity than clinical examination, but the relationship between EP changes and disease progression has been incompletely evaluated, with few exceptions [4, 11]. In fact neurophysiological changes have been correlated with clinical changes of the tested pathways but not with the whole neurological status; moreover, most authors did not use multimodal evoked potentials.

In our study disease progression was evaluated, comparing initial and final Kurtzke's scores: in this connection a relationship was found between neurophysiological pattern and clinical course. Clinically stable patients showed only slight changes at the end of follow-up, according to the concept that MS involves a progression of lesions with time, even in apparently stable patients. In patients with progression of the disease, responses were more delayed with respect to stable patients even at the beginning of the study and more severely increased at the end. In other words, it seems to us that EPs give an objective measure of disease activity being correlated with clinical progression.

Responses severely delayed and responses increasing with time were mostly observed in progressive MS, also suggesting a possible prognostic value of neurophysiological tests.

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Clinical and Immunological Findings After Treatment with Thymostimulin in Multiple Sclerosis

A. R. Giovagnoli,[1] *L. Provinciali,*[1] *C. Bartocci,*[2] and *M. Montroni*[2]

1 Istituto delle Malattie del Sistema Nervoso, Università di Ancona, Ancona, Italy
2 Instituto di Clinica Medica Università di Ancona, Ancona, Italy

Introduction

Even if the pathogenesis of multiple sclerosis (MS) has not yet been clarified, interest is focussed on the role of the immune system in causing the disease. In recent years many studies have investigated the status of the immune system during MS.

Most previous reports stressed the correlation between T-lymphocyte subsets and the clinical course. The most consistent findings are:

1. the reduction in the total number of circulating and suppressor T-lymphocytes in acute exacerbations or in chronic progressive forms of the disease [1];
2. the increase in the helper/suppressor T-cell ratio in clinical and subclinical relapses [2];
3. the reduction in and the impairment of the suppressive function of the T-lymphocytes in clinically definite MS [3];
4. the increase in T-suppressor lymphocytes during recovery from acute exacerbations [4]; and
5. the association between low levels of complete T-cells, including both the helper and suppressor subsets, and the presence of progressive disability in relapsing-remitting and chronic MS [5].

It was pointed out that drugs which do not improve the lymphocytic state in MS cannot modify the long-term disability produced by the disease; such drugs include steroids which mainly alter the T-helper cells.

Using these assumptions as a starting point, we carried out a therapeutic trial using thymostimulin (Tpl-Serono), a crude extract of calf thymus affecting the maturation of the T-lymphocytes in different ways, relying on its possible influence on the T-cell subset derangement described in MS. Clinical and immunological changes were evaluated during thymostimulin therapy.

Patients and Methods

Ten out of 30 patients (average age, 33.4 years; 8 males, 2 females; average duration of illness, 7.5 years) were selected according to the presence of alterations of the lymphocytic assessment (total and suppressor T-cell reduction). Nine had definite MS whereas one had probable MS as defined by McDonald and Halliday's criteria. One patient left the trial after the first phase of treatment and was excluded.

A 6-month i.m. treatment with thymostimulin was administered (100 mg/day for 15 days, 50 mg/day for 3 months, 50 mg twice a week for the last 3 months) after obtaining informed consent.

The T-cell count was assessed (total lymphocytes, OKT3, OKT4, OKT 8 subsets, and T4/T8 ratio using monoclonal antibodies) and clinical parameters were evaluated using the Kurtzke Scale before the trial began and then 3, 6, and 12 months later. At the beginning, all patients had stable neurological deficits; during the trial five patients showed an exacerbation of the disease (three acute, two progressive) and were treated with steroids and ACTH for a short time after the end of the thymostimulin administration period.

None of the patients was suffering from infectious diseases.

We considered the changes in the lymphocytic assessment with particular regard to the total and suppressor T-cell subpopulations and the modifications in the disability scores.

Because of the extreme variability in the normal T-cell populations, we paid particular attention to the serial changes in each case and to the agreement between the clinical and immunological values.

A statistical analyses was performed using the t-test and the Wilcoxon Rank test.

Results

The patients were used as their own controls and the data obtained at each observation were compared with the previous and following checks, thus considering two periods, during and after the treatment.

During the treatment four patients showed an improvement in their lymphocytic condition with an increase in the total and suppressor T-cells. Of these four, two patients showed a clinical improvement during the same period. The remaining five patients showed no significant changes in their immunological or clinical data during the thymostymulin therapy (Table 1).

In the 6 months following the treatment immunological values worsened in four patients; in this period the clinical follow-up showed acute relapses in three patients and a progressive deterioration in two patients (Table 1).

Table 1. Comparison of clinical and immunological data[a]

Patients	First period (during treatment)		Second period (after treatment)	
	Lymphocytic Assessment	Kurtze Score	Lymphocytic Assessment	Kurtze Score
C. G. (Definite MS)	→	→	→	→
D. M. (Definite MS)	↑	↑	↑	↑
F. M. (Definite MS)	↑	→	↓	↓
M. G. (Definite MS)	↑	↑	↑	↑
N. D. (Definite MS)	→	→	↑	↓
P. A. (Definite MS)	→	→	↓	↓
R. M. (Definite MS)	↑	→	↓	↓
Z. L. (Definite MS)	→	→	↑	↓
P. L. (Probable MS)	→	→	↓	↑

[a] ↑ improved, ↓ worsened, → stationary

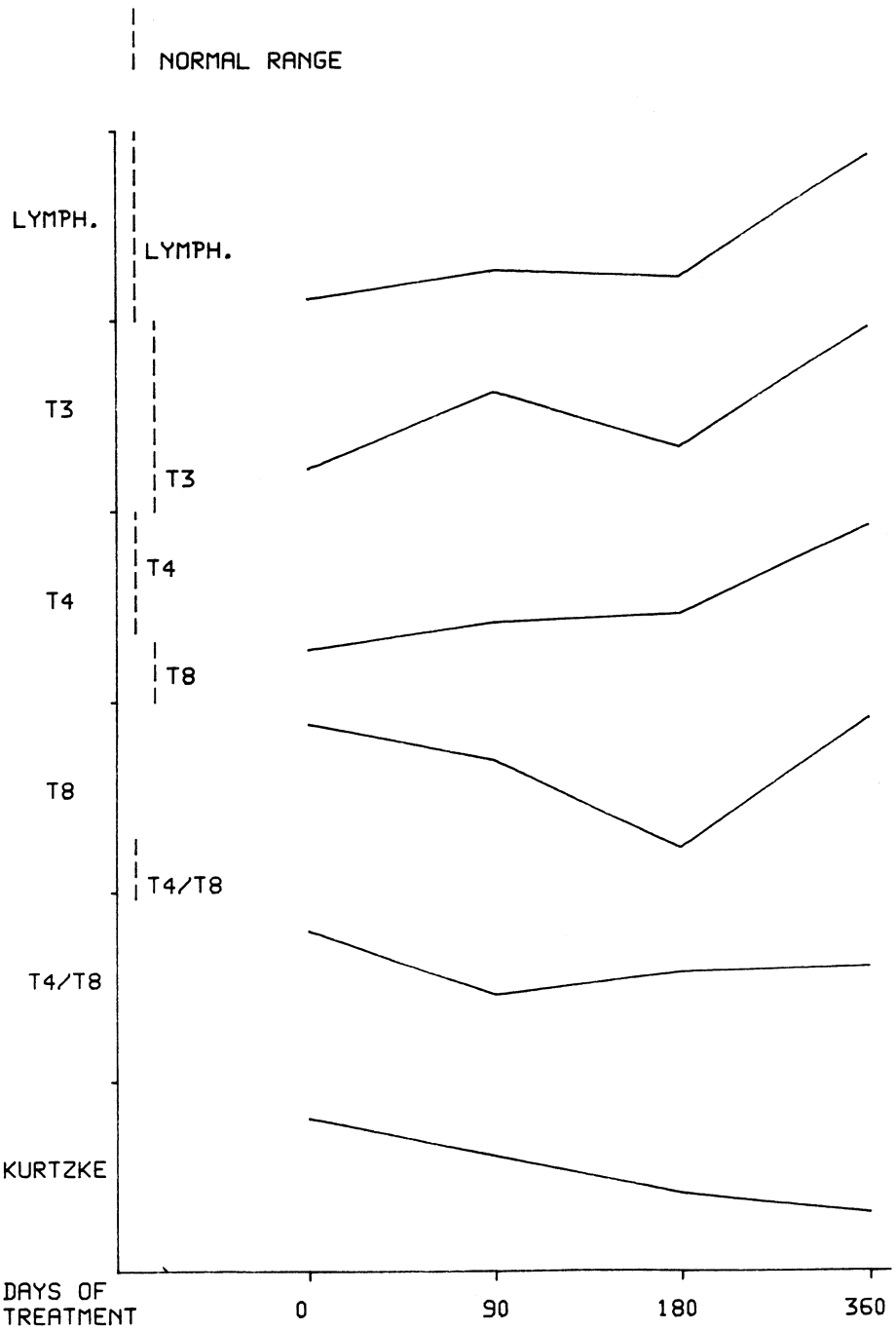

Fig. 1 Longitudinal assessment of clinical and immunological parameters

On the whole, the laboratory and clinical observations during the treatment period showed the same trend in seven patients (two improved, five stationary); nevertheless, there was an immunological improvement in two patients without a similar clinical change. After the therapy the same trend was observed in six patients (three worsened, two improved, one stationary) and three patients showed contradictory clinical-immunological parameters (two cases of immunological improvement related to clinical impairment and one case of immunological impairment without clinical changes) (Table 1).

The clinical parameters of all the patients did not differ statistically between each evaluation; likewise, the t-test showed no significant differences in the immunological values of the whole group (Fig. 1).

No side effects were found during thymostimulin treatment.

Conclusions

Apart from the individual changes, thymostimulin therapy did not significantly change the number and intensity of the relapses, the global neurological deficit, and the lymphocytic state. In most patients clinical and immunological parameters showed the same trend; when this did not happen, an improvement in the lymphocytic state did not usually correspond with a better clinical course. However, the variability in the MS evolution and in lymphocytic subsets needs a larger number of patients and a longer period of study to obtain more reliable results.

This preliminary study showed the usefulness of comparing the clinical and immunological data to evaluate drug effects in MS.

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Treatment of Multiple Sclerosis with a Mega Dose of 6-Methylprednisolone

S. Stecchi,[1] *R. Piperno,*[2] *G. A. Magagni,*[1] *B. Miccoli,*[1] *M. Franceschini,*[2] and *C. G. Montagna*[1]

1 Divisione Recupero e Rieducazione Funzionale Ospedale M. Malpighi, USL 28, Bologna, Italy
2 Servizio Recupero e Rieducazione Funzionale Ospedale Trevi, USL, 5. Foligno (PG), Italy

The administration of a large dose of 6-methylprednisolone in acute multiple sclerosis (MS) was introduced some years ago, after the encouraging results in the treatment of renal transplantation rejection, lupus, rheumatoid arthritis, and other self-immune disease [1, 2, 3]. The treatment protocol most widely adopted is daily i. v. infusion of 1 g 6-methylprednisolone for 3–7 days. However, this treatment regimen has not yet been clearly defined. Generally speaking, 6-methylprednisolone "pulse„ administration seems to be justified when usual treatment regimens cease to be effective. The purpose of the study was to assess this treatment protocol during exacerbations in MS patients with "remittent with sequelae" form and high exacerbation rate. Patients presenting a longer plateau and a clear reduction in clinical response to usual doses of steroids (8–12 mg dexamethazone/day) were selected. The treatment regimen of 500 mg/day of 6-methylprednisolone was chosen on the basis of a preliminary pilot study, since clinical response seems equivalent at this dose or at 1000 mg. Drug withdrawal after 3–5 days induced rapid recurrence of symptoms within a short period in almost all the pilot observations. It was therefore decided to administer an initial pulse dose of 6-methylprednisolone for 3 days to be gradually reduced to 40 mg over 18 days, and subsequently further decreased until withdrawal over a 2-month span (Table 1).

Material und Methods

Nineteen MS patients with "remittent" form at the onset of the disease and "remittent with sequelae" (EDSS > 3 when nonexacerbating) at the time of treat-

Table 1. Treatment with high-dose 6-methylprednisolone

1. 1000–500 mg i. v./day by slow infusion in saline solution for 3–4 days	
2. Gradual decrease to 40 mg in 18 days	
3. Further reduction until drug withdrawal	
Mean duration of treatment: 51.68+15.61 days	
Side effects	
1. Insomnia and hyperphagia	11 cases
2. Gastralgia	8 cases
3. Psychotic episodes	2 cases

Table 2. Rating in functional groups [4]

Functional	Before trial	After trial	At 6-month follow-up
Pyramidal	2.68±0.95	2.16±1.06	2.16±1.17
Cerebellar	2.29±1.14	1.84±1.12	22.6±1.88
Brain stem	1.16±1.42	0.84±1.26	0.79±1.03
Sensibility	2.32±1.97	2.11±2.05	1.79±1.96
Bowel/bladder	0.63±0.60	0.79±1.23	0.63±0.68
Visual	1.24±1.56	1.11±1.56	1.00±1.56
Psychic	1.63±1.30	1.63±1.30	1.63±1.30
Other	0.16±0.37	0.11±0.32	0.05±0.23
EDSS grade	5.23±1.57	4.44±1.79	4.31±1.97

ment were examined. The clinical diagnosis of "proven" MS was made according to Schumacher et al. Disease progression showed subsequent exacerbations with a tendency toward a progressively longer plateau. Mean age was 37.0±8.4 years with a mean age at disease onset 28.7±6.3 years. Average disease duration was 8.2±3.7 years. The mean progression index was 0.8±0.5. Of our 19 patients 6 were male and 13 female. The Follow-up was at 6 months. Neurological rating was assessed according to Kurtzke's scales for functional groups [4]; the overall EDSS was scheduled at the beginning and stop of treatment as well as at the 6-month follow-up. Administration of "pulse" large-dose 6-methylprednisolone was usually started within 24–48 h of overt signs of disease exacerbation. The mean duration of treatment (pulse plus tailing off) was 51.68±15.61 days. While in hospital, patients underwent gastroenterological examination, chest X-ray, check-up of the main chemical-clinical parameters for kidney and liver function, as well as electrolytic balance examination. During treatment chemical-clinical parameters and body weight were checked twice weekly. Control culture of the urine was performed periodically.

Discussion

The side effects of treatment were insomnia and hyperphagia in 11 cases, gastralgia in 8 cases, and psychotic episodes in 2 cases. All side effects were well controlled with symptomatic treatment. The mean scores in the functional groups (Table 2) show a certain nonstatistically significant improvement between the first and second assesment. The clinical picture stabilizes, thus consolidating the improvement at the 6-month follow-up. Similarly, the degree of disability, shows stabilized improvement at the 6-month follow-up. In the author's opinion the fact that no patient has shown exacerbation in the 6-month follow-up period is noteworthy since all cases presented a high exacerbation rate prior to treatment. This has enabled the rehabilitation programs to be carried out for a better functional recovery [5].

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Long-Term Hyperbaric Oxygen in Chronic Progressive Multiple Sclerosis: A Placebo-Controlled Double-Blind Randomized Study with Evoked Potential Evaluation

S. Barbieri, C. Pirovano, G. Cislaghi, G. Albonico, G. Oriani, and *C. Mariani*

Istituto di Clinica Neurologica Università di Milano, Milano, Italy

Introduction

The immunosuppressive action of hyperbaric oxygen (HBOX) has been proved by experimental studies in allergic encephalomyelitis [1] and other pathologies. Since 1970, clinical studies, most of them uncontrolled and nonblind, have been set up to evaluate the possible efficacy of HBOX in patients with multiple sclerosis (MS). The results have been controversial: some trials reported a positive action of HBOX [2] while others have not shown any clinical improvement [3].

Since definite MS is a chronic disease, it is not reasonable to assess the efficacy of any therapy on the basis of a short-term treatment. In this paper the effects of a prolonged HBOX therapy are evaluated by means of both clinical and neurophysiological invetigations in a selected population of 22 patients with clinically definite multiple sclerosis group A1 (CDMSA1), according to Poser et al. [4].

Materials and Methods

All patients had chronic stable disease: there was absence of exacerbations or of changes in DSS [5] score in the 6 months preceding the onset of therapy. Disease duration had to be longer than 2 years; its mean value was 13.25 years. Treatment with steroids or immunosuppressants was allowed only if patients were receiving it regularly for more than 6 or 12 months respectively. No new drugs were permitted. Patients with a DSS score higher than 5 or with contraindications to hyperbaric therapy such as active pulmonary disease, glaucoma, otitis, sinusitis, seizure disorder, or abnormal EEG were excluded. The patients were divided into two age- and sex-matched groups, one receiving 100% oxygen and the other "air" (20% O_2) at 2.3 atmospheres absolute (ATA). Treatment was carried out for 90 min at a time during 20 exposures in a multiplace chamber. There were five "booster" exposures monthly in the six successive months. Neurologic evaluation, with grading of muscle strength and of stretch reflexes and with DSS and FSS [5] score, was made before, soon after, and 6 months after the end of the therapy. Pre- and postreatment study of PSVEPs (pattern-shift visual evoked potentials), BAEPs (brain stem auditory evoked potentials), and SEPs somatosensory evoked potentials) was performed in all patients.

Results

Clinical Studies

After the first month of therapy no significant clinical variations were found in both groups between pre- and posttreatment mean values. Also the number of patients improved, unchanged, or worsened was not significantly different in the two groups (Tables 1, 2). After 6 months of therapy still no significant clinical variations were found in both groups while the number of improved patients, although not statistically significantly different, seemed to be higher in the treated group (Table 3, 4).

Table 1. DSS and FSS scores after 1 month of therapy

		HBOX		AIR	
		B	A	B	A
DSS score		3.36	3.45	2.90	3.09
FSS score		6.82	7.00	5.36	5.72
Pyramidal	functions	2.54	2.36	2.36	2.36
Cerebellar	functions	0.81	0.54	0.90	0.90
Brain stem	functions	1.36	1.54	1.09	1.36
Sensorial	functions	1.27	1.81	0.90	1.09
Sphincteric	functions	0.63	0.54	0.00	0.00

B, before treatment; A, after treatment

Table 2. Pre- and posttreatment variations. Number of patients improved (A), unchanged (B), or worsened (C)

		HBOX			AIR		
		A	B	C	A	B	C
DSS score		2	6	3	0	9	2
FSS score		2	4	5	2	6	3
Pyramidal	functions	2	9	0	0	11	0
Cerebellar	functions	3	7	1	1	9	1
Brain stem	functions	1	8	2	1	7	3
Sensorial	functions	0	8	3	1	8	2
Sphincteric	functions	1	10	0	0	11	0

Table 3. DSS and FSS score after 6 months of therapy

		HBOX		AIR	
		B	A	B	A
DSS score		3.36	3.00	2.90	2.87
FSS score		6.82	6.45	5.36	6.00
Pyramidal	functions	2.54	2.18	2.36	2.00
Cerebellar	functions	0.81	0.54	0.90	0.75
Brain stem	functions	1.36	1.45	1.09	1.75
Sensorial	functions	1.27	1.81	0.90	1.25
Sphincteric	functions	0.63	0.45	0.00	0.13

B, before treatment; A, after treatment

Table 4. Pre- and posttreatment variations. Number of patients improved (A), unchanged (B), or worsened (C)

		HBOX			AIR		
		A	B	C	A	B	C
DSS score		5	5	1	3	6	2
FSS score		4	4	3	3	6	2
Pyramidal	functions	6	4	1	3	7	1
Cerebellar	functions	4	6	1	1	10	1
Brain stem	functions	2	6	3	1	7	3
Sensorial	functions	1	6	4	1	7	3
Sphincteric	functions	2	9	0	0	10	1

Neurophysiological Studies

The mean PSVEP P100 latency in the HBOX group, after the 1 month of therapy, was significantly shortened (103.8 vs. 108.8 m: $P < .001$) while no significant difference was evident in the air group. In the same way also the latencies of BAEP III and V waves were shortened, even though the level of significance was lower (3.21 vs. 3.29 m: $P = 0.02$; 4.85 vs. 5.08 m: $P = 0.05$). No changes were seen in the latencies of SEPs (N13 and N20 for the upper and N22 and P38 for the lower limb were taken into account). To rule out a possible direct effect of HBOX on the conduction along the central pathways, two groups of controls have been investigated: one was receiving HBOX therapy for other diseases (Sudeck's atrophy and osteomyelitis) (group 1) and the second was formed by normal subjects who voluntereed for the study (group 2). In both groups no variation of PSVEP P100 latency was evident after HBOX therapy (group 1: 100.58 vs. 103.12 ms, $P = \text{NS}$; group 2: 99.35 vs. 99.78, $P = \text{NS}$).

Discussion

This study shows a beneficial effect of HBOX treatment on the conduction along the CNS pathways revealed by evoked potential studies. This positive effect was evident soon after the 1 month of therapy and was maintained also after 6 months from the beginning of the study. In previous reports the effect of HBOX on the CNS conduction measured with evoked potentials has been controversial. The improvement of PSVEP P100 latency and BAEP III and V waves latencies seen in this study might be due to the higher partial pressure of oxygen adopted (2.3 ATA in mask). It was decided to adopt this pressure because the positive effect obtained by the increased diffusion of oxygen to the tissues is greater than the flow reduction due to the vasoconstriction seen at this pressure (that is about 25%). The possible direct effect of oxygen on the CNS conduction is excluded by the control groups formed by normal subjects and by patients undergoing HBOX for different pathologies. None of the patients have suffered from undesired side effects of oxygen at 2.3 ATA. It is

known that the toxic effect of HBOX on the respiratory chain of the mitochondria is observed at a pressure higher than 2.8 ATA.

Concerning the clinical status of the patients, it is evident that no significant variation of DSS or FSS score was present right after the 1 month of therapy and after a 6-month follow-up. Nonetheless, although statistical significance was not reached, it is felt that, after a 6-month follow-up, the number of patients treated with HBOX that showed an improvement of DSS and FSS score was greater than the number seen in the control group.

It is reasonable to assume, according to other authors [3], that, in this particular case where minor modifications are expected (slowing of the progression of the disease), Kurtzke's scales are probably not accurate enough. Moreover, since our patients were selected according to a DSS score of lower than 5, the problem of the grading of the scale in the first 5 degrees is that the interval between the degrees is too large to allow a fine discrimination in the clinical status of the patients. The homogeneity of the scoring between the pyramidal and the sensory functions is also questionable.

In conclusion our study shows a beneficial effect of HBOX on MS patients indicated both by an improvement of conductions along CNS pathways and by an increased number of subjects with a lower DSS and FSS score after 6 months of therapy.

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Psychotherapy in Patients with Multiple Sclerosis

M. G. Landoni, L. Mendozzi, S. Bianchi, and *C. Locatelli*

Centro Studi Sclerosi Multipla, University of Milano, Hospital of Gallarate, Italy

The term "psychotherapy" is extensive, and referring to a privileged physician-patient relationship. The physician, in particular a specialist, stands as a cognitive and secure reference point for the patient. The MS patient is unconsciously incorporated, with this physician or with the institution to which the specialist belongs, after the first favorable encounter, into a "family", with specific connotations of privacy, continuity, confidence in the information he or she receives, and trust in the relationship. Within this "family" the physician and patients must learn to live together and decodification of symptoms and phantasies of recovery can be performed. A specific type of psychotherapy is not proposed here, experience of psychoanalysias with these patients being extremely rare.

In these cases, the psychotherapeutic relationship has a peculiar characteristic that consists of sharing as much as possible all the information and news about the disease between patient and neurologist. The more gradually objectively, truthfully, and realistically supplies the patient with the data in his or her possession, the more concrete and advantageous the relationship with the patient becomes.

Psychotherapy Group

As with other chronic and disabiling diseases, MS causes changes, often global, in the patients is life. The patient has to face work, affective, and social problems, in addition to the specific problems connected with the functional deficiences (motor, sensorial, visual, and sexual disturbances). Moreover, MS, because of its variable course and remitting-relapsing phases, requires adjustment to the changed physical conditions; this creates difficulties in programming the patient's life, with recurrent hopes and disappointments.

Therefore, the disease imposes a new reality and obliges the patient to revise future expectations. Frequent consequences are crises of identity, loss of role, and disesteem. Depressive reactions are frequent, and when suicide is not attempted refusal or escape from the necessity to adapt is often imagined. The disease inflicts a narcissistic wound on the self and a libido investment on the body, particularly on the ill part. As a consequence, the patient withdraws from objectual relationships and projects his or her self-nonacceptance on to other people. Therefore, the patient

qualitatively reduces social connections and conflictual relationship modalities are established. The retirement is frequently worsened by healthy people who refuse the disabled.

In this situation, the group therapy has an elective goal: to take patients away from isolation and introduce them into a social situation where they feel they belong to a group. They compare themselves with other patients and find they have common problems: this is a therapeutic factor which reduces their anxiety. As they improve their esteem they recognize in themselves resources for helping others. Under the guidance of a psychotherapist, they can learn a higher introspective capacity in the game of identifications, counteridentifications, and projections. So the patient recovers his or her identity, reaching a new balance either by strengthening the healthy parts or reducing the energy wasted in correcting or denying his or her handicaps. Exploiting interpersonal relationships, the MS patient reaches a new existential situation of self-gratification and self-realization.

Furthermore, group psychotherapy seems useful for some MS patients. The ten categories of Irving Yalom for the classification of the therapeutic factors which allow the patients to undergo beneficial group psychotherapy are: information, infusion of hope, universality, altruism, correctional interention of the family, development of the socialization techniques, imitative behavior, interpersonal group cohesion, and catharsis.

Taking into account the disability that MS involves, psychotherapy has the aim of rehabilitating the patients: it aims to, partially or completely, reestablish the conditions of physical and psychological health as well as of social integration.

Multiple sclerosis patients are usually young adults who must fight with a chronic progressive, unpredictable, disabling disease: it is important to consider this aspect when diagnosis is given and communicated. The first rehabilitative intervention recognizes two main aims: to inform and to give psychological help. Therefore the formation of sociotherapy groups is important.

Sociotherapy groups for hospitalized patients constitute an incentive, a start for more complex and structurized interventions. A frequent finding, especially among patients affected for some years, is a progressive process of withdrawal from family and of coercive social relationships. The sociotherapy group enables the patient to face the uncomfortableness and the worries of the disease and molds the patient into having a more mature and responsible connection with other MS patients and with society. An important factor is that the renouncement of school, working activities, friendships, and social life is not correlated with the severity of the neurological damage but to the emotional problems related to the experience of disability. The patient thinks him- or herself not to be up to the task of being a parent or a spouse, but he or she is unlikely to accept his/ha reduced value in family and working activities.

Self-nonacceptance sometimes produces a claiming behavior toward the social context which frequently, gives pathological answer to the problems of disabled people. The patient is sometimes scarcely tolerated when neurological symptoms worsen, requiring hospitalization and removal from the family for prolonged times. Sometimes the patient's defense or refusal disguises itself as the clothes of the hiperprotection or pity. An crucial point of "psychological management" is that it extends to nonhospitalized patients, giving psychological support.

The results from our experience show that "psychological management" is important for MS patients: it should be considered a rehabilitative and therapeutic instrument along with pharmacological therapy, physiotherapy, and social assistance. MS patients are affected by an organic disease which has important psychological implications: furthermore, patients can contribute to maintenance or an increase of the effects produced by the disease, hindering efforts toward recovery.

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Cognitive Impairment in Multiple Sclerosis

L. Mendozzi, M. G. Landoni, A. Ghezzi, and *C. L. Cazzullo*

Centro Studi Sclerosi Multipla, Università di Milano Ospedale di Gallarate, Gallarate, Italy

Introduction

Among the symptoms of multiple sclerosis (MS) an alteration of the cognitive functions has been noticed by several authors (for a review see ref. [1]). The relevance, nature, and frequency of the cognitive impairment are not yet well clarified. Different tests (Wechsler Adult Intelligence (WAIS), Luria Nebraska Neuropsychological Battery (LNNB), Halstead-Reitan, others) have been employed. Our study was carried out in order to evaluate the possible defect of cognitive functions in patients affected by MS in relation to neurological impairment and disease duration.

Subjects and Methods

Eighteen patients (6 females and 12 males) affected by definite MS [2] were examined; they were selected according to their disease duration: < 5 years (8 patients), > 10 years (10 patients). The age ranged between 30 and 44 years (mean 38.2 years). In order to avoid neurological damage influencing LNNB results, patients with a disability status [3] higher than 4 were excluded. None presented psychiatric disorders as defined by DSM III [4]. Patients were tested by WAIS and LNNB. IQ and cognitive impairment were assessed by WAIS with respect to standardized normal values. The LNNB includes 269 items grouped into 11 scales: motor, rhythm, tactile, visual, receptive speech, expressive speech, writing, reading, arithmetic, memory and intellectual process; it enables the presence of brain damage to be revealed and the cortical focus of the lesion to be localized with good approximation. The final scores were compared with the critical level (CL): this is calculated for the single patient considering his/her school level and age. Any scale exceeding the critical level was considered abnormal.

Results

Comprehensive results in the tests are reported in Table 1. In the group with a low disease duration, IQ ranged from 71 to 111, the mean value being 79 (middle-low level): the percentage of deterioration was 17.1. In the group with disease duration

Table 1. Clincial characteristics of MS patients

Disease duration	Patient	Sex	Education (years)	IQ	Deterioration	LNNB Abnormal scales
> 5 years	L.G.	M	13	108	23%	–
	C.L.	F	11	83	11%	Rhythm, memory
	M.A.	F	12	111	12.5%	–
	G.C.	F	9	71	44%	Rhythm, memory, visual, intelligence, speech
	M.P.	F	19	84	9%	Not completed
	B.G.	M	8	107	18%	–
	C.M.	M	11	91	2%	–
	C.V.	M	15	108	2%	–
< 10 years	S.F.	M	8	87	6%	–
	N.G.	M	11	110	7.5%	Rhythm
	T.A.	F	5	90	3%	–
	T.P.	F	11	78	48%	Rhythm, memory Intelligence process
	A.P.	M	8	99	2.5%	–
	T.M.	M	13	99	12.5%	Rhythm, visual
	N.F.	M	8	89	3%	Memory
	G.S.	M	18	100	5%	Rhythm
	M.L.	M	13	100	22%	Rhythm
	P.G.	M	18	93	14%	Rhythm, memory, arithmetic

over 10 years the mean IQ was 93.1 (range 78–110), the percentage of deterioration being 11.7. Three patients presented a deterioration ratio over 20%. Testing by LNNB, abnormal results were observed in 50% of cases. The Rhythm scale was affected in eight patients followed by the memory scale in five cases and by the visual scale in two cases. LNNB was abnormal in 2/7 patients (28.6%) with disease duration < 5 years, one case refusing to complete the test, and in 7/10 patients with disease duration > 10 years (70%).

Discussion

Testing by WAIS, intellectual deterioration was found in a small number of MS cases (3/18: 2/8 of the group with disease duration < 5 years, 1/10 of the group with disease duration > 10 years). The results in the LNNB showed abnormalities in a higher number of patients. The rhythm scale was particularly affected, suggesting a

defect of attention and of concentration. According to Golden [5], a defect of the rhythm scale indicates a dysfunction of the right hemisphere provided that speech is not affected. A memory defect was detected in five patients, agreeing with other reports [6, 7]. There was not a constant correspondence between LNNB and WAIS results: however, the two patients who had the highest deterioration (G. C., T. P.) also showed the highest abnormality in the LNNB.

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