Progress in Multiple Sclerosis Research

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Preface

At the annual meetings of the International Federation of Multiple Sclerosis Societies (IFMSS) the representatives of the national MS societies of numerous countries assemble for discussions and reports on work accomplished, under way, and to be undertaken in the future. In addition to publicity work, education, and patient services, there has been an increasing interest in obtaining first hand, reliable, and realistic information not only on the medical problems relevant to diagnosis, treatment, and the management of MS, but also on progress in the difficult field of MS research. For this purpose the International Medical Advisory Board (IMAB) was founded.

In the past years its members have organized a number of symposia in conjunction with the IFMSS meetings in New York, Copenhagen, Barcelona, Toronto, Amsterdam. The program of the Göttingen symposium, relevant in its focus to the 1978 meeting of the IFMSS in Hannover, had the double purpose of bringing together scientists from many countries with active MS societies, and to inform the organizers and members of these societies, and thereby also the MS patients, of problems and progress in a number of areas of MS research. The program was conceived as a survey of newer work undertaken in basic and experimental MS research and, on the clinical side, as a reassessment of the prerequisites in the diagnosis of MS, the value of laboratory tests, some therapeutic approaches, and organizational principles in the management of MS.

Symposia with such a wide range of topics are to a large extent precluded from probing deeper into details and controversial questions. On the other hand, they may serve as a platform for contacts and an exchange of information by researchers with very different approaches, one of the ways of avoiding the "factory blindness" that might lead us away in pursuing the ultimate goal of MS research: to identify the cause or causes and to find ways of curing this disease.

Acknowledgements. We are grateful to the Deutsche Forschungsgemeinschaft, which has sponsored and essentially financed this symposium as a part of its research program on "Multiple Sclerosis and Related Diseases of the Nervous System"; to the German Multiple Sclerosis Society for financial help; to the National Multiple Sclerosis Societies of the United States of America, Great Britain, Japan and a number of other nations for providing travel funds for scientists from their respective countries and to the administration of the University and the City of Göttingen for helping us in many ways.

We owe special thanks to the Hertie Foundation for the Advancement of Science for generously providing funds to ensure publication at a reasonable price, and finally to the Springer-Verlag for the effort and work devoted to the publication of this book.

Göttingen, Summer 1980 H. J. BAUER · S. POSER · G. RITTER

Welcome Address

It is my privilege to extend the welcome of the government of Lower Saxony to the participants of this international symposium. We feel honored that so many eminent researchers on multiple sclerosis have assembled here in Göttingen on the eve of this years meeting of the International Federation of Multiple Sclerosis Societies, scheduled to be held in Hannover next week. I consider it is an excellent idea to have such a symposium in conjunction with this meeting of the IFMSS – to provide the representatives of the national MS Societies with the most recent information concerning the latest research results. Furthermore I would like to add my satisfaction as a former vice-president of the Deutsche Forschungsgemeinschaft, the German Research Society, that the DFG has acted as sponsor for this important get-together of outstanding researchers.

I am also pleased, as is my government, to present the new Medical Center of this University as the site for your symposium.

It appears to me, and I say this without a trace of arrogance, that there are two good reasons for having this meeting here. First, research on the Nervous System has an important place in Göttingen: in the University as well as in the Max-Planck-Institutes. Secondly, research on MS and awareness of the medicosocial problems involved in this very grave disease of the central nervous system have been a growing concern to the government of Lower Saxony for a number of years. On the basis of epidemiological studies carried out in the Göttingen area, we can extrapolate, that there are over 6000 MS cases in Lower Saxony alone, the estimated figure for all of West Germany being between 50000 – 100000 cases. But MS is not only numerically an important disease of the central nervous system. It is an illness manifesting very often early in life, and usually lasting for many years. This generates medical, socioeconomic, and psychological problems of a magnitude not adequately expressed by statistics.

We – I mean the general public – are looking to the researchers for advances in treatment of MS. The researchers who are probing into the mysteries of the pathology of MS with fundamental theoretical and experimental investigations, who also cooperate on the practical level (i.e., in the application of newly found research results) with all those who are concerned with the management of MS and with the care of the persons afflicted with this grave disease.

We look to you with the hope that your work will be blessed with success. Realistic researchers, and for many years I was one of them, know that your task is very difficult and that there is a long way ahead of you. As always in research, as it gets under way, new questions arise before all the old ones have been answered. Well, the experienced among you will know and the younger ones among you will learn that it is the quality of the questions that largely determines the quality of the research. May therefore the exchange of information during your symposium give not

only useful answers, but also new, better, and fertile questions, whose challenge will engender new research, leading the way hopefully towards the ultimate solution of the problem of multiple sclerosis. This is my wish which I have the honor and the privilege to express to you on behalf of my government.

Professor Dr. Dr. E. PESTEL
Minister for Science and Art of Lower Saxony

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List of Abbreviations

The following abbreviations are used to indicate both noun and adjectival forms, e.g., EMG stands for electromyography and electromyographic.

ACTH	corticotrophin
ADE	acute disseminated encephalitis
CFA	complete Freund's adjuvant
CNS	central nervous system
CSF	cerebrospinal fluid
DSS	disability status scale
EAE	experimental allergic encephalomyelitis
EAN	experimental allergic neuritis
EMG	electromyography
E-UFA	erythrocyte-unsaturated fatty acid test
IEF	isoelectric focusing
IFA	incomplete Freund's adjuvant
LCM	lymphocytic choriomeningitis virus
MBP	myelin basic protein
MEM-LAD test	macrophage electrophoretic mobility-linoleic acid depression test
MHC	major histocompatibility complex
MLD	metachromatic leukodystrophy
MLR	mixed lymphocyte reaction
MS	multiple sclerosis
ON	optic neuritis
PNS	peripheral nervous system
PPD	purified protein derivative (of tuberculin)
RBN	retrobulbar neuritis
SDE	subacute demyelinating encephalomyelitis
SSPE	subacute sclerosing panencephalitis
VER	visual-evoked response

Experimental Models in Demyelinating Disease

Applications of Chronic Relapsing Experimental Allergic Encephalomyelitis to the Study of Multiple Sclerosis

C. S. RAINE[1], U. TRAUGOTT[1], and S. H. STONE[2]

Background

Research into the precise analysis of the march of morphological events and the possible fluctuations in immunologic response in multiple sclerosis (MS) has been somewhat impeded by the apparent lack of a truly chronic, nonfatal, relapsing demyelinating analog in animals. For many years, neuropathologists have applied the highly pertinent model of acute experimental allergic encephalomyelitis (EAE) to the analysis of short-term pathogenetic mechanisms. While serving a pioneering role in autoimmune demyelination research, this model had in its acute, severe nature, distinct drawbacks in its relevance to MS.

In 1965, Stone and Lerner [14] reported on the production of a form of chronic EAE in juvenile, inbred Strain 13 guinea pigs, a model which had, perhaps more than any other thus far described, distinct clinical and pathologic similarities to MS. Other strains and age groups of guinea pigs were also investigated by Stone and his colleagues but it was found that due to increased mortality or failure to develop signs, chronic relapsing EAE can only be reproduced reliably in Strain 13 guinea pigs [14, 15]. This model was subsequently analyzed extensively at the clinical and morphologic levels and found to bear many resemblances to patterns in MS [3, 6, 7, 12]. Our morphologic studies allowed us to conclude that chronic relapsing EAE might afford the neuroscientist new avenues for MS research. Based on this, we embarked upon a series of studies relevant to the therapeutic approach to MS [9, 10, 18, 19], and similar parameters in MS [18].

The present synopsis of chronic relapsing EAE will first detail the induction protocol, then outline briefly our recent applications of the model to MS, and finally, will tabulate those facets of this disease which render it a potentially valuable tool for investigations of pathogenetic and therapeutic mechanisms in MS.

The Model

Induction

Chronic relapsing EAE depends upon the use of juvenile Strain 13 guinea pigs. Animals sensitized as adults invariably develop acute, fatal EAE 2–3 weeks post inocu-

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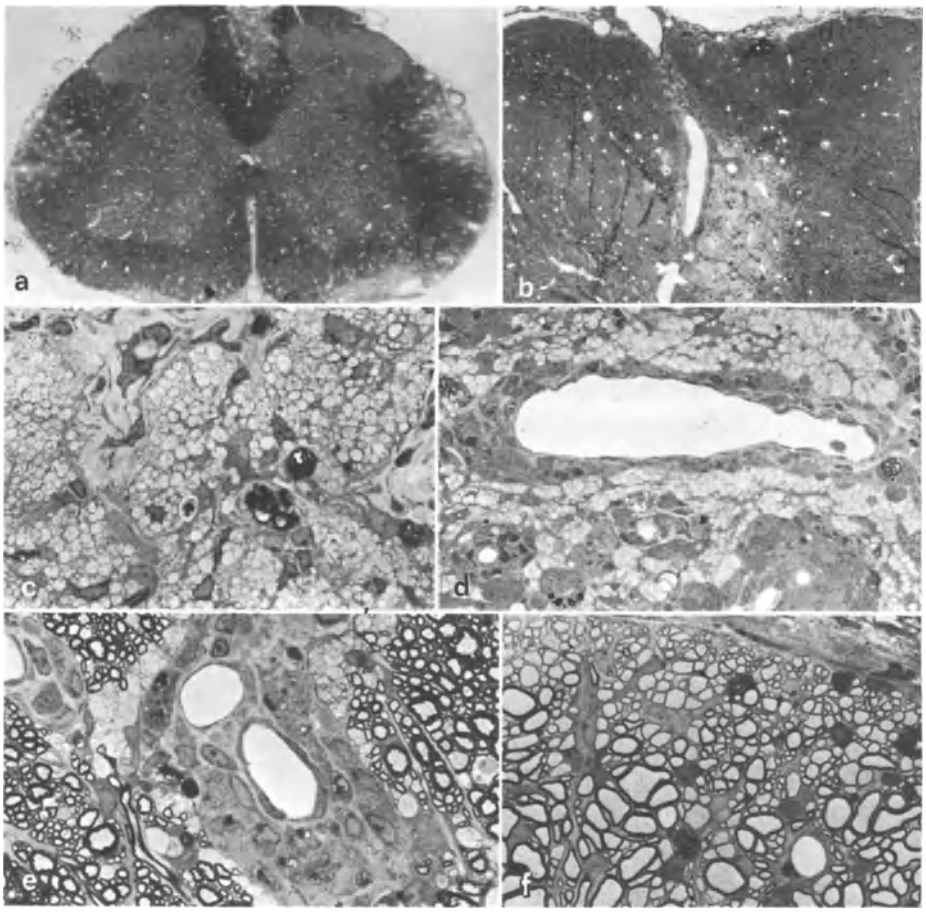

Fig. 1. a Chronic EAE, 16 weeks, 2 relapses. Note the disseminated nature of the lesions in the spinal cord at L_7. Chronic plaques are seen in the dorsal columns, anterior columns, and the right lateral column, while a recent, acute plaque is seen in the left lateral column. ×20. **b** Chronic EAE, 20 weeks PI, 2 relapses. A demyelinated plaque (*center*) in a dorsal column at L_7 is shown. A dorsal horn lies to the left. ×120. **c** Slightly higher magnification from the plaque shown in Figure 1 b. Note the fibrotic blood vessels, macrophages, gliosis, and many transversely sectioned naked axons. ×245. **d** Another section from the lesion shown in Figure 1 a. Acute perivascular inflammation (note the densely staining plasma cells) and recent demyelination manifested by tightly-packed axons are seen. ×190. **e** Chronic EAE, 22 weeks PI, 2 relapses. A recent perivascular cuff with a surrounding rim of acutely demyelinated axons is shown from an area of previously unaffected white matter. ×756. **f** Suppressed chronic EAE, 8 weeks PI. A subpial lesion shows early remyelination of all fibers and fibrotic changes in the leptomeninges. ×480

lation (PI) [7, 14]. Juvenile strain 13 guinea pigs (less than 250 g body weight) are given a single intracutaneous inoculation in the nuchal area with an emulsion containing 0.25 ml of a 50% suspension of isogeneic spinal cord in 0.25 ml complete Freund's adjuvant containing 2.5 mg *M. tuberculosis.* Fuller details are given elsewhere [7, 14].

Clinical Features

Contrary to other forms of EAE, juvenile strain 13 guinea pigs sensitized for chronic relapsing EAE do not usually display signs at 2–3 weeks, although previous morphologic study has revealed subclinical demyelinating lesions in the CNS at this time [7]. The first overt clinical signs develop about 8–12 weeks PI. They are less severe than those of acute EAE, are rarely fatal, and generally remit, albeit incompletely. Neurologic signs consist of weight loss, incontinence, and paraparesis, which sometimes relapses or progresses slowly to quadriparesis. Animals with this disease have been maintained for more than 3 years PI, during which time 1–5 relapses have been recorded in some.

Pathology

The CNS of Strain 13 guinea pigs at various stages of chronic relapsing EAE have been analyzed morphologically and compared to patterns described in MS [4]. The CNS of animals displaying signs at 8–12 weeks PI show both recent and old lesions, the latter having developed during the prolonged latent period. There is considerable topographical similarity between chronic EAE and MS demyelinated plaques, but in general, the EAE lesions are less extensive and less well demarcated. They do, however, possess a paraventricular distribution in the brain and in the spinal cord, the entire white matter can be affected (Fig. 1 a, b). Also, there tends to be more axonal sparing in chronic EAE lesions, a feature possibly related to the shorter timespan of the disease (Fig. 1 c). Lesions occurring in the CNS of animals with a recent relapse show chronic demyelinative activity in the center, upon which is superimposed active inflammation and ongoing demyelination at the margins (Fig. 1 d, e). Other morphological features of relevance to MS include heterotopic gliosis and Schwannosis [11], fenestrated endothelial cells [13], and axon-glial membrane specializations [5].

Taken in concert, the morphologic analysis of chronic relapsing EAE, a known autoimmune response to CNS antigens, supports the concept of an immunologic pathogenesis for MS lesions. Relapsing clinical disease can invariably be matched in chronic EAE by recent inflammation in the CNS and remission by remyelination. In addition, evidence for subclinical disease is also present in this model. Ongoing investigations will examine the immunocytochemical aspects of the disease process and compare them with similar studies on MS [2].

Suppression

Previous work from this laboratory has demonstrated that both acute and chronic EAE in strain 13 guinea pigs can be successfully suppressed with a regimen of injections of myelin basic protein (MBP) in incomplete Freund's adjuvant (IFA) [8]. This study demonstrated, among other things, that while the disease in suppressed animals was curtailed clinically, at the morphologic level, identical CNS changes developed initially in both unsuppressed and suppressed animals. All unsuppressed

adult animals succumbed to the acute disease, all unsuppressed juveniles eventually came down with chronic EAE, but suppressed animals from both groups displayed only transient signs, if any, and these completely remitted. Thus, in suppressed EAE guinea pigs, the structural changes did not entirely complement the clinical findings. This was the subject of subsequent studies [16], which show that in acute EAE, there occurs a significant decrease in circulating "early" T cells (a population considered to reflect cell-mediated immunity) with the onset of signs. At the same time, animals in which acute EAE has been suppressed, demonstrate, during the period of transient signs, only a minor decrease in early T cells which then rise and remain at elevated levels throughout the suppression period. We believe that the decreases with clinical signs are due to migration of lymphocytes from the circulation to the CNS compartment (this has been proven to be the case in later studies) [20], while we have suggested that the increased levels of early T cells in suppressed animals might reflect a population of MBP-generated suppressor cells.

We then turned our attention to the suppression of chronic, relapsing EAE. For these experiments, the full details of which are presented elsewhere [9, 19], four groups of juvenile strain 13 guinea pigs were sensitized for chronic EAE. The first group (27 animals) was left to develop chronic relapsing EAE over a 27-month period (unsuppressed group). Animals from this and the other three groups were sampled periodically for morphologic study and at regular intervals, 1–3 weeks apart, were bled for study of circulating lymphocyte populations.

The second group (4 animals) was sensitized for chronic EAE, and then, 12 months later, rechallenged with whole CNS/CFA (unsuppressed, rechallenged group).

The third group (20 animals) was sensitized for chronic EAE and then given a regimen of MBP/IFA 1–5 weeks later to suppress the disease (suppressed group). These animals were observed for up to 27 months PI.

The fourth group (6 animals) was sensitized for chronic EAE and then, 1–5 weeks later, each animal was given a course of injections of MBP/IFA to suppress the disease. At 12 and 26 months PI, these animals were challenged for a second time with CNS/CFA to test the permanence of the suppression (suppressed, rechallenged group).

The results of these experiments show that unsuppressed animals develop typical relapsing disease beginning 8–12 weeks PI. Also, the CNS changes are identical to those described previously (Fig. 1 a–e). The lymphocyte studies show that the clinical relapses coincided with significant decreases in circulating early T cell levels (Fig. 2 a). The unsuppressed rechallenged group displayed an episode of acute disease 2–4 weeks after the second challenge. This episode and the previous relapses were also marked by corresponding decreases in circulating early T cells. In both these groups of animals, during remissions, early T cell values rose to a level at the upper limit of the normal range.

The suppressed chronic EAE animals showed no clinical signs over the 27-month period of study. Surprisingly, the CNS of these animals displayed evidence of extensive remyelination of lesions which had developed previously but which were not detectable clinically (Fig. 1 f). Early T cell levels rose during suppression to significantly high levels in comparison to normals and remained elevated throughout (Fig. 2 b). The suppressed, rechallenged group also demonstrated no clinical

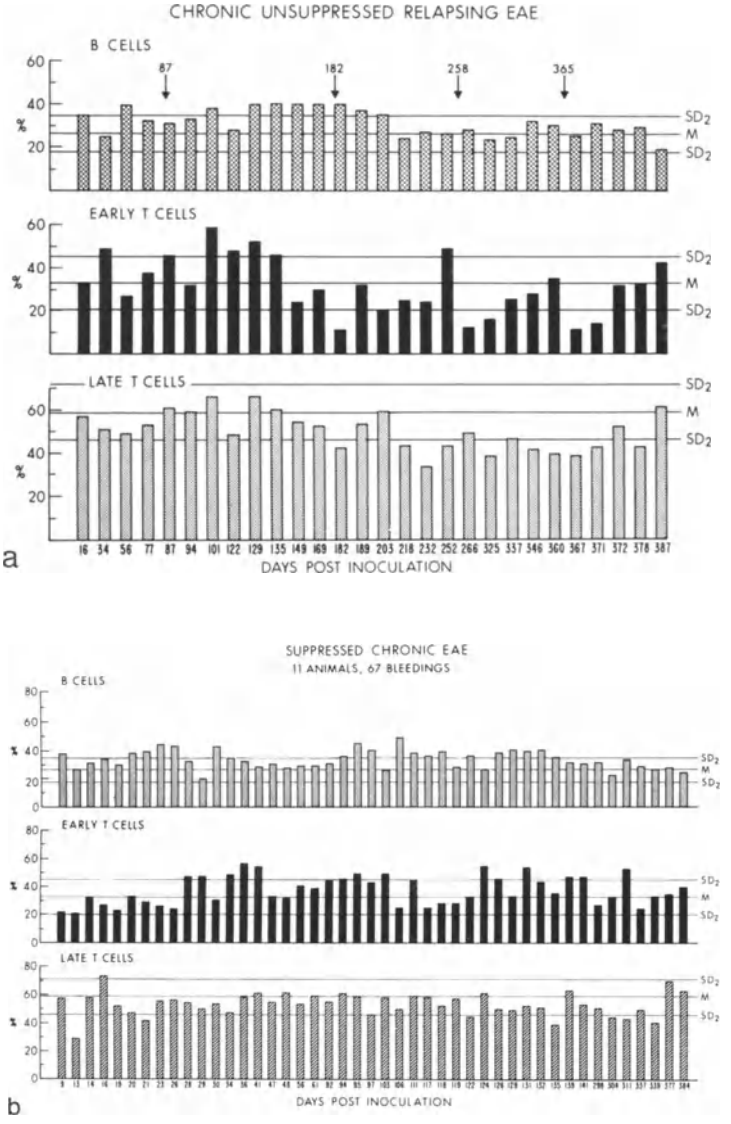

Fig. 2. **a** Histograms of circulating lymphocyte levels from one representative unsuppressed, chronic EAE strain 13 guinea pig, based upon 28 separate bleedings. The actual values are compared with the mean (M) ± 2 standard deviations (SD) obtained from 11 uninoculated, normal strain 13 guinea pigs [16]. This animal had four clinical exacerbations, indicated by the *arrows*. A delayed onset of disease occurred on day 87, followed by three relapses (days 182, 258 and 356). Although B cells and late T cells displayed minor changes only, early T cells demonstrated significant decreases ($P < 0.01$) coinciding with relapses. During remissions, early T cells return to normal levels. **b** Circulating B-cell, early and late T cell values from 11 suppressed chronic EAE guinea pigs plotted against the mean value and 2 SD from a group of 11 normal, uninoculated guinea pigs. Since it is not possible to illustrate all bleedings due to space limitations, only selected points are shown and for example, 22 bleedings taken between days 141 and 298, have been omitted. Note the consistent elevation of early T cells between days 28 and 41 PI. Later on, early T cell values are normal or slightly elevated. B cells and late T cells are in the normal range

disease, even after rechallenge, indicating that the suppression was permanent over the period studied.

Thus we have shown that chronic relapsing EAE can be successfully suppressed with MBP/IFA and that this suppression is permanent. Interestingly, we noted strong correlation between clinical, pathologic and lymphocyte patterns. Future experiments will test the efficacy of non-neural antigens upon chronic relapsing EAE and also whether the disease can be suppressed after several episodes have occurred. The latter would approximate more accurately the situation in MS. The results of our suppression experiments should encourage the use of MBP in therapeutic trials in MS.

Lymphocyte Studies

The studies on circulating lymphocyte populations described above in the suppression experiments stemmed originally from similar studies carried out on strain 13 guinea pigs sensitized for acute EAE [16]. In addition to the determination of quantitative changes, we have more recently been investigating the responsiveness of early T cells to MBP both within the circulation and within CNS infiltrates. It was shown that with the onset of signs the decrease in circulating early T cells can be accounted for by a corresponding migration of early T cells to the CNS [20]. When samples of lymphocytes from the circulation and CNS infiltrates in acute EAE were tested for reactivity against MBP [1], it was found that in the CNS most early T cells respond to MBP. This observation might indicate that MBP-reactive early T cells become concentrated in the CNS by their migration from the circulation, a speculation which could not be supported by the T cell rosette enhancement technique [17].

Similar studies on the comparative levels of circulating and CNS-associated lymphocytes have not yet been possible in MS. However, we have completed one longitudinal study on circulating early T cell levels in stable and active MS which compared the values with samples from other neurological disease (OND) and normals [18]. We found that in general, early T cells were lower in MS than in controls. Also, during exacerbations in MS, early T cells decreased further. When aliquots of these lymphocytes were tested for their reactivity to MBP, while positive reactivity was seen in both the OND and MS group (but not in normals), MBP reactivity was higher during exacerbations. Whether this MBP-reactivity serves as a measurement for antigen specificity will only be proven by tests with different antigens. In addition, there remains the possibility that the reactivity represents nonspecific stimulation, such as that obtained after exposure to Concanavalin A.

Therefore, the similarities at the present immunologic level between EAE and MS suggest possible common pathogenetic mechanisms. Further comfirmation of this suggestion will be sought by comparing circulating and CNS-associated lymphocytes from chronic relapsing EAE and MS for MBP reactivity.

Pertinence of Chronic Relapsing EAE to MS Research

Chronic relapsing EAE is pertinent to MS research because:

1. It is age and strain dependent.
2. Only a single sensitization is necessary.

3. It has a long, latent period before clinical onset.
4. The disease course is protracted and progressive.
5. The disease is rarely fatal.
6. Relapses are common.
7. Demyelinated plaques are large and of different ages.
8. Axons are spared and remyelination is seen.
9. The use of an inbred strain has immunogenetic advantages.
10. The prolonged disease course renders this a useful model for therapeutic trials.
11. The model is appropriate for lymphocyte studies.
12. Sensitization in the nuchal area does not impair clinical evaluation.

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Chronic Relapsing EAE: Its Application to Study of Human Inflammatory Demyelinating Diseases

H. M. WISNIEWSKI and H. LASSMANN[1]

In 1933, Rivers et al. [23] reported that they had observed an encephalomyelitis with myelin destruction in 2 of 8 monkeys that had received repeated IM injections of aqueous emulsion and alcohol-ether extracts of normal rabbit brain. Two years later, Rivers and Schwentker [22] confirmed these original observations, stressing that 7 of 8 monkeys developed demyelinating encephalomyelitis. The procedure used by Rivers et al., followed by confirming reports by Ferraro and Jervis [3], involved repeated (46–85) IM injections of aqueous emulsions and alcohol-ether extracts of normal brain. This technique produced morphological lesions of various ages and sizes, and gave a very low mortality rate. However, it was very time consuming (3 inoculations a week, up to 85 injections) and difficult to apply to small animals. Since the goal of these experiments was to determine what component of brain tissue causes demyelinating encephalomyelitis, there was a great need for a method which would shorten the time and procedure required to induce the disease. This was solved when Freund and McDermott [5] demonstrated a new technique of emulsifying antigens in various adjuvants in aquafor, paraffin oil, and heat-killed tubercle bacilli. This technique facilitated the experimental production of encephalomyelitis and the inception of its symptoms with one or two injections of brain emulsion in Freund's adjuvant [15]. In subsequent years, using Freund's adjuvant, it was found that the antigen responsible for the induction of EAE is present in the myelin sheath and was eventually characterized as an encephalitogenic basic protein [4, 7, 29]. Although the introduction of Freund's adjuvant accelerated the identification of the antigen responsible for induction of EAE, it also clouded the relation of EAE to MS by producing an acute encephalomyelitis with a mortality rate close to 100%. Therefore many investigators considered EAE to be an acute monophasic disease almost always inducing death, and, in contrast to MS, characterized by exacerbations, remissions, and negligible death rate. It should be stressed, however, that during the time of extensive use of adjuvant and studies of the acute phase of EAE, sporadic papers were published [1, 2, 19, 25, 31] describing chronic and recurring episodes of EAE in animals sensitized with antigen and with Freund's adjuvant. However, such reports were treated as exceptions to the rule that EAE is an acute and mortal disease. In 1950 Freund et al. reported chronic EAE in young guinea pigs, due to the use of CNS tissue and a not acid-fast strain of mycobacterium as adjuvant. However, only 4 of 21 animals developed chronic disease.

A major breakthrough was achieved when Stone and Lerner [28] introduced a highly reproducible model of chronic EAE by sensitization of newborn Strain 13 guinea pigs. However, this report stressed a slow, uninterrupted and progressive dete-

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rioration from onset of clinical signs (30–80 days after sensitization) to paralysis and death. A relapsing clinical course was sometimes noted in animals sensitized using this procedure [18, 20]. In recent publications, fluctuations of the disease are reported to be more frequent [21]; however, no clinicopathological correlation is available in these animals; also, brain lesions were reported to be rare and were not documented.

By minor variations of the inoculum and by changing the inoculation site, we were able to induce a chronic disease with predictable remissions and relapses in 80% of Strain 13 and 38% of Hartley guinea pigs [10]. This model is different from the previously reported in several essential aspects:

1. It shows a regular occurence of an acute EAE episode 8–12 days after sensitization followed by a clear-cut remission clinically as well as pathologically.

2. It shows a synchronicity of the pathological events in the demyelinating lesions in the first relapse and their clear-cut correlation with duration of clinical symptoms.

3. It demonstrates the large size (macroscopically visible) and the regular occurrence of demyelinating lesions within the brain and optic nerve in addition to the spinal cord lesions in the chronic stage of the disease.

This report deals with the clinical, pathological and immunological changes observed in 127 Strain 13 and 125 Hartley guinea pigs sensitized according to the procedure described by Wisniewski and Keith [30].

Clinically, several disease patterns of EAE were observed: acute fatal, chronic progressive with or without delayed onset, chronic relapsing, and chronic relapsing with progressive course. In Strain 13 guinea pigs, the chronic relapsing course was the most frequent. The relative incidence of the different disease types depended upon the strain of animals used and the age at the time of sensitization [11].

Pathohistological examination of animals during different stages of the EAE model showed very interesting differences in inflammatory reaction. During the first 3 days of clinical symptoms of the acute episode, a variable but generally large number of polymorphonuclear leukocytes were found within the lesions. In most severely affected animals, the pattern of hyperacute EAE with hemorrhages, fibrin deposition, and necrosis together with an inflammatory infiltrates nearly exclusively composed of polymorphonuclear leukocytes was observed [10]. Immunofluorescence studies in these areas showed deposition of immunoglobulins together with complement (C_3). The amount of complement found in these lesions corresponded well with the severity of the hyperacute reaction pattern [6]. Later stages of the acute episode (4–10 days after onset of clinical symptoms) showed pathohistological alterations closely resembling those described in ordinary EAE. During the first remission, perivascular cuffs of lymphocytes and mononuclear cells were found without invasion of the tissue by inflammatory cells and without active demyelination. During the second attack and all further relapses, lymphocytes and mononuclear cells dominated and invaded the parenchyma, leading to large plaques of demyelination (Figs. 1 a, b, c and 2 a, b). With immunofluorescence technique, no complement deposition was found in these lesions. However, presence of immunoglobulin containing cells throughout the whole chronic stage of the disease was noted.

Clinicopathological correlation was good during the acute and early chronic stage of the disease (first episode, first remission, and first relapse) [10]. The synchronic-

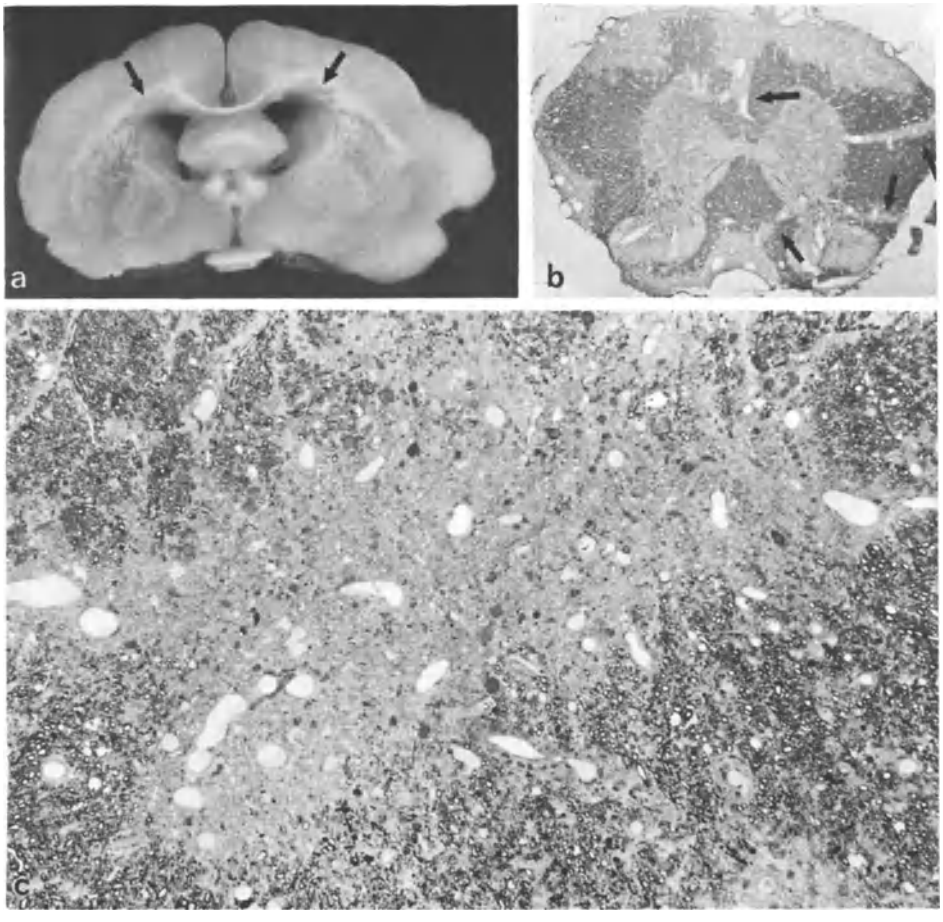

Fig. 1. **a** Hartley guinea pig; first relapse, general slice through the brain in the level of the optic chiasm. Note the large grey discolored lesion periventricularly in the centrum semiovale (↑) ×3.5. **b** Hartley guinea pig; first relapse, spinal cord. Large demyelinated lesion in the anterior and posterior and lateral columns. Perivascular demyelination in anterior, posterior, and lateral column (↑) ×15. **c** Hartley guinea pig; first relapse, centrum semiovale. Large confluent demyelinated plaque with fingerlike perivascular demyelination in the periphery. Toluidine blue. ×400

ity of evolution of demyelinating lesions, especially with regard to myelin degradation products and their clear-cut correlation with clinical duration of the first relapse in the 30 animals studied in this stage of the disease, strongly indicates the absence of subclinical disease during the first remission. However, one out of 15 animals sampled during the first remission showed evidence of subclinical demyelinative lesions in the brain, but not in the spinal cord, and two other animals sampled very late in the remission showed the beginning of tissue invasion by mononuclear cells from a few vessels in the spinal cord. These results indicate the importance of pathohistological correlation in immunological studies. During the later relapses of the

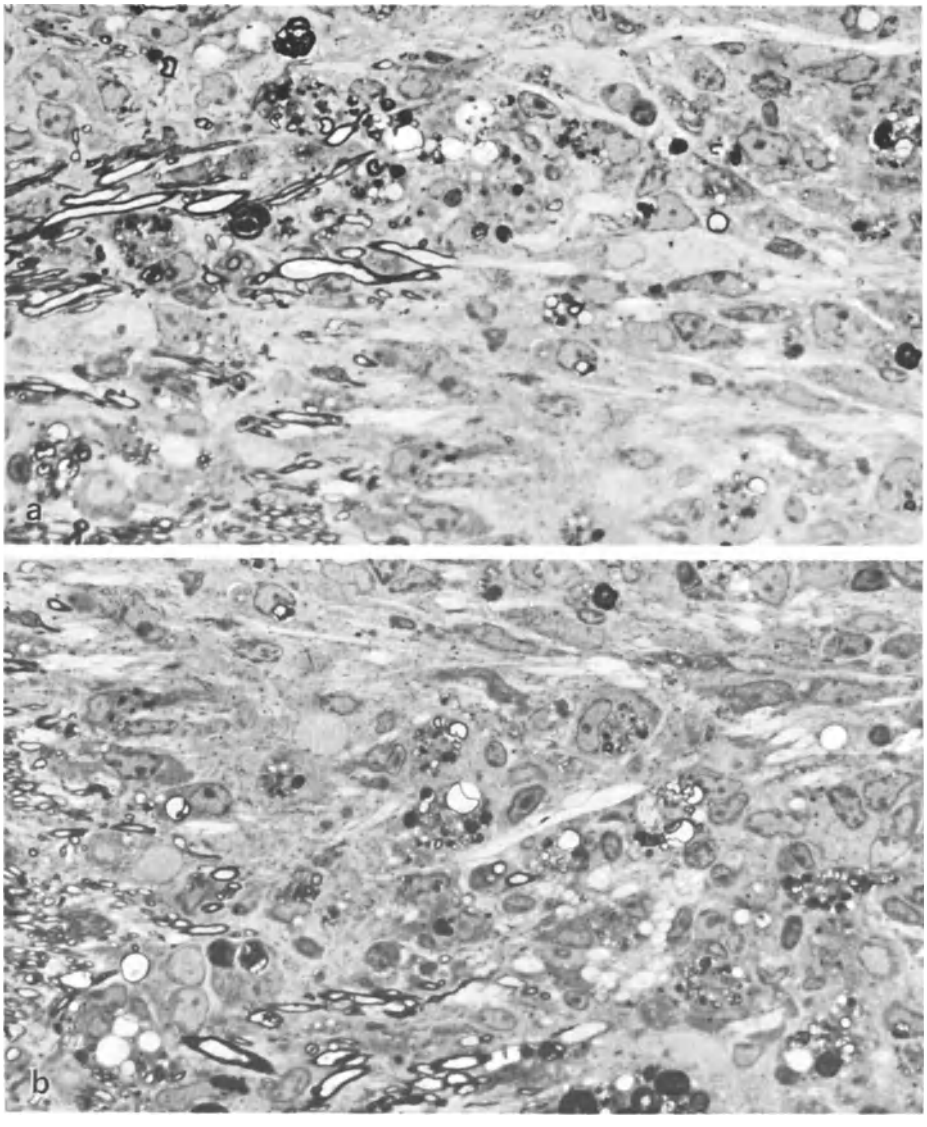

Fig. 2. **a** Hartley guinea pig; first relapse, centrum semiovale. Edge zone of large actively demyelinating plaque with variable amount of recent myelin debris. In the center a segmentally demyelinated nerve fiber can be seen. Toluidine blue. ×840. **b** Hartley guinea pig; first relapse, centrum semiovale. Periphery of a large plaque. Early and late stages of myelin debris and reactive gliosis. Toluidine blue. ×840

disease (4–12 months after sensitization), a variable number of inactive and active plaques was found. However, the clinicopathalogic correlation was poor. In the late chronic stage (12–24 months after sensitization), definite clinical relapses were infrequent, but when present, correlated better with pathology, because of the generally lower disease activity.

The pathology of the demyelinating lesions in the chronic stage of the model was characterized by primary demyelination with axonal preservation, gliosis,and oligodendrocyte loss. In later chronic stages of the disease, peripheral remyelination in the spinal cord and shadow plaque formation in the brain was noted. The demyelinating lesions started in perivenous distribution, but with increasing size large confluent demyelinated plaques with or without activity in the borders were found. The topographical distribution of the lesions within the brain and spinal cord closely resembled the pattern found in multiple sclerosis. We also noted that, similarly as in MS, some vessels in old demyelinated plaques revealed increased permeability [8].

Thus, depending on the stage of the disease when the animals died or were killed, pathological lesions were found closely resembling those described in the different types of human inflammatory demyelinative diseases (acute and subacute hemorrhagic leukoencephalitis, acute perivenous leukoencephalitis, and acute and chronic multiple sclerosis). Thus in our opinion, these data stress the close pathogenic relationship of these different diseases, as has been postulated by several previous authors [9, 13].

Studies on the morphological sequence of myelin degradation revealed that the Luxol fast blue positive myelin degradation products were rapidly transformed into PAS-positive, ultrastructurally polymorphic debris, without formation of sudanophilic neutral lipid droplets. This is of particular interest because of the description of abundance of PAS-positive material with similar ultrastructure in human inflammatory demyelinative diseases [16, 17, 26, 27]. The reason for the lack of a sudanophilic stage in chronic relapsing EAE is not yet clear; however, in EAE in monkeys, the occurrence of sudanophilic degradation products has been described [24, 26]. In Wallerian degeneration in the CNS, the sudanophilic stage, although present, is much less pronounced in small rodents, as compared to larger animals and man [12]. Therefore, species differences may account for differences of macrophage ability to handle cholesterol esters and triglycerides.

It is generally believed that myelin basic protein (MBP) is responsible for the induction of EAE. Based on this assumption, we investigated the stimulation of peripheral lymphocytes by MBP. However, our data indicate that a positive correlation with respect to sensitization to MBP is present only in the acute episode of the disease. In addition, up to now we have been unable to induce chronic EAE with plaque-like demyelination by sensitization with MBP. Our studies on neuritogenic activity and chemical properties of guinea pig anterior and posterior root myelin also indicate that antigens other than MBP play an important role in the recurrence of EAE and the formation of large demyelinated plaques [14].

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Refinement of the EAE Model Utilizing Various Synthetic Adjuvants and Bacterial Fractions: An Attempt to Produce a Chronic Model of EAE

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Introduction

Although there is a great difference between the EAE model and MS, it is still worthwhile to scrutinize and refine the EAE model to gain an insight into an immunopathological aspect of MS and find a clue to making its modulation possible. In previous papers [4, 5], we have demonstrated that EAE can be induced in guinea pigs by a combination of a synthetic determinant heptapeptide and a synthetic adjuvant peptide in the form of incomplete Freund's adjuvant (IFA). In this paper we describe an attempt to further simplify this model, by which a use of hydrocarbons and detergents inherent in the adjuvant method is not necessary. Secondly, we describe a reevaluation and analysis of suppression of EAE induction, performed by presensitization with complete Freund's adjuvant alone, as originally reported by Cunningham and Field in 1965 [2].

Materials and Methods

Immunization and Pretreatment

Outbred male Hartley guinea pigs, weighing 350–400 g, were used throughout this study. Myelin basic protein (MBP) was prepared from white matter of bovine brain as described previously [5]. This preparation caused a 100% incidence of EAE in guinea pigs at a dose of 10 μg in complete Freund's adjuvant (CFA). In the present experiments, 30 μg of MBP were used for EAE induction using CFA containing 100 μg of heat-killed *Mycobacterium tuberculosis*, human virulent Aoyama B strain unless otherwise specified.

For prevention and challenge treatments, 0.25 ml volumes of aqueous solutions of antigen and/or mycobacteria (or test materials) were emulsified in a syringe with 0.1 ml of Arlacel A and 0.4 ml of Bayol F, whereupon 0.1 ml portions were injected into the indicated sites of guinea pigs.

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Evaluation of clinical signs of EAE was performed as follows: 0 = no apparent change; 1 = body weight loss and a slight paralysis or paresis; 2 = paralysis accompanied by slight diarrhea and/or incontinence; and 4 = intensive paralysis accompanied by diarrhea and incontinence. Challenged animals were weighed and inspected daily.

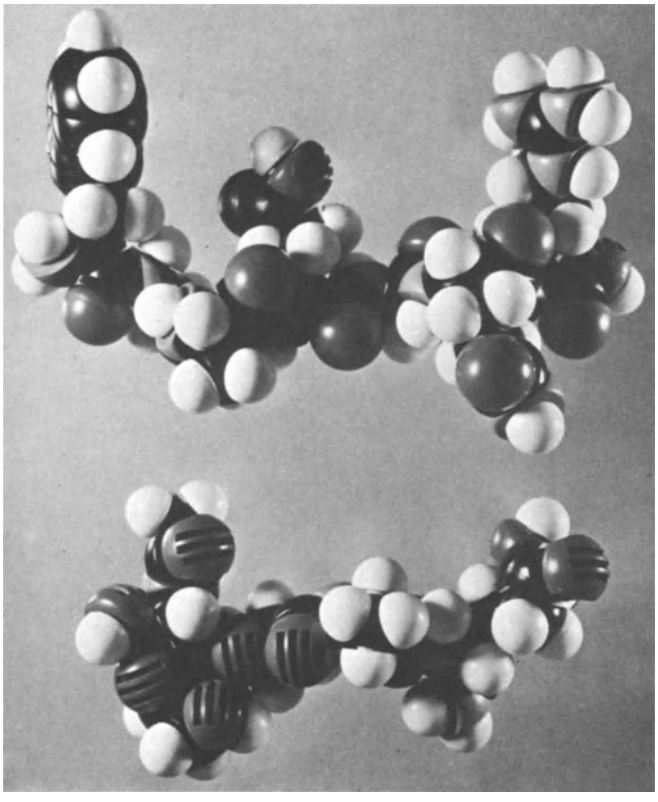
Liposome Experiments

In this experiment, a synthetic adjuvant, N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP) was acylated with various straight or branched chain fatty acids at C_6-OH of muramic acid residue as later mentioned and those acylated MDP are designated as AcMDP. Synthetic dipalmitoyl lecithin (0.6 ml, 15.4 mg/ml, chloroform), 0.6 ml of cholesterol (5.8 mg/ml, chloroform), 0.6 ml of dicetyl phosphate (Plainview, New York) (1.1 mg/ml, chloroform) and 0.75 ml of synthetic 6-O-(acyl)-N-acetyl-muramyl-L-alanyl-D-iso-glutamine (AcMDP) (2.88 mg/ml, chloroform-methanol 1:1, v/v mixture) were combined in a small conical flask, evaporated to dryness with a rotary evaporator, and then left in vacuo in a desiccator for 2 h. To the dried thin film of the above lipidic materials, 1.0 ml of aqueous MBP or synthetic determinant decapeptide (P-10) solution (1.0 mg/ml, PBS, pH 7.2) was added and mixed vigorously with a vortex mixer. The liposome suspension was further sonicated for 5 min in a sonic bath (Bransonic 220, U.S.A.) and each 0.2 ml of the suspension was administered to a guinea pig. The liposome consisted of lecithin, cholesterol, and dicetyl phosphate in a 2.0:1.5:0.2 molar ratio. Each guinea pig thus received 200 µg of MBP (or P-10) and 360 µg of AcMDP, respectively. As a control, 0.5 ml of aqueous MBP or P-10 (2.0 mg/ml, PBS, pH 7.2) were added dropwise into 0.5 ml of clear aqueous solution of AcMDP (4.18 mg/ml, PBS, pH 7.2). A turbidity developed, finally resulting in a suspension. The suspension was sonicated for 10 min in a sonic bath (80 kc/s, Laboratory Supplies Inc., New York) and each 0.2 ml of the suspension, which contained 200 µg of MBP or P-10 and 418 µg of AcMDP, respectively, was administered into a guinea pig. The amino acid sequence of the synthetic P-10 used is H-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg-OH [5].

Results

Simplication of EAE Model

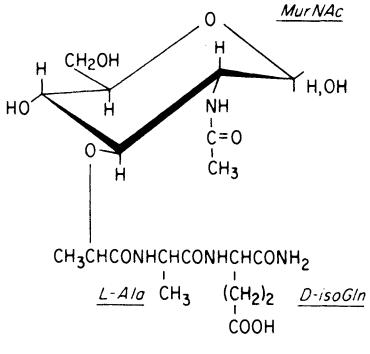
In 1976 we demonstrated a simplified EAE model in which EAE could be induced in guinea pigs by a combination of synthetic encephalitogenic peptides and muramyl dipeptides (MurNAc-L-Ala-D-isoGln, MDP) effectively at a dose on the microgram level [4, 5]. In this system, the synthetic heptapeptide, H-Trp-Gly-Ala-Glu-Gly-Gln-Arg-OH, was the shortest peptide causing EAE (Fig. 1). MDP is also effective in monkey EAE (Ohtani et al., unpublished data). This refined system, however, still needed hydrocarbon and a detergent in the form of Freund's type adjuvant. Therefore, further simplication was attempted by administration of antigen in the



a

H-Trp-Gly-Ala-Glu-Gly-Gln-Arg-OH

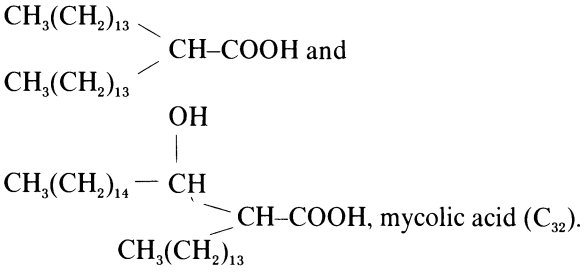
ENCEPHALITOGEN



b ADJUVANT

Fig. 1. Minimum structural units **(a)** and structural formula **(b)** for encephalitogen and for adjuvant in the induction of EAE in guinea pigs. Encephalitogen (*upper*): H-Trp-Gly-Ala-Glu-Gly-Gln-Arg-OH; Adjuvant (*lower*): MurNAc-L-Ala-D-isoGln

form of liposomes, in which no such oily admixture is necessary. For this purpose, MDP was acylated at C_6-OH of a muramic acid residue by either a straight-chain C_{18} fatty acid, stearic acid, or C_{30} or C_{32} α-branched long-chain fatty acids, i.e., 2-tetradecylhexadecanoic acid (C_{30}) or 3-hydroxy-2-tetradecyloctadecanoic acid (C_{32}, mycolic acid). These acids have the following structure:



These AcMDP were incorporated into liposome membranes containing MBP or P-10, and administered to the animals. Up to the present we have succeeded in producing EAE in the case of 6-O-stearoyl MDP (MBP antigen: clinical, 2/4; histological, 2/4; P-10: clinical 0/5; histological, 0/5) and also in the case of 6-O-C_{30}-MDP (MBP antigen: clinical, 3/5; histological 2/5; P-10: clinical, 1/5; histological, 0/5). In the former case, liposome antigen was first administered in foot pads of hind legs and an *i.p.* booster injection was given 24 days after the first injection. In the case of MBP, clinical signs (weight loss and evident paralysis, but no diarrhea and incontinence) appeared 6 days after the booster. Histopathological examination revealed disseminated lesions characteristic of experimental allergic encephalomyelitis, consisting in one case of perivascular cuffs and compact or discrete cell infiltration into the nervous parenchyma. Myelin sheath was found to disintegrate in the foci with dense cell infiltration. These changes were predominately distributed in the hippocampus, diencephalon, brain stem, and the cerebellar white matter. A tiny focus was also present in the anterior column of the thoracic cord. In another case, a slight cell infiltration was observed in the fimbria of the hippocamus and the septal nuclei. None of the other cases showed histologically positive lesions. No clinical and histological positives were observed with an *i.v.* booster (0/5). P-10 administration failed to induce EAE either by *i.p.* booster or by *i.v.* booster.

In the latter case (6-O-C_{30}-MDP), where the liposomal antigen was administered first in the foot pads of the hind legs, weak but distinct paralysis occurred 7 days after injection (MBP, 1/5; P-10, 1/5). But after a foot pad booster injection given 41 days after the first sensitization, 3 out of 5 animals developed severe paralysis, diarrhea, and incontinence when MBP was injected, and two of them, which were histologically positive, died. Among the three surviving animals, two were dubiously positive on histological examination. With injection of P-10, however, no such change occurred after the booster, and the only animal that became infected recovered, survived, and was sacrificed 5 weeks after the booster. However, this case was histologically negative. Experiments with C_{32}-MDP failed to induce EAE. Liposomes using either stearylamine instead of dicetyl phosphate or bovine brain sphingomyelin instead of dipalmitotyl lecithin also failed. Mere injection of a PBS suspension of antigen (MBP and P-10) and AcMDP produced no EAE positive clinical or histological results.

Prevention of EAE Using Bacterial Cell Components

In 1965 Cunningham and Field reported a protection against EAE in guinea pigs by pretreatment with CFA alone [2]. Since only a few studies have appeared since then, we extended this study.

As shown in Table 1, a single foot pad injection of CFA containing 100 μg of a human virulent strain of mycobacteria, Aoyama B, effectively protected guinea pigs from EAE. However, a loss in body weight was observed in some of the animals. This is consistent with the report of Cunningham and Field [2]. The protection lasted for up to at least 26 weeks, while pretreatment immediately followed by a challenge after 3 days failed to prevent EAE.

The degree of protection seemed to depend on the injection site (Table 2). A combination of a pretreatment by foot pad injection and a challenge by intradermal injection on back skin was found to be most effective and reproducible. We then tested prevention with various strains of bacteria (Table 3). The results showed that a hu-

Table 1. Prevention of EAE by pretreatment with CFA [a]

Exp. No.	Interval (weeks)	Clinical EAE	(Survival)
1	1	0/5	(5)
2	2	0/5	(5)
3	3	0/5	(5)
4	4	0/5	(5)
5	13	0/5	(5)
6	26	0/4	(4)

[a] CFA: mycobacteria, human virulent strain Aoyama B; 100 μg (f.p.)
Challenge with MBP in CFA; 30 μg (i.d.b.)

Table 2. Dependency of prevention on injection sites [a]

Exp. No.	Sites		Clinical score (mean)	Clinical EAE	(survival)
	Prevention	Challenge			
1	f. p.	i. d. b.	body wt. loss only	0/5	(5)
2	f. p. (L)	f. p. (R)	3.3	4/4	(1)
3	f. p.	i. d. b.	body wt. loss only	0/4	(4)
4	f. p. (L)	f. P. (R)	4.0	4/4	(0)

[a] Aoyama B strain, 100 μg, was used for prevention in exp. No. 1 and 2, and BCG, 100 μg in exp. No. 3 and 4
f. p. (L): a foot pad of left hind leg; f. p. (R): a foot pad of right hind leg; i. d. b.: intradermal injection on back
30 μg of MBP was used for challenge a week after the pretreatment

Table 3. Prevention with various strains of bacteria [a]

Bacteria strains	Onset of clinical signs (days)				Clinical score				Clinical EAE	(survival)
	Animal no.				Animal no.					
	1	2	3	4	1	2	3	4		
Aoyama B	–				– [b]				0/5	(5)
BCG	–				– [b]				0/4	(4)
Myc. rhodochrous (ATCC 184)	14	12	12	12	4	1	4	2	4/4	(0)
N. corynebacteriodes (ATCC 14898)	16	12	–	–	1	4	0	0	2/4	(2)
N. corallina (ATCC 14347)	12	16	14	12	1	2	2	1	4/4	(2)
Stm. gardneri (ATCC 23911)	17	14	12	17	2	2	2	1	4/4	(3)

[a] Pretreatment: CFA containing 100 μg of each bacteria injected on foot pads of hind legs; Challenge: MBP (30 μg) in CFA by i. d. b. route a week after the pretreatment

[b] Only loss of body wt. observed

Table 4. Prevention with various subfractions of cell walls [a]

Subfractions of cell walls	Onset of clinical signs (days)					Clinical score					Clinical EAE	(survival)
	Animal no.					Animal no.						
	1	2	3	4	5	1	2	3	4	5		
1 (MDP)	15	12	12	21	16	1	3	1	4	1	5/5	(0)
2 (LD-3Y-01) [b]	12	15	14	15	–	2	2	2	3	0	5/5	(0)
3 (WSA) [c]		12	~	15			3	~	4		5/5	(0)
4 (NCCM-1-1) [d]	14	6	14	–	–	1	1	1	0	0	3/5	(2)
5 (NCCM-1-2) [e]	12	12	15	–	–	3	2	2	0	0	3/5	(1)
6 (SGCM-1-2) [f]	14	27	15	–	–	2	1	1	0	0	3/5	(1)

[a] Prevention: 100 μg of each subfraction were used (f. p. injection) Callenge: 30μg of MBP (i. d. b. injection)

[b] LD-3Y-01: cell wall glycopeptide, bis disaccharide peptide subunit dimer from *Lactobacillus plantarum* [5]

[c] WSA: water-soluble adjuvant prepared by endopeptidase degradation of the cell walls of *Staphyrococcus epidermidis* (free of specific structure, and glycan portion remained intact)

[d] NCCM-1-1: a cell wall component of *Nocardia corinebacteriodes* prepared with M-1 enzyme; having a structure of (mycolic acid)-arabinogalactan-peptide glycan

[e] NCCM-1-2: a cell-wall component similary prepared as in the above NCCM-1-1; peptide glycan portion alone

[f] SGCM-1-2: a cell wall component of *Streptomyces gardneri* prepared with M-1 enzyme; having a structure of a phosphorous-containing specific structure linked to a peptide glycan cleaved

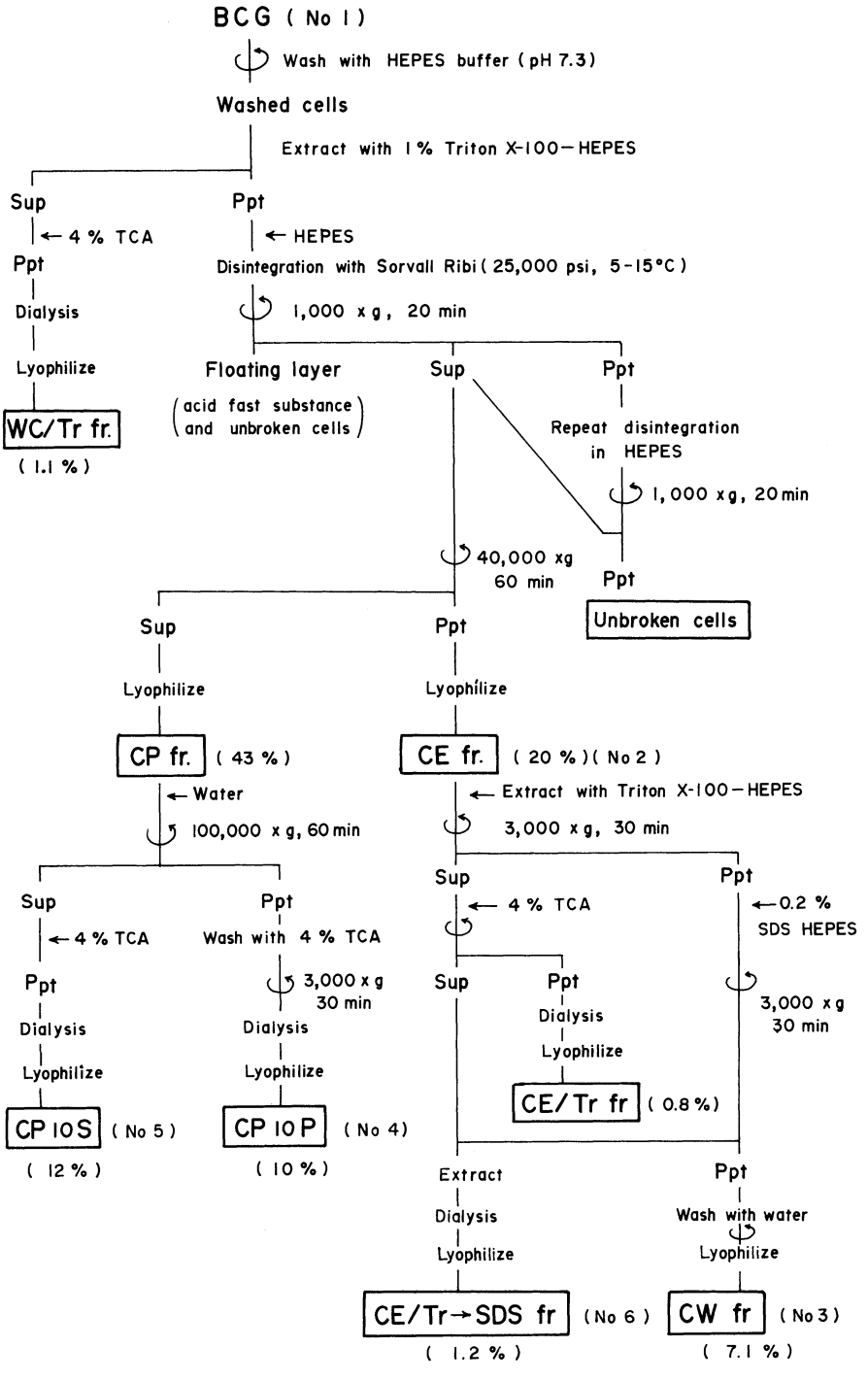


Fig. 2. Fractionation of BCG cell components [7]. No. 1 WCB, whole cells; No. 2 CE, from cell envelope; No. 3 CW, from cell walls; No. 4 CP10P, water-insoluble cytoplasmic components; No. 5 CP10S, water-soluble cytoplasmic components; No. 6 CE/Tr→SDS, from detergent-soluble cell envelope fraction

Table 5. Prevention with various subfractions of BCG [a]

Subfraction No.	Onset of clinical signs (days)				Days of death				Clinical score				Clinical EAE	(survival)
	Animal no.				Animal no.				Animal no.					
	1	2	3	4	1	2	3	4	1	2	3	4		
1 (WCB)	–	–	–	–	–	–	–	–	–	–	–	–	0/4	(4)
2 (CE)	15	–	–	–	19	–	–	–	1	–	–	–	1/4	(3)
3 (CW)	–	–	–	–	23	–	–	–	–	–	–	–	1/4	(3)
4 (CP10P)	15	15	14	–	15	15	14	22	3	2	–	1	4/4	(1)
5 (CP10S)	–	–	–	–	–	–	–	–	–	–	–	–	0/4	(4)
6 (CE/Tr-SDS)	19	15	14	–	19	19	16	20	1	2	3	–	3/4	(0)
7 (H_{37}Rv-CW)	–	–	–	–	–	–	–	–	–	–	–	–	0/4	(4)
8 (PPDs)	12	16	15	14	17	17	19	16	1	1	1	2	4/4	(0)
9 (control)	19	16	14	17	20	19	15	19	3	3	4	1	4/4	(0)
10 (Aoyama B)	–	–	–	–	–	–	–	–	–	–	–	–	0/4	(4)

[a] Prevention: 100 μg of each subfraction were used (f. p. injection); Challenge: 30 μg MBP, i. d. b. 2 weeks after the pretreatment; Control: Challenge alone; For subfraction, see legend in Fig. 1

man virulent strain of *Mycobacterium tuberculosis*, Aoyama B, was most potent, followed by BCG.

Preventive activity in several cell wall constituents was then tested, since it has been clearly established that immunoadjuvant activity resides in cell walls. As shown in Table 4, activity was found in NCCM and SGCM fractions. It is noteworthy that no activity was found in MDP and its related compound, LD-3Y-01.

To find where the chemical principle responsible for prevention resides, we performed a careful and mild fractionation of BCG cell components (Fig. 2) [7]. Our results are summarized in Table 5. Although the results did not definitively show a distinct localization of activity due to a drawback in using foot pad pretreatment and challenge assay method, a consideration of the entire clinical course as well as of the days of onset and of death seems to indicate that activity resides mainly in a water-soluble cytoplasmic fraction (CP10S) and to a lesser extent in cell walls but not in PPD_S. In this connection it is of note that a purified cell wall fraction ($H_{37}R_V$–CW) of a human virulent strain, H_{37} R_V, which was prepared by a pronase treatment, was quite potent, and that CP10S showed a high delayed-type hypersensitivity upon skin testing [7].

Discussion

The present study clearly shows that EAE can be induced without the help of Freund's adjuvant, if antigen is administered in an anionic form of liposomes containing AcMDP. It should be emphasized that in this simplified model the disease develops earlier than usual EAE, usually within a week after the last sensitization. Recently Coon and Hunter [1] reported that the immunological response to bovine serum albumin can be switched from a humoral to a cell-mediated type by increasing the lipophilicity of antigen by acylation with a medium-chain fatty acid.

Prevention of EAE using CFA alone is unique in its simplicity and long-lasting effect (among the various types of protections so far reported). Our study shows that a mechanism different from the usual type of immunological potentiation by adjuvant is in operation, since a strong adjuvant, MDP, and related compounds failed to give this preventive effect. Orbach-Arbouys and Poupon [6] and Germain et al. [3] report that spleen cells from BCG-treated animals inhibit PHA responsiveness, mixed lymphocyte reactions, graft-versus-host reactivity, and tumor cell growth in vitro, in which an involvement of T cells and/or macrophages was suggested. Although the mechanism of the present EAE-preventive effect is unknown, this model is valuable for an understanding of the underlying mechanism of MS relapses and may lead to a unique relapsing EAE model. Our study also indicates that the chemical principle responsible for this prevention may reside in a cytoplasmic fraction of mycobacteria as well as in cell walls and that this active substance is enriched in human type strains of *Mycobacterium tuberculosis*. A study is now in progress to identify this principle.

Conclusion

1. Myelin basic protein and possibly its determinant decapeptide can elicit EAE, when administered in the form of anionic liposomes consisting of lecithin, cholesterol, dicetylphosphate and muramyldipeptide which is acylated at C6-OH of muramic acid with long-chain fatty acids.
2. EAE induction with myelin basic protein is effectively prevented by a single injection of Freund's complete adjuvant alone. The preventive effect is long lasting. Human type strains of mycobacteria (Aoyama B and H_{37} R_v) were more potent than BCG and several other adjuvant-active bacteria.
3. Chemical principle responsible for prevention was surveyed on subfractions of BCG and found to reside mainly in a water-soluble cytoplasmic fraction but not in PPD_s. The cell walls were also effective but to a lesser extent. Other subfractions (detergent-soluble cell envelope and water-insoluble cytoplasmic fractions) and synthetic adjuvant-active muramyl peptide and its related compounds were not effective.
4. Prevention with mycobacterial cell components may provide a way to establish a new chronic EAE model and to get an insight into the pathogenesis of the chronic form of EAE.

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Discussion

W. Arnason: It seems to me that there are three major themes that emerge from these papers. The first is the development of chronic EAE, and I have a slide of chronic EAE that I'd like to show.

The first mention of EAE was made by Rivers. This is taken from a paper that Rivers published in 1933 about a monkey with chronic EAE. This was the first monkey that ever developed EAE, and it comes from the original paper describing EAE. Rivers did not have any Freund's adjuvant, and it was the development of Freund's adjuvant which led to the development of acute EAE, and which I think probably misdirected many of the efforts in terms of EAE for many, many years. In these early lesions, shadow plaques and gliosis were also seen. Freund's adjuvant and tubercle bacilli generally have two effects: (1) they augment the immune response, and (2) they turn it off. They are potent augmentors of suppression, and I think that is perhaps what Dr. Nagai was showing when he demonstrated that he could suppress EAE with complete Freund's adjuvant. Finally, it seems to me that the report by Dr. Wisniewski that the antigen in chronic EAE may not be basic protein is of greatest interest, because I think the same may hold for multiple sclerosis itself. The data that basic protein is the antigen responsible for MS, at least to me, seems at present unpersuasive. Finally, the point that Dr. Raine made, that there seemed to be alterations in T-cell numbers, and possibly in T-cell regulator function in the chronic and relapsing EAE model, is a point of some importance which I think will be elaborated during the course of this meeting in terms of MS itself.

T. Yonezawa: In studies on EAE and EAN, selection of animal species for the experiment is important, since each species develops specific types of allergic changes. Among the various kinds of animals, monkeys develop widespread lesions characterized by a distribution similar to that of multiple sclerosis and by marked exudative and necrotic tendency. The histology of these lesions is characterized by fibrinoid necrosis of blood vessels, serofibrinous exudation, hemorrhage, and infiltration of polymorphonuclear leukocytes. Nervous tissue elements are necrotized. Electron-microscopic pictures of these lesions show that the alterations are not demyelinative, but degenerative and necrotizing. In other words, myelin destruction is characterized by slippage of myelin lamella. Axons are swollen, and no neurofibrils and neurotubules are seen. Inflammatory cells participating in these lesions are mainly neutrophils.

Most of the animals die in this acute disease process, before demyelinating change takes place. No matter how immunization procedures are modified (such as by using a different encephalitogenic substance), animals develop the same identical alterations. If the animals survive the acute stage, perivascular leukocytic infiltration

is replaced by monocytes and lymphocytes and characteristic demyelinating changes begin to occur.

In monkeys with EAN, exudative processes are still severe, and the lesions are often hemorrhagic and necrotic. Histological examination shows moderate edema, hemorrhage, and perivascular infiltration of neutrophiles. However, exudative processes in monkey EAN seem to be less severe, compared to the lesions in EAE. If these diseased monkeys survive the acute exudative stage, they may develop demyelinative changes, i.e., perivascular infiltrates are replaced by monocytes and lymphocytes. In these lesions, invasion of macrophages into myelin sheaths and splitting of myelin lamella are common features of demyelination.

In rabbit and guinea pigs, however, exudative processes seem to be only temporal and minimal. Edema and hemorrhage are exceptional. Histologically, perivascular infiltrates are composed of lymphocytes and monocytes. Infiltration of polymorphonuclear leukocytes is observed only occasionally. Electron-microscopic examination reveals demyelinative changes in the lesions, represented by splitting of myelin lamella caused by infiltrating macrophages, vesicular dissolution of myelin, and autophage of myelin in the myelin-associated cells.

Immunological analysis also shows species differences. Serum factors, i.e. complement-dependent demyelinating and myelination-inhibiting antibodies, are absent in monkeys with EAE and EAN. Our previous work suggests that these antibodies are derived from galactocerebroside as a hapten antigen. When rabbits were inoculated with galactocerebroside and carrier protein together with CFA, they developed both demyelinating and myelination-inhibiting antibodies. However, monkeys are not susceptible to this hapten antigen, and this may be related to the fact that they do not develop demyelinating antibody with EAE and EAN.

Compared to the serum factor, sensitized lymphocytes from animals with EAE and EAN are all found to actively participate in demyelination, regardless of species difference, developing both lymphotoxic and macrophage-activating factors.

Y. Nagai, T. Uchida, S. Takeda, F. Ikuta: The chemical nature of EAN antigen is not known, although PNS myelin itself can specifically cause EAN at least in rabbits. None of three proteins, P0, P1 and P2 (Eylar's nomenclature), which constitute major PNS myelin proteins, developed an EAN-inducing potency, when their highly purified preparations were used as antigen [1, 3], except that P1 protein having an amino acid sequence nearly identical with myelin basic protein (MBP) of CNS tissues regained the EAE-inducing potency. We have found that specific EAN-inducing activity is successfully reconstituted by complexing P2 protein of PNS myelin with PNS-derived gangliosides [2]. This suggests the necessity of EAN activity of a specific membraneous conformation of antigenic molecules, in sharp contrast to the segmental nature of EAE antigen, by which not only MBP having a largely randomly coiled disordered structure is EAE-active, but also its short peptide fragments are still potent (Table 1). In fact, in the preceding paper (see Nagai et al., this volume) we showed that the shortest EAE determinant active in guinea pigs is a tryptophan-containing heptapeptide. Thus, the next important step is to elucidate whether the EAN determinant resides in a particular conformational region of P2 protein, in specific molecular species of gangliosides, or in both.

Table 1. Antigenic difference in EAE and EAN

	EAE	EAN
Antigen	Myelin basic protein (MBP)	P2 protein-ganglioside complex
Molecular form	Largely randomly-coiled disordered structure	Protein-lipid complex β-structure ($\sim$60%)
Antigenic form	Segmental (or linear-bound)	Conformational (membraneous)
Determinant	Species-specific peptide fragments	a particular conformational region of P2, specific gangliosides, or both

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Theiler's Virus Infection of the Central Nervous System of Mice: An Animal Model of Virus-Induced Demyelination

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Historical Perspective

In the 1930s, Max Theiler recovered a virus from the central nervous systems (CNS) of spontaneously paralyzed mice [11]. Experimental transmission of the virus to other mice by intracerebral (IC) and intranasal routes of inoculation produced similar paralytic disease. It was subsequently shown that Theiler's murine encephalomyelitis viruses are enteric pathogens which usually cause asymptomatic intestinal infections [8]. These viruses are now known to be small, nonenveloped viruses containing RNA, and are classified as picornaviruses. The clinical disease and the histopathology of the CNS lesions described by Theiler so closely resembled human poliomyelitis that for many years this infection served as an analog of human polio in a small laboratory animal.

Theiler also demonstrated that surviving mice had chronic CNS infection [11]. In fact, this was the first report of a chronic virus infection in an animal host. In 1952, Daniels and co-workers discovered extensive areas of myelin destruction in the spinal cords of four mice sacrificed several months after infection with Theiler's virus [2]. Therefore, it was recognized that this persistent infection is associated with a chronic CNS pathology.

Pathogenesis of Theiler's Virus Infection

During the past several years, we have studied the pathogenesis of Theiler's virus infection in young adult mice. CNS disease is regularly produced by inoculating mice IC (but not IP) with any of several strains of the Theiler's viruses. For many years the strains of Theiler's viruses which produced chronic CNS infection (designated T0 strains) could only be maintained by brain-to-brain passage in mice, i.e., they could not be successfully grown in cell culture. Recently, it has been shown that these viruses can be adapted to grow and produce cytopathic changes in L929 and BHK21 cells [4]. With this development came the discovery that the T0 strains form small plaques under agar overlay (in contrast to other more virulent strains which produce large plaques) [4, 5]. It is now clear that the exact pattern of CNS disease in mice depends in part on whether the T0 strains of virus have been adapted to cell culture.

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CNS Disease Produced by Brain-Derived Virus

After IC inoculation the majority of mice develop flaccid paralysis (designated early disease) within 1–3 weeks, and usually only a small percentage die. The majority of animals recover from paralysis but subsequently develop a spastic gait disorder within 1–3 months (designated late disease) [3]. Mice with late disease manifest a distinctive waddling and spastic gait, and minimal stimulation, such as jarring the cage, results in loss of balance and prolonged extensor spasms. Although the clinical manifestations of late disease are progressive, affected mice generally can be kept alive for many months.

Infected mice have a brief period of viremia and only limited virus growth in extraneural organs. However, during the first 3 weeks after infection, there is logarithmic virus growth in the CNS, and virus antigen is found in the cytoplasm of neurons and possibly other cell types in the gray matter of the spinal cord, brain stem and thalamus by fluorescent antibody staining. After 1 month, low levels of infectious virus are still detectable in the spinal cord, but the titer declines and then disappears with time. Virus antigen has not been observed in the CNS after 1 month.

Although serum-neutralizing antibodies are present after 1 week, there is an unusually gradual rise of antibody titers over the first 2 months of infection. In addition, the kinetics of the cellular immune response to virus is unusual [9]. Spleen cell reactivity is not detectable until 2 months after infection, but, once established, is present throughout the first year after intracerebral inoculation in parallel with CNS virus replication.

The histopathology of this infection is limited to the CNS. Initially, gray matter involvement predominates, principally consisting of patchy areas of neuronal necrosis, neuronophagia, and microglial cell proliferation in the spinal cord, brain stem, and thalamus. As these lesions resolve toward the end of the first month, spinal cord leptomeningeal and white matter involvement commences and gradually increases in severity. This involvement is characterized by focal collections of mononuclear cells and some plasma cells in the leptomeninges, in perivascular sites, and extending into the white matter. Epon-embedded, toluidine blue-stained sections have shown that myelin is the primary structure damaged in the white matter [1]. Individual demyelinated axons as well as clusters of axons are found scattered amidst the inflammatory cells, and numerous macrophages containing myelin debris are readily identified in these lesions. Remyelinating axons and astrocytic gliosis become more prominent with time. Ultrastructurally, stripping of myelin lamellae by mononuclear cell processes und vesiculation of myelin independent of mononuclear cells are the morphologic events accompanying myelin destruction. In addition, oligodendrocytes do not appear to be infected, since normal oligodendrocytes are frequently identified in the vicinity of naked axons, whereas degenerating oligodendrocytes have not been observed. Virus particles have not yet been seen in the CNS by ultrastructure, but this is not unexpected considering the relatively low virus titers and the small size of these virions.

CNS Disease Produced by Tissue-Culture Adapted Virus

In general, use of tissue culture-adapted T0 strains of Theiler's virus leads to the occurrence of late disease in mice without antecedent early disease [7]. In this case, late disease occurs after a long incubation period, lasting one or more months. The clinical manifestations, pathological changes, and CNS virus growth of this infection closely resemble that produced by brain-derived virus as already described. We are not certain why adaptation of the T0 strains to cell culture tends to eliminate the early phase of poliomyelitis. While this probably merely represents attenuation of the original virus, it has revealed a unique virus-host interaction for a picornavirus, a slow infection. Theiler's virus disease now satisfies the major criteria for slow infections set forth by Sigurdsson in 1954 [10].

Immunosuppression of Infected Mice

Immunosuppression of experimental viral infections is now a recognized method for characterizing the role of the immune response in host defense and determining the mechanisms of cellular injury. Therefore, the effect of immunosuppressive drug treatment of Theiler's virus infection has been studied [6]. The net result of immunosuppression was overall potentiation of the infection with a significant increase in mortality. Virus replication was enhanced because of slower virus clearance, and this resulted in longer maintenance of higher virus levels in the CNS. There was a substantial increase in microglial proliferation and neuronal necrosis in brains and spinal cords of immunosuppressed Theiler's virus-infected mice. Taken together, these findings indicate that Theiler's virus causes a cytolytic infection of neurons and possibly other cells in the gray matter. Thus it is reasonable to think that potentiation of a cytolytic infection in the gray matter of infected, immunosuppressed mice caused the increase in mortality.

In contrast to the augmented gray matter involvement, immunosuppression caused a dramatic reduction in mononuclear inflammatory cells in the spinal cord leptomeninges and white matter. Since the induction of the inflammatory response in CNS virus infections appears to be immunologically specific, a lack of parenchymal inflammation would be expected if such treatment sufficiently suppressed host immunity. More important, immunosuppressive treatment prevented actual parenchymal damage, i.e., demyelination, from occurring, suggesting that the white matter lesion is immune mediated. The effector mechanism of immunological injury to myelin remains to be elucidated.

Theiler's Virus Infection as an Animal Model for Multiple Sclerosis

We feel that Theiler's virus infection in mice is an excellent animal model for studying the pathogenesis of virus-induced demyelination and is a good analog of multiple sclerosis for the following reasons: (1) demyelination is the sole structural change

during the chronic phase of infection, and neurological disease in the mouse can be directly attributed to this lesion; (2) active and inactive demyelinating lesions are commonly present in the same animal, suggesting that there may be recurrent demyelination; (3) using tissue-culture adapted virus, mice only develop late disease (the demyelinating stage of this infection) and this occurs after a prolonged incubation period; and (4) myelin breakdown appears to be immune mediated. It should be pointed out that clinical disease in Theiler's virus infection is progressive rather than relapsing, that there is often a severe leptomeningitis, and that demyelination is essentially limited to the spinal cord. In contrast, in multiple sclerosis, there is usually a distinctive relapsing course, inflammation is not commonly present in the leptomeninges, and patchy areas of demyelination are distributed throughout the CNS. The relative paucity of myelin in the cerebral hemispheres of rodents in comparison to those of primates may be responsible for this difference in lesion distribution.

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Acute and Recurrent Demyelination Induced by Wild-Type and Temperature-Sensitive Mutants of Mouse Hepatitis Virus. Electron-Microscopic Studies

P. W. LAMPERT [1], M. V. HASPEL [1], and M. B. A. OLDSTONE [2]

Weanling mice infected with the neurotropic JHM strain of mouse hepatitis virus (MHV), a coronavirus, develop an acute demyelinating encephalomyelitis [1]. Demyelination occurs as a result of virus-induced destruction of oligodendrocytes [6]. The severity of the myelitis is related to virus dose, inoculation route, age, and strain of mice [9]. Further, the manifestation of the disease is significantly altered by inoculation of temperature-sensitive (ts) mutants of MHV [3]. The attenuated viruses produce fewer fatalities, while retaining the ability to destroy oligodendrocytes. This report describes the morphologic changes leading to acute and recurrent demyelination in the spinal cord of mice infected with wild type and ts mutants of MHV.

Mouse hepatitis virus (JHM strain) was plaque purified in NCTC-1469 cells. The infectious titer, expressed as plaque forming units (PFU/ml), was determined by plaque assay on NCTC-1469 cell monolayers after 48 h at 34° C. The virus was grown overnight in NCTC-1469 cells at 34° C in the presence of 5-azacytidine (12 μ g/ml) or 5-Fluorouracil (150 μ g/ml). These mutagen concentrations resulted in a 100-fold reduction of progeny virus. Mutants, selected for an inability to produce multinucleated syncytia at 39.5° C, were plaque purified an additional time. Four week old BALB/c St mice were inoculated by the intracerebral (IC) route with 0.05 ml of virus. In other experiments, mice were infected by intranasal instillation of virus. Following fixation with Bouin's solution, tissues were embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Alternatively, mice were perfused via the heart with 2.5% phosphate-buffered glutaraldehyde. Blocks of the spinal cords were embedded in araldite, and sections were stained with paraphenylenediamine. For electron microscopy, thin sections were stained with uranyl acetate and lead citrate and examined using a Siemens 101 electron microscope.

Four representative ts mutants, ts7, ts8, ts11, and ts15, were studied in animal experiments. The mutants were highly attenuated (Table 1). Whereas 2 PFU of wt virus killed 50% of the mice, 10,000 PFU of mutant virus were required to induce the same mortality. Further, mice infected with wt virus usually died within 1 week, whereas those inoculated with ts mutants did not succumb until 8 to 11 days after IC inoculation. Lesions also developed after intranasal virus administration. The degree of demyelination produced by wt virus was dependent on virus dose. A reduction in dose resulted in more survivors but also in a decreased incidence of demyelination. By contrast, infection with ts8 and ts11 produced a high incidence of demyelination and a low mortality. However, ts7 and ts 15 had little effect, despite in vivo virus replication. For electron microscopy, we studied mice sacrificed during

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the first week at daily and later at weekly intervals from 2 to 126 days after infection with a ts8 mutant.

The spinal cord of mice infected with either wt virus or ts8 mutants revealed similar lesions. Changes were noted as early as 4 days after infection. Demyelinated areas developed throughout the white matter of the spinal cord, with some preference for anterior columns and root entrance zones. Widespread subpial demyelination was often noted but there was no clear relationship of the lesions to vessels. Early alterations of oligodendrocytes were observed within the edematous white matter in

Table 1. The neurovirulence and induction of demyelination by mouse hepatitis virus

Virus	Infectious dose [a]	Survival [b]	Demyelination [b]
wt	10	3/12	2/2
wt	2	6/12	2/6
wt	0.4	9/11	1/9
ts 7	10,000	9/12	2/9
ts 8	10,000	151/143	19/24
ts 8	1,000	12/12	6/12
ts 11	10,000	16/23	12/16
ts 15	10,000	21/25	1/21

[a] Plaque-forming units (PFU)/ml
[b] 11 days after infection, number positive/total number studied

the absence of cellular infiltrates. Oligodendrocytes were identified by connections of their plasma membranes with myelin lamellae (Fig. 1). The infected cells underwent proliferative changes prior to degeneration. The hypertrophic cells were replete with microtubules and filaments. Abundant aggregates of electron-dense granules were scattered throughout the cytoplasm. These granules most likely represent viral RNA, since they condensed to form the core of virus particles that budded into dilated cisterns of endoplasmic reticulum. The spheroid virus was about 80 to 100 nm in diameter. The inner granular or translucent core was surrounded by an outer membrane studded with fuzzy projections of variable length which constitute the "corona." The fuzzy outer rim was less well visualized around virus particles of the ts8 mutant. The infected hypertrophic oligodendrocytes were further characterized by multiple unusual connections of their plasma membranes with myelin sheaths [8]. Degenerating cells with vacuolated cytoplasm were usually devoid of visible virus particles. After degeneration of the oligodendrocytes, their related disintegrating sheaths were removed by macrophages derived from hematogenous mononuclear cells that invaded the damaged tissue by passing between endotheloid cells of venules. The macrophages stripped the disintegrating myelin sheaths off axons (Fig. 1). The cells penetrated into the sheaths at nodes of Ranvier. Multinucleated macrophages were noted but these cells revealed no virus. There was no evidence of virus-induced fusion of infected oligodendrocytes.

Mice that survived the acute disease recovered rapidly. Surviving oligodendrocytes can multiply and form new myelin sheaths around the denuded axons [5]. Af-

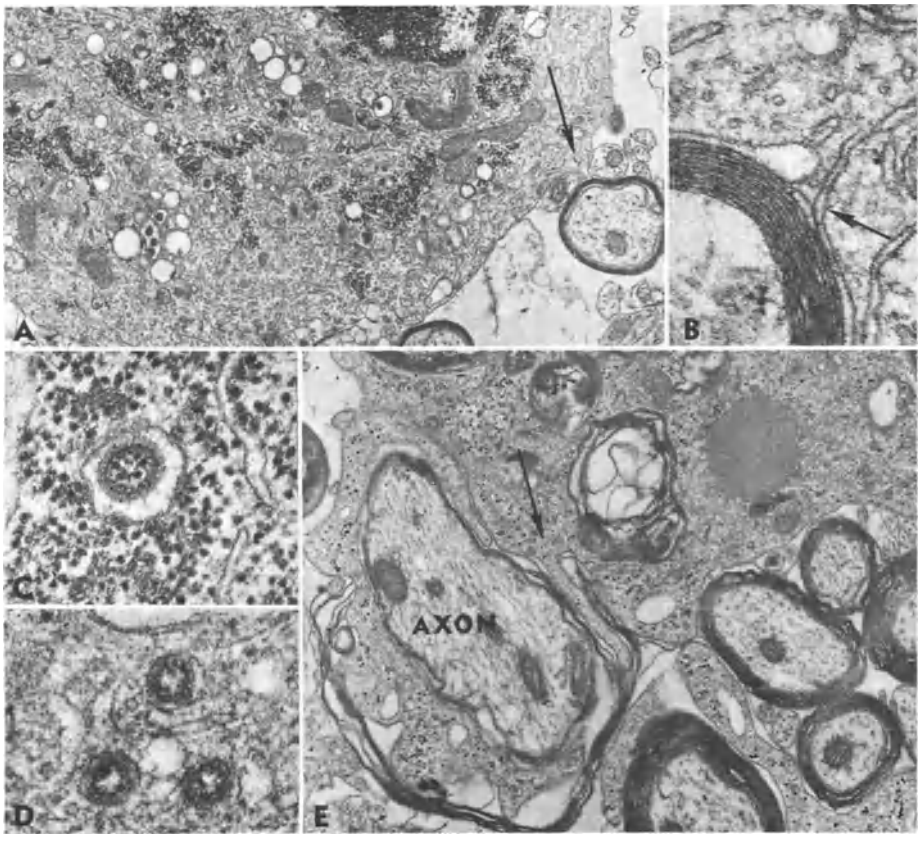

Fig. 1 A–E. Acute infection with mouse hepatitis virus. **A** Infected hypertrophic oligodendrocyte revealing its connection (*arrow*) with a myelin sheath. Mature virus particles are seen within dilated endoplasmic reticulum. × 14,000. **B** Connection (*arrow*) of the plasma membrane of an infected oligodendrocyte with the outer lamella of a myelin sheath. × 70,000. **C** Wild type virus in a cistern of endoplasmic reticulum. × 100,000. **D** Virus particles of the ts8 mutant in endoplasmic reticulum. × 70,000. **E** A myelin sheath is stripped off an axon (*arrow*) by a macrophage. × 20,000

ter several months, lesions were recognized only by finding fields containing axons that were surrounded by thinner than normal myelin sheaths (Fig. 2). Late demyelination association with persistent infection has been described in mice as late as 16 months after wt virus infection [4]. We observed recurrent demyelination within remyelinated areas as early as 57 days after infection with ts8 mutant. In focal areas, macrophages were seen stripping myelin off axons (Fig. 2). Oligodendrocytes in the vicinity of these lesions contained doughnut-shaped structures within endoplasmic reticulum which resembled type A oncornavirus particles [2]. These structures were budding into cisterns of endoplasmic reticulum. Occasional cylindrical shapes were noted. They averaged about 70 nm in width and always displayed a clear core surrounded by two distinct membranes. Mature C-type particles were not observed. It is of interest, however, that oncornavirus infection has been shown to damage oligo-

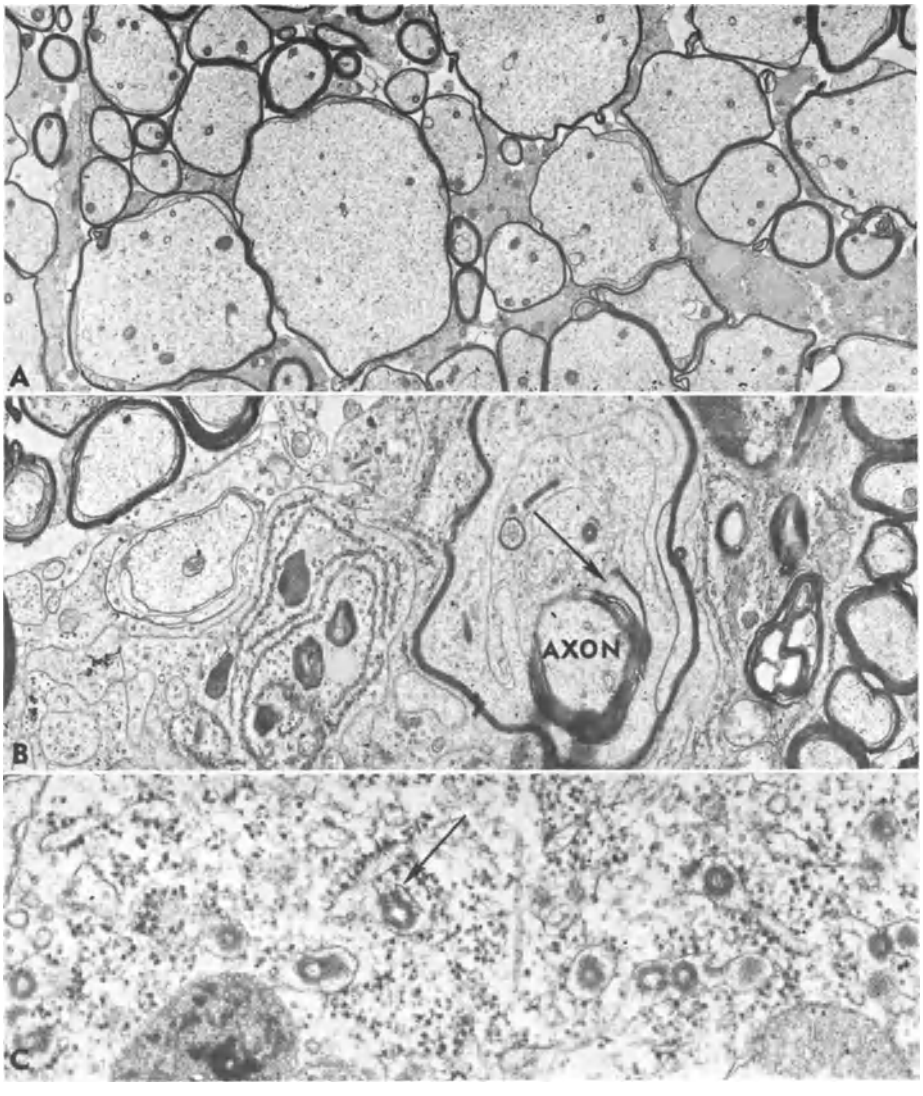

Fig. 2 A–C. Remyelination and demyelination 57 days after infection with ts8 mutant of mouse hepatitis virus. **A** Axons are surrounded by thin, newly formed myelin sheaths. × 10,000. **B** Stripping (*arrow*) of myelin by macrophages occurs within a field containing remyalinated axons. × 18,000. **C** Oligodendroglial cytoplasm filled with doughnut-shaped, A-type oncornavirus particles within the endoplasmic reticulum. Note cylindrical shape of one of the particles (*arrow*). × 70,000

dendrocytes leading to primary demyelination [7]. Studies are in progress to explore the possibility of an activation of latent oncornavirus in proliferating oligodendrocytes.

In summary, ts mutants of mouse hepatitis virus with enhanced demyelinating ability have been isolated. Demyelination is due to virus-induced destruction of oli-

godendrocytes. The cells first undergo hypertrophy showing many unusual plasma membrane connections with myelin lamellae. The removal of disintegrating sheaths follows a nonspecific pattern characterized by stripping of myelin sheaths by invading macrophages. During recovery, new myelin sheaths are formed by proliferating oligodendrocytes some of which contain structures resembling A-type particles of oncornavirus. Recurrent demyelination is observed in areas of remyelination in the spinal cord of mice recovering from MHV infection.

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Coronavirus Infection in Rats

K. NAGASHIMA [1], H. WEGE [2], R. MEYERMANN [3], and V. TER MEULEN [2]

Summary

Murine coronavirus JHM infection in rats can be accompanied by different types of demyelinating central nervous system (CNS) diseases. Infection of suckling rats results in an acute, disseminated encephalomyelitis affecting both neurons and oligodendroglia cells. After an average incubation period of 2–3 weeks, weanling rats develop a predominantly subacute demyelinating encephalomyelitis (SDE) characterized by perivascular cuffings, demyelinated foci, and destruction of oligodendroglia cells, leaving the neurons and axons intact. After 3–8 months, some of the weanling rats which do not after 3 weeks display any CNS affection develop a neurological disease with neuropathological changes similar to SDE. However, in addition to demyelination, remyelination is detectable in this disease pattern. Infectious coronavirus can be isolated from all diseased animals. The infection of weanling rats with coronavirus JHM offers the possibility to explore experimentally the pathogenetic mechanisms responsible for a virus-associated demyelinating process.

Introduction

Demyelinated lesions occur in several virus-induced CNS diseases of man and animals [12]. The etiologic agents have been identified for most of the diseases, but the pathogenetic mechanisms leading to the neurological changes are unknown. Therefore, a laboratory animal model using a virus for which a detailed knowledge of biochemistry and replication exists would be useful for the analysis of the virus-host relationship in demyelination. The murine coronavirus strain JHM meets these requirements. The essential structural features of the virion are known [14, 15], a persistent infection in tissue culture can be established [6, 10] and ts mutants are available which induce demyelination at high frequency [4]. In mice, JHM infection is accompanied by an acute disseminated encephalitis with a short incubation period and rapid death [1, 5, 16]. In weanling rats, however, JHM virus infection leads to a subacute demyelinating encephalomyelitis [1, 7, 8]. Based on this observation, the infection of weanling rats with JHM virus was further investigated. In the present communication the different diseases of rats occurring after JHM infections are described.

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Materials and Methods

Animals

Suckling (3–7 days of age) and weanling (20–25 days of age) rats, strain CHBB/THOM, were obtained specific-pathogen free from Thomae, Biberach, Germany.

Virus

JHM virus was passaged by intracerebral inoculation in suckling mice and rats and a 20% brain suspension was used for the inoculation of animals.

Animal Inoculation

Weanling and newborn rats were inoculated into the left brain hemisphere. The animals obtained approximately 5×10^4 TCID 50 of JHM virus.

Virus Isolation

After dissection under aseptic conditions, the specimens were washed in cold PBS with antibiotics and homogenized in a glass douncer to give a 15% (w/v) suspension. The homogenates were absorbed for 1 h on monolayers of Sac(-) cells (0.3 ml/petridish 20 ccm), washed, and incubated with 5 ml Eagle's minimal essential medium containing 5% fetal calf serum. Cultures which did not show a CPE characteristic of JHM infection after 48 h were passaged. Sac(-) cells are a mouse moloneysarcoma line obtained from Dr. Mussgay, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, Germany.

Immunofluorescence Studies

Cryostate section (8 μ thick) were fixed for 10 min in acetone and stained using the indirect immunofluorescence technique. Antiserum against JHM was prepared in mice. FITC-labeled anti-mouse immunoglobulin was obtained from Microbiological Associates, Maryland, USA. The sera were absorbed with brain powder from uninfected animals prior to use.

Light and Electron Microscopy

The animals were anesthetized with ether and perfused with a fixative consisting of 2% paraformaldehyde and 2.5% glutaraldehyde buffered to pH with phosphate. For both light and electron microscopy, coronal sections of brain, spinal cord, and optic

nerve were made alternatively. The tissue for light microscopy was postfixed in 10% formalin and embedded in paraffin. The specimens were stained with hemytoxylin-eosin, the Klüver-Barrera method for myelin and the Glees-Marsland method for axons. Blocks for electron microscopy were postfixed in 1% osmium tetroxide buffered with phosphate, stained en bloc with 2.5% uranyl acetate, dehydrated, and embedded in epon. Thick sections (1 μm) were taken from all regions sampled, stained with 1% toluidine blue, and examined by light microscopy. Thin sections of selected areas were cut with a Reichert OmU-3 ultramicrotome, placed on formvar-coated grids, and stained with lead citrate. The preparations were examined with a Zeiss 10B electron microscope.

Results

JHM Infection in Suckling Rats

Out of 78 animals infected, 57 became moribund with an incubation period of approximately one week. Neuropathological examinations revealed that the main lesions were situated in the cerebral cortex and brainstem. The lesions found in the brain were usually necrotic with polymorphonuclear leukocyte infiltrations. Neuronal necrosis, neurophagia, and glial nodules were frequently encountered. The lesions in the brainstem showed multiple large demyelinating plaques. Viral antigens were demonstrated by immunofluorescence both in neurons and glial cells. Electron-microscopic examination revealed intact virus particles in the neuronal and glial cytoplasm [7]. Infectious JHM virus was isolated from diseased animals.

JHM Infection in Weanling Rats

JHM inoculations into weanling rats were followed by neurological diseases which developed either 2–3 weeks or 3–8 months after virus inoculation (Table 1). The clinical signs consisted of hindleg paralysis or spastic tetraplegia and seizures lasting up to 1 week. Both groups of animals were neuropathologically examined, and our observations are described in the following sections.

Subacute Demyelinating Encephalomyelitis (SDE)

Those weanling rats which 2–3 weeks after inoculation developed a neurological disease revealed neuropathological lesions mainly located in the brainstem, the optic

Table 1. Coronavirus JHM infection in weanling rats

Number of animals infected	Number of diseased animals	Onset of neurological disease after inoculation				Number of animals without clinical disease
		2–3 weeks	3 months	6 months	8 months	
120	36	33	1	1	1	84

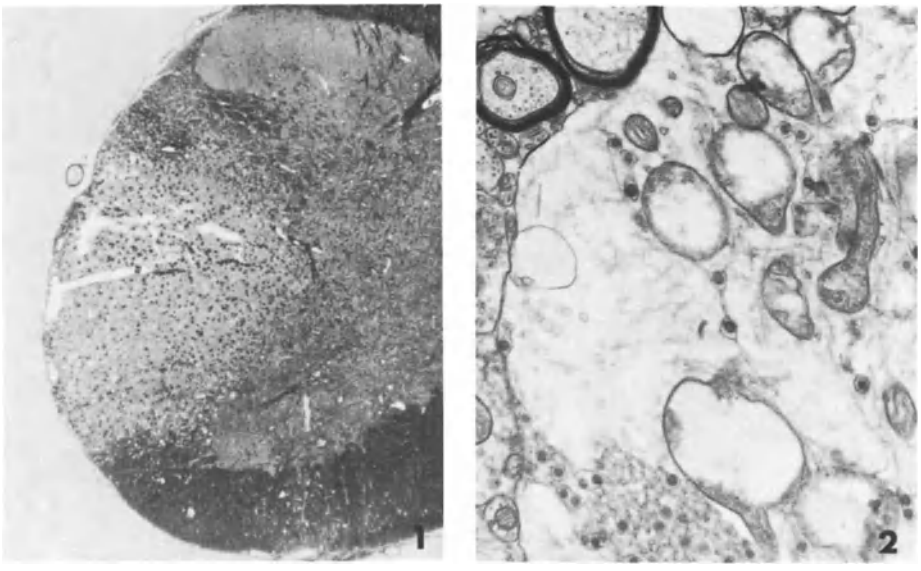

Fig. 1. Demyelinating lesion of the spinal cord from an animal which developed clinical signs 18 days after infection. Widespread lesion in the right lateral funiculus. Dark cells are macrophages loaded with lipid material. Note the spongy state in the anterior funiculus. Toluidine blue staining. x 40

Fig. 2. Part of the hypertrophically degenerating cell found in a pontine spongy state of an animal with subacute onset. Intact virus particles are visible in vesicles or tubulovesicular structures. Note the increased amount of microtubules in this degenerating cytoplasma. x 25,000

nerve, and the spinal cord (Fig. 1). The lesions consisted of widespread loss of myelin sheaths with preservation of axons and neurons, presenting a primary demyelination [8]. Lymphoid cells, plasma cells, and monocytes were found scattered in the lesions and massively around blood vessels inside or near demyelinated foci. Neither neuronal necrosis, nor giant cell formation, nor inclusion bodies were observed. Immunofluorescent studies showed that viral antigen was confined to glial cells. Neurons were always unstained, even in areas where the lesions involved the pontine nucleus. Despite the large amount of viral antigen, intact virus particles were rarely observed. They were only detectable in hypertrophically degenerated cells (Fig. 2). These degenerating cells were encountered in the spongy state of the white matter. A morphological investigation of the initial demyelinating lesions suggested that the hypertrophically degenerating cells might originate from both astrocytes and large oligodendrocytes [8]. Infectious virus could be isolated from all diseased rats.

Demyelinating Disease with a Long Incubation Period

3 animals developed clinical signs 3, 6, and 8 months after inoculation [9]. In these animals the predominant neuropathological finding consisted of primary demyeli-

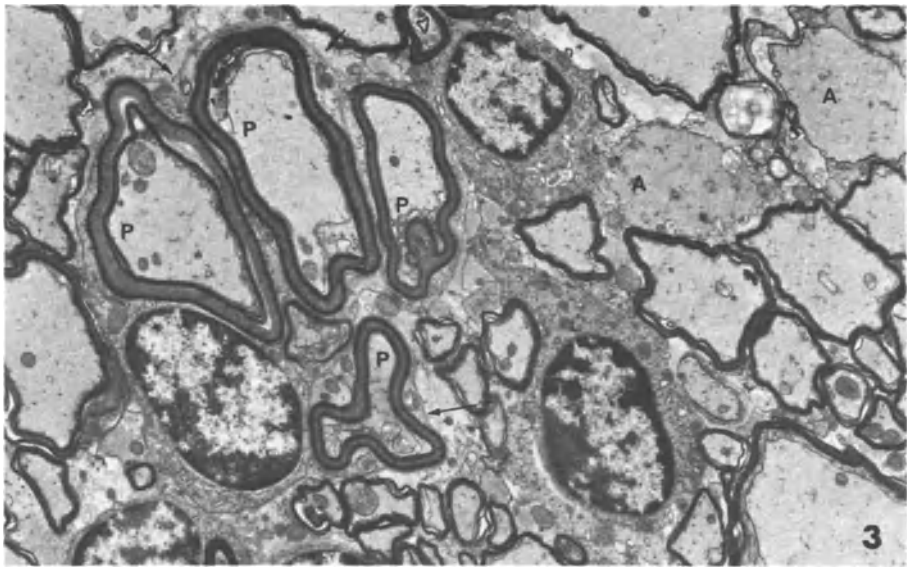

Fig. 3. Small-cell focus found in an animal which developed clinical signs 8 months after infection. Two naked axons (*A*) are seen. Well-developed myelin sheaths (*P*) are surrounded by the basement membrane→). These myelin sheaths correspond to the PNS-type of remyelination. Myelin sheaths without the basement membrane are thin, compared to the diameter of axons. These myelin sheaths correspond to CNS-type of remyelination. x 20,000

nation with a mononuclear inflammatory cell response. Demyelinating plaques were usually large and distributed both in the deep cerebral white matter, the brainstem, and the spinal cord. Neurons and axons were well preserved within the lesions, which were morphologically similar to those of SDE.

In the animal which developed clinical signs 8 months after inoculation, foci consisting of an aggregation of small cells and intact myelin sheaths were found in the white matter of the pons, and spinal cord. Electron-microscopic examination of these foci revealed numerous thinly myelinated axons and small aggregated cells. Naked axons and incompletely myelinated axons were also observed (Fig. 3). Moreover, the well-developed myelin sheaths were surrounded by a basement membrane. These morphologically distinct features of myelin sheaths are similar to those observed during remyelination [2, 3]. Myelin sheaths surrounded by the basement membrane correspond to the PNS type of remyelination and those without basement membrane to the CNS type of remyelination. The observation of both demyelination and remyelination in this animal suggests a recurrent disease process.

Infectious virus was isolated from the cace with a 3-month incubation period. Viral antigen was detected by immunofluorescence only in glial cells of the white matter of the pons, and spinal cord. The other two animals were sacrificed by perfusion for ultrastructural studies. Virus particles could not be detected by electron microscopy in these cases. However, it is conceivable that the selected areas were not focussed to the sites of virus replication.

Animals Without Recognizable Clinical Signs

In animals which did not display clinical signs, no active lesions were found. However, in 10 rats out of 24 examined, small-cell foci, and in two additional animals, a slight hydrocephalus was observed. The small-cell foci were similar to those found in the diseased animals with long incubation periods. They were found in the cerebral crus, pons, cerebellar peduncles, and spinal cord. Electron microscopic study of the small-cell foci revealed a state of PNS- and CNS-type remyelination (Table 2). Infectious virus could not be isolated.

Table 2. Summary of the main neuropathological findings of JHM infection in weanling rats

Number of animals	Neurological disease with clinical signs	Incubation Period	Inflammation		Demyelination	Remyelination
			Perivascular Cuffing	Diffuse		
33	+	2–3 weeks	+	+	+	–
1	+	3 months	+	(+)	+	–
1	+	6 months	+	(+)	+	–
1	+	8 months	+	(+)	+	+
10	–	6–8 months	–	–	–	+

Comments

Intracerebral inoculation of JHM virus in rats leads to different CNS disorders, depending on the age of the animals at the time of infection. Acute encephalomyelitis occurs in suckling rats and infects both neurons and glia cells. The infection spreads so rapidly that the animals die within 24 to 48 h after infection which is clinically of an acute nature. The disease in weanling rats, however, is different from the acute case. Small and large sharply demarcated demyelinating lesions are located in the deep cerebral white matter, optic chiasma, pons, and spinal cord, accompanied by perivascular infiltrations consisting of plasma cells and monocytes. Virus particles and antigen can only be detected in glia cells and not in neurons, suggesting a selective vulnerability of glia cells for JHM virus at this age of the animals.

In addition to these diseases, a more chronic CNS infection can develop in rats months after JHM inoculation. The neuropathological findings are similar to those observed in SDE, except for the occurrence of remyelination which consists of the PNS and CNS type. It is noteworthy that infectious virus can be isolated from these animals, suggesting a state of virus persistency. Remyelination of both PNS and CNS type first appears in rats at least 2–3 months after demyelination [3]. Therefore, the finding of this type of remyelination in clinically silent animals suggests that these animals had a subclinical demyelinating disease some months prior to investigation. In other experiments, weanling rats infected with JHM virus developed, after 6 to 8 months, a chronic progressive paralysis characterized by hydrocephalus and myelomalacia [7, 8]. In these cases no direct evidence for a participation of

JHM virus in this disease was found. The neuropathology of this progressive paralysis differed completely from the late demyelinating disease.

At present it is not known which mechanisms are responsible for the different CNS diseases observed. It has been shown that susceptibility of mice for a mouse hepatitis virus infection depends not only on a genetic predisposition, but also on factors such as age [10, 11, 13]. On the other hand, properties of a virus preparation may also play a role, since certain temperature-sensitive mutants of JHM virus are inducing a more pronounced demyelinating disease than the wild type virus [4].

Further studies combining virological, immunological, and biochemical analysis of JHM infection in vitro and in vivo should lead to a better understanding of the virus-cell and virus-host interaction in a demyelinating disease.

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Search for Viral Nucleic Acids in Multiple Sclerosis Brain

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Introduction

A viral etiology for multiple sclerosis has been suggested for many years, since epidemiological observations tend to call for an agent which is acquired early in life and which after a long period of latency initiates a disease process. Various virological studies have compared the frequency of antibodies against common viruses in the serum and cerebral spinal fluid of MS patients to matched controls and patients without neurological diseases. Although measles virus seemed to be the prime candidate for a causative agent in MS, increased antibody titers against other common viruses such as herpes simplex virus, cytomegalovirus, varicella, and adenovirus, as well as influenza C, parainfluenza 3, mumps, and rubella viruses have also been reported in MS cases. In addition, oligoclonal IgG bands in CSF material are present in approximately 90% of MS patients, suggesting a local antibody production directed against viral and other unknown antigens [8]. These observations have prompted many attempts to isolate infectious virus from MS brain, but the different agents recovered could not be linked etiologically or pathogenetically to this chronic CNS disorder.

During the last decade, virological techniques have been developed to detect viral genomic information in organ material. In principle, viral nucleic acids, especially of DNA viruses, can be detected either by in situ hybridization or by reassociation kinetics in biopsy or autopsy material. The high resolution capacity of the latter technique provides a useful method to look for viral DNA in cell material, as has been demonstrated for Epstein-Barr virus in lymphocyte cell lines and tumors [7, 13] or for JC virus infection in progressive multifocal leukoencephalopathy [4].

In the present study, this biochemical approach was chosen to detect viral nucleic acids in MS brain. In a first attempt, biochemical probes were prepared from herpes simplex and adenovirus. Both viruses can persist for a long time in human tissue [1, 5], and herpes simplex virus in particular has recently been implicated as a candidate causing MS.

Materials and Methods

Brain Material

Brain material from MS patients and controls were kindly supplied by Dr. R. Meyermann of the Department of Neuropathology, University of Göttingen. Brain tis-

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sue was obtained within 10 h after death and frozen immediately at autopsy. MS brain areas with and without plaques were chosen for DNA extraction.

Preparation of Viral DNA and Cellular DNA of Brain Material

Adenovirus type 2 DNA was kindly provided by Dr. W. Dörfler, Institute for Genetics, University of Cologne. Herpes simplex virus type I (Thea) was grown in Hela cells with MEM plus 5% fetal calf serum. Viral DNA was labeled by adding 2 μCi thymidine (sp. act. 15–25 Ci/mmol) per ml to infected cell cultures. Virus particles were purified [10] from tissue culture fluids by low- and high-speed centrifugation. Pellets homogenized by a Dounce homogenizer were banded in 17–33% (w/v) neutral sucrose velocity gradients in a Beckman SW 27 rotor for 30 min at 20,000 rpm at 4° C. Virus particles were disintegrated by 1% sarcosyl and 50 μg/ml Proteinase K (Merck, Germany) on top of a 20–35% (w/v) neutral velocity sucrose gradient in 10 mM Tris-HCl, 10 mM EDTA, pH 7.4 for 1 h at 37° C. The gradient was run in a Beckman SW 27 rotor for 14 h at 20,000 rpm at 23° C. Fractions containing DNA were sedimented in a Beckman 50 Ti rotor for 15 h at 45,000 rpm at 23° C. This DNA was adjusted with CsCl to a density of 1.710 g/cm^3 and spun to equilibrium in the Beckman 50 Ti rotor for 60 h at 30,000 rpm at 23° C. DNA banding in a single homogeneous band at a density of 1.711 g/cm^3, dialyzed against 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, served as a substrate for in vitro labeling.

For cellular DNA isolation, frozen brain material was disintegrated by grinding with sterilized sea sand, treated with proteinase K (50 μg/ml) and SDS (1%) in 10 mM Tris-HCl, 1 mM EDTA, pH 8.1 for 1 h at 37° C, followed by three extractions with Tris-HCl saturated phenol, pH 8.1. After extensive dialysis, the DNA was treated with DNase-free RNase for 1 h at 37° C, phenol extracted again, and dialyzed against 10 mM Tris-HCl, 1 mM EDTA, pH 7.4.

In Vitro Labeling of Herpes Simplex and Adenovirus DNA

Viral DNA was labeled by the nick-translation procedure according to Rigby et al. [9]. 0.25–0.5 μg DNA were labeled with 0.5 mCi ^{32}P thymidine triphosphate (300–350 Ci/mMol, Amersham Buchler, Scotland), using 1.5 U polymerase I of *Escherichia coli* (Boehringer, Germany) and DNase I (Merck, Germany) 10 ng/ml in a buffer of 10 μM of each of the three triphosphates (ATP, GTP, CTP) in 10 mM KH_2PO_4, 50 mM Tris-HCl, 7.5 mM $MgCl_2$, and 0.73 mM mercaptoethanol for 60 min at 17° C. After extraction with phenol, the ^{32}P-DNA was separated by a Sephadex G 50 column in 10 mM Tris-HCl, 1 mM EDTA, 0.1% sarcosyl, pH 7.4 buffer. The DNA was stored at – 70° C until use. The product of the reaction had a single stranded length of about 6 S as determined from its sedimentation in alkaline sucrose gradients.

Determination of Reassociation Kinetics of Viral DNA According to Britten and Kohne [2]

Less than 2.5×10^{-4} μg of labeled DNA was mixed with 500 μg of cell DNA, lyophilized, and dissolved in 500 μl 0.1 M Tris HCl, pH 7.4, 10 mM EDTA 0.05% sarcosyl, and 1 M NaCl. The DNA was fragmented to 5 S by sonication, denatured by heating for 10 min at 109° C, and allowed to reanneal at 68° C. At various intervals, specimens were frozen at – 20° C. After completion of the test, single-stranded and duplex DNA was separated by hydroxyapatite (Biogel HTP DNA grade) chromatography. Single-stranded DNA was eluted with 0.14 M sodium phosphate 0.4% SDS, and double-stranded DNA was eluted with 0.4 M sodium phosphate, 0.4% SDS.

For determination of the reassociation rate of a given DNA, the second order rate plot proposed by Wetmur and Davidson [12] was used.

Results

Brain material from ten multiple sclerosis patients was available. From these MS cases, three were diagnosed neuropathologically as acute MS, three revealed a chronic progressive course, and one showed changes typical of relapses and remissions (Table 1). The control brain tissue was derived from patients who died of an acute demyelinating encephalitis of unknown etiology, encephalitis with minor inflammatory changes, amyotrophic lateral sclerosis and non-CNS system disease. Virological and serological data of sera or CSF specimens from these patients were not available.

Table 1. Data of patients with multiple sclerosis and encephalitis

Clinical diagnosis	Histopathology	Age (years)	Sex
Chronical MS	Chronic-progressive MS	24	F
MS and pituitary gland adenoma	Non secreting adenoma	63	M
MS with remissions	MS	46	F
Chronic-progressive MS	Chronic-progressive MS	28	M
MS	Chronic-progressive MS	38	M
MS	MS	34	M
Acute MS	Acute MS	20	F
Acute MS	Acute MS	40	F
Acute MS	Acute MS	22	M
MS	MS	58	F
Acute viral encephalitis of unknown aetiology	Acute demyelinating encephalitis	24	F
Viral encephalitis of unknown aetiology	Minor inflammatory changes	18	M

Radioactive-labeled viral DNA was reassociated with cellular DNA of MS brain and control brain material in order to detect homologous viral DNA in these tissues. Since the rate of DNA reassociation is directly proportional to the total concentration of DNA in the measured probe, any increase in the total amount of homologous viral DNA would increase the annealing reaction. As a control, a known concentration of ^{32}P-labeled viral DNA was allowed to reassociate in the presence of 1 mg/ml calf thymus DNA in each experiment. The control reassociation rate was then compared to the rate of ^{32}P-labeled viral DNA with DNA of brain material (1 mg/ml).

Any detectable amount of viral genomic DNA in the MS brain material would increase the concentration of viral DNA and therefore increase the reassociation rate over the radioactive control. This is shown in reactions with various amounts of cold viral DNA mixed to the ^{32}P-labeled homologous viral DNA (Figs. 1, 2). According to the specific activity of the labeled viral DNA, the ratio of calf thymus DNA to labeled viral DNA is equivalent to a fixed amount of genomes per cell (Table 2). Figure 1 shows the reassociation kinetics of ^{32}P-HSV-DNA with cellular DNA from MS and control brain. No increase of the reassociation over the control reaction of ^{32}P-HSV-DNA with calf thymus DNA alone was noticed with any brain DNA. This indicates the absence of HSV type I DNA in MS brain tissue above 0.02 genome equivalents per cell. The same reactions were observed with ^{32}P-labeled adeno type 2 DNA showing no adenovirus DNA of a concentration of 0.04 virus genome equivalents per cell.

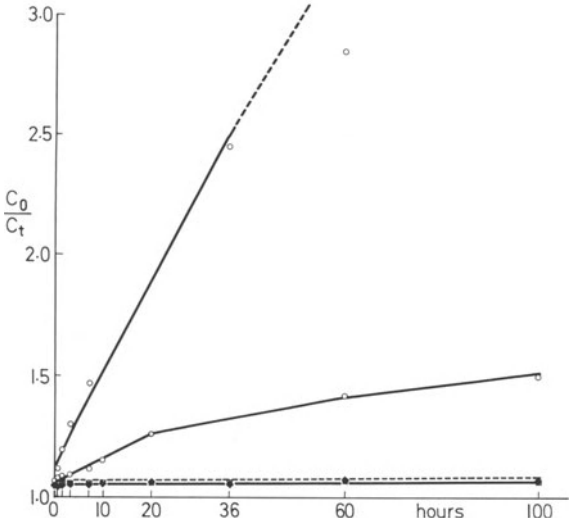

Fig. 1. DNA-DNA reassociation kinetics of ^{32}P-labeled herpes simplex virus type I DNA (specific activity 4.63×10^7 cpm/μg) with DNA of different sources. (◆) 500 μg cellular DNA extracted from brain material of MS or control patients. (○) 500 μg calf thymus DNA and 16 ng (1.5 genome equivalents/cell) unlabeled HSV I DNA; (○) 500 μg calf thymus DNA and 139 ng (10 genome equivalents/cell) unlabeled HSV I DNA; (□) 500 μg calf thymus DNA and ^{32}P-labeled HSV DNA alone. All reactions were carried out at a final DNA concentration of 1 mg/ml. Each point represents a total of 900 cpm

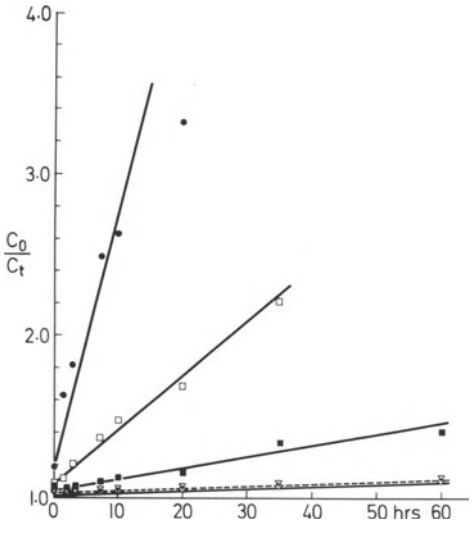

Fig. 2. DNA-DNA reassociation kinetics of ^{32}P-labeled adenovirus type 2 DNA (specific activity 1.65×10^8 cpm/μg) with DNA of different sources. (▽) 500 μg cellular DNA extracted from brain material of MS patients or control patients; (■) 500 μg calf thymus DNA and 0.2 ng (0.08 genome equivalents/cell) unlabeled adenovirus DNA; (□) 500 μg calf thymus DNA and 2.0 ng (0.4 genome equivalents/cell) unlabeled adenovirus DNA; (●) 500 μg calf thymus DNA and 20 ng (4 genome equivalents/cell) unlabeled adenovirus DNA; (○) 500 μg calf thymus DNA and ^{32}P-labeled adenovirus DNA alone. All reactions were carried out at a final DNA concentration of 1 mg/ml. Each point represents a total of 1000 cpm

Table 2. Sensitivity of DNA-DNA reassociation kinetics with adenovirus and herpes simplex virus

Viral DNA	Specific activity (cpm/μg)	Sensitivity [a] (^{32}P-virus DNA copies per cell detectable)
Adeno virus type 2	1.65×10^8	0.04
Herpes simplex virus type 1	4.63×10^7	0.02

[a] $\text{Sensitivity} = \frac{\text{ng viral } ^{32}\text{P-DNA}}{\text{ng cellular DNA}} \times \frac{\text{mol. wt. of diploid mammalian cell DNA}}{\text{mol. wt. of virus DNA}}$

See L. D. Gelb et al. [6]

Comments

In the present study, MS brain material was examined for genomic information of herpes simplex type I and adenovirus type 2 by DNA-DNA reassociation kinetics. These two DNA-containing viruses were looked for with respect to their ability to produce persistent infections in brain tissue. Brain material from ten MS patients was available. Among these cases, acute as well as remitting courses of the disease were found. With a threshold level for the detection of 0.02 genome equivalents per cell for HSV and 0.04 genome equivalents per cell for adenovirus, no evidence for the presence of viral genomic DNA was available. The possibility of detecting viral information by the applied technique depends on the molecular weight and specific activity of the radioactive-labeled viral DNA as well as on the amount and proportion of viral and cellular DNA in the test specimen [6]. Since plaque and other brain areas were selected for DNA extraction, it is assumed that viral nucleic acids of the

two viruses would have been detected within the limitations of this assay if a persistent infection existed. With regard to the specific activity of the viral DNA, a further increase of the radioactive label could not be achieved without degradation of the viral nucleic acid.

The techniques applied here have proven useful in the detection of herpes simplex virus DNA in brain material from cases of herpes simplex encephalitis (Dörries et al., in preparation). It could be shown that within the area of the encephalitis up to 20 genome equivalents per cell were present using a probe with a sensitivity similar to that which was applied in the present study. In the periphery of the encephalitic foci, a decrease of activity was noted which could be correlated to the neuropathological changes. Our data suggest that in the brain material examined from the different MS cases neither herpes nor adenovirus is present in amounts detectable by biochemical methods. Whether viral genetic information can be obtained by complementation experiments using virus mutants as has been shown for HSV infection in human trigeminal ganglia awaits further investigation [3, 11].

Acknowledgments. This work was supported by the Deutsche Forschungsgemeinschaft, Schwerpunkt "Multiple Sklerose und verwandte Erkrankungen", Az 270/16.

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A Possible Coronavirus Isolation from Multiple Sclerosis Autopsy Material

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Introduction

While epidemiologic and serologic evidence suggests a viral etiology of multiple sclerosis (MS), virus isolations have yet to be confirmed. Our laboratory has possibly isolated a coronavirus from two MS patients. Virus was isolated from two of nine MS patients' brains at autopsy. This paper reports the isolation methods, the attempts to identify the virus as a coronavirus, and a preliminary serologic survey investigating the presence of antibody to this virus in MS and control populations.

Case Report and Isolate No. 1

The patient was a 55-year-old woman with a 28-year history of MS. Her most recent MS symptoms were related to brain stem dysfunction. An autopsy performed within 4 h following death revealed typical MS plaques in the cerebrum, cerebellum, brain stem, and spinal cord. Histologic sections of medulla and pons showed areas of demyelination surrounded by reactive astrocytes.

Fresh, unfrozen brain stem from autopsy material was prepared as a sterile 10% homogenate and inoculated intracerebrally (IC) (0.03 ml) into ten weanling BALB/c mice. Seven of the ten mice died between 2 and 6 months after inoculation. Some moribund mice exhibited seizures, myoclonic jerks, and limb paralysis. When fresh brain stem material from a mouse dying 99 days after inoculation was inoculated IC into another group of weanling BALB/c mice, the incubation period was decreased to approximately 50 days. Mouse brain stem material obtained from these mice was inoculated into newborn BALB/c mice IC with resultant disease and death in 12 days. Subsequently, on serial passages, the incubation period decreased to 3–5 days. The freezing of infected weanling and newborn mouse brain material during early passage attempts usually resulted in a loss of transmissibility, whereas fresh, unfrozen mouse brain material consistently produced illness.

Histologic studies in the few moribund adult mice have been negative. However, in newborn BALB/c mice, the agent induced multifocal areas of spongy change with relative sparing of neurons and axons. After the fourth passage of this isolate in newborn mice, necrotizing thymic, hepatic, and enteric lesions were noted. These lesions were not present in earlier passages.

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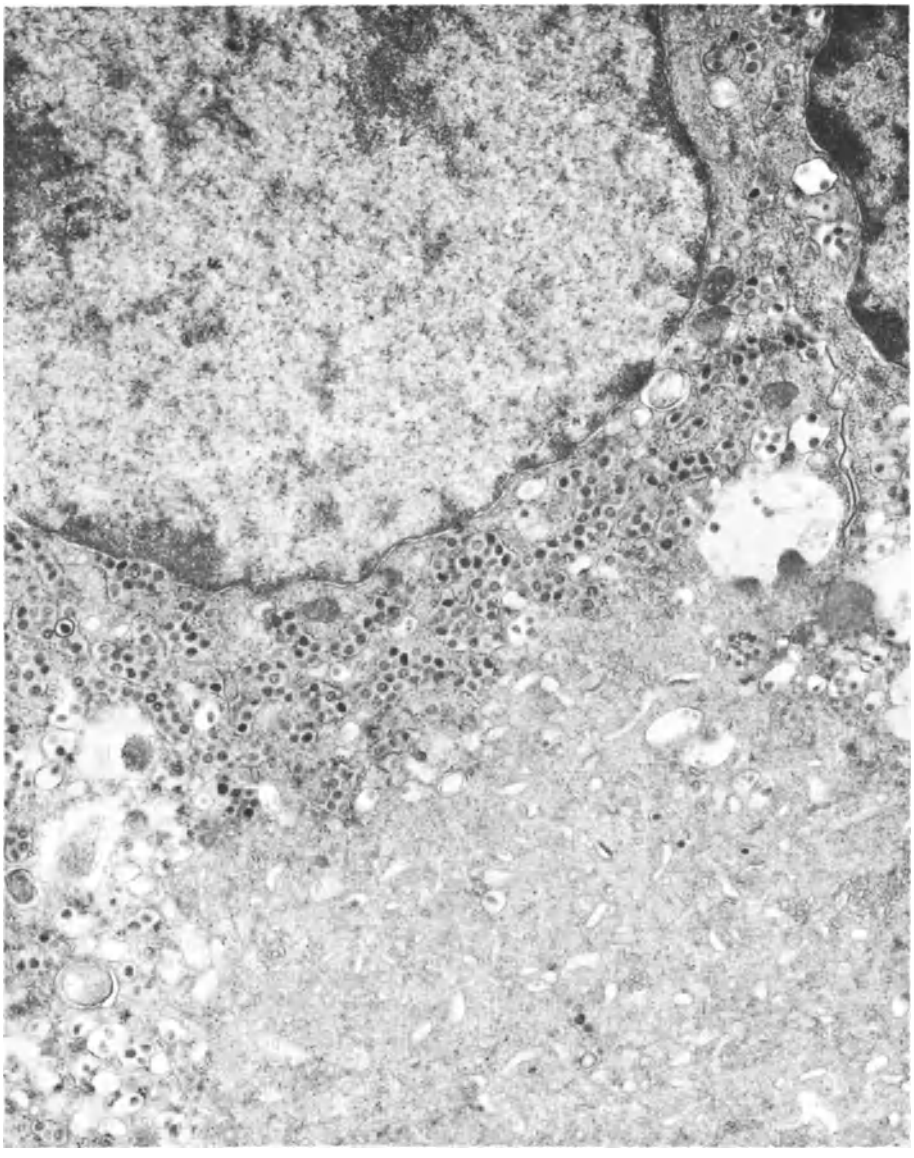

Fig. 1. Electron microscopy of a giant cell with Isolate I reveals 80–120 nm diameter viral particles in vacuoles and among profiles of the smooth endoplasmic reticulum. x 28,375

Electron-microscopic examination of livers from infected suckling mice has revealed numerous 80–120 nm virus-like particles within vacuoles. Rarely, brains from the same infected suckling mice revealed similar structures in spongy areas.

Serologic studies on IC-inoculated weanling mice showed no consistent antibody response to pneumonia virus of mice, Sendai virus, reovirus type 3, mouse hepa-

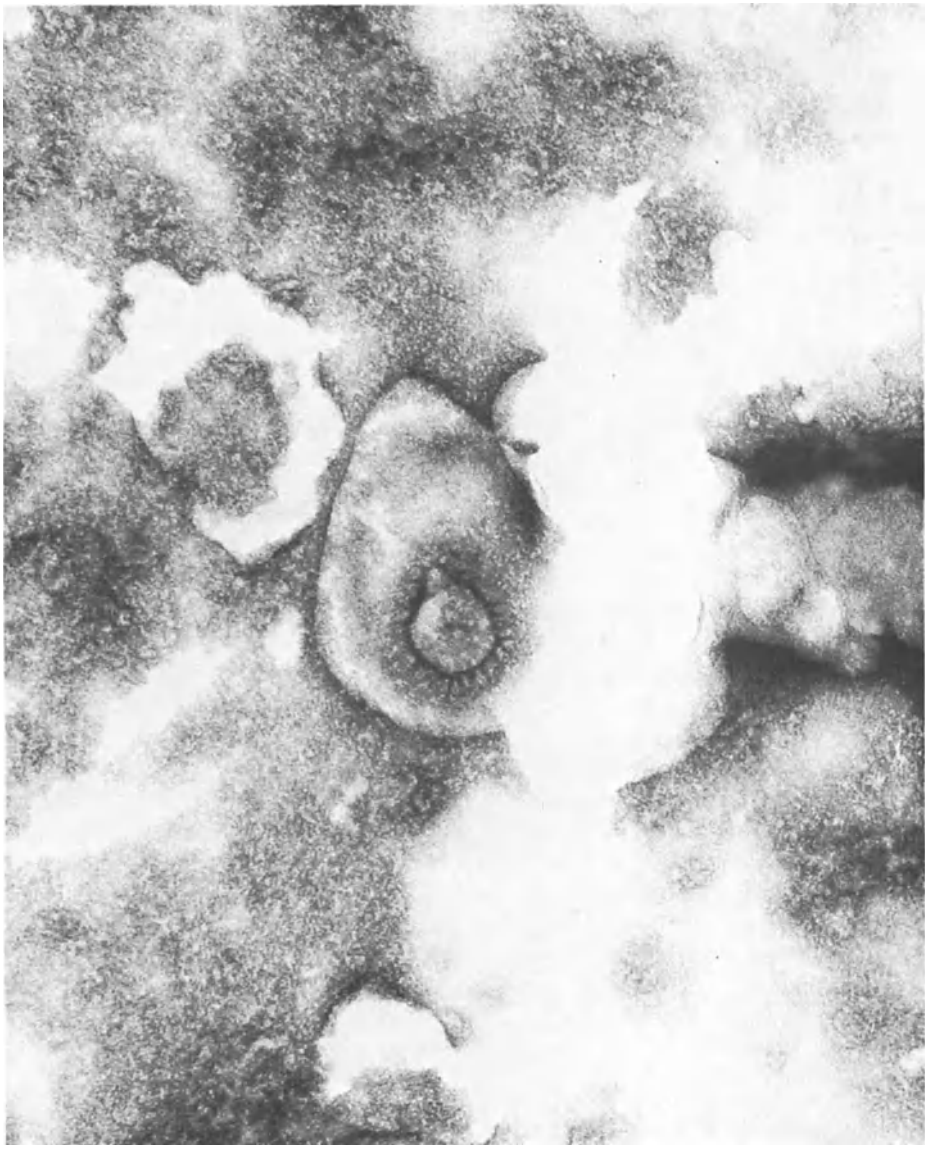

Fig. 2. Negative stain electron microscopy of virus Isolate I reveals the pleomorphic 80–100 nm core surrounded by approximately 10 nm club-shaped spikes. x 180,000

titis virus, mouse encephalomyelitis virus, K virus, polyoma, ectromelia, minute virus of mice, adenovirus, or lymphocytic choriomeningitis virus when assayed by Microbiological Associates Laboratory, Bethesda, Maryland, USA. No cytopathic effect was noted when infected brain material homogenate was inoculated into tissue cultures of Rhesus monkey kidney, African green monkey kidney, human embryonic lung, NCTC-1469, mouse L cells, primary bovine embryonic kidney, primary

rabbit kidney, W138, BSC-1, MDCK (canine kidney), HELA, HEP-2, VERO, rabbit kidney, and primary human amnion.

When infected suckling mouse brain homogenates were inoculated into 17CL-1 cells (a spontaneously transformed 3T3 cell line), syncytial formation (giant cells) was observed. Electron microscopy on these cultures revealed viral particles similar to those seen in infected suckling mouse liver and brain (Fig. 1). Negative stain electron microscopy on infected 17 CL-1 cells revealed virus particles resembling coronavirus (Fig. 2).

Case Report and Isolate No. 2

The patient was an 89-year-old woman with a 40 to 50 year history of neurological problems involving mainly corticospinal tracts with resultant weakness. Also, she demonstrated cerebellar dysfunction and extraocular movement paresis. Her course was slowly progressive for the last 30 years without exacerbations or remissions. An autopsy performed within 4 h following death revealed bilateral pneumonia and demyelinated areas in the periventricular white matter, brain stem, cerebellum, and spinal cord. A mild glial reaction was noted around most demyelinated areas.

Fresh, unfrozen autopsy material was prepared as a sterile 10% homogenate and inoculated into weanling BALB/c mice IC and 17 CL-1 cells as described in case report 1. Fresh material from the frontal lobe, occipital lobe, parietal lobe, cerebellum, midbrain, pons, cervical spinal cord, thoracic spinal cord, lumbosacral spinal cord, and lymph nodes was used in both mice and 17 CL-1 experiments. All mice remained healthy but on subculture No. 12, synctial formation was noted in the 17 CL-1 cells inoculated with deep frontal lobe material. Coronavirus-like particles, similar to those seen as Isolate I were noted by electron microscopy in the tissue culture cells. However, negative stain electron microscopy of tissue culture supernatant has not been successful in revealing virus particles, probably due to low virus concentration in the supernatant. The other eight central nervous system (CNS) areas from this patient's brain which were inoculated into mice and 17 CL-1 cells produced no viral isolation.

Other Isolation Attempts

Although no littermate controls were inoculated with fresh, unfrozen non-MS autopsy material at the time of the initial transmission studies, numerous other mice within the colony have been inoculated with human brain material. This includes two MS patients whose brain material was frozen before inoculation into mice. Since the first virus isolation, we have inoculated 97 areas of CNS tissue from five additional MS patients into weanling mice and 17 CL-1 cells. No virus has yet been detected in this material. Also, we have inoculated fresh, unfrozen material from 28 areas of the CNS from 12 non-MS patients into mice and 17 CL-1 cells with negative results.

Serologic Studies

A plaque neutralization assay was developed using the second virus isolate to evaluate the serum and spinal fluid antibody concentrations to this virus in MS patients and a control population. Serum was diluted 1 : 20 and spinal fluid was diluted 1 : 2 for these tests. Greater than 50% plaque reduction was considered as evidence for the presence of antibody to the virus. Spinal fluid from five of 140 (3.5%) MS patients (including the two virus-positive patients) had antibody to the second virus isolate. No spinal fluid from 43 control patients neutralized the virus. Serum from 85 of 102 (83%) MS patients neutralized the virus at a 1 : 20 dilution, whereas only ten out of 55 (18%) serums from control patients neutralized the virus (Table 1).

Table 1. Neutralization of virus isolate II by serum and spinal fluid from MS and control patients

	Serum[a]	*CSF*[b]
MS patients	85/102 (81%)	5/140 (3.5%)
Control patients	10/55 (18%)	0/43 (0%)

[a] Serum: Number of serums (1 : 20 dilution) demonstrating >50% plaque reduction against Isolate No. 2 / the total number of serum samples tested

[b] CSF = Number of cerebrospinal samples (1 : 2 dilution) demonstrating >50% plaque reduction against Isolate No. 2 / the total number of CSF samples tested

Discussion

The isolation of coronavirus-like agents using different techniques and two MS patients presents several unanswered questions. First, what were the origins of the isolate? Second, if the isolates did originate from the patients, what is their relationship to the pathogenesis of multiple sclerosis? Lastly, why should these isolation attempts have been successful when previous attempts have failed?

There are several possible explanations for the origin of these isolates. First, they might be contaminating agents from sources other than patient brain material. Endogenous mouse coronaviruses are notorious for contaminating isolation and transmission studies. The second isolate is unlikely to be a mouse contaminant virus, since coronavirus particles have not been found in 17 CL-1 cells. Despite the precautions taken, the isolates might represent laboratory contaminants.

The possibility that the isolates are coronaviruses from the MS patients is supported by several facts. First, the presence of neutralizing antibody to this virus in

the spinal fluid of the two virus-positive MS patients indicates possible viral specific antibody production within the CNS of these patients. Second, a statistically significant, higher percent of MS patients shows antibody to this virus in the serum when compared to control patients' serum. Third, coronaviruses are an attractive candidate virus for MS, since mouse hepatitis virus, another coronavirus, causes a demyelinating and remyelinating disease when inoculated IC into mice [2]. Finally, coronavirus particles have been previously identified by electron microscopy in brain tissue of one MS patient previously [1].

If the virus originated from the MS patients' brains, its relationship to the pathogenesis of multiple sclerosis would still remain questionable. Activation of a coronavirus may have been related to a terminal event or to a latent infection which is unrelated to multiple sclerosis. Additional isolations from MS patients in other laboratories are essential before an association can be made between a coronavirus and MS.

The success of our virus isolations may be related to the marked sensitivity of the virus to freezing, since the virus was not isolated when brain material was frozen. Many viruses lose some degree of infectivity after freezing. However, most viruses are numerous enough so that a loss of 90% of the infected particles would not hamper isolation attempts. However, if only small numbers of infectious virus particles are present, freezing the material may decrease the number of particles enough to prohibit isolation. Therefore, the use of fresh brain material is a possible methodological advance which may increase the chances of the recovery of a virus(es) in MS.

Emphasis should be placed on the relative rarity of our isolations. To date we have inoculated 99 fresh CNS homogenates from seven MS patients into mice or tissue culture cells and have isolated the virus in only two of these attempts. We have found antibody to the virus in only five of 140 spinal fluids from MS patients. If coronaviruses play a role in MS, an explanation for our lack of repeated success in isolations may be that the virus resides in a latent or defective state in the CNS.

While we do not claim to have isolated an agent related to MS, we do hope that this report will stimulate other investigators to use fresh unfrozen brain material from multiple CNS sites in future isolation attempts in MS. Coronaviruses should be looked for specifically in future isolation attempts from other laboratories.

Acknowledgments. This research was supported by a grant from the Kroc Foundation of California and by Veteran's Administration Grant No. 1169.

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Defective Viral Infection: The Rescue of Virus from Antigen-Negative Cells

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There is epidemiologic evidence for a viral infection in multiple sclerosis (MS). Over the years, frequent attempts to rescue virus and demonstrate viral antigens in the brain of MS patients have failed. There have been a number of isolates, but none have been found to play a role in the etiology of the disease. We have been studying an animal model of viral-induced acute and chronic demyelination. The virus, a mouse coronavirus, JHM, is a neurotropic strain of mouse hepatitis virus. In order to better understand JHM virus chronic infection, we have developed an in vitro carrier culture system of persistently infected mouse neuroblastoma cells [6].

In neuroblastoma cells, we are able to study specific cellular functions in relationship to persistent viral infection. Analysis of mouse neuroblastoma JHM-infected carrier cultures (N_J cells) did not detect any changes in the released virus populations even after 40 subcultures of the cells. The properties of the virus continued to be consistent with those of the parental virus which in the experimental animal produces acute demyelination. There was no evidence for the production of temperature-sensitive mutants, defective interfering particles, or interferon [6].

To determine the effect of antibody on the in vitro infection, N_J cells were passaged four times in the presence of antiviral antiserum. The presence of specific antiviral antibody ablated the synthesis of infectious virus as well as the expression of cell surface viral antigen. The majority of the cells retained intracytoplasmic viral antigen as detected by the indirect fluorescent antibody technique [8]. Removal of the antibody did not result in the reinitiation of infectious virus production or expression of cell surface viral antigen, even after ten passages.

The persistently infected cells were cloned in the presence of antiviral antibody in order to get a population of identical cells derived from a single cell. Single cells from the persistently infected cultures were added to individual wells of microtiter plates and grown in the presence of antiviral antibody. Following isolation, the clones were propagated in the absence of antibody. Removal of the antibody did not result in the reinitiation of production of infectious virus or expression of cell surface viral antigen, even after ten passages.

Eighteen clones derived from single cells were established from the original culture. They appeared to be morphologically identical to uninfected neuroblastoma cells. Electron-microscopic examination revealed that the clones contained no viral particles consistent with the morphology of JHM virus, and the doubling times of the cell clones derived from the infected cultures and uninfected cells were similar. Eleven of the isolated cell clones had viral antigen detectable by immunofluorescence.

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The antigen-positive clones were refractory to superinfection by JHM virus. Ordinarily, 24 h after infection of C-1300 neuroblastoma cells with 0.1 plaque forming units per cell (PFU/cell) of JHM virus, 80% of the cells show cytopathic effects, and by 96 h, the culture is destroyed. In the N_J culture, free of antibody and producing infectious virus, the cytopathology of dying cells was consistently around 40%. Superinfection of the N_J cells produced a steady 40% destruction even 96 h post infection. By contrast, the antigen-positive cell clones (nonproducers of infectious virus) had no detectable cytopathology with superinfection at 24 h and a very minimal amount, roughly 10%, at 96 h, a time when control neuroblastoma cells would be totally destroyed. Three clones were also found to be antigen negative. These cells were similar in all other ways to the antigen positive cells. These clones also resisted superinfection by 0.1 PFU/cell of JHM virus in the same fashion as the antigen positive clones.

To determine the nature of the viral antigens, two clones, designated S-1 (antigen positive) and S-3 (antigen negative), were radiolabeled with ^{3}H-amino acids and analyzed by polyacrylamide gel electrophoresis (PAGE) [7]. PAGE patterns indicated no differences between the S-1, S-3, or uninfected neuroblastoma cells. Using this technique following infection of uninfected neuroblastoma cells with JHM virus, one can distinguish at least three structural proteins as early as 10 h post infection. Cytoplasmic extracts tested by complement fixation tests using anti-JHM virus and polyvalent anti-mouse hepatitis virus antisera (Microbiological Associates, Rockville, Maryland) were also negative. Membranes derived from virus-infected cells have been effective immunogens in eliciting antiviral antibody responses in other viral systems [6]. However, neither membranes from S-1 nor S-3 cells when used as immunogens in the A/J mice elicited any anti-JHM virus antibody. The sera were negative when tested by microneutralization, immunofluorescence, and complement fixation tests. In addition, the antiserum which produced the positive immunofluorescence in the S-1 clone did not react by complement fixation with the membrane antigens from the S-1 clone. These studies suggest that the antigen-positive cells did not have sufficient viral protein expressed to be differentiated from the host cell proteins by PAGE or serological studies.

The S-1 antigen-positive clone and S-3 antigen-positive clone were resistant to superinfection by the JHM viruses previously described. This was initially the only evidence that the S-3 clone might be infected, since over 100 single cell clones of uninfected neuroblastoma were all found to be susceptible to JHM virus. When the S-1 and S-3 cell clones were fused to DBT and 17 CL-1 cells, both permissive for JHM virus, infectious virus was recovered. The fusing agent was polyethylene glycol [4]. Virus was recovered from S-1 clones in five of seven separate attempts using either DBT or 17 CL-1 as the indicator cell. In all fused cultures destined to yield infectious virus, cytopathology was first evident 48 h post fusion and by 96 h involved all of the permissive cells in the culture, while sparing all of the latently infected neuroblastoma cells. To our surprise, virus was also rescued in three of six attempts from the S-3 antigen-negative cells, two times using DBT cells as the permissive cell and once using 17 CL-1 cells. No virus was recovered following fusion of DBT to DBT, DBT to 17 CL-1, or 17 CL-1 to 17 CL-1 cells.

The yields of infectious virus in all the cultures was approximately 10^6 PFU/ml when titered on monolayers of DBT cells. The clones were also fused with UV-inac-

tived Sendai virus to DBT, 17 CL-1, and NCTC 1469 cells, but no infectious virus was detectable in three attempts [3]. It is of importance that the first evidence of CPE was seen at about 48 h post fusion, and by 96 h all the permissive cells were involved. This would suggest that the viral genome needed 24–48 h to generate viral proteins, to assemble infectious virus, and to produce cytopathic effect. This is in contrast to other systems, particularly SSPE, where almost immediately after fusion infectious virus can be found. The latter instance is consistent with an assembly defect. The time lapse in the JHM virus system suggests that the defect in viral maturation is more consistent with a translational problem. The fusion could have derepressed the viral genome. The virus could not be rescued by other techniques [1, 9]. Cells derived from the clones were also cocultivated with approximately equal numbers or tenfold excess of indicator cells at 32°, 37°, and 39° C without the release of infectious virus. The nature of the viruses designated S-1 JHM and S-3 JHM was of interest in that studies indicated no evidence of restricted growth at 37° or at 39°; however, the virus rescued after all the fusions of either S-1 or S-3 clones were restricted at low temperatures, namely 32° C. We have tentatively characterized this as a cold-sensitive mutant of JHM virus (Stohlman, Sakaguchi, and Weiner, unpublished data).

Neuroblastoma cells have been shown to express neurospecific functions, such as neurite formation, following an increase of intracellular cyclic adenosine-3′5′monophosphate (cAMP). This differentiation of neuroblastoma cells is also characterized by a decrease in DNA synthesis, a failure to divide, and the presence of increased protein synthesis, particularly the production of specific neuroenzymes related to neurotransmitters [5]. In addition, elevation of intracellular cAMP can cause an increase of viral antigen production and causes accelerated death in a measles-infected Vero cells (Katz and Weiner, unpublished data).

When S-1 and S-3 clones were treated with 0.5 mM of cAMP and 0.5 mM of theophylline, a phosphodiesterase inhibitor, both clones died within 96 h of treatment. No expression of differentiation, as defined by neurite formation, was seen, nor was there evidence of either increased viral antigen or production of infectious virus. Control cultures of uninfected neuroblastoma cells responded to the drugs by the expression of a differentiated morphology in approximately 40% of the cells, and cells remained in culture for 1 week or more. In the carrier culture producing infectious virus (N_J), the response to cAMP is also inhibited, but the dramatic effect of cell death seen following treatment of S-1 and S-3 clones does not occur.

The importance of these experiments to the study of MS is that a virus which is known to produce demyelination in its natural host can be induced by the presence of low levels of antibody to become increasingly defective. The defectivity is such that viral antigens are undetectable by conventional techniques, even in the presence of complete viral genome. Although there are numerous examples of the rescue of DNA and RNA viruses from antigen-negative cells lacking virus-induced cytopathic effect, such reports with RNA viruses whose replication does not require a DNA intermediate are rare. The JHM virus is a positive-stranded RNA virus [2] and latency with such viruses may indeed be unique. Of further importance to work on MS is that the cells harboring the viral genome will function normally until they are asked to perform a specific task. In the case of the neuroblastoma cells, the specific function we have analyzed is the expression of neurites. We have not yet mea-

sured the specific neuroproteins or neurospecific enzymes in the neuroblastoma cells to see what effect the viral genome has on synthesis, but work is in progress. It is of further importance that the effect on cellular function and the eventual cell death are not dependent on the production of either viral antigen or infectious virus. Although the mechanism is not clear, the latent virus genome might interfere with differentiation by affecting cellular gene expression. One could further postulate that if such a system were to exist in an oligodendroglia, a latent viral genome could produce cell death at times when there is high metabolic requirements demanded of the oligodendrocyte or stress due to other extrinsic factors.

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Some Properties of Cortical Nerve Cells of Adult Rats Bulk Isolated After Brain Perfusion

H.-H. ALTHAUS[1] and W. B. HUTTNER[2]

Introduction

Several methods for the bulk separation of nerve cells have been developed during the last 20 years. Basically, two procedures are modified in one or another step [5, 6]: First, by mechanical disaggregation of the brain tissue by chopping and then sieving; and secondly, by incubating the brain tissue between the two mechanical steps, with or without exogenous enzymes.

Various objections have been raised regarding the use of this technique in meaningful biochemical studies on nerve cells.

Objections

There are several objections to the above-mentioned technique:

1. The neurones consist of neuronal perikarya largely shorn of processes. The loss of processes directly at the perikarya leads to a disruption of the plasma membrane, which is inevitably accompanied by a leakage of intracellular substances like amino acids, enzymes, RNA and mitochondria; in addition, an influx of non-neuronal substances may occur.

2. The use of trypsin during incubation may produce harmful effects on the plasma membrane of the neurones. It has been shown that trypsin attacks proteins of the plasma membrane, e.g., glycoproteins. Furthermore, it has been suggested that trypsin enters the cell. Moreover, contamination of trypsin by desoxyribonuclease may alter the amount of DNA.

3. Those procedures which provide at least some neurones retaining their processes use almost exclusively 10–30-day-old rats as starting material. The morphological appearance of bulk-isolated neurones deteriorates when adult rats are used.

4. Some procedures are carried out at quite unphysiological pH values, e.g., pH 4.7 or pH 6.

5. Large variations with regard to cell morphology and cell yield occur using the same technique; e.g., Rose states that his method has "a fair bit of noise in the system", while Norton points out the "rather fragile nature of the method".

Additional objections relate to the purity of the obtained cell fraction as well as the representativeness of these fractions for the cerebral neurones. Furthermore, the

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substance used in density-gradient centrifugation led to problems, since, for example, sucrose can enter the cell and may lead to an inhibition of 5*-nucleotidase or succinatedehydrogenase.

Perfusion Method

In order to meet this criticism a new method was developed [1, 4]. The procedure involves (Fig. 1) a vascular perfusion of the rat brain in situ with an oxygenized hexose medium containing 0.1% collagenase/hyaluronidase, pH 7.4. The perfusion lasts for 20 min with a flow rate of 4 ml/min when rats weighing 150–180 g are used. Thereafter, the brain is rapidly removed from the skull, placed on a cooled plate, whereupon the cortices are minced with a razor blade, yielding an initial cell-tissue suspension which already contains free neurones. Further disaggregation is induced through sieving this suspension. The resulting cell suspension is fractionated on a discontinuous Ficoll-Fluorocarbon gradient, and the nerve cells are collected above the fluorocarbon cushion. This method can be used for animals of different ages and species.

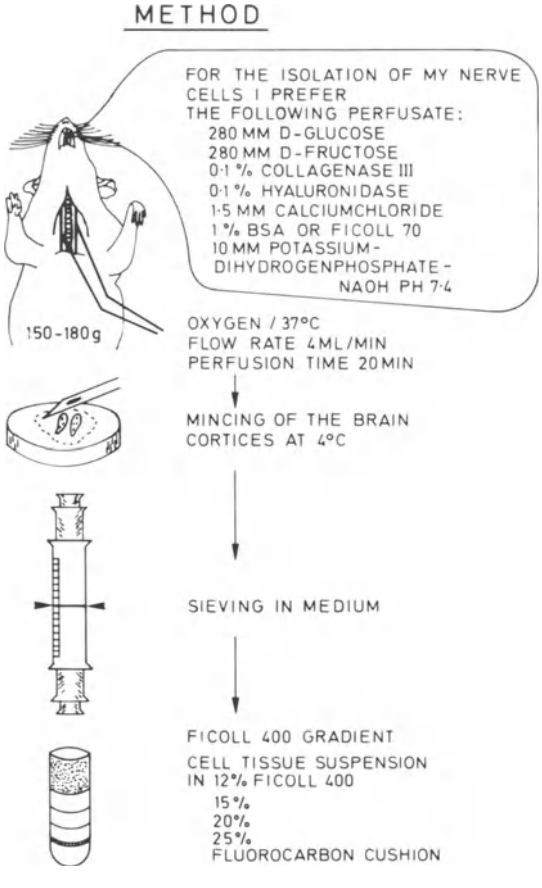

Fig. 1. Scheme of the perfusion method

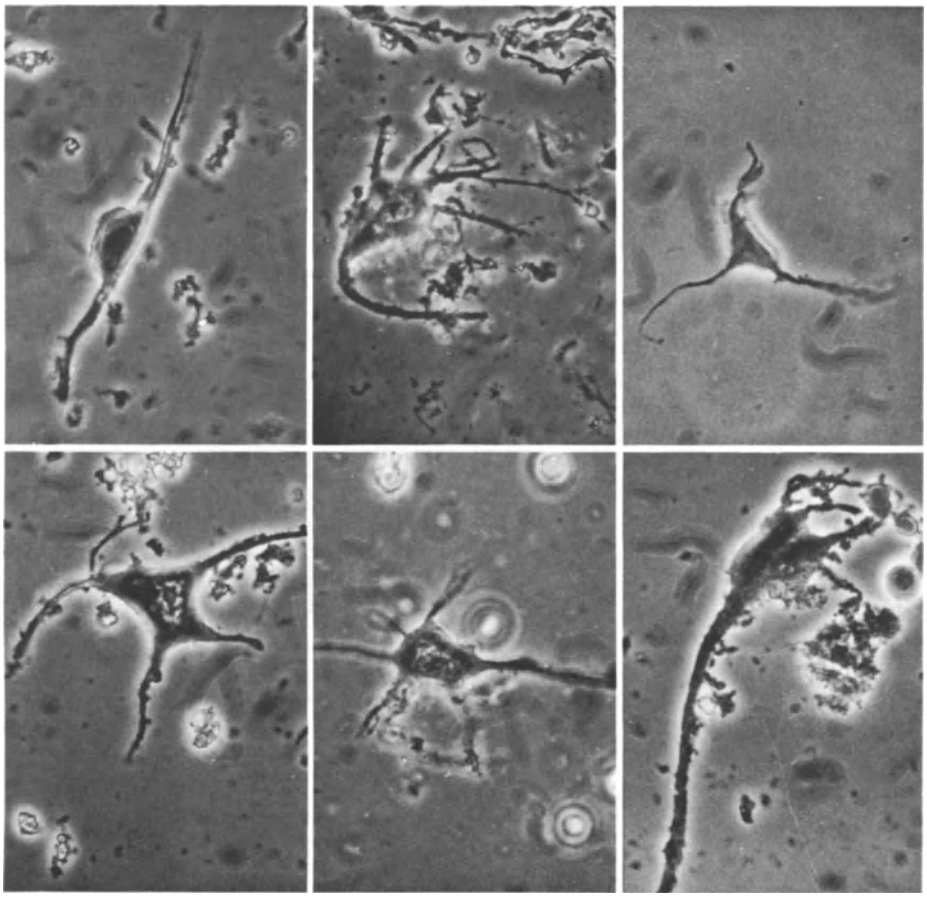

Fig. 2. Phase-contrast photomicrographs of single neurones obtained from the initial cell-tissue suspension. ×400

Results

Morphological Results

The perfusion leads to a pronounced softening of the brain tissue. This effect is produced by a swelling of glial processes and a disruption of the basement membrane of the capillaries, thus causing a predisaggregation in situ. As a result of this treatment, well-preserved neurones can be observed even in the initial cell tissue suspension. Under phase contrast, 60–70% of the neurones are classified as retaining their proximal part of processes [1].

A variety of cell types can be distinguished (Fig. 2). The TEM reveals an intact plasma membrane to which synaptic boutons adhere [4].

Biochemical Results

The cell yield of 17×10^6 neurones/g rat brain compares well with other published data. The protein (210 pg/cell) and the lipid (95 pg/cell) content is significantly higher than previously published values, which might reflect the better-preserved morphology of the cells. The activities of two important enzymes – Na^+-K^+ATPase (800 nmol ADP/mg protein × h) and adenylate cyclase (7.4 nmol cAMP/mg protein × h) – are higher than that of other nerve cell preparations. The amino acid composition (dansylated AA plus microchromatography) shows the presence of all putative neurotransmitters. The determination of amino acids in neurones from distinct areas is currently being undertaken.

Hints of Viability

Ninety-five percent of the isolated neurones exclude trypan blue, and these neurones respire well. At least these criteria are not very meaningful for cell vitality. Moreover, the neurones phosphorylate actively and the cells can be maintained in culture for several weeks. Some of the cells start to regenerate their fibers after a period of a week, and this can be observed by cinematographic analysis [2].

Conclusion

The appropriate perfusion of rat brain with a hypertonic hexose solution containing collagenase/hyaluronidase produces a predisaggregation of the neurones in situ. This greatly facilitates the subsequent mechanical dissociation of the nerve cells. A 60–70% portion of the isolated neurones can be classified as retaining processes. A variety of cell types can be distinguished, and these retain synaptic complexes at their intact plasma membrane and are viable, which means they are suitable for tissue culture. This procedure meets the above-mentioned objections, for (1) it is performed at physiological pH, (2) enzymes with damaging effects on the neuronal plasma membrane are avoided, (3) it is highly reproducible, and (4) it may be used with animals of different ages and various species [3].

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In Vitro Demyelination by Lymphocytes and Lymphokines from Patients and Experimental Animals

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Demyelinating change is a characteristic feature of demyelinating diseases. The mechanism involved has been thought to be cell-mediated immune processes characterized by activation of macrophages, which invade and phagocytize the myelin sheaths and the autophage of myelin debris in the myelin supporting cells. In addition, degenerative changes in sheaths, such as vesicular dissolution, are often observed.

In vitro studies on the demyelination processes using myelinated cultures [3, 11] have been performed using the aids of circulating antibody or sensitized lymphocytes [1, 4, 10].

Our previous works [10, 12] have disclosed that the circulating antibody in experimental allergic encephalitis and neuritis produces myelinotoxic change and activation of macrophages in vitro. Furthermore, it was found that galactocerebroside is the hapten antigen responsible for this circulating antibody [6, 13]. However, animals sensitized with galactocerebroside with carrier protein and complete Freund's adjuvant did not develop both clinical and pathological EAE, although anticerebroside antibody and demyelinating antibody were elevated [13]. On the contrary, basic protein from myelin sheaths and encephalitogenic peptide did not develop ciculating antibody, bud produced clinical and pathological lesions characteristic of EAE and EAN. These results indicate that the pathognomonic significance of the antibody in the demyelinating diseases is still uncertain.

On the other hand, sensitized lymph node cells or lymphocytes from peripheral blood seem to be more responsible for the development of demyelinating changes. The success of passive transfer of this disease to recipient animals with sensitized lymphocytes seems to be especially conclusive evidence for the participation of lymphocytes in the occurrence of this disorder [2, 7]. Though in vitro studies with sensitized lymph node cells or lymphocytes from peripheral blood have been performed by several authors [1, 4, 10], details of the mechanism of in vitro myelin destruction have not yet been explored, and no correlation to in vivo sequences has been established. The present report will deal with the alteration of myelinated cerebellar and ganglion cultures by the application of sensitized node cells or lymphocytes from EAE and EAN, and from patients' peripheral lymphocytes. Furthermore, in vitro studies on the possible effective agent, lymphokines extracted from lymphocytes, are analyzed.

Materials and Methods

Guinea pigs, rabbits, and Japanese monkeys were sensitized with homogenate of peripheral nerve, white matter, myelin fraction, or myelin basic protein (MBP) to-

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gether with complete Freund's adjuvant (CFA) on the foot pads. About 2 weeks to 20 days after inoculation, the animals developed clinical EAE or EAN.

Lymph node cells obtained from swollen lymph node at the knee were washed with saline solution and applied to the myelinated cerebellar or ganglion cultures from rat or mouse being suspended in routine feeding medium (10^5–10^6 cells/ml).

Node cells from some of the animals were cultivated in Eagle's medium supplemented with 3% fetal bovine serum (10^6 cells/ml), to which concanavalin A (40 μg/ml), myelin fraction, or MBP had been added. After cultivation for 48 h, the supernatant was gel-filtered through Sephadex G-200. Each fraction, in an amount of 10 ml, was concentrated to 0.5 ml and applied to myelinated cultures that were being diluted with feeding media (1:1). At the same time these fractions were applied to cell line cultures (L cell) to study toxicity. In addition, a macrophage migration inhibition test was performed using peritoneal macrophages from guinea pigs.

Active fractions for the above-described cytotoxicity were pooled and further purification was performed using a DEAE-cellulose column according to a technique described by Russell et al. [8]. After condensation, each fraction was again applied to myelinated cultures. Cytotoxicity to L cells and a migration inhibition test were also examined.

Using the Ficoll technique, lymphocytes were separated from the peripheral blood of patients suffering from Guillain-Barre syndrome and polyneuritis of varied etiology. Lymphocytes were applied to the ganglion cultures.

Alterations in these cultures were followed using an ordinary light microscope. When needed, the cultures were stained with Bodian's silver impregnation for axon, and Sudan black for myelin sheaths, or fixed for EM study.

Results

Effects of Sensitized Lymph Node Cells on the Myelinated Cultures

Morphological alterations of cultures after the application of sensitized node cells were classified into two types: cytotoxic change, and activation of macrophages and phagocytosis. The first change was characterized by fusiform swelling of myelinated fibers, often similar to that seen in Wallerian degeneration. Neurons often developed granular alteration, although distinct degenerative alteration was not seen in myelin supporting cells. These changes were often interpreted as demyelinative alteration. However, these should be considered as toxic change, and swelling of nerve fibers was more likely to be the Wallerian type of degeneration. When toxicity was low, this type of cytotoxic change showed simple fusiform swelling of nerve fibers and the continuity of axon was well maintained. When the effect was severe, neuron somas became granular and necrotic. Nerve fibers and myelin supporting cells were also necrotized in a similar fashion. Intermediate toxicity caused granulation of neurons and Wallerian degeneration.

The second change was characterized by an active phagocytosis of myelin by macrophages present in the cultures. No myelin alterations such as rippling, swelling, or interruption were observed. Once activated, macrophages came into contact

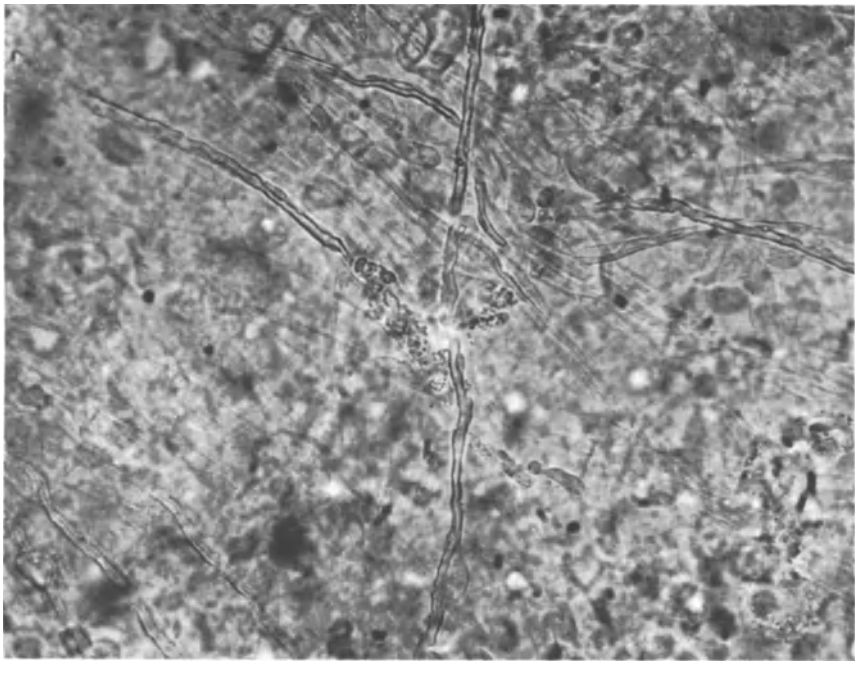

Fig. 1. Phagocytic activity of macrophage, enhanced by application of lymph node cells from an EAN rabbit. 36 h after application (living)

with myelin sheaths, the cells stuck to the fiber, and myelin sheaths were gradually phagocytized. Although these phenomena, Wallerian degeneration and phagocytosis, were observed in different fibers in the same culture, the former type of change was usually more prominent. An important indication that activated phagocytosis occured is the fact that the latter change took place in healthy myelin fibers which did not show any changes suggesting Wallerian degeneration. Degenerative changes of axons were minimal. On rare occasions, electron-microscopic examination revealed a slight accumulation of neurofilaments.

Thus, morphological changes after the application of sensitized lymph node cells were divided into two types, cytotoxic change and activated phagocytosis. Target cells in the former change were not specific, involving many kinds of tissue elements. In the latter change, however, tissue damage was very specific, involving only myelin sheaths. In these cases direct contact of applied node cells with myelin sheaths was not clearly demonstrated. Furthermore, development of toxic or phagocytic change did not occur until at least 2 days after the application. Furthermore, the lymphocyte effect did not require complement in the feeding media.

Effects of Lymphokines Liberated by Cocultivation with Mitogen or Antigen

The culture media of sensitized node cells, to which concanavalin A or myelin components had been added, were gelfiltered. The optical density of each fraction is

shown in Figure 2. This was obtained from a culture medium of EAN node cells stimulated with basic proteins of peripheral nerve. Each fraction was applied to the myelinated cultures. Fractions 23 to 25 in Figure 2 had a toxic effect on the ganglion cultures. The alterations in the cultures were characterized by swelling of nerve fibers in mild cases, and by neuronal granulation and necrosis in severe cases.

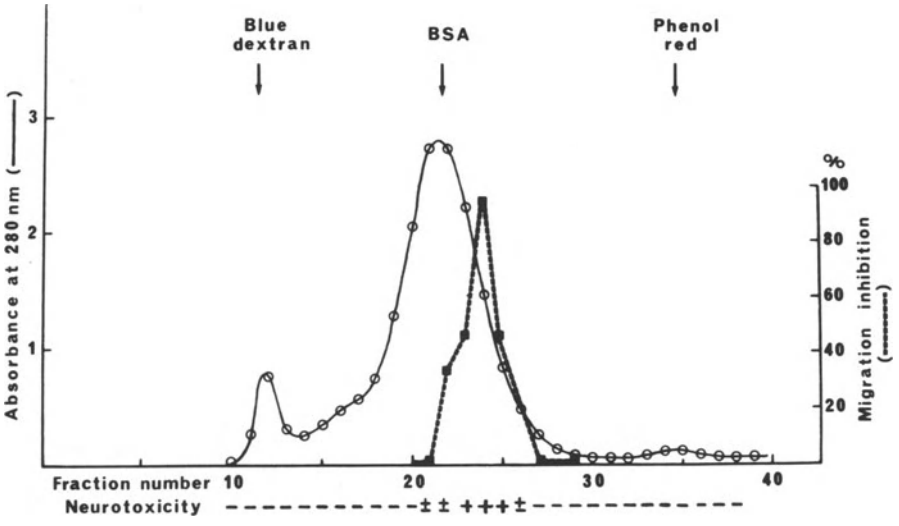

Fig. 2. UV absorbance, cytotoxicity, and migration inhibitory ratio of gel filtrates. Fractions number 1–9 were omitted, since these were regarded as a starting buffer. Cytotoxicity and migration inhibition were most active in fractions 23–25. Molecular weight was estimated to be roughly 44,000, slightly smaller than bovine serum albumin (BSA)

These changes were very much like those observed in cultures applied with sensitized node cells. However, phagocytic activity of macrophages was not clearly demonstrated in these cultures. But a migration-inhibiting factor for macrophages was demonstrated in fractions 23–25, as shown in Figure 1. The location of the cytotoxicity and migration-inhibiting factor seemed to be the same as described above. Further separation of these factors was not successful using a DEAE cellulose column according to the technique of Russel et al. [8]. These two factors were always found in the same fraction. The molecular weights of these toxic factors in monkey and rabbit were almost the same and were estimated to be about 44,000. Lymphotoxin from different EAN animals, such as rabbit and monkey, showed the same change.

Regardless of the different stimulants, whether mitogens or antigens, the morphological alterations caused by lymphokines were identical. However, lymphocytes from adjuvant-sensitized animals did not yield lymphotoxin for nerve culture by stimulation with myelin, while stimulation with mitogen showed release of lymphotoxin. On the contrary, lymphocytes from EAN animals released lymphotoxin by stimulating either mitogen or antigen. Identical relations and results were obtained using lymphokines from EAE animals.

Discussion

One of the most remarkable phenomena in demyelinating disease is demyelination. The mechanism involved is still the subject of arguments. In vitro studies have shown that demyelinating changes can be produced by application of the circulating serum factor and purified IgG from EAE and EAN animals. But since this serum factor has not been found in EAE animals sensitized with encephalitogenic peptide or MBP, the pathognomonic significance of this antibody for the development of the disease has been questioned. Furthermore, the circulating demyelinating factor was not found in EAE monkeys sensitized with white matter and adjuvant. In searching the antigenic substance, galactocerebroside has been implicated as a responsible hapten antigen. Sensitization of animals with galactocerebroside and carrier protein together with CFA caused antibody elevation, although the animals did not show any clinical and pathological abnormalities. Due to the negative results of passive transfer of the disease with circulating antibody and the absence of antibody in EAE and EAN monkeys, the significance of circulating antibody in the disease processes remains unknown.

Compared to the serum factor, sensitized lymph node cells produced cytotoxic change, and consequently myelin destruction and enhancement of active phagocytosis of macrophages. Degenerative changes in vitro produced by sensitized lymph node cells seemed to be identical to those produced by lymphokine released from lymphocytes. As described above, the two active factors were located in the same fraction. This may be the reason why phagocytic changes by activated macrophages in cultures were camouflaged by cytotoxic alteration. However, cytotoxic changes always exceeded changes in macrophage activity. At present, these results are not easy to apply to in vivo demyelination. There must be more intervening factors between the in vivo and in vitro sequences. However, the effects of sensitized node cells and lymphokines released from sensitized lymphocytes, possibly T cells, were identical. Therefore, it is very possible that a major part of the T-cell function in the demyelinating processes is cytotoxic effect and macrophage activation. Both of these were directed toward destruction of myelin sheaths, and these cellular factors were always seen, regardless of species differences between experimental animals and the type of encephalitogenic compound. When lymph node cells from EAN guinea pigs were labeled with tritiated thymidine and administered to inbred guinea pigs, labeled lymphocytes and nonlabeled monocytes or macrophages accumulated in the lesions of recipient EAN, suggesting that the lesion was initiated by sensitized lymphocytes together with activated macrophages (unpublished data). Therefore, it is suggested that these T lymphocytes initiate the disease processes. Active agents from sensitized lymphocytes are considered to be lymphokines. At present, the significance of B cells in the demyelination processes is not known. In particular, immunoglobulin against basic protein has no ability to demyelinate or activate macrophages. Further study of the subpopulation of lymphocytes in the allergic process and in B-cell function is required.

Summary

Lymph node cells from EAE and EAN animals such as monkeys, rabbits, and guinea pigs were applied to cerebellar and ganglion cultures from rats and mice. The alterations produced were classified into two types: cytotoxic changes and enhancement of macrophage activity. Lymphokines, liberated from sensitized node cells which were inoculated with mitogen or antigen, showed the same effects on the cerebellar and ganglion cultures, suggesting that the active agent in the sensitized node cells and lymphokine is identical. A cytotoxicity test using L cells and a macrophage-migration inhibition factor seemed to result in the same lymphokine fraction, the molecular weight of which was estimated at 44,000.

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The Virus-Host Relationship in CNS Model Cells

K. KOSCHEL [1]

Virus-Host Relationships in General

In principle, there are three different courses that the virus infection of a cell may take: (1) The cell may be destroyed by a cytocidal virus; (2) The cell may be transformed into a tumor cell by a tumor virus; and (3) The cell may be persistently infected by a non-cytocidal virus.

All three possibilities may be realized in the central nervous system (CNS). We were primarily interested in whether a noncytocidal persistent infection could, by itself, impair specific functions of CNS cells. This question is not easy to answer when studying intact animals, for in many cases, the immunological response elicited in the host might contribute to the symptoms observed and it is hard to say whether the dysfunctions observed are primarily due to the viral infection or are secondary to immunological causes. Furthermore, special biological cell functions are hard to assess in vivo.

However, in some instances, virus infections seemingly provoke no immunological reaction and cells are not destroyed by the virus. How could one then explain the resulting pathological phenomena? It seemed possible that CNS-specific biochemical functions per se might be influenced by persistent virus infection.

This possibility could best be investigated in vitro. The first prerequisite for studying an impairment of such CNS-specific functions in the absence of any immunological processes is suitable cells exhibiting the specific functions which one would like to investigate. Such cells could be (1) primary cells of the embryonic CNS in culture which can differentiate, (2) CNS-derived tumor cells, e.g., neuroblastoma or glioma cells, and (3) cell hybrids derived from these cells.

The second prerequisite is the establishment of a persistent virus infection in the cells. So far, the influence of a noncytocidal persistent virus infection on specific biochemical functions has only been investigated using tumor cell lines or hybrid cells. To date, very few data are available and this field might open up completely new vistas for the study of the virus-host relationship in CNS cells.

What Can We Investigate?

Using the appropriate cell lines and primary cultures, it might be worthwhile to study the influence of persistent virus infection on the following parameters:

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1. Synthesis of neurotransmitters, their release and degradation, and the transport of precursors or the reuptake of the released transmitters.
2. Differentiation with respect to CNS cell parameters.
3. Morphological aspects, e.g., the influence of virus infection on storage vesicles for neurotransmitters, tubulin- and actin-like proteins, and neurite extension.
4. Synaptogenesis.
5. Neuroreceptor functions and electrophysiological parameters.

Until now, only the influence of the persistent infection of mouse neuroblastoma cells with lymphocytic choriomeningitis virus (LCM) on the actylcholine synthesizing enzyme, acetylcholine transferase, and the degrading enzyme, acetylcholine esterase, have been studied. Oldstone et al. found that these enzyme activities are decreased by about 50% in infected cells without any influence of the virus on cell growth, cloning efficiency, and macromolecular synthesis [7]. In my laboratory, we have studied two additional cell systems infected by two different viruses with respect to the reactions mediated by membrane receptors for specific hormones and neurotransmitters.

One cell line studied was of glial origin; here we used the C 6 rat glioma cell [1], in which we established persistent infections with SSPE- and canine distemper virus (CDV). The other cell was the mouse neuroblastoma × rat glioma cell 108 CC-15 (NG 15) [4], which we infected with rabies virus (Hep Flury Strain).

Materials and Methods

The C 6 Cell SSPE and CDV System

C 6 cells were cultured in plastic tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) in a 10% CO_2/90% air atmosphere and were passaged by trypsinization. Infection of C 6 cell monolayers with SSPE virus (Lec Strain) was performed using a multiplicity of 0.2 infectious units per cell in DMEM containing 10% FCS.

After 24 h, the virus suspension was removed, and the cells were passaged normally. The state of infection of the cell culture was determined using indirect immunofluorescence with a patient's anti-SSPE serum and an FITC-conjugated anti-human γ-globulin antibody.

The 108 CC-15 Hybrid Cell/Rabies Virus System

The hybrid cells (a gift of Dr. B. Hamprecht, Munich) were grown at 37° C in plastic flasks in DMEM containing FCS, the HAT selection additions [5], and 0.02 M glucose and 3×10^{-2} M $NaHCO_3$ (osmolarity 330–340 mOsm) in a water-saturated atmosphere of 90% air and 10% CO_2 and were passaged by trypsinization (0.005% trypsin). Virus infection was performed with rabies HEP Flury strain at a multiplicity of 3 infectious units per cell. After virus adsorption for 45 min at 37° C in a sus-

pension of 3×10^7 cells/ml DMEM containing 1% FCS and 25 µg/ml DEAE-Dextran (Pharmacia, Uppsala), the cells were seeded onto petri dishes and allowed to grow in fresh medium. The percentage of infected cells in culture was estimated by determination of intracytoplasmic (RNP) and membrane-bound viral antigens using the direct immunofluorescence technique with specific FITC conjugated antisera.

Receptor-Mediated Adenylate Cyclase Reactions and cAMP Determinations

For receptor tests, infected cells and uninfected controls were freed from growth medium, washed twice, and incubated as monolayer cultures with the receptor-specific substances dissolved in serum free DMEM. After 10 min, the incubation medium was removed and displaced by 5% trichloroacetic acid (TCA) for cell denaturation. The content of the dishes was collected and centrifuged at 2000 *g*. The pellet was redissolved in 1 M NaOH and protein was determined using the method of Lowry et al. [6].

The supernatant was freed from TCA by ether extraction, lyophilized, and cAMP determined using the method of Gilman [2]. Specific cAMP concentrations were calculated as pmol cAMP per mg protein.

The C 6 Cell/Paramyxovirus System

The parameter that we studied was the β-adrenergic receptor of the cells. Incubation of uninfected cells with catecholamines is followed by a fast rise in the intracellular cAMP level [3]. In the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and catecholamines, we found a 100 to 1000-fold increase in cAMP level after 20–30 min. Persistent infections by SSPE-virus and canine distemper virus (CDV) could be established. The time dependence of viral antigen appearance in the cell population could be demonstrated using immunofluorescence technique (Figs. 1, 2).

During the time shown, the C 6/SSPE system produced infectious virus. In the C 6/CDV system we could detect viral membrane antigens and infectious virus only at the beginning. In later passages, neither viral membrane antigens nor infectious virus particles were observed either inside or outside the cells. However, there was an intracellular accumulation of cytoplasmic viral antigens, especially RNP. Cells taken late in the stage of viral persistence did not show morphological changes or changes in the growth rate when compared with the uninfected one. Even so, in the infected cells, the β-receptor mediated cAMP response (pmol cAMP/mg protein) to catecholamines was reduced to about 50–20% of the control of uninfected cells. The dose-response treshold of the catecholamine used (D,L-isoproterenol) was unchanged.

Because infected cells showed neither an enhanced cAMP release into the medium nor more rapid degradation of the cyclic nucleotide formed, we conclude that the

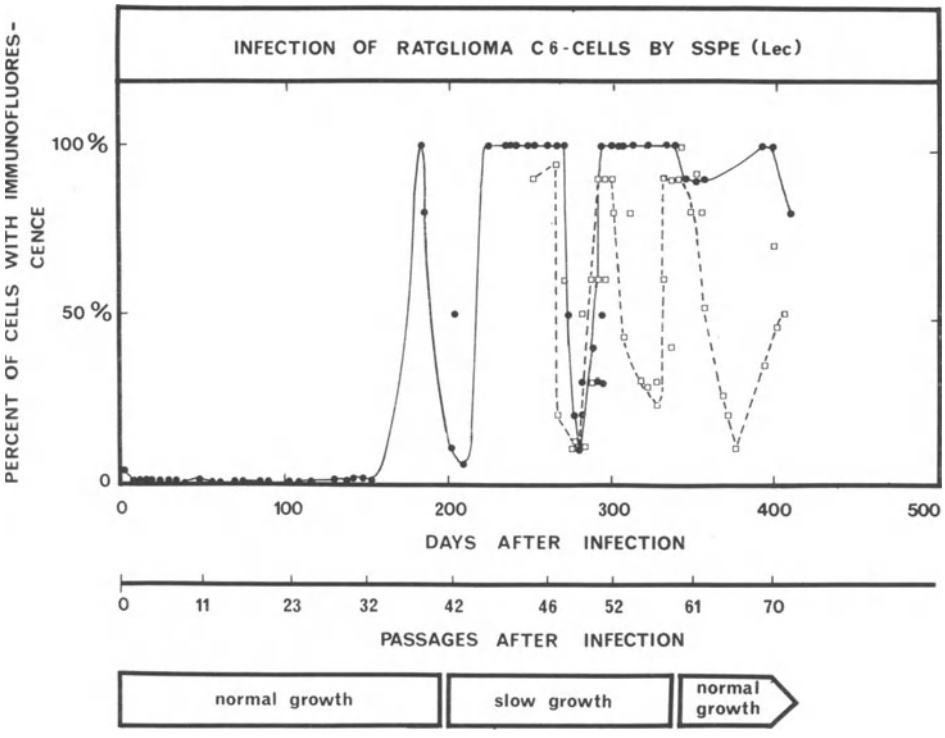

Fig. 1. Time course of viral antigens in persistently SSPE virus infected C 6 rat glioma cells. (●) total immunofluorescence, viral antigens. (□) viral membrane antigens, immunofluorescence

synthesis of cAMP itself is reduced in infected cells. The analysis of the kinetics of the fluoride-stimulated adenylate cyclase in a cell-free system (not documented here) has shown the specific activity of the enzyme to be diminished. The enzyme activity is sufficiently reduced to explain the reduced cellular cAMP response after incubation with catecholamines. Michaelis-Menten kinetics show that the binding of the substrate ATP to the enzyme is unchanged. It is therefore possible that, in the infected cell, only the number of enzyme molecules is lowered. Further studies are necessary to clear up this point. The other system used in our experiments was the 108 CC-15, hybrid cell/rabies virus system.

The 108 CC-15 Hybrid Cell/Rabies (Hep Flury) Virus System

Rabies virus can efficiently infect mouse neuroblastoma × rat glioma cell hybrids. Within a timespan of 24–48 h, nearly all cells in the culture show an increase in viral membrane antigen on the cell surface and an accumulation of viral RNP antigen in the cytoplasm. The cells produce infectious virus. A persistent infection can develop in the cell culture (Fig. 3).

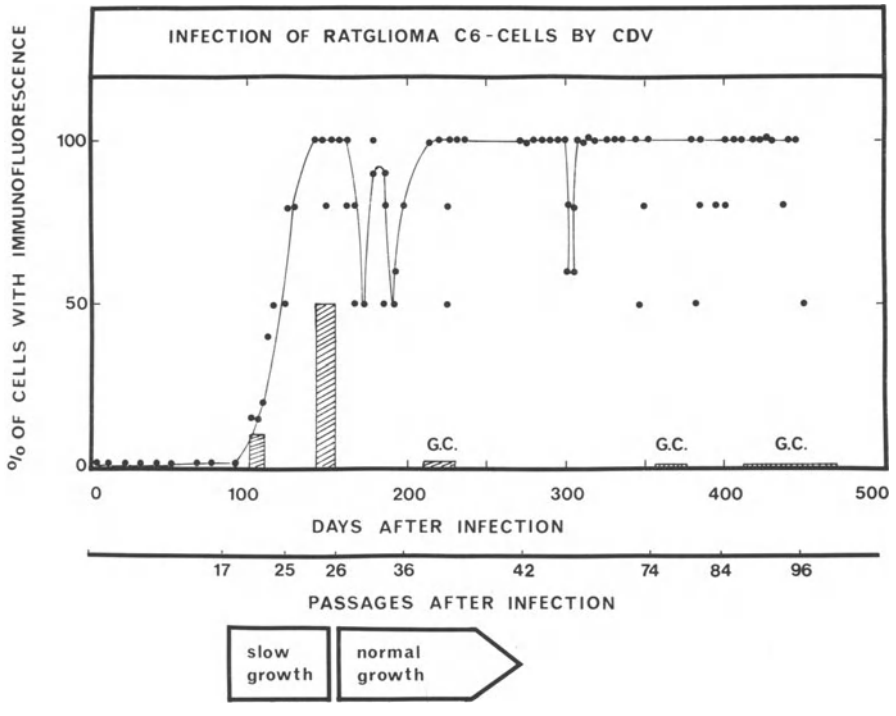

Fig. 2. Time course of viral antigens in persistently CDV infected C 6 rat glioma cells. Columns labeled with G. C. indicate percentage of giant cells in the culture and also show the percentage of cells with viral membrane antigens on their surface. The percentage of cells which show viral antigens in the cytoplasm is indicated by (●)

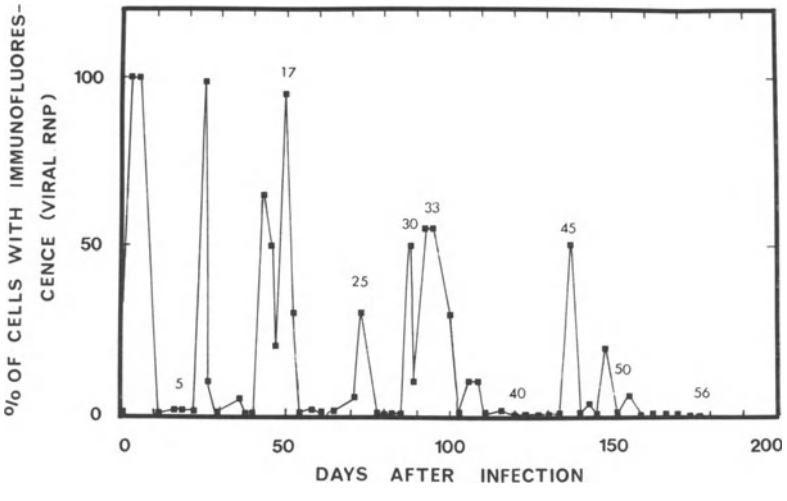

Fig. 3. Time course of viral RNP antigen in persistently rabies virus (Hep Flury) infected mouse neuroblastoma × rat glioma hybrid cells. The *numbers* inside the figure indicate passage numbers of the cell culture

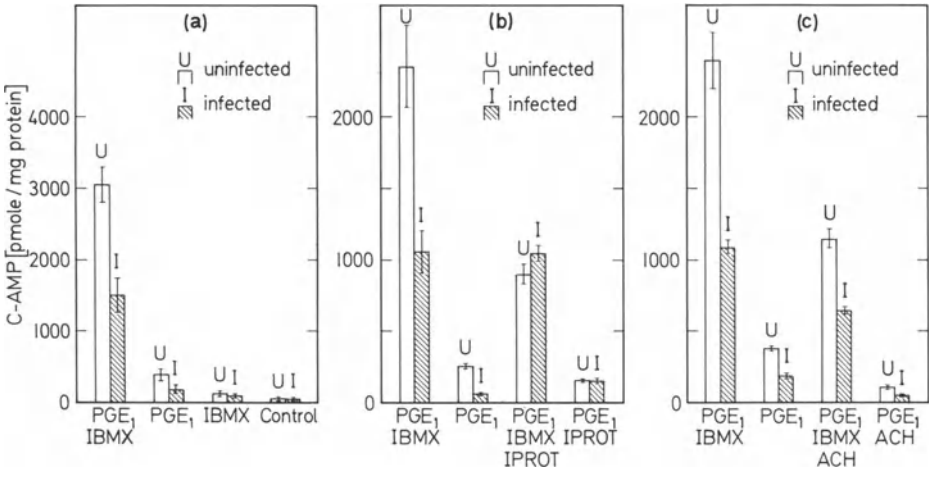

Fig. 4. Specific intracellular cAMP concentrations in uninfected and rabies virus (Hep Flury) infected 108 CC-15 hybrid cells after incubation with different hormones and neurotransmitters: **a** Cells incubated with 2.5×10^{-5} M prostaglandin E_1 (PGE_1) with and without the addition of 1×10^{-3} M IBMX (values shown with SD). **b** Cells incubated with 2.5×10^{-5} M prostaglandin E_1 (PGE_1) alone (with and without the addition of 1×10^{-3} M IBMX) and simultaneously with PGE_1 and 2.5×10^{-5} M D,L-Isoproterenol (IPROT) in presence and absence of IBMX (values shown with SD). **c** Cells incubated with 2.5×10^{-5} M PGE_1 alone (with and without addition of IBMX and simultaneously with PGE_1 and 1×10^{-5} M acetylcholine (ACH) with and without IBMX (values shown with SD)

As is known from other cell systems infected with rabies virus, viral processes exhibit a certain periodicity, as shown for the viral RNP antigen in Figure 3. To circumvent the objection that changes observed in infected hybrid cells might be due to a loss of chromosomes after long periods of cultivation, we used the cells 48–72 h after infection by the rabies virus in our experiments. The growth rate of the cells is unchanged after infection. Papers by Hamprecht et al. and Nirenberg et al. have shown that the 108 CC-15 hybrids are suitable model cells for cholinergic neurons (see [4] for references).

Among the many known characteristics of these cells, we chose a series of hormone- and neurotransmitter-receptor mediated reactions to investigate the influence of rabies infection. These receptor reactions affect intracellular cAMP level in two differing ways.

One type of receptor activates the synthesis of cAMP after binding of an agonist (PGE_1-, PGF2α- and adenosine-receptor). The second type of receptor inhibits, by binding of an agonist, the stimulation of cAMP synthesis normally caused via the first type of receptor. Inhibition occurs if the cells are incubated simultaneously with the appropriate agonists for both types of receptors. In our cells, the inhibiting receptors are the α-adrenergic receptors, opiate receptors, and muscarinic acetylcholine receptors. Figure 4 shows the receptor response of infected cells compared to the uninfected cells in culture after incubation for 10 min. By inhibiting the cAMP-destroying phosphodiesterase with IBMX, one obtains higher intracellular cAMP levels. However, the relationship between the columns is unchanged. Figure 4 a shows that the PGE_1-receptor reaction is reduced by about 50%. By analogy, the PGF2α

and the adenosine receptor responses are reduced by about 40% and 30%, respectively. Figure 4 b shows that the α-adrenergic receptor response with D,L-isoproterenol in combination with PGE_1 is reduced by 90%. By analogy, the opiate receptor response with methadone in combination with PGE_1 is reduced 90%.

The muscarinic acetylcholine receptor (Fig. 4 c) and the opiate receptor in combination with PGF2α (not shown) and adenosine (not shown) are not influenced by virus infection.

The different behavior of the receptor-mediated signal transfer in rabies virus-infected cells leads us to conclude that the infection has either influenced the receptor sites or their coupling to the adenylate cyclase. Adenylate cyclase activity, as measured by the in vitro incubation of membrane preparations in the presence of fluoride, is not impaired in infected cells (not documented here). In this respect there exists a difference between the rabies-infected hybrid cells and the paramyxovirus-infected C 6 glioma cells, where we observed a reduction of specific enzyme activity. Both cases, however, lead to the same result: a virally induced dysfunction of the membrane receptor-mediated signal transfer in the receptor/adenylate cyclase system. Such dysfunctions could explain the phenomena of pathological disorders of cell communication in the CNS even in the absence of cell destruction.

Acknowledgments. The work was supported by the Deutsche Forschungsgemeinschaft, grant SFB 105.

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Attempts to Transmit Multiple Sclerosis to Newborn and Germ-Free Nonhuman Primates: A Ten Year Interim Report

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Relatively few reports of attempts to transfer multiple sclerosis (MS) to primates have been made. Hudson and Grinker [6] inoculated Rhesus monkeys, but observed them for only 12 months, and found no pathology. Schaltenbrand [9] inoculated many monkeys and a few men. In the monkey he occasionally obtained an illness which could not be distinguished from a spontaneous leukoencephalomyelosis, a naturally occurring illness of this species. The Laboratory of Slow, Latent, and Temperate Viruses at the National Institutes of Health in the United States inoculated two adult chimpanzees and 12 adult Rhesus monkeys with MS brain material intracerebrally (IC) and IV. Two of these animals died in 1969 and none of the 12 remaining have developed disease relating to the inoculation in 11 years [4]. The report by Gibbs and Gajdusek is the only attempt to transmit MS in primates in which the inoculated animals were observed for more than 12 months.

We began attempts to transmit MS to nonhuman primates in early 1969. Newborn animals were used almost exclusively, because their immunologic capabilities are much less than those possessed by adult animals. This is attested to by their increased susceptibility to a variety of viral infections and by the greater ease with which tumors can be transplanted in newborn animals [8]. Later, in 1973, the program incorporated newborn nonhuman primates that were maintained as long as possible in a germ-free (GF) state. GF animals were used because evidence was emerging at that time that they were more susceptible to certain infections. For example, Heberling et al. [5] had reported that GF baboons inoculated with monkeypox experience a fatal infection, whereas conventionally reared baboons experienced only a mild infection and all recovered.

Five varieties of nonhuman primate species were inoculated: chimpanzees, baboons, marmosets, cebus, and squirrel monkeys. It seemed important to inoculate a wide variety of species because of evidence indicating marked differences among various species in susceptibility to a variety of human viruses. For example, measles produces a mild and inapparent infection in most primates but an overwhelming and often fatal disease in marmosets [2]. The chimpanzee and New World monkeys are susceptible hosts for kuru, but transmission to baboons and some other simians has not been successful [3].

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Materials and Methods

Recipients

The animals inoculated were 53 nonhuman primates: 32 baboons, 7 chimpanzees, 5 marmosets, 5 cebus, and 4 squirrel monkeys.

The numbers of animals inoculated with MS material and those inoculated with control brain or remaining uninoculated are indicated in Tables 1 and 2, respectively. All of the baboons and chimpanzees and two of the marmosets were inoculated in the newborn period, usually 24 to 48 h after birth. The remaining marmosets, cebus monkeys, and squirrel monkeys were adult animals.

Donors

Donors were eight patients with MS and four patients without MS. Five MS specimens were postmortem brain – the autopsy being done in less than 4 h in all except

Table 1. Monkeys inoculated with MS material

Total	Total	Surviving		Dead or sacrificed	
		No.	Follow-Up (mean)[a]	No.	Time to death (mean)[a]
Baboons (N. B.)	25	12	92	13	36
Chimpanzees (N. B.)	6	3	77	3	19
Marmosets (N. B.)	2	–	–	2	3
Marmosets (adult)	3	–	–	3	15
Cebus M. (adult)	3	0	0	3	38
Squirrel M. (adult)	2	1	48	1	43
Total	41	16	86	25	29

[a] Mean survival and time to death in months

Table 2. Control monkeys

Total	Total	Surviving		Dead or sacrificed	
		No.	Follow-Up (mean)[a]	No.	Follow-up (mean)[a]
Baboons (N. B.)	7	4	96	3	26
Chimpanzees (N. B.)	1	–	–	1	19
Marmosets (N. B.)	–	–	–	–	–
Marmosets (adult)	–	–	–	–	–
Cebus M. (adult)	2	–	–	2	39
Squirrel M. (adult)	2	2	49	–	–
Total	12	6	80	6	36

[a] Mean survival and time to death in months

one. Three MS specimens were biopsies: one of the brain and two of lymph nodes. Brain tissue was selected so that adjacent frozen sections showed lesions with some activity (e. g., perivascular round cell infiltrates). Ten percent suspensions preserved at – 70° C prior to inoculation were used. In two inoculations, cerebrospinal fluid frozen at the bedside from a patient with acute MS was used.

Five animals were inoculated with cells from tissue culture explants of MS material – two with explant cells from two different MS brains and three with cells from explants prepared from MS lymph nodes from three different patients.

In all, 41 animals were inoculated with MS material – 35 were primary donors and 6 with blind-passage brain material from MS-inoculated animals that developed neurological symptoms during the course of the study. Twelve animals served as controls – four received non-MS brain material IC and eight remained as uninoculated controls. Inoculations were made IC in all cases.

Results

Clinical

To this date, neurological symptoms have developed in six MS-inoculated animals – 17% of those 35 animals receiving material from primary donors, and in none of 12 controls. The symptoms were epilepsy in four, mental subnormality and failure to thrive in one, and intension tremor of the arm in one.

The general mortality rate has been high – 25 of the 41 MS-inoculated animals have either died or have been sacrificed because of severe illness (61%), whereas exactly 50% of the 12 control animals have died. Most of the deaths in both groups have been due to various kinds of infection (Table 3).

Table 3. Causes of death (21 MS-inoculated monkeys)

Epilepsy	2
Bronchopneumonia	3
Gastroenteritis	3
Septicemia (staph, aureus)	1
Meningitis (pneumococcus)	1
Pulmonary hemorrhages and skin rash	1
Small bowel necrosis (intususception)	1
Nephritis	1
Sacrificed	4
Unexplained	8
Total	25

Four animals were reared in the GF state, and maintained GF for several months, after being inoculated with MS explant cells in the newborn period. All of these animals have died. Three had severe bacterial infections, although these infections sometimes occurred many months following discontinuance of the GF state (Table 4).

Table 4. Germ-free animals

Animal	Inoculum	Time germ-free	Survival Time	Cause of death
Baboon (1012)	MS brain biopsy explant	6 months	27months	Suppurative dermatitis; septicemia, thymic atrophy
Chimpanzee	MS lymph node explant cocultivation with recipient amnion cells	2 months	19 months	Pneumonia enteritis
Marmoset (11)	MS node explant	3.5 months	4 months	Pneumonia
Marmoset (10)	MS node explant	1 month	1 month	Intussusception bowel; pulmonary fibrosis

Pathology

Neuropathological examination was performed in 31 animals. Twenty-five had been inoculated with MS material. Histology of control and experimental animals has usually shown either no abnormality, or nonspecific minor changes. In no instance has a demyelinating lesion been seen.

Several of the animals have shown nonspecific changes including subependymal gliosis, especially in the floor of the fourth ventricle; this was noted in one baboon who developed a right-arm intention tremor during the study. One other animal had ependymal granulations. There was unilateral dilatation of a lateral ventricle in one baboon, probably a congenital anomaly.

Another baboon was sacrificed because of status epilepticus 1 month after inoculation, and had rare glial nodules in both the grey and white matter – a type of lesion indicative of discrete focal necrosis and seen in viral infections, bacteremic states, and trauma. Two baboons showed swelling and mucinoid degeneration of the oligodendroglia, a nonspecific change seen commonly when death occurs after prolonged coma.

Of special interest was the finding in three chimpanzees and one marmoset of basophilic concretions, with staining characteristics of mucopolysaccharides – with iron and calcium deposits – in the basal ganglia and periventricular areas. All had been inoculated with MS material. However, there was no associated necrosis, cell loss, or reactive gliosis. Similar lesions were seen in three chimpanzees inoculated as newborns with 6/94, a parainfluenza virus, by Lief et al. [7]. In another animal reported by these authors, however, the concretions were found in the cortex as well as in the basal ganglia and were associated with widespread cortical cell loss and gliosis. These authors considered it possible that the more marked pathology was an exaggerated example of the same process that produces the benign concretions. They thought it possible that it was related to periventricular leukomalacia of infancy as described by Banker and Larroche [1].

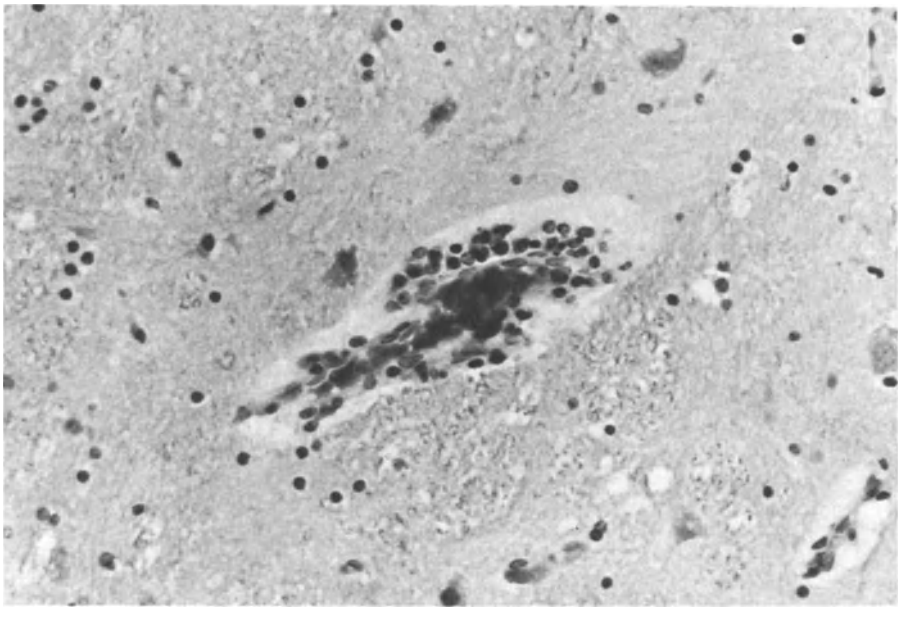

Fig. 1. Perivascular infiltrate of mononuclear cells and phagocytes in the basal ganglia of a squirrel monkey inoculated with MS material IC 4 years prior to death. No demyelinating lesions were seen

One squirrel monkey, dying of undetermined cause 4 years after inoculation with MS material showed rare areas of perivascular infiltration with mononuclear cells and phagocytes in both the grey and white matter, suggesting an infectious process; however, there was no associated clinical abnormality, and no demyelinating lesions were seen (Fig. 1).

Discussion

If MS is due to a virus, and if the demyelinating lesions are the *usual* response to that virus, then this study, to date, must be regarded as negative. If, on the other hand, the typical lesions of MS develop only as an *occasional* response to a virus infection, due to special host factors, then perhaps some of the neuropathological changes so far seen in this study may be significant.

We believe that this is the largest MS transmission study ever attempted in nonhuman primates. We have used a variety of species and have inoculated animals with the best available MS material, at a time of life when they should have been maximally susceptible to virus infection. On the whole, we conclude tentatively that this study lends little support to the idea that MS is a disease of viral etiology. It will, of course, be necessary to follow the surviving animals for longer periods, and the results to date do not exclude this possibility.

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Laboratory Investigations in Relation to the Etiology and Pathogenesis of MS

Introduction

H. J. BAUER [1]

There is as yet no specific diagnostic laboratory test for MS. However, a battery of cerebrospinal fluid (CSF) findings – mononuclear pleocytosis, normal or only slight to moderate elevation of total protein, but a pronounced increase of IgG – the demonstration using the formulas of Ganrot and Laurell, Link, or Tourtellotte that this is of CNS origin, and the appearance of subfractions, oligoclonal bands in the gamma range (Lowenthal and coworkers), represent a combination which is highly pathognomonic for MS and of decisive value in establishing clinical diagnosis in cases with atypical symptomatology and course. Mononuclear pleocytosis, with the appearance of plasmocytes, is the earliest alteration found by routine examination of CSF. In the majority of cases it is a transitory finding, followed within 6–12 weeks by an IgG increase of persistent nature. The IgG increase in CSF is found in 60–80% of all MS cases, and oligoclonal bands have been demonstrated in 70–90% of CSF specimens from MS patients.

These findings reflect immunological phenomena in the course of MS, and beyond their diagnostic significance and potential value in assaying the process activity, they are valuable entrées for the exploration of immune reactions leading to demyelination. This motivated the selection of laboratory investigations in relation to the etiology and pathogenesis of MS as one of the main topics of this symposium.

Inevitably, immune reactions of humoral or cellular nature in the CNS, as reflected by CSF findings, must involve the extraneural immune systems; the CSF alterations in all probability are secondary to the immunopathological events that take place before the demyelinating process is set off and maintained within the CNS.

Consequently, the examination of humoral and cellular components of the blood using serological, virological, biochemical, and morphological techniques today holds a dominating position in laboratory studies investigating the pathogenesis and possibly the etiology of MS. The following reports exemplify some of the important approaches and results in this area of MS research.

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Production of Interspecies Hybridomas Between CSF or Blood Lymphocytes from Patients with Neurological Diseases and Mouse Myeloma Cells

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Lymphocytes obtained from cerebrospinal fluid (CSF) of cases of multiple sclerosis and aseptic meningitis have been shown to produce oligoclonal immunoglobulins (IgG and IgA) in culture [10]. However, it is difficult to analyze these immunoglobulins for their antibody specificity because the lymphocytes that secrete them have a short life span in vitro [10]. On the other hand, somatic cell hybrids between antibody-secreting lymphocytes of mice and P3×63 Ag8 mouse myeloma cells continue to produce antibodies against a variety of antigens even when maintained in culture for long periods [6]. Therefore, we fused lymphocytes from human CSF with mouse myeloma cells in an attempt to obtain interspecies hybrids that would produce human globulins and antibodies directed against a specific antigen. To increase our chances of obtaining viable hybrids, we also fused lymphocytes from peripheral blood of patients with mouse myeloma cells. These experiments were undertaken in the knowledge that it would be difficult to produce hybrids expressing functions of human immunocytes, since the number of B lymphocytes in CSF or blood committed to react against a specific antigen is small in contrast to the number of such cells in the spleen of mice immunized against a specific antigen [6].

Patients and Methods

Patients

As shown in Table 1, the cells used for hybridization were obtained from the CSF of two patients and the blood of three patients. Patients AH and IB had a sudden onset of impaired vision in one eye, defective color vision, and central scotomas in the visual field 6 days (AH) and 2 months (IB) prior to the time when blood (AH) and CSF (IB) were obtained. The clinical diagnosis of optic neuritis was based on the symptoms and ophthalmologic findings. The CSF showed a mononuclear pleocytosis of 54 cells/mm^3 for patient AH and 40 cells/mm^3 for patient IB. The total protein content of the CSF was slightly elevated, and electrophoresis revealed an oligoclonal pattern in the gamma globulin region of IB's CSF. Patient TA suffered from three attacks of neurologic symptoms including sensory disturbance, spastic paraplegia, and ataxia over a period of 1½ years. He was recovering from the third attack when his blood was drawn. On several occasions his CSF showed a mononuclear pleocytosis ranging from 40–60 cells/mm^3. The total CSF protein was

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Table 1. Source of human lymphocytes[a]

Patient	Age and sex	Clinical diagnosis	Time after onset	Cells	Number of cells fused with P3×63 Ag8
OA	23 M	Herpes zoster	2 weeks	CSF	4.1×10^6
				BL	5×10^7
TA	25 M	MS	1½ years	BL	5×10^7
AH	25 F	Optic neuritis	6 days	BL	5×10^7
IB	22 F	Optic neuritis	2 months	CSF	5.8×10^6

[a] CSF = Cerebrospinal; BL = Blood Lymphocytes; M = Male; F = Female

within a normal range but on electrophoresis an oligoclonal pattern was seen in the gamma globulin region.

The fourth patient, OA, had clinical symptoms of herpes zoster with pain sensation and vesicles in the region of the right external ear. In addition, he had peripheral facial paralysis, impaired neurogenic hearing, and no vestibular function on the right side. CSF showed a mononuclear pleocytosis of 294 cells/mm^3; the number of cells had decreased to 84 cells/mm^3 at the time the CSF was obtained for fusion purposes. The total protein and glucose were within a normal range; there were no oligoclonal bands on electrophoresis. The titer of herpes zoster antibodies was 1 : 2 in CSF and 1 : 20 in blood 4 days prior to the time CSF and blood cells were obtained for fusion purposes.

Fifty milliliters of CSF were obtained from patient OA by one lumbar puncture. The sample was immediately centrifuged at 1500 rpm in an International centrifuge for 7 min at room temperature, and the cells were washed once with minimal essential medium (MEM) and used for fusion. Patient IB was tapped five times every 2½ hours, 40 ml of CSF being obtained on each occasion. The CSF samples were processed in the same way as in the case of patient OA, but the cells from each of the five samples were resuspended in 0.5 ml of MEM and kept in a shaking water bath at 37° C until all the samples were collected. The cells were then pooled, centrifuged at 1500 rpm for 7 min and used for fusion with P3×63 Ag8 cells.

White blood cell counts of all four patients were within a normal range at the time blood was collected. Fifty milliliters of heparinized venous blood were drawn from patients OA, TA, and AH, and lymphocytes were obtained by separation on Ficoll-Hypaque gradient [4].

Fusion Procedure

The number of human lymphocytes mixed with P3×63 Ag8 mouse myeloma cells is shown in Table 1. In the case of CSF lymphocytes, the ratio of human cells to mouse myeloma cells was 1 : 10 and in the case of blood lymphocytes, 1 : 2.

Fusion with PEG was performed according to the procedure previously described. The fused cells were placed in T-25 Falcon Flasks in HAT medium [12] containing 10% fetal calf serum and 10% horse serum and incubated at 37° C in 95% air/5% CO_2.

Assay for Human Globulins

Hybrid cells were labeled with ^{3}H-leucine for 24–48 h. The supernatant was precipitated at 4° C for 1 h with rabbit anti-human γ or μ and goat anti-mouse γ globulin. These samples were added to a prewashed *Staphylococcus Aureus* pellet and precipitated at 4° C for 30 min. The immune precipitates were washed and dissolved in 2% SDS, 10% glycerol, 80 mM Tris-HCl, pH 6.8, 0.1 M DTT, PMSF, and Brom phenol blue before electrophoresis. The samples were heated to 80° C for 10 min. Electrophoresis was performed in 11% discontinuous slab gels as described by Laemmli [7]. Gels were embedded in DMSO-PPO, dried and autoradiographed on Kodak RP-5 X-ray film.

The presence of human IgG in hybridoma medium or mouse ascites produced by hybridoma was also determined in competitive microradioimmunoassays [5] in which binding between ^{125}I-labeled human IgG and rabbit anti-human $F(ab^{1})_2$ of IgG was inhibited by the hybridomas.

Karyologic Analysis

Chromosomes of hybrid cells were banded by the trypsin-Giemsa method according to a modification of the procedure described by Seabright [2, 11].

Isozyme Analysis

Hybrid cultures were assayed for the expression of human glucose-6-phosphate dehydrogenase (EC1.1.149) by a previously described method [8].

Results

Frequency of Hybrid Production

Cultures obtained by fusion of CSF cells of patient OA with P3×63 Ag8 mouse myeloma cells were transferred from T-25 flasks to FB16-24TC Linbro plates 36 h after fusion. Fused cells obtained from other patients were kept in T-25 flasks.

Table 2 shows the hybridomas produced by the different combinations of human and mouse cells. Within 2 weeks after fusion of blood lymphocytes from patients OA, TA, and AH with mouse myeloma cells, we observed small colonies of cells larger than the parental P3×63 Ag8 cells in all T-25 flasks. In cultures obtained by fusion of CSF cells of patient OA with mouse myeloma cells, colonies of hybrid cells were observed in 2 out of 18 wells 5 weeks after fusion. In cultures obtained by fusion of CSF cells of patient IB with mouse myeloma cells a few colonies of hybrid cells appeared in one T-25 flask within 5 days after fusion. Many of the colonies degenerated within the next few days, therefore a feeder layer of WI-38 human fibroblasts was added to the flask in order to support growth of the hybrid cells.

Table 2. Hybridomas produced by fusion of human lymphocytes with P3×63 Ag8 mouse myeloma cells[a]

Patient	Origin of lymphocytes	Ratio of culture vessels showing presence of hybrid cells	No of clones produced	G6PD		Presence of human chromosomes
				Human	Mouse	
OA	CSF	2/18[b]	18	+	+	+
	Blood	4/4	60	+	+	+
TA	Blood	4/4	not cloned	+	+	+
AH	Blood	4/4	20	+	+	+
IB	CSF	1/4	not cloned	+	+	+

[a] CSF = cerebrospinal fluid; G6PD = glucose-6-phosphate dehydrogenase
[b] Refers to wells of Linbro plate. All other cultures were kept in T-25 flasks

Formation of hybrid colonies was then observed and the feeder layer was removed by addition of 10^{-4} M oubain to which human cells, but not mouse or mouse × human hybrids, are sensitive [1].

No difficulties were encountered in maintaining viable cultures of all hybrids in the course of numerous cell transfers. The hybrid cells were grown in HAT medium in a suspension culture at 37° C and transferred to fresh medium every 5 days.

Karyological and Isozyme Analysis of Hybrid Cultures

All hybrid cultures retained the entire complement of parental P3×63 Ag8 mouse cells. The hybrid lines segregated human chromosomes, retaining 5 to 7 human chromosomes as shown in Figures 1 and 2 for the hybrid produced by CSF lymphocytes of OA patient with P3×63 Ag8 cells. Isozyme analysis for the presence of human glucose-6-phosphate dehydrogenase (G6PD) showed presence of the human form of the enzyme in all hybrids investigatd (Fig. 3, Table 2) confirming the presence of human chromosome X, which was also detected by karyological analysis (Figs. 1, 2).

Production of Human Immunoglobulins by Hybrid Cultures

As shown in Table 3, media of the hybrid cultures obtained by fusion of OA blood lymphocytes with mouse myeloma cells showed the presence of human IgM. This culture was cloned [3], and one out of 40 clones showed the presence of human IgM as detected by SDS polyacrylamide gel electrophoresis. This clone was found to produce 30 μg of human IgM/ml of culture medium by a Biorad immunofluorescent (Biorad Laboratories, Immuno-Fluor, Richmond, California) quantitative assay for human IgM. No human IgA or IgG was detected using this method.

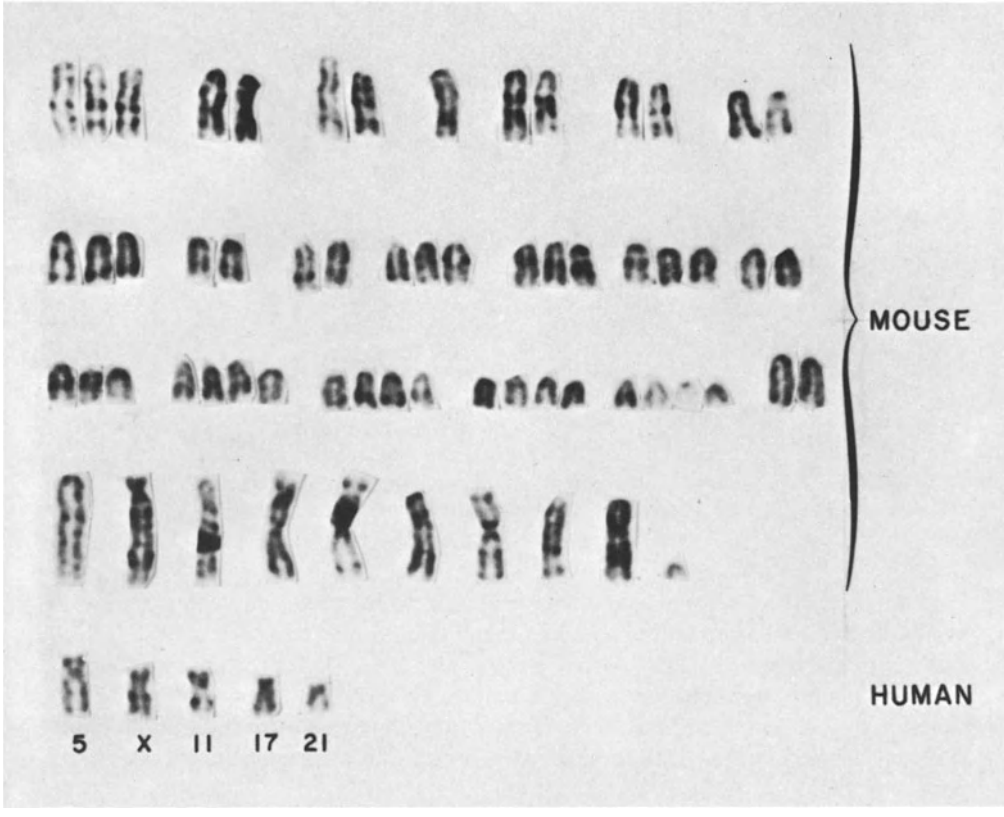

Fig. 1. Karyotypes of hybrid cell OA CSF × P3X63Ag8. This hybrid contains a complete mouse chromosome complement and 5 to 7 human chromosomes

Table 3. Immunoglobulins secreted by hybridomas

Source of lymphocytes		Secretion of human immunoglobulins by hybrid cells		
Patient	Cells	IgM	IgG	IgA
OA	CSF	–	tr[a]	–
	Blood	+	–	–
TA	Blood	–	–	–
AH	Blood	–	–	–
IB	CSF	–	–	–

[a] tr = trace

In the case of hybrid cultures between OA-CSF lymphocytes and mouse myeloma cells, ascitic fluid obtained by inoculation of pristane-primed nude mice with one of the hybrid clones was assayed in a competitive radioimmunoassay (see Patients and Methods) against human IgG and found to show 70% inhibition at a 1 : 10 dilution. Medium obtained from mouse P3×63 Ag8 myeloma cultures did

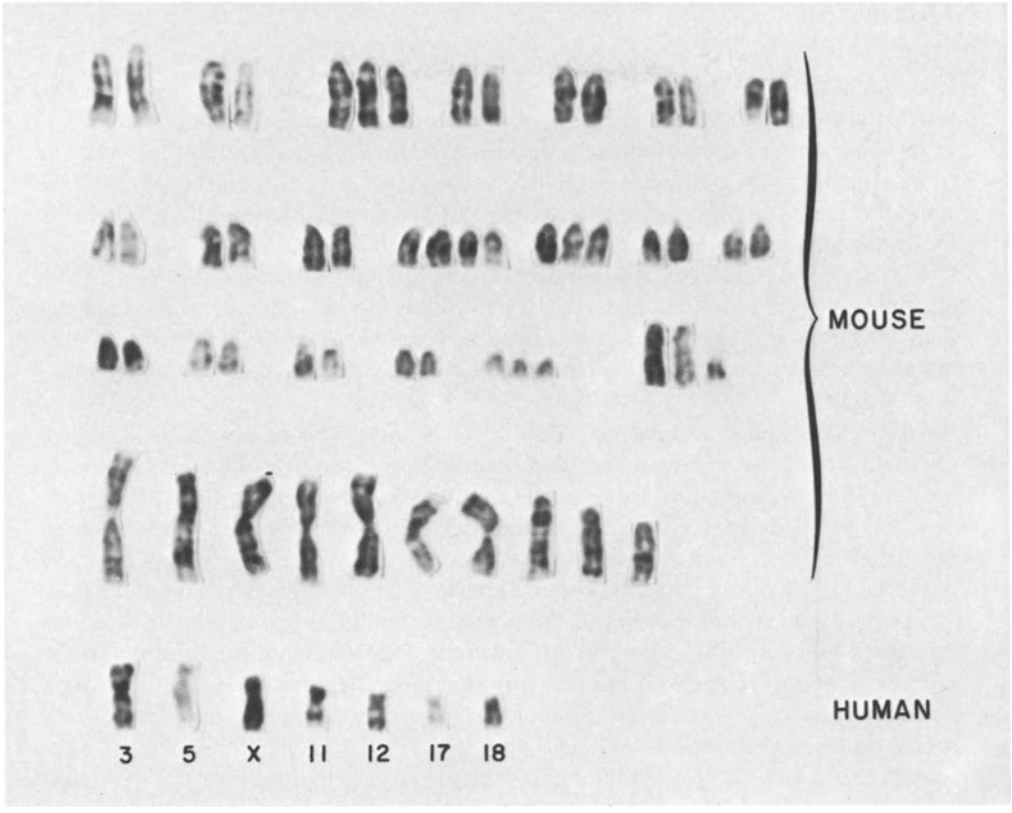

Fig. 2. See legend to Figure 1

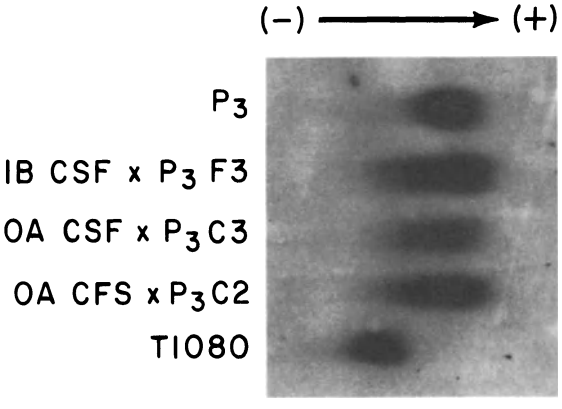

Fig. 3. Zymogram of glucose-6-phosphate dehydrogenase on starch gel electrophoresis. Extracts of the parental mouse cells P3 and a human control cell T1080 show G6PD separation. The hybrid cultures IB CSF × P3 F3, OA CSF × P3 C3, and OA CSF × P3 C2 show positive expression of human G6PD

not inhibit this reaction. Thus, we consider that this hybrid produces at least traces of human IgG (Table 3). The immunofluorescent Biorad assay also exhibited traces of human IgG; less than 0.1 μg of human IgG/ml was detected in the supernatant of a clone of the OA-CSF × P3 × 63 Ag8 hybrid. None of the other hybrids was found to produce any of the subclasses of human globulin detectable in our assays.

Discussion

In contrast to the facility with which intraspecies hybrid cultures are produced between mouse splenocytes and P3×63 Ag8 cells [6], production of interspecies hybrids between mouse myeloma cells and human lymphocytes resulting in secretion of human immunoglobulins is a relatively infrequent event. Nevertheless, the results of this study indicate that it is possible to produce viable somatic cell hybrids by fusion of human blood or CSF lymphocytes with P3×63 Ag8 mouse cells, and that these hybrids secrete at least two classes of human immunoglobulin heavy chains. Since clones of hybrids originating from OA blood lymphocytes produce human IgM in large quantities and segregate human chromosomes, it should be ultimately possible to assign the gene for production of IgM to a specific human chromosome [3].

Hybrid cells produced by the fusion of mouse myeloma cells with lymphocytes obtained from the CSF of patients with neurological disease can be used for studies on the range of antibodies produced by immunocytes resident in the CNS. Up to now, the antibody specificities of the oligoclonal IgG of CSF in MS have not been identified, since antibodies against viruses account, at best, for a very small fraction of the IgG [9]; hybrids produced with CSF cells of MS patients may help to characterize these specificities. Studies of the immunoglobulin(s) secreted by hybrid cultures produced with CSF cells from MS cases may permit identification of the major immunoglobulin(s) present in the CSF of the donors. An anti-idiotype against such globulins could be produced and used to examine an MS patient for the presence of a specific idiotype in his CSF (and blood), during the course of the illness. The cross-reactivity of this anti-idiotype with idiotypes obtained from other cases of MS could also be assessed to determine whether a characteristic idiotype is present in CSF and/or blood of other patients.

Hybrids formed with MS CSF cells may aid in another aspect of MS research. The symptoms of optic neuritis in patient IB of this study may represent the onset of MS. Thus, examination of the antibodies produced by the hybrid cultures using IB-CSF cells may reveal the nature of the antigen evoking the immune response in these lymphocytes and, perhaps, shed some light on the etiologic agent active in the initial stages of MS.

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Antibodies to Myelin Basic Protein in Cerebrospinal Fluid of Patients with Multiple Sclerosis

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Summary

Antibodies of the IgG class directed against myelin basic protein (MBP) were detected by solid phase radioimmunoassay in the cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS). Comparison with controls at equivalent IgG concentrations indicated that the activity detected was due to antibody rather than to nonspecific adherence of IgG. Absorption and blocking studies showed that the reaction was antigen specific for MBP. Antibody was found more frequently in patients with active disease, and less often in patients in remission. There was no correlation with total CSF IgG or the presence of oligoclonal bands on electrophoresis. These findings support the occurrence of an autoimmune response in MS, though its role in pathogenesis of the disease remains obscure.

Introduction

Multiple sclerosis (MS) has certain pathological and immunological features in common with experimental allergic encephalomyelitis (EAE), an autoimmune disease of the central nervous system (CNS) induced by inoculation of animals with MBP in complete Freund's adjuvant [18]. Cell-mediated and humoral immune responses to the sensitizing antigen occur concomitantly with development of the experimental disease [5, 15]. In MS, investigators have reported cell-mediated immunity in vitro to MBP [8, 11], and antibodies to myelin or to crude CNS antigen preparations [13, 21]. However, antibodies specific for MBP have been found neither in serum nor in cerebrospinal fluid (CSF) [5, 10, 12, 22].

Primary myelin destruction occurs in MS, and MBP or a fragment of MBP is present in the CSF of patients with the disease [2, 25]. The IgG concentration of CSF is increased in approximately two thirds of clinically definite cases, and oligoclonal IgG bands are seen on agarose gel electrophoresis in approximately 90% [6]. The excess immunoglobulin is produced within the CNS and often contains antibodies to one or more viruses [17, 23]. Most of the oligoclonal bands, however, cannot be removed by absorption with viral antigens [24], and may represent antibodies to other exogenous or autoantigens.

Using a solid phase radioimmunoassay (RIA) recently developed for detection of antibodies to MBP [20], we have found such antibodies in the CSF of patients

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with MS when compared with a control population of patients with other neurological diseases. The presence of these antibodies indicates for the first time that a humoral autoimmune response to a well-defined myelin antigen may occur in this common but poorly understood demyelinating disease.

Materials and Methods

CSF was obtained by aseptic lumbar puncture from 22 patients with clinically definite MS as defined by McDonald [14] and from 20 control patients with other neurological diseases (viral meningoencephalitis [2], idiopathic epilepsy [4], acute stroke [3], Guillain-Barre syndrome [2], amyotrophic lateral sclerosis [3], and miscellaneous non-inflammatory CNS diseases [6]. IgG concentrations were determined by rocket electrophoresis. Oligoclonal immunoglobulin bands were determined as described by Johnson et al. [7].

Guinea pig MBP was prepared according to the method of Deibler et al. [4]. It was electrophoretically pure on polyacrylamide gels and highly encephalitogenic in Lewis rats. RIA was performed as described by Randolph et al. [20] with slight modifications. Tissue culture tubes were coated with MBP at 20 µg/ml in borate buffered saline, pH 8.0. Serial dilutions of CSF were then incubated in duplicate tubes, followed by ^{125}I-labeled staphylococcal protein A (SPA, Pharmacia) containing approximately 30,000 CPM per ml. The percent of ^{125}I-SPA bound was determined in a gamma counter. Binding curves were constructed by plotting percent ^{125}I-SPA bound by serial dilutions of CSF against IgG concentrations. MS and control fluids were compared in the range of 0.2 to 1.0 µg IgG per tube, since all samples were diluted at least 1 : 5 in buffer to maintain pH and to prevent excessive nonspecific adherence. Significance of differences in means of combined data were determined by a *t*-test. Individual samples were designated positive if their binding activities were 2 SD above the composite control mean.

To establish the dependence of binding activity upon IgG, CSF was absorbed with rabbit anti-human IgG coupled to polyacrylamide beads. CSF IgG and anti-MBP activity were then determined. Specificity of the reaction was evaluated by absorption of CSF with MBP, with calf thymus histone (another highly basic protein), or with bovine serum albumin (BSA). Aliquots of the three proteins at concentrations of 10 to 1000 µg/ml were mixed in solution with equal volumes of CSF for 1 h at 37° C followed by 2 h at 4° C, and the CSF-protein mixtures were then assayed by RIA. Further specificity of CSF for MBP was shown by inhibition of binding with rat antibody to MBP. IgG fractions were prepared from pooled sera of rats hyperimmunized with guinea pig MBP and from normal rats by DEAE cellulose column chromatography. Eluates were concentrated, tested by immunoelectrophoresis for IgG, and by RIA for antibody to MBP. Fractions of normal and hyperimmune IgG were then diluted serially and incubated in MBP-coated tubes. After removal of unreacted rat IgG, CSF was added followed by ^{125}I-labeled rabbit anti-human IgG, and the extent of blocking by the rat antibody was determined.

Results

Comparison of MS and control CSF at equivalent IgG concentrations was essential because of elevated IgG levels in most cases of MS and the tendency of human IgG to adhere to MBP through nonimmune binding at the F_c portion of the molecule [1]. At all concentrations, mean binding activity was significantly greater in MS than in control CSF ($p < 0.05$ at 1.0 µg and $p < 0.01$ at the other concentrations, Fig. 1). ^{125}I-SPA binding by controls was attributed to nonspecific adherence of IgG, since dilutions of normal human IgG gave a binding curve corresponding to the mean values for control CSF. Activity in excess of this level represents the reaction of antibody with its specific antigen.

In Figure 2, MS and control CSF are compared at a single concentration of IgG, in this case 0.8 mg per reaction tube (or 0.8 mg/100 ml). At this concentration, 18 control fluids and 20 MS fluids were available for analysis. Binding activity was greater in the patients with active MS than in those in remission ($p < 0.05$) and in both MS groups compared with controls ($p < 0.01$).

Using the criterion of 2 SD above control levels to define a positive antibody level, the results shown in Table 1 were obtained. Six of 8 MS patients in acute exacerbation and 4 of 5 with gradually progressive disease were positive, while 4 of 9 patients in remission were positive. Within the MS group, IgG concentration of undiluted CSF was unrelated to antibody level; several patients with relatively low IgG (5–7 mg/100 ml) had as much antibody activity as others in whom total IgG

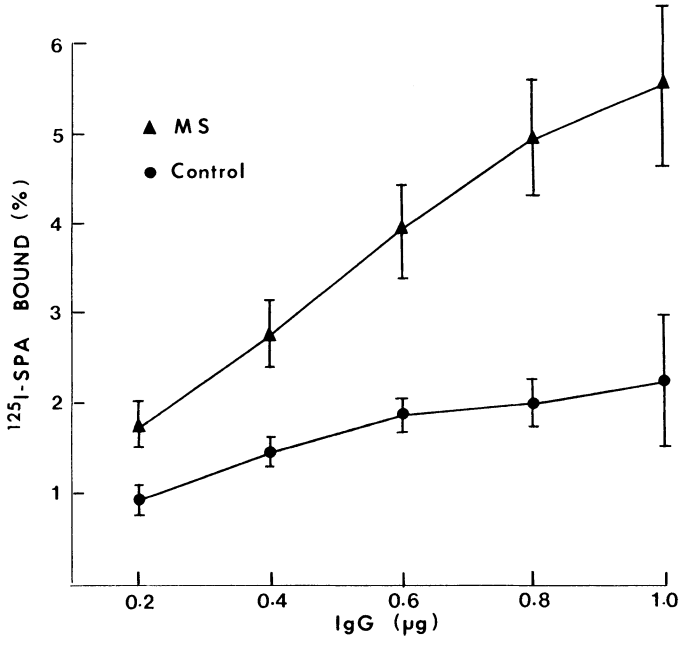

Fig. 1. Anti-MBP activity (mean percent ^{125}I-SPA bound ± SE) for MS and normal CSF at concentrations of diluted IgG from 0.2 to 1.0 mg/100 ml. Composite data derived from binding curves constructed for CSF samples from 22 MS patients and 20 controls

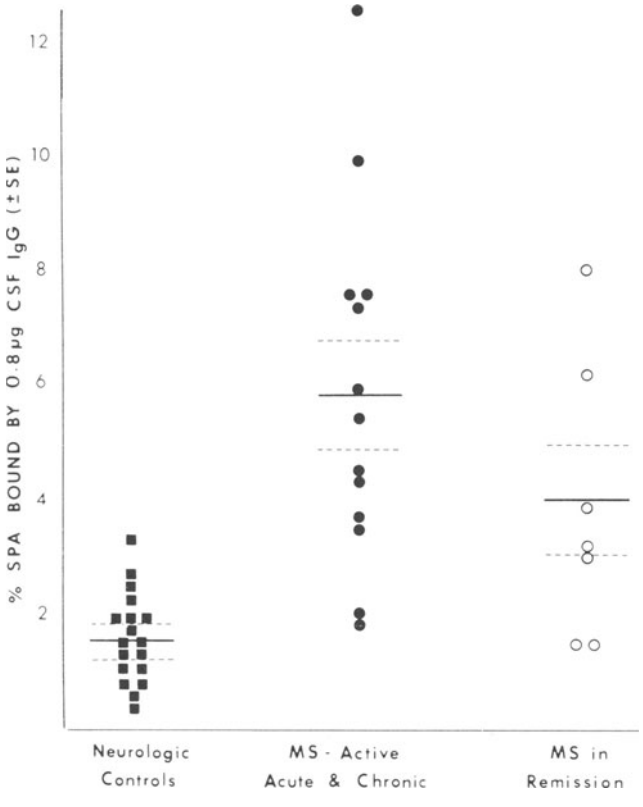

Fig. 2. Antibody to MBP at a single concentration of CSF IgG. Patients with active MS and patients with MS in remission are compared with controls

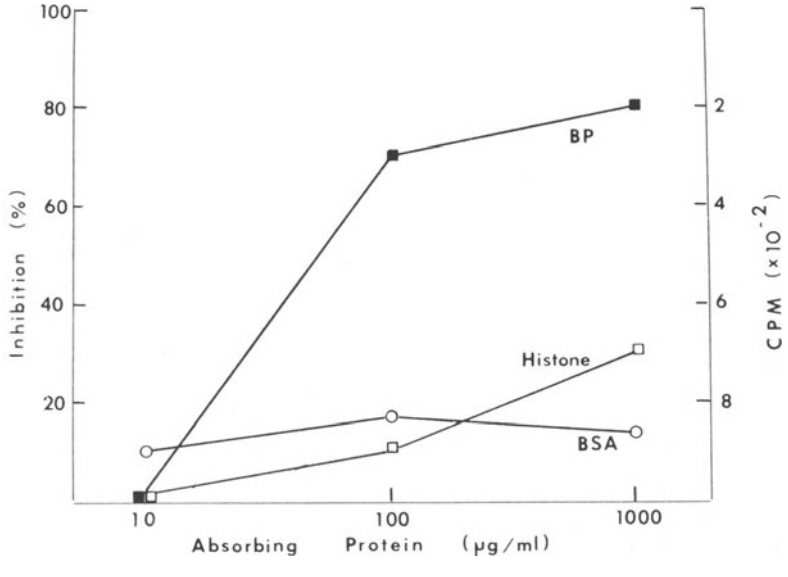

Fig. 3. Inhibition of MS CSF antibody by absorption of CSF with MBP. Unabsorbed CSF bound 900 CPM or 7.5% of added ^{125}I-SPA. No significant inhibitory effect by MBP or other proteins was seen with control CSF

Table 1. MS Patients: Clinical status and laboratory data

Patient (N=22)	Duration (years)	Clinical status			Cortico-steroids	IgG (mg/100 ml)	Oligoclonal bands	Anti-MBP RIA
		Acute	Prog.	Static				
P.K.	4	+			–	6.0	+	+
C.G.	1	+			–	6.5	+	+
B.F.	7	+			–	6.0	+	+
R.H.	13	+			–	5.4	+	+
S.V.	9	+			–	4.3	+	+
W.F.	2	+			–	12.9	+	+
M.V.	10	+			–	21.4	+	–
C.W.	27	+			+	12.0	+	–
L.P.	3		+		–	10.0	+	+
B.S.	6		+		+	5.4	+	+
P.S.	5		+		–	4.1	–	+
L.S.	5		+		–	6.7	–	+
A.D.	7		+		–	11.3	+	–
G.S.	25			+	–	11.7	+	+
J.T.	9			+	–	3.8	+	+
D.T.	19			+	–	13.8	+	+
L.C.	8			+	–	5.6	–	+
R.F.	1.5			+	–	5.6	+	–
J.H.	10			+	–	27.0	+	–
R.B.	29			+	–	10.4	+	–
R.B.	4			+	–	4.0	–	–
R.R.	26			+	–	2.3	–	–

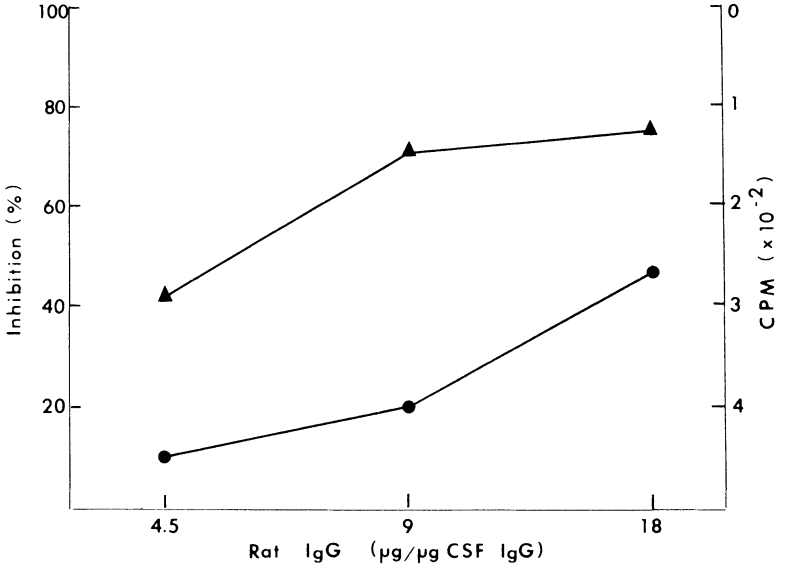

Fig. 4. Inhibition of MS CSF binding to MBP by blocking with hyperimmune rat IgG. MS CSF alone bound 554 CPM or 1.85% of ^{125}I-anti-human IgG

was two to three times higher. When compared with the presence of oligoclonal IgG bands, 11 of 17 patients with bands had antibody to MBP by RIA, while 3 of 5 without bands were positive. Similarly, there was no correlation with duration of disease.

When CSF was absorbed with immobilized anti-human IgG, removal of anti-MBP activity was directly proportional to the amount of IgG removed. With sufficient anti-IgG, 100% of the activity was abolished, indicating that all of the binding activity detected by the RIA was associated with IgG.

Incubation of CSF with MBP, calf thymus histone, or BSA prior to RIA resulted in absorption of over 70% of binding activity by MBP, but only 10 to 20% by histone or BSA (Fig. 3). In blocking experiments with hyperimmune rat IgG there was marked inhibition of CSF binding activity (Fig. 4).

Discussion

Two features of this study which may help to explain the ability to detect antibodies to MBP in contrast to previous negative studies [5, 10, 12, 22] are the sensitivity of the solid phase technique and the use of iodinated SPA in place of anti-human IgG. In preliminary studies we, like Randolph et al. [20], compared the solid phase assay with a sodium sulfate precipitation method for detection of antibodies to MBP [3] and found it to be much more sensitive both with sera from immunized rats and with human CSF. Although antibody could be detected in some MS fluids using ^{125}I-anti-human IgG, ^{125}I-SPA gave consistently higher values. It should be noted that binding activity of human CSF was low in comparison with immune rat sera which bound up to 35% of ^{125}I-anti-rat IgG in this system. A further increase in sensitivity might have been seen if human MBP had been used as antigen. Extensive homology exists among basic proteins from many species with respect to their antigenic activity [19], however, the extent of cross-recognition of anti-human MBP with guinea pig MBP has not been documented. Some cross-reactivity was apparent in this study, yet the use of human MBP might yield even more striking results.

Although we allowed for nonspecific binding of IgG to MBP by comparing MS and control CSF at equivalent IgG concentrations, it is possible that nonimmunoglobulin constituents of CSF may have interfered with the assay to some extent. It has been shown, for example, that alpha-2 macroglobulin in rat, rabbit, and human sera [15, 16] and in human CSF [10] bind to MBP. However, alpha-2 macroglobulin is not detected by SPA, which reacts only with immunoglobulins, principally IgG [9].

The specificity of the reaction for MBP was documented by absorption of antibody activity with MBP, but not by other proteins, and by showing that rat antibody to MBP would block the activity of MS CSF. Moderate blocking activity seen at high concentrations of normal rat IgG was probably related to nonspecific adherence of IgG to MBP. Use of F_{ab} or $F_{(ab)_2}$ fragments of hyperimmune and normal IgG should result in a lower level of nonspecific blocking.

The studies presented here strongly suggest that the activity measured in MS CSF is IgG antibody directed against MBP. Since MBP itself is found in the CSF of

MS patients, it is plausible that antibodies to it might be present as well. Moreover, it is possible that a portion of the antibody is bound to antigen in the form of immune complexes, which may account for the low activity observed in some patients and perhaps for the inability of previous investigators to detect it. The presence of anti-MBP antibodies in MS tends to strengthen the hypothesis that autoimmunity is a feature of the disease, though its role in pathogenesis remains unknown.

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Multiple Sclerosis De Novo Central Nervous System IgG Synthesis: Measurement, Antibody Profile, Significance, Eradication, and Problems

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Abstract

Three independent means may be used to detect and measure de novo central nervous system (CNS) IgG synthesis: the application of an empirical formula, the results of an IgG isotopic test, and the analysis of cerebrospinal fluid (CSF) for IgG oligoclones. The empirical formula and the isotope test provide a quantitative measure of the synthesis rate in mg per day. In contrast, the detection of CSF IgG oligoclones, presently a qualitative test, supports the presence of de novo CNS IgG synthesis, providing the serum does not contain IgG oligoclones and the blood-brain barrier (BBB) to protein is not excessively damaged.

Based on facts that (1) de novo CNS IgG synthesis (determined by measuring production rates and analyzing CSF oligoclonal bands) occurs in over 90% of patients classified as having clinically definite multiple sclerosis (MS); (2) that myelinotoxic factors, presumably antibodies, have been detected in CNS extracts and CSF; and (3) that antibodies to myelin basic protein have been detected in the CSF, it is hypothesized that de novo CNS IgG synthesis is related to the production of MS lesions. Accordingly, clinical studies have been designed to eradicate de novo CNS IgG synthesis in MS patients. Results showed that steroids administered systemically and intrathecally modulated or normalized the rate of CNS IgG synthesis whereas cytosine arabinoside given intravenously or intrathecally did not. Gamma radiation to the CNS (150 rads per day for 12 days) enhanced synthesis modestly, but the effect could not be blocked by steroid treatment. None of the putative treatments eradicated CSF oligoclonal bands, even though by inspection they were modulated. The neurologic functions of the severely afflicted patients who participated in these studies were unchanged. Problems concerning the relevance of de novo CNS IgG synthesis to lesion formation and concerning its eradication as a test of an effective treatment are pesented along with possible solutions.

Introduction

The purpose of this report is to present current concepts of de novo CNS IgG synthesis in MS patients after reviewing its scientific basis and discussing three independent methods of detection. Because de novo CNS IgG synthesis is present in

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over 90% of clinically definite MS patients, its absence should cast some doubt on the diagnosis of MS.

The antibody nature of MS de novo CNS IgG synthesis is also reviewed. Virus antibody studies are difficult to interpret because methods used to determine de novo CNS antibody synthesis are qualitative. Many cases of MS show an abnormally high titer for more than one viral antibody and not all cases show the same viral antibody rise. These results may be explained by the hypothesis that preprogrammed B cells are recruited nonspecifically to the multifocal sites of the MS CNS immune reaction and accordingly, presence of virus in the CNS is not necessary.

Because a myelinotoxic factor, presumably antibody, and antibodies to myelin basic protein in CSF and CNS have been reported, it is reasonable to assume that de novo CNS IgG synthesis is related to lesion formation. Accordingly, we have designed clinical trials to eradicate de novo CNS IgG synthesis. These clinical trials are briefly reviewed. After interpreting results to date, remaining problems are stated and possible solutions are presented.

Scientific Basis of De Novo CNS IgG Synthesis

The predominant characteristic of the CSF profile indicative of MS is an elevated IgG value. Various hypotheses have been postulated regarding the source of the increased IgG in MS. Evidence from studies done by many laboratories [31–36, 43] suggest that the central nervous system–cerebrospinal fluid (CNS-CSF) compartment, surrounded by its BBB to protein, can perform as an immunologic unit and can synthesize IgG independently of the systemic immune system [37, 38]. This is termed de novo central nervous system IgG synthesis [36]. In health, the source of CNS-CSF IgG is exclusively the blood. The blood is also a source of CNS-CSF IgG when there is damage to the BBB. (In this case, the elevated IgG concentration is accompanied by a proportional elevation in albumin [1]. It is postulated that in MS extra and perivascular lymphoplasmyocytes synthesize IgG within the CNS in multifocal regions, probably at edges of active plaques of demyelination [9, 24, 28, 39]. After IgG is secreted into the extravascular space of the CNS by these immune cells, it sinks by diffusion into the CSF, a fast moving (5 dl/day) lacuna of CNS extracellular space, to be mechanically transported into blood via macropinocytes of the arachnoid villi. With provisos, the presence of CSF oligoclonal IgG may serve as evidence for MS de novo CNS IgG synthesis [36].

Detection and Quantification of De Novo CNS IgG Synthesis

Three means may be used to detect and quantify daily de novo CNS IgG synthesis in MS: (1) an empirical formula based on physiological principles governing the

1 Albumin, a natural marker of the BBB, is synthesized and secreted into the blood only by the liver. It is maintained in the blood at a uniform concentration in both health and disease [31, 32]. Albumin is perfused by the circulatory system throughout the CNS vasculature. Like IgG, albumin is excluded from certain areas by the tight junction of the vasculature. In certain capillary beds, however, in particular those of the choroid plexus, where the tight junctions do not exist, a small amount of albumin and IgG filters into the CNS-CSF

passage of albumin and IgG across the BBB; (2) a radioactive IgG exchange test; and (3) identification and analysis of CSF IgG oligoclones through electrophoresis.

The Empirical Formula

An empirical formula [35, 43] has been devised to calculate the amount of de novo CNS IgG synthesized daily in MS based on theory of the sink action of IgG into the CSF and the operation of the BBB to proteins. The method is based on assumptions that the excess albumin in the CSF is a quantitative marker of a damaged BBB and that for each mole of excess albumin which passes the damaged barrier, one mole of IgG diffuses into the CSF, as well as on knowledge that 5 dl of CSF are formed and absorbed each day in humans.

With the empirical formula method, the amount of excess IgG is first determined, then the value is corrected for the amount of serum IgG which has passed the BBB. Using the formula shown below, a value is derived which, if it exceeds the normal mean plus 2 standard deviation value, reflects synthesis of IgG in the CNS.

$$\text{de novo CNS IgG}_{SYN} = \left[\left(\text{IgG}_{CSF} - \frac{\text{IgG}_S}{369}\right) - \left(\text{Alb}_{CSF} - \frac{\text{Alb}_S}{230}\right)\left(\frac{\text{IgG}_S}{\text{Alb}_S}\right) 0.43\right] \times 5$$

Where de novo CNS IgG_{SYN} represents the IgG in mg per day; IgG_{CSF} is the concentration (mg per dl) found in the patient's CSF, and IgG_S is the patient's serum IgG concentration (mg per dl). The number 369 is a ratio constant which quantitatively determines the proportion of CSF IgG which normally passes by filtration from the serum into the CSF across the intact BBB [32, 35]. It is the quotient of the average normal serum IgG concentration divided by the average normal CSF IgG concentration. Thus, $IgG_S/369$ is the IgG which is expected to cross from the serum to the CSF based on the patient's serum IgG concentration and the normal CSF/serum ratio.

Alb_{CSF} is the albumin concentration (mg per dl) found in the patient's CSF, while Alb_S is the patient's serum albumin concentration (mg per dl). The number 230 represents a constant which determines the proportion of CSF albumin which normally passes by filtration from the serum into the CSF across an intact BBB [32, 35]. It is the quotient of the average normal serum albumin concentration divided by the average normal CSF albumin concentration. Thus, $Alb_{CSF}-(Alb_S/230)$ is a term which represents the excess CSF albumin which has crossed a damaged BBB.

This term is then multiplied by $(IgG_S/Alb_S) \times (0.43)$ to convert the excess CSF albumin to excess CSF IgG (on a molar basis) [1] which has crossed a damaged BBB with the albumin, assuming a one-to-one molecular equivalence [32, 35]. To calculate the daily IgG synthesis rate, this entire equation is then multiplied by five to convert from concentration in mg per dl to the amount present in 5 dl which, on the average, is formed each day [35].

This empirical formula was used to calculate the synthesis rate in normal individuals from whom the ratio constants for IgG and albumin were derived. A gaussian

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1 $\frac{\text{Albumin}}{\text{IgG}} = \frac{69{,}000 \text{ daltons}}{160{,}000 \text{ daltons}} = 0.43$ [25]

distribution was obtained and the average synthesis rate was minus 3.3 mg per day, with a 95% confidence limit of – 9.9 to + 3.3 [35].

Radioactive IgG Exchange Test

A second method for detecting and quantifying the daily de novo CNS IgG synthesis rate in MS is the isotope exchange test where radiolabeled IgG is injected into the blood and at steady state, about the 5th day, it is possible to calculate the mg of IgG which originates from the CNS [35, 43]. Direct tracer studies employing intravenous ^{131}I-IgG have been carried out in 9 clinically definite MS patients to validate the empirical formula [35, 43] described above. Using serial CSF and serum samples obtained simultaneously at multiple points over a 21-day period, dynamic equilibrium was determined by graph and the amount of IgG in mg per day originating from an extravascular source (presumably the CNS) was calculated following equilibrium. Daily production of CSF extravascular IgG as calculated by the empirical formula was compared with results from the radiolabeled IgG exchange test. The concordance for the two methods from a low rate of synthesis (approximately 5 mg/day) to a very high rate (120 mg/day) was excellent [35, 43]. A second double radiolabeled IgG study was carried out on two patients to further validate the formula and to determine if the MS BBB processes normal serum IgG differently from IgG derived from autologous IgG sera. Results showed that the MS BBB processed normal serum IgG in the same way as IgG derived from autologous MS serum [43]. Accordingly, the empirical formula, which requires only one sample of CSF and matched serum, can reliably and validly estimate de novo CNS IgG synthesis rate in MS.

Identification and Analysis of CSF IgG Oligoclones

A third method for detecting de novo CNS IgG synthesis in MS is identification and analysis of CSF IgG oligoclones. Either Panagel or agarose gel electrophoresis may be used; however, the conventional agarose gel method [17] has disadvantages. CSF must be concentrated several hundred times and it is not possible to distinguish between IgG subfractions and other electrophoretically similar non-IgG proteins (i. e., gamma and beta trace proteins). Panagel electrophoresis, which is 20 times more sensitive than conventional agarose electrophoresis, involves application of 3 µg of IgG followed by electrophoresis, then immunofixation by layering a monospecific IgG antiserum over the separated proteins, followed by staining with Coomassie blue. Preliminary concentration of CSF is not required when the IgG concentration exceeds 10 mg per dl. When the IgG concentration is less than this value, concentration by ultrafiltration in collodion bags is necessary (average recovery of IgG is 90%). Multiple applications of as many as five 3 µl samples of neat CSF, totaling 3 µg of IgG, have been found to be equivalent to preliminary concentration. The addition of IgG immunofixation validates cathodic proteins as being IgG. Using Panagel electrophoresis, from 4 to 10 IgG oligoclones have been identified in CSF from MS and subacute sclerosing panencephalitis (SSPE) patients [6, 18].

To study the association of the daily rate of de novo CNS IgG synthesis with the presence of CSF IgG oligoclonal bands [20], paired CSF, and serum samples from patients with MS and with other neurologic disorders were electrophoresed in Panagel. The albumin and IgG concentrations from which CNS IgG synthesis was calculated were measured by electroimmunodiffusion [40]. An abnormal BBB was defined by a CSF albumin $\geqq$ 35 mg per dl (mean + 2 standard deviations). Results showed a significant positive correlation between CNS IgG synthesis and the presence of CSF oligoclones in all patients with a normal BBB or a modestly abnormal BBB (albumin $\leqq$ 130 mg per dl). A lack of correlation was found when damage to the BBB was more severe (albumin > 130 mg per dl); in this case oligoclones were never demonstrated, even though the formula indicated CNS IgG synthesis. It is likely that oligoclones do exist in the CSF when de novo IgG synthesis is detected, but are masked by serum polyclonal IgG which has passed the severely damaged BBB.

One caveat is in order when considering the association of CSF IgG oligoclones and de novo CNS IgG synthesis [21]: The presence of CSF IgG oligoclones is not always a sign of de novo IgG synthesis. If IgG oligoclones exist in the blood as they do in multiple myeloma, it is possible that similar electrophoretically separated IgG oligoclones are present in the CSF due to diffusion from blood to CSF. To minimize misinterpretation, it is recommended that a matched serum sample be analyzed.

Two other methods now being used to identify and measure CSF IgG oligoclonal bands are isoelectric focusing and capillary isotachophoresis. Kjellin and Vesterberg [15], Delmotte and Gonsette [8], and Laurenzi and Link [16] have applied isoelectric focusing to CSF gamma globulins. Findings indicate that isoelectric focusing may be more discriminative than conventional agarose gel electrophoresis.

Delmotte [7] has used capillary isotachophoresis to quantitate both CSF and serum IgG oligoclonal bands. Preliminary results showed oligoclonal fractions of IgG in the MS CSF that were not in sera, particularly in the cathodic IgG region. Confirmation of these results and application of a formula similar to the empirical de novo CNS IgG synthesis rate formula might permit a type of quantitation not yet available for CSF IgG oligoclonal bands.

Correlation of De Novo CNS IgG Synthesis and Clinically Definite Multiple Sclerosis

A variety of clinical parameters have recently been correlated with daily de novo CNS IgG production as determined by empirical formula for 70 normal subjects and 127 MS patients [36]. IgG production rate was more than 2 standard deviations above normal in 92% of patients with clinically definite MS as determined from criteria established by Schumacher et al. [27]. The average IgG production (mg per day) was 29, with a range of 0 to 207. IgG production rates were higher in the more acute cases of MS and tended to increase with age, with duration of disease, with number of relapses, and with elevated CSF leucocytes.

Analysis of CSF IgG oligoclones in the same group of MS patients showed frequency of banding corresponding to the rate of de novo CNS IgG synthesis. Our re-

sults with Panagel electrophoresis are in agreement with those of Johnson and Nelson [13] who also employed Panagel.

From these data, the absence of de novo CNS IgG synthesis in a case suspected of having MS on clinical grounds should cast some doubt on the diagnosis of clinically definite MS.

Antibody Nature of De Novo Synthesized CNS IgG

The usefulness of de novo CNS IgG synthesis measures as an aid to the clinician in diagnosing MS and monitoring the effect of putative therapies is well established (see following Sect.). However, a most important question is still to be answered. What are the specific antibodies which make up the IgG synthesized de novo in the MS CNS? Or, stated differently, are there antigens in the CNS which cause the multifocal MS CNS immune reaction to make antibodies? The answer to this question should reveal the etiology of MS.

Since Adams and Imagawa [1] showed increased measles antibody titers in serum and CSF from patients with MS, many investigators have implicated viral [5, 10, 11, 26] antigens in the CNS as the cause of de novo CNS IgG synthesis. It is our opinion that Arnadottir et al. [2] best present the current status of viral antibodies research in MS patients. Their design included a longitudinal study (5 years) of rubella, measles, and respiratory syncytial virus antibodies in serial serum and CSF specimens from 20 MS patients, using solid-phase radioimmunoassay. Albumin and immunoglobulin-G levels were also measured to check the integrity of the BBB and to detect de novo CNS IgG synthesis. All patients had IgG production in their CNS. In 15 patients, a local antibody production against one or more of the viruses studied was evident. In 8 patients, fluctuations in CNS viral antibody synthesis were evident. No correlation was found between these changes and the clinical course of the disease. They concluded that de novo antibody synthesis in MS is only partially against any given virus, and in most patients the bulk of the CSF IgG is against antigens other than those studied.

To further refine understanding of the antibody nature of MS CSF, Nordal, Vandvik and Norrby have reviewed the literature and introduced a sensitive qualitative imprint electroimmunofixation technique [22]. They found evidence of de novo CNS antibody synthesis, for one or more common viruses in a given patient, without a pattern; a result which is similar to studies mentioned above.

In 1978 [36] we offered the following hypothesis to explain local CNS synthesis of viral antibodies: Circulating immunocytes of the B-lymphocyte type, preprogrammed in the core immune network to common virus antigens through natural exposure or immunization, are recruited from the blood to the CNS by nonspecific chemotaxic mechanisms generated by a preexisting MS immune reaction. After arrival within the BBB, they develop into antibody forming cells and secrete antibody that they have previously been programmed to synthesize (see Fig. 1).

According to this hypothesis, the core immune system's response to antigenic challenge can be duplicated in the MS CNS without the presence of viral antigens; and the profile of de novo CNS IgG should contain the antibodies to common viru-

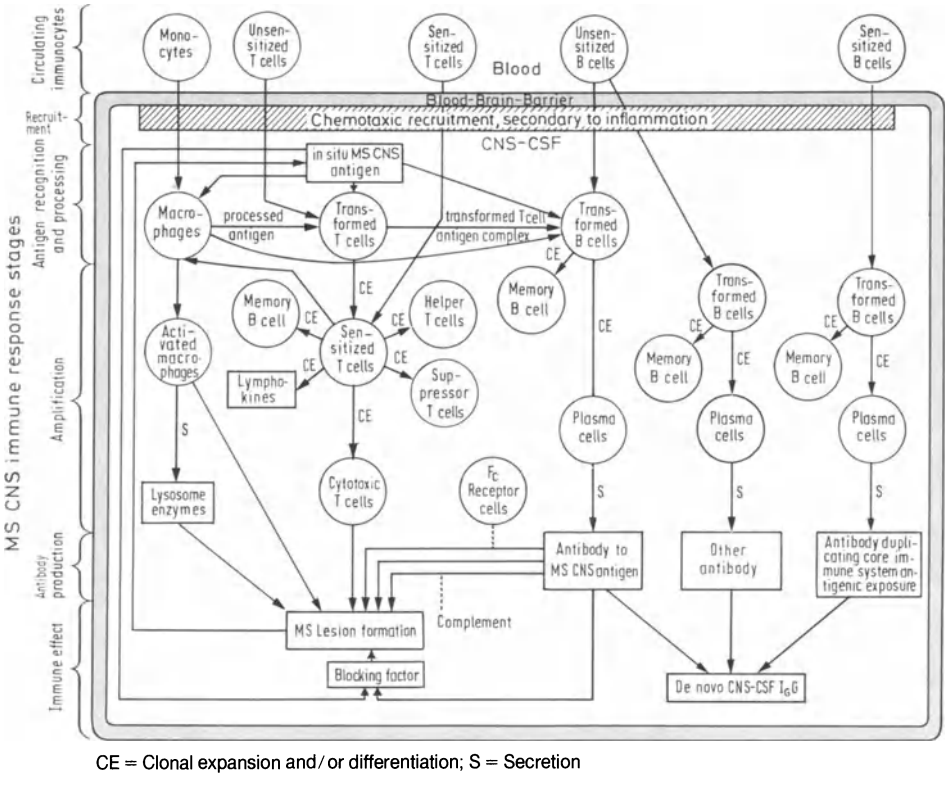

Fig. 1. Immunokinetic model for Multiple sclerosis. Circulating B cells, T cells, and monocytes are recruited from blood to CNS probably by nonspecific chemotaxic mechanisms generated by a pre-existing MS immune reaction. The self-perpetuating cycle involves cellular and humoral immune mechanisms, as well as hydrolase demyelination. Included in the cycle are: antigen recognition, monocyte-macrophage activation, lymphoid cell (B and T cells) interactions, B cell to plasma cell conversions generating autoimmune antibody, lysosomal enzymes capable of hydrolyse destruction of myelin, and antigen-antibody complexes possibly blocking destruction of myelin. In addition to the cycle are 2 epiphenomena pathways that may account for part of the IgG produced in the CNS-CSF of MS patients. First, after recruitment of circulating B cells which have been sensitized through natural exposure or immunization, antibodies may be synthesized in the CNS even when antigen is not present. Second, IgG may be produced through CNS synthesis of *other* antibody following chemotaxic recruitment of sensitized B cells

ses (such as measles, Epstein-Barr virus, mumps, influenza, vaccinia, or polio) as have been found in MS by other investigators. Thus, a portion of the de novo CNS antibody synthesis may be an epiphenomenon to the etiology of MS.

It is also hypothesized that another portion of the de novo CNS antibody profile is an autoantibody whose antigen is probably associated with the myelin sheath complex, thus associated with MS lesion formation. The scientific basis for the presence of in situ CNS autoantibody has evolved over the past decade. In 1970, Kim et al. [14] showed that concentrated CSF from MS patients, extracts from plaque, shadow plaque, and periplaque areas demonstrated a high degree of myelinotoxicity when applied to myelinated cultures of mouse cerebellum, whereas extracts of nor-

mal appearing white matter of MS patients, normal brain extracts, and normal concentrated CSF registered substantially lower toxicity. Since this myelinotoxic action was thermolabile (presumably complement dependent) and was correlated to IgG concentration, we suggested that antimyelin antibodies similar to those present in MS serum [4] exist in CSF and brain (especially plaque areas) of MS patients. Since that time Webster's group [30] and Link's group [29] have applied unconcentrated MS CSF to tadpole optic nerve system and confirmed myelinotoxic activity. Also Frick and Stickl [12] and Pantich et al. [23] have shown that antibodies to myelin basic protein exist in MS patient's CSF.

Many advances have been made in recent years to resolve the antibody nature of MS de novo CNS IgG synthesis. However, important problems remain. There is need to develop a technique to express antibodies in mg of protein, so that the empirical formula can be used to calculate mg of antibody synthesized de novo by the CNS. This has been done for SSPE [19]. If it could be done for MS, it would be possible to determine the percent of de novo CNS IgG synthesis which is due to antibodies to common viruses. Hence, the unaccounted for IgG, presumably due to the MS antigen located in the CNS, could be calculated. If the situation in MS is comparable to that in SSPE, between 50–70% of de novo CNS IgG synthesis should be accounted for by the MS antigen.

Use of De Novo CNS IgG Synthesis to Evaluate Putative Therapies

Based on facts that (1) de novo CNS IgG synthesis (determined by measuring production rates and analyzing CSF oligoclonal bands) occurs in over 90% of patients classified as clinically definite MS; (2) that myelinotoxic factors, presumably antibodies, have been detected in CNS extracts and CSF [4, 14, 29, 30]; and (3) that antibodies to myelin basic proteins have been detected in the CSF [12, 23], it has been proposed that de novo CNS IgG synthesis is related to the production of MS lesions. Accordingly, clinical studies have been designed to attempt to eradicate de novo CNS IgG synthesis in MS patients.

Three studies have been completed to date [42, 44, 45, 46]. Results showed that steroids administered systemically and intrathecally modulated or normalized the rate of CNS IgG synthesis [3, 44] whereas cytosine arabinoside given intravenously or intrathecally did not [46]. Gamma radiation to the CNS (150 rads per day for 8 or 12 days) at 1800 rads enhanced synthesis modestly, which could not be blocked by steroid treatment, whereas at 1200 rads there was suppression [41, 45]. None of the putative treatments eradicated CSF oligoclonal bands, even though by inspection they were reduced in intensity. Clinically, the neurological function of the severely afflicted patients who participated in these studies was unchanged.

From our clinical trials so far, it appears likely that a mature nonreplicating cell in the CNS, such as a plasma cell, is responsible for MS de novo CNS IgG synthesis. This hypothesis is based on the fact that the cytotoxic effect of and cytosine arabinoside is to inhibit the cell reproductive cycle by preventing DNA synthesis. Therefore, since this putative treatment had no significant effect, it is warranted to conclude

that DNA synthesis is not necessary for de novo CNS IgG synthesis. The interpretation is in concordance with histological observations that plasma cells, non-replicating cells, exist in the MS CNS and that mitoses are never seen in the MS CNS immune reaction [24]. Since a recognized effect of high dose steroids on the immune reaction is an ability to block IgG synthesis, subsequent clinical trials will include drugs such as lomustine which inhibit protein synthesis or RNA synthesis, and readily pass the blood-brain barrier [12 A].

Thus, de novo CNS IgG synthesis rates, as expressed by empirical formula and corroborated by electrophoretic analysis of CSF oligoclonal IgG, can be used as measures to follow the effect of putative therapies, especially if MS is an autoimmune in situ CNS disease. On the other hand, we also believe, that de novo CNS IgG synthesis can also be used to follow putative therapies if MS is a viral disease. If the treatment were a virocidal agent and the CNS antigen load were decreased, the CNS immune reaction would be expected to attenuate, as manifested in part by a decrease in de novo CNS specific viral antibody synthesis.

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Evaluation of the Blood/CSF Permeability Coefficients and of the Intrathecal Synthesis of IgG by Capillary Isotachophoresis

P. DELMOTTE [1]

Although a considerable amount of time and effort has been spent on the study of the immunological response on the cellular level in multiple sclerosis (MS), no clear-cut pattern has emerged until now. The difficulties in standardizing the cellular tests and the overlapping of results between MS and other neurological diseases have prevented their use for diagnostic purposes.

However, it is a well established fact that the qualitative and quantitative study of the humoral immunological response within the central nervous system (CNS), remains, until now, one of the most important parameters for the diagnosis of inflammatory diseases of the CNS, and this is especially true for MS [5].

For this purpose, several techniques have been proposed and used: zone electrophoresis, immunoelectrophoresis, rocket electrophoresis, and isoelectric focusing, to name only some of them.

During the last 2 years, we have been experimenting with capillary isotachophoresis of the serum and cerebrospinal fluid (CSF) proteins. We have been able to show that this new electrophoretic technique offers some interesting advantages: (1) exactly controlled working conditions lead to very reproducible results; (2) protein fractions are detected by their UV absorbance under dynamic equilibrium conditions; and (3) peak areas of separated protein fractions are a direct measure of the absolute amount of protein present.

A detailed description of the entire experimental setup is beyond the scope of this presentation. For technical details see Delmotte [1].

The lower limit of detection lies around 10 ng of protein.

Unconcentrated CSF can be used, but its high salt content adversely influences the separation results.

We concentrate the CSF about 10 times and 4 µl of this concentrate are injected. For serum, only 0.6 µl are used.

Figure 1 shows the isotachophoretic separation patterns of the serum and CSF of the same normal individual. By manipulation of the composition of the spacer mobility gradient, one can get a clear-cut separation of albumin and at the same time a mobility subfractionation of the immunoglobulin G fraction. The non-UV absorbing zones are due to amino acids injected together with the sample. As already mentioned, the integrated peak areas are a direct measure of the absolute amount of protein present in a zone.

By taking into account the injected volumes of serum and CSF, and also the concentration factor, blood/CSF permeability coefficients can be calculated using the integrated peak surfaces.

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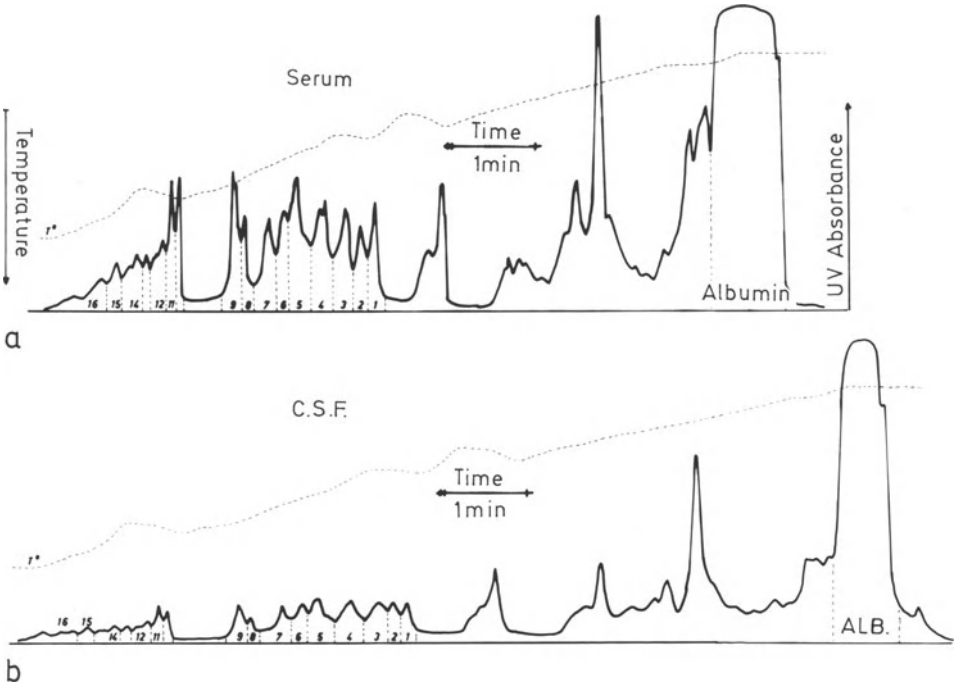

Fig. 1. **a** Capillary isotachophoresis of serum. **b** Idem of CSF from the same individual

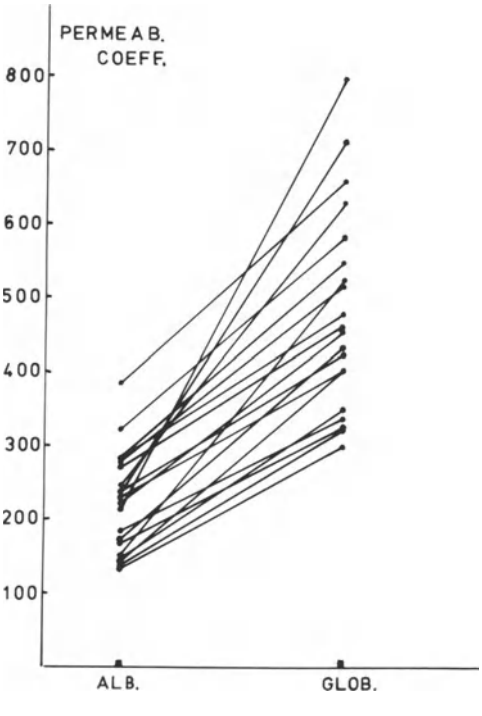

Fig. 2. Blood/CSF barrier permeability for albumin and IgG from 20 normal individuals as obtained by capillary isotachophoresis

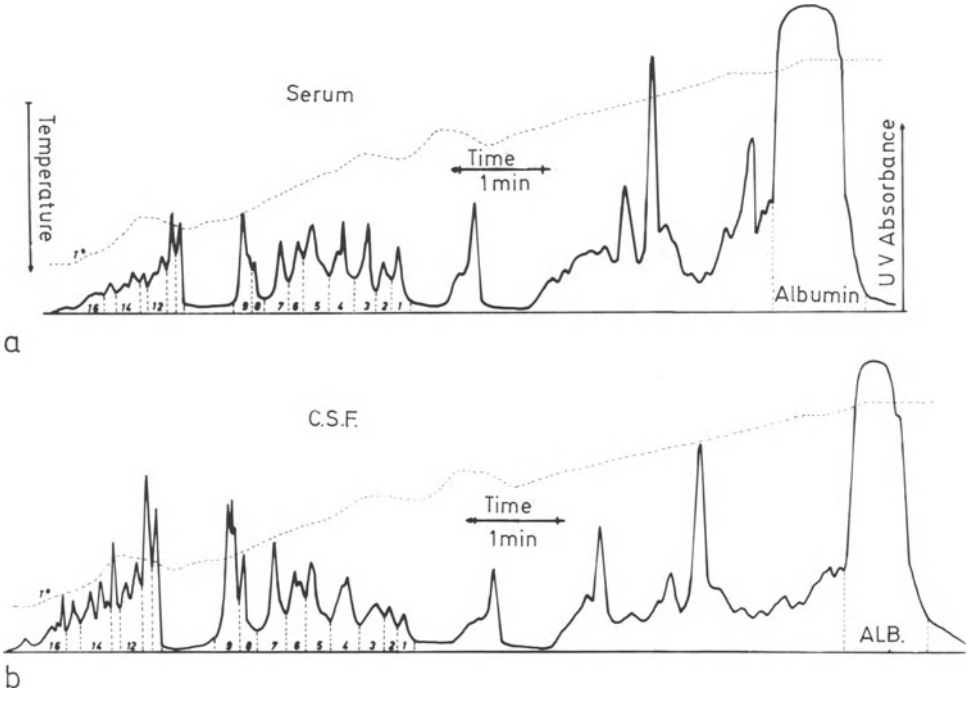

Fig. 3. **a** Capillary isotachophoresis of serum from a MS patient. **b** Idem of CSF from same patient

The results obtained for a group of 20 normal individuals are presented in Figure 2. The mean and range of the permeability coefficients for albumin and for total IgG correlate well with results obtained by immunological determination of the same fractions [2, 5].

The most important feature of capillary isotachophoresis lies in the fact that the heterogeneous population of immunoglobulin G molecules, with isoelectric points ranging from around pH 6.8 to 8.6 can be reproducibly subfractionated in about 15 fractions. This subfractionation is based solely on differences in electrophoretic mobility and the delimitation of the fractions depends exclusively on the composition of the spacer mobility gradient used.

The integration of the peak surfaces of the individual immunoglobulin subfractions in serum and CSF permits us to calculate the barrier permeability for all of these subfractions. Considering the fact that all immunoglobulin G molecules have the same molecular size, it is evident that these permeability coefficients must be nearly the same and of the same value as calculated for the total IgG's. All capillary isotachophoretic determinations carried out on serum and CSF of normal individuals have confirmed this observation.

The situation is entirely different when serum and CSF of patients suffering from certain neurological disorders are submitted to the same experiments. Figure 3 shows the separation patterns of serum and CSF from a typical MS patient. As might be expected from the overall composition of the two fluids and from the fact

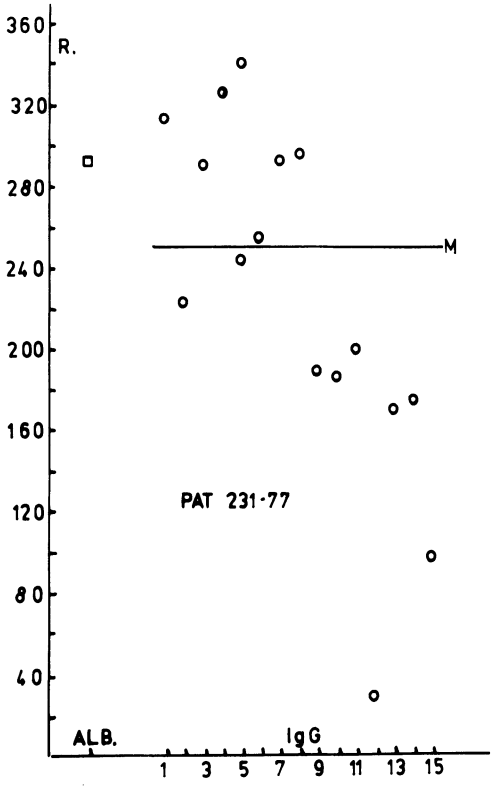

Fig. 4. Blood/CSF barrier permeability for albumin and IgG subfractions from a MS patient

that the same mobility spacer gradient is used, the qualitative aspect of the two patterns is grossly the same.

However, the integration of the individual immunoglobulin subfractions brings to light some striking differences. The permeability coefficients for the fastest moving IgG subfractions were exactly as could be expected from the permeability coefficient calculated for albumin. But, as can be seen from Figure 4, most, but not all, of the slower moving fractions show dramatically lower values for the permeability coefficients. The open square is the value for albumin and the open circles are the values for the IgG subfractions. The permeability coefficient for total IgG is indicated by the line.

In this case, the value for total IgG left no doubt about the presence of intracerebrally synthesized IgG. However, it is well to draw attention to the striking differences in values for the permeability coefficients for the different IgG subfractions.

An entirely different picture is shown in Figure 5. Here, the permeability coefficient calculated for total IgG was about twice the value calculated for the albumin fraction: as this result fell within the range of values found for normal individuals, there was absolutely no evidence for intrathecal synthesis of IgG.

However, the permeability values of the IgG subfractions leave no doubt in this case. The fastest moving fractions have values in accordance with the results found for albumin, and also well above the value calculated for total IgG. But for the slower moving fractions, the values are dramatically different and leave not the slightest doubt about the intrathecal synthesis of IgG.

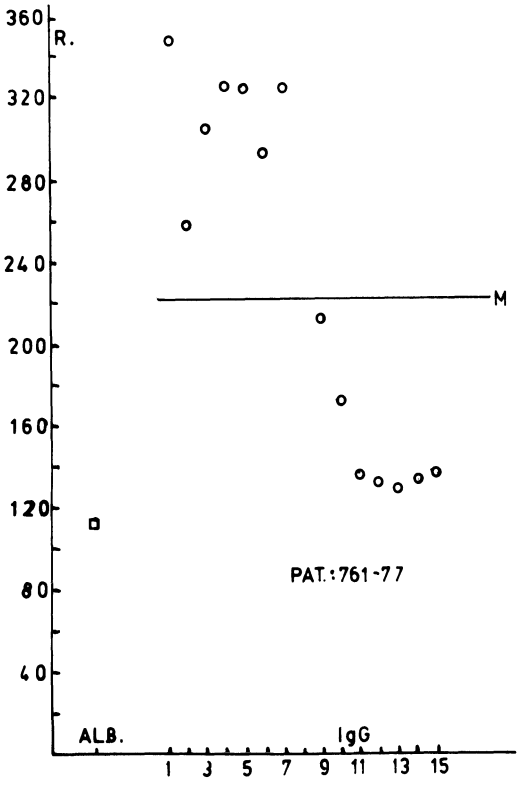

Fig. 5. Blood/CSF barrier permeability for albumin and IgG subfractions from a MS patient

The same quantitative approach can be used to estimate the amount of intrathecally synthesized IgG. Instead of using as a baseline value the permeability coefficient of albumin, we now can use as a baseline value the mean permeability coefficient of the IgG subfractions which have the highest permeability coefficient [4].

For a group of about 70 clinically confirmed cases of MS we found in most cases a percentage of intrathecally synthesized IgG of between 20 and 40% of total IgG, with cases reaching values as high as 65%.

Even in cases with very low total CSF protein and having at the same time a low percentage of IgG, capillary isotachophoresis can detect local synthesis of IgG.

Isoelectric focusing in thin layers of acrylamide of the CSF IgG's, has proven to be the most sensitive method for detecting the presence of oligoclonal IgG fractions in CSF. Parallel studies between isoelectric focusing and capillary isotachophoresis have shown a 100% correlation between these two methods for the detection of local IgG synthesis. Capillary isotachophoresis has the added advantage of speed and quantitation.

In conclusion we can state the following: (1) Capillary isotachophoresis gives not only an overall qualitative picture of the protein composition of serum and CSF, but the separation pattern can be quantitatively interpreted. (2) Total analysis time for one individual takes only 1 h; (3) The reproducible subfractionation of the IgG's gives not only very interesting information for diagnostic purpose, but at the same time has brought additional strong evidence for the intracerebral synthesis of IgG's in some neurological diseases, especially MS.

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CSF-Oligoclonal Bands in Multiple Sclerosis

R. M. SCHMIDT and V. NEUMANN [1]

Summary

The present paper is concerned with a correlation between clinical parameters of multiple sclerosis (MS) and different agar gel electrophoresis patterns in cerebrospinal fluid (CSF). Agar gel electrophoresis clearly represents the most sensitive method for the demonstration of oligoclonal bands in CSF.

The determination "oligoclonal aspect" has been proposed by Laterre [5]. By this term he designated the discontinuous course of the γ-globulin zone and the existence of several γ-fractions in agar gel electrophoresis.

In this paper we shall discuss the following points:

1. The frequency of CSF γ-globulin fractions and augmentation of the γ-globulin bands in relapsing, chronic-progressive, benign MS and in the late form of polysclerosis with special consideration of age and sex.
2. The frequency of CSF γ-globulin changes in relation to exacerbation and interval of MS. This paper is an attempt to prove that the agar gel electrophoresis of CSF is the most useful method for establishing the diagnosis of MS.

Materials and Methods

Two-hundred and twenty-five MS cases were classified as clinically confirmed, whereas the benign forms were considered as probable. The diagnosis of MS was made according to Bauer et al. [1] and Poser et al. [11].

Agar Gel Electrophoresis

Five ml of CSF were concentrated 500 times by means of negative-pressure ultrafiltration, using the commercial collodium bags from Membranfilter Gesellschaft, Göttingen. Electrophoresis of the concentrates was carried out in agar gel according to Wieme [18].

Statistical calculations for the different groups were done using an f-test. Corresponding to the resulting values, we employed the empirical or the approximative t-test. In the individual groups, the calculations were made with the actual values obtained. If we did not obtain a separation of the fractions, we reduced the total number of patients and did not set the omitted value equal to zero. The calculating evaluation was done using a computer of the type R 300.

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Frequency of CSF γ-Globulin Fractions and Augmentation of γ-Globulin Bands

We classified the MS patients into relapsing, chronic-progressive, benign, and late forms, and formed groups of 20 to 30 year-old males and females, 30 to 40 year-old males and females, and over 40 year-old male and female patients.

Table 1 shows the frequency and the augmentation of the CSF γ-globulin fractions from 110 relapsing MS patients, taking into consideration age and sex. In 100% of the cases we found the appearance of the γ_3-, in 81% the appearance of the γ_2-, and in 87% of the cases the γ_4-globulin fractions. An augmentation of the γ_3-globulin bands was observed in about ⅔ of the cases in the various age groups. The γ_2- and γ_3-globulin fractions were augmented in more than 50% of the cases on the average. Within the relapsing MS group, the demonstrable differences of the above values due to age and sex are very small, but since the groups were so small, further statistical evaluation was impossible.

Table 2 gives a survey of the frequency of occurrence and augmentation of CSF γ-globulin fractions in 75 chronic-progressive forms of MS with respect to age and sex. Altogether, we found in 99% of the cases γ_3-, in 90% γ_2-, and in 91% γ_4-globulin fractions. The γ_3-globulin band was increased in over 50% of the cases, the γ_2 globulin band in less than 50%, and the γ_4-globulin band in more than ⅔ of the cases. In the benign form of MS and in late polysclerosis there was no possibility of grouping according to age and sex. We found the γ_3 fraction in almost all the cases, the γ_2-band in 85%, and the γ_4 fraction in 55–65% (Table 3). In ⅔ of all the cases, one can see an augmentation of the γ_3-globulin band. The γ_2-globulin band was augmented in 50% of the cases, and the γ_4-globulin fraction in ⅔ of the cases.

In relapsing MS, the distribution of the CSF γ_2-, γ_3-, and γ_4-globulin bands with special consideration of age and sex is shown in Table 4. In almost half of the cases

Table 1. Frequency and augmentation of the CSF γ-globulin fractions from relapsing multiple sclerosis patients considering age and gender

Relapsing sclerosis multiplex			Frequency %		Augmentation	Fraction														
age (years)	Gender	Number of patients	γ_1			γ_1'			γ_2			γ_3			γ_4			γ_5		
20 to 30	♀	20	18	90	6	18	90	7	17	85	13	20	100	17	19	95	14	12	60	6
20 to 30	♂	20	18	90	6	17	85	5	16	80	8	20	100	13	17	85	10	9	45	6
30 to 40	♀	20	17	85	5	16	80	6	16	80	11	20	100	16	15	75	10	12	60	8
30 to 40	♂	20	10	90	–	17	85	6	17	85	10	20	100	12	18	90	11	8	40	5
Over 40	♀	20	19	95	6	17	85	8	15	75	12	20	100	17	17	85	10	15	75	8
Over 40	♂	10	8	80	1	10	100	6	9	90	6	10	100	7	9	90	7	5	25	3
Total		110	98	89	24	95	87	38	90	81	60	110	100	82	95	87	62	61	36	55

Table 2. Frequency and augmentation of the CSF γ-globulin fractions from chronic-progressive MS patients considering of age and gender

Chronic-progressive sclerosis multiplex			Frequency % Augmentation			Fraction														
Age (years)	Gender	Number of patients	γ_1			γ_1'			γ_2			γ_3			γ_4			γ_5		
20 to 30	♀ u. ♂	6	5	83	–	6	100	1	6	100	5	6	100	3	4	67	1	3	50	3
30 to 40	♀	20	19	95	1	19	95	5	16	80	11	20	100	18	18	90	16	15	75	6
30 to 40	♂	9	8	89	1	9	100	1	8	89	3	8	89	6	7	78	4	1	11	1
Over 40	♀	20	18	90	3	14	70	5	19	95	14	20	100	18	19	95	14	15	75	9
Over 40	♂	20	18	90	4	20	100	11	18	90	10	20	100	17	20	100	13	12	60	3
Total		75	68	91	9	68	91	23	67	90	43	74	99	62	68	91	48	46	61	22

we found an augmentation of the γ_2-, γ_3-, and γ_4-globulin fractions. A small accentuation of the increase of the γ_2-, γ_3-, and γ_4-globulins can be seen in the group of 20 to 30 year-old patients.

In the chronic-progressive forms of MS, the distribution of the CSF γ_2-, γ_3-, γ_4-globulin fractions, inclusive of the γ_3- and γ_4-globulin bands for the 30 to 40 year-old group of older patients is very similar (Table 5). On the average they are augmented in half of the cases with an accentuation of the female sex. In a statistical comparison of 16 different age and sex groups of the above-mentioned courses of MS, we could, with the help of the approximative or empirical *t*-test, observe significant results in the γ_1-, γ_2-, and γ_3-globulin bands of the relapsing and chronic-progressive

Table 3. Frequency and augmentation of the CSF γ-globulin fractions in the benign and late form of sclerosis multiplex

Benign sclerosis multiplex		Frequency % Augmentation			Fraction															
Number of patients	Age	γ_1			γ_1'			γ_2			γ_3			γ_4			γ_5		
20	♀ u. ♂	19	95	5	17	85	4	17	85	12	19	95	17	13	65	8	11	55	6
Late form of sclerosis multiplex																				
number of patients	Age	γ_1			γ_1'			γ_2			γ_3			γ_4			γ_5		
20	♀ u. ♂	16	80	1	14	70	5	17	85	9	20	100	14	12	60	6	11	55	8

Table 4. Distribution of the CSF γ_2-, γ_3- and γ_4-globulin bands in relation to age and gender in relapsing sclerosis multiplex

Age	Gender	number	No. fraction path	only γ_2	only γ_3	only γ_4	$\gamma_2+\gamma_3$	$\gamma_2+\gamma_4$	$\gamma_3+\gamma_4$	$\gamma_2+\gamma_3+\gamma_4$
20 to 30	♂	20	3	1	0	0	0	0	2	10
	♀	20	1	0	0	0	0	0	0	12
	Σ	40	4	1	0	0	0	0	2	22
30 to 40	♂	20	2	0	1	1	2	0	0	6
	♀	20	0	0	2	1	0	0	0	8
	Σ	40	2	0	3	2	2	0	0	14
Over 40	♂	12	2	0	0	0	0	0	0	5
	♀	20	0	1	0	0	0	0	0	8
	Σ	32	2	1	0	0	0	0	0	13
Σ	♂	52	7	1	1	1	2	0	2	21
	♀	60	1	1	2	1	0	0	0	28
	Σ	112	8	2	3	2	2	0	2	49

Table 5. Distribution of the CSF γ_2-, γ_3- and γ_4-globulin bands in relation to age and gender of chronic-progressive sclerosis multiplex

Age	Gender	Number	No. fraction path	Only γ_2	Only γ_3	Only γ_4	$\gamma_2+\gamma_3$	$\gamma_2+\gamma_4$	$\gamma_3+\gamma_4$	$\gamma_2+\gamma_3+\gamma_4$
30 to 40	♂	10	2	1	1	0	1	0	2	2
	♀	20	1	0	0	0	2	0	6	9
	Σ	30	3	1	1	0	3	0	8	11
Over 40	♂	20	2	0	3	0	2	0	5	7
	♀	20	0	0	3	1	1	0	1	12
	Σ	40	2	0	6	1	3	0	6	19
Σ	♂	30	4	1	4	0	3	0	7	9
	♀	40	1	0	3	1	3	0	7	21
	Σ	70	5	1	7	1	6	0	14	30

forms. These differences especially concern the patients aged 30 to 40 years and the over 40 group. A representation of the individual results would trespass the limits of this paper. A publication on this subject will be prepared.

To summarize, it must be said that the agar gel electrophoretic γ-globulin spectrum with respect to age and sex does not show statistically significant differences between the relapsing, chronic-progressive, benign, and late forms of MS, even if only significant differences could be found in individual comparisons of the different age and sex groups.

Frequency of CSF γ-Globulin Changes in Relation to Exacerbation and Interval of Multiple Sclerosis

In a further investigation group, we compared the CSF γ-globulin bands of 20 patients during exacerbation and in the interval stage. Here, no significant differences in the γ-globulin bands could be detected, although reduced γ-globulin values could be observed in individual cases during the exacerbation. This finding of lowered relative CSF γ-globulin concentrations in relation to exacerbations of MS may be due to increased consumption or to a temporary blockade in synthesis.

In MS patients over 40 years old, especially in females, the γ_2–γ_4-globulin values, for instance, in the chronic-progressive course, show a slightly increased quantitative and qualitative aspect, as can be seen from Table 5. We think that these differences not only originate from the disease, but possibly from age-conditioned augmented immunoglobulin values observed in the serum.

Table 6 gives the age- and sex-dependent values of immunoglobulins of normal persons, which were found recently by a member of our team using radial immunodiffusion.

The oligoclonal bands in the γ_3-, and γ_4-globulin range, which are not specific for the disease but contribute significantly to the confirmation of the diagnosis, might correspond to an overrepresentation of the immunoglobulin G molecules and might produce structural deviations in the normal immunoglobulin G molecule, which are represented physicochemically in a separate fractionation.

Table 6. IgG-concentration (mg/100 ml; IE/ml) in blood serum at 280 healthy reference persons in relation to age and genus

	♂			♀			
Age	n	$\bar{x} \pm s$ (mg/100 ml)	$\bar{x} \pm s$ (IE/ml)	n	$\bar{x} \pm s$ (mg/100 ml)	$\bar{x} \pm s$ (IE/ml)	P
20 to 30	26	692 ± 270	80 ± 31	32	889 ± 364	102 ± 42	< 0,05
31 to 40	71	882 ± 316	101 ± 36	14	933 ± 278	107 ± 32	n.s.
41 to 50	67	908 ± 328	104 ± 38	7	838 ± 203	96 ± 23	n.s.
51 to 60	26	1055 ± 504	166 ± 58	12	843 ± 277	97 ± 32	n.s.
Over 60	16	1257 ± 395	145 ± 45	9	887 ± 253	102 ± 29	< 0,01
Total	206	917 ± 372	105 ± 43	74	885 ± 305	102 ± 35	n.s.

Summary

We found three basic results:

1. The high incidence of positive results in oligoclonal bands in 90–95% of definite MS cases and the low incidence in other diseases (3%) emphasizes the diagnostic value of agar gel electrophoresis.
2. Agar gel electrophoresis is the most sensitive electrophoretic technique presently available for the demonstration of discrete immunoglobulin G bands in CSF. We

do not agree with Delmotte et al. [3] that in MS only 70% of the clinically confirmed cases show a restricted microheterogeneity of the γ-globulins in the CSF.

3. For clinical diagnosis, the relative or absolute levels of γ-globulins in CSF are less important than the electrophoretic morphology.

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CSF Findings in Patients with Multiple Sclerosis and Optic Neuritis

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There have been numerous studies of routine CSF parameters (cells, total protein, IgA, IgM, absolute and relative IgG content) with different symptom constellations and the course of MS (1–5). In this report the CSF findings in patients with MS and optic neuritis were registered using a detailed documentation system of clinical symptoms and CSF data; a comparison of the results was made.

Material and Methods

Three groups of patients were chosen:

1. Patients with isolated optic neuritis ($n = 43$).
   Twenty-three of these patients (53%) developed other symptoms of MS in the further course of disease. In the other cases the etiology could not be clarified. The duration of illness was less than 12 weeks with the exception of five patients who had recurring optic neuritis.
2. MS cases with exclusively spinal symptoms throughout the course of disease ($n = 21$; primarily remitting 8; primarily chronically progressive 8; primarily remitting, later chronically progressive 5). The mean duration was 5.3 years.
3. MS cases with disseminated symptoms.
   This is a miscellaneous group of 210 patients with various courses of MS varying in symptoms and duration of disease (primarily remitting 58%; primarily chronically progressive 12%; primarily remitting, later chronically progressive 30%). The mean duration of illness was 7.1 years.

The CSF total protein was determined by the biuret method (normal up to 45 mg per 100 ml). Immunoglobulins of CSF were estimated by means of radial immunodiffusion technique (Behring-Werke Marburg; normal ranges with IgG 2.1–4.3 mg/100 ml, IgM 0–0.2 mg/100 ml and IgA 0–1.0 mg/100 ml). The relative IgG content is corresponding the CSF-IgG/total protein ratio (IgG × 10/total protein; normal up to 1.5).

CSF cells were counted in the Fuchs-Rosenthal chamber.

Results and Discussion

The results of this study are given in Table 1. The colloid curve was found to be pathologic in the majority of MS patients (group 2 and 3 – 81% and 90% respectively) but only at 47% of the optic neuritis cases.

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Table 1. The percentage of normal and pathologic values of the different CSF parameters in optic neuritis (A), MS with spinal (B), and disseminated (C) symptomatology

	Total protein			CSF cells			IgG (absolute)			IgG (relative)			IgM			IgA		
	n	normal	pathologic	*n*	normal	pathologic	*n*	normal	pathologic	*n*	normal	pathologic	*n*	normal	pathologic	*n*	normal	pathologic
Optic neuritis	43	38 88.4%	5* 11.6%	42	26 61.9%	16 38.1%	38	24 63.1%	14** 36.9%	38	31 81.6%	7*** 18.4%	37	36 97.3%	1 2.7%	37	35 94.6%	2 5.4%
MS (spinal symptomatology)	21	13 61.9%	8 38.1%	21	10 47.6%	11 52.4%	13	2 15.4%	11 84.6%	11	3 27.3%	8 72.7%	13	11 85.0%	2 15.0%	–	–	–
MS (disseminated symptomatology)	210	138 65.7%	72 34.3%	209	94 45.0%	115 55.0%	206	35 17.0%	171 83.0%	205	87 42.4%	118 57.6%	204	196 96.0%	8 4.0%	198	182 91.9%	16 8.1%

significant differences between group A and group C (B):
* $P < 0.01$ ** $P < 0.005$ *** $P < 0.001$

Compared with MS cases presenting disseminated and exclusively spinal symptoms and signs, optic neuritis differs significantly from both MS groups with respect to the absolute IgG increase (more than 80% in the MS groups vs 37% in the optic neuritis) and to the quotient IgG ÷ total protein (about 60% in the MS groups versus 18% in the optic neuritis group). There is also a significant difference concerning the total CSF protein increase (about 40% in the MS groups versus 12% in the optic neuritis group), whereas the difference in pleocytosis (55% in the MS groups and 38% in the optic neuritis group) is less pronounced.

We did not ascertain any statistical differences between the two groups of MS with disseminated and spinal symptomatology.

These findings suggest that the CSF immune reaction characterizing MS is (still) absent in the majority of the optic neuritis cases, possibly due to the fact that (1) they represent a very early stage of the disease and (2) some cases of optic neuritis may not be a manifestation of MS. The fairly high percentage of pleocytosis in the optic neuritis group, contrasting with the absence of IgG rise and pathologic colloidal curve in the majority of these cases, indicates that increase of CSF cells is the initial finding in the chain of immunological reactions identifiable in the CSF. This should also be remembered in early stages of MS presenting with polytopic symptomatology.

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Oligoclonal Cerebrospinal Fluid Immunoglobulin in Relation to Measles and Parainfluenza Antibody Response in Multiple Sclerosis

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Introduction

The concomitant occurrence of (1) a local production of oligoclonal IgG as estimated based on kappa and lambda light polypeptide chain imbalance (KL/LL chain imbalance), and (2) measles and parainfluenza type III (p. infl. type III) specific antibody response in the cerebrospinal fluid (CSF) of many multiple sclerosis (MS) patients prompted an analysis of the correlation between these phenomena [1, 3, 5, 8, 9, 11, 12, 13].

Materials and Methods

Quantitation of CSF and serum IgG, IgA, IgM, and KL and LL polypeptide chains and albumin was performed by single radial immunodiffusion (RID) on 10-fold concentrated CSF samples and 20-fold diluted serum samples [6]. The descriptions given by Link [5] regarding the choice of pooled blood donor serum as standard and regarding the quantitation of KL and LL chains, were followed [3,4]. CSF IgG index was calculated according to the formula: $\frac{\text{CSF IgG}}{\text{CSF albumin}} : \frac{\text{Serum IgG}}{\text{Serum albumin}}$. In or-

Table 1. Frequencies of immunoglobulin abnormalities in the CSF in MS

KL/LL Chain imbalance		IgG Increased above			IgG Index
<0.85 Ratio > 1.18		3.2 mg%	12% of prot.	24% of alb.	$\frac{\text{CSF IgG} \cdot \text{Serum IgG}}{\text{CSF alb.} \cdot \text{Serum alb.}}$ >0.73
% of MS cases with abnormal values (above or below[b] normal limits)	52%[a]	57%	64%	48%	51%
		67% (57% + 64%)		65% (64% + 48%)	
		together 68%			
Additional % of cases	–	+	30%	+	0% +
Together of cases	52%		82%		82%

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der to visualize multiple discrete bands in the gamma globulin region of an electrophoretic run, agar and agarose gel electrophoresis of *unconcentrated* CSF samples was performed according to Kerenyi et al. [2]. Indirect immunofluorescence was employed to search for IgG content in the cytoplasm of the CSF cells sedimented in Sayk's apparatus [10, 15]. To determine the titer of measles and p. infl. type I and type III specific antibody in unconcentrated CSF and serum samples, the hemagglutination inhibition (HI) microtest was performed [11, 12].

Results obtained in the group of 100 patients classified as definite or probable MS according to McAlpine et al. [7] were referred to four control groups, including the normal control group of neurotics and the group of 35 active neurosyphilis cases (Table 3).

Results

CSF immunoglobulin abnormalities in the MS group are shown in Tables 1–3.

The upper normal limits of IgG, IgM, and IgA absolute (mg%) and relative (% of total CSF protein and % of CSF albumin) level in the CSF of normal control group of neurotics was determined (Table 1). The upper normal limit of IgG index values was equal to 0.73. All CSF KL/LL chain ratio values in the control neurotics group fell into the range of 0.86–1.16. Oligoclonal Ig response recognized from discrete bands in the gamma globulin region on agar gel electrophoresis of unconcentrated CSF was seen in 84% of MS cases (Table 2). The usefulness of this electrophoretic method is compared with the diagnostic value of KL/LL chain ratio estimation. The KL/LL ratio shift proved to be very specific for MS CSF immunoglobulin. The oligoclonal Ig pattern recognized from the KL/LL chain ratio imbalance was encountered only in the CSF of MS patients – in 52% of MS cases (Table 3). Other

Table 1

IgM Increased above			IgA Increased above			Oligoclonal gamma globulin on agar gel electrophoresis
0.05 mg%	0.29 of prot.	0.52% of alb.	0.7 mg%	2.7% of prot.	4.5% of alb.	
	30%	27%		30%	30%	84%
27%	27%	17%	25%	23%	28%	
together	30%		together	31%		
	0%		+	4%		+ 9%
	82%			86%		95%

[1] This group of 52 cases of MS with KL/LL chain ratio imbalance comprises 14 cases with normal IgG level and 9 cases with normal IgG, IgM, and IgA level

[2] Refers only to KL/LL ratio reduction < 0.85

Table 2. Oligoclonal CSF IgG character recognized from discrete bands in the gamma globulin region on agar gel electrophoresis of *unconcentrated* CSF[a]

	Oligoclonal CSF IgG		Number of cases with		
	Number of cases (*n*)	%	2 bands (*n*)	3 bands in gamma globulin region (*n*)	4 bands (*n*)
Group I: Multiple Sclerosis	61 (73)	84%	32	27	2
Group II: Neurotics	0 (20)	0%	0	0	0
Group III: Other CNS diseases	11[b] (39)	28%	10	1	0

( ) Number CSFs tested

[a] Electrophoresis was performed according to Kerenyi [2]

55 Clinical diagnosis: Tumor cerebri–3 cases; encephalomalacia, SSPE, and Gullan-Barré Syndrom – 1 case: Syphilis of the CNS – 5 cases

Table 3. The characteristics of CSF and serum lg with respect to KL/LL chain ratio in the MS group as compared to controls

	CSF KL/LL ratio		Incidence of CSF KL/LL ratio imbalance		Serum KL/LL ratio	
	$\bar{X}$ ±sd	Range	KL/LL >1.18	KL/LL +<0.85	$\bar{x}$ ±sd	range
Group I: MS	1.16±0.47 (100)	0.50 – 2.68	31 (100)	+21=52 =52%	0.98±0.06 (46)	0.85 – 1.15
Group II: Neurotics	1.01±0.08 (25)	0.86 – 1.16		0	1.00±0.10	0.85 – 1.15
Group III: Neurologic Diseases with CSF total protein < 40 mg%	0.98±0.09 (25)	0.86 – 1.16		0	0.97±0.07	0.85 – 1.15
Group IV: Neurologic diseases with CSF total protein > 40 mg%	1.02±0.07 (11)	0.93 – 1.17		0	1.01±0.07	0.86 – 1.16
Group V: Syphilis	102±0.2 (35)	0.85 – 1.16		0	not tested	

CSF immunoglobulin abnormalities: multiple Ig bands on agar gel electrophoresis, increased IgG index value, increased IgM, IgG, and IgA absolute and *relative* level expressed as percent of total CSF protein and percent of CSF albumin – although characteristic of and very frequent in MS (Table 1), were less specific than KL/LL chain ratio shift. Nevertheless, the increase of CSF IgG index value, and CSF IgG, IgA, and especially IgM *relative* level was largely confined to MS and neurosyphilis cases [Wajgt, unpublished data].

On the whole, CSF immunoglobulin abnormalities were encountered in 95% of MS cases studied (Table 1). Increased CSF relative Ig level and CSF IgG index value, oligoclonal CSF Ig pattern recognized from discrete bands on agar gel electrophoresis and from KL/LL chain derangement as opposed to polyclonal serum Ig character [Wajgt, unpublished data], and the presence of IgG-laden cells in the CSF of MS patients evidenced by the indirect immunofluorescent technique, all indicate intrathecal Ig production in MS cases.

Presence of Measles and P. Infl. Type I and Type III HI Antibody in Serum and CSF Samples in MS and Control Groups

Serum measles and p. infl. type III antibody were found with a comparably high frequency (above 91% of positive sera) in all groups studied (Table 4). However, the mean titer of positive samples was significantly higher in the MS group ($P < 0.01$). Serum p. infl. type I antibody was demonstrated in a relatively lower frequency in the MS and control groups, and the differences in mean titer between groups were insignificant (Table 4).

CSF measles and p. infl. type III antibody were found more frequently in the MS group than in the controls (47% for measles and 41% for p. infl. type III of positive CSF in the MS group). These differences were significant at a $P < 0.001$ and $P < 0.02$ level, respectively. The mean titer of measles-positive CSF samples, equal to 1.122, was significantly higher ($P < 0.05$), and the mean titer of p. infl. type III antibody, equal to 0.697, was much higher in the MS group than in the control neurotics and other neurological diseases (OND) group, with CSF total protein level below 40 mg% (Table 5).

P. infl. type I CSF antibody was demonstrated in much lower frequency in all groups (in only 12% of positive CSF samples in the MS group), and the mean antibody titer was even lower in the MS group than in the control neurotics group (Table 5). The correlation coefficient for serum and CSF measles HI antibody titer in the MS group ($r = 0.24$) was lower than in all other groups together ($r = 0.50$). In the case of p. infl. type III HI antibody titer, this correlation coefficient was low both in the MS group ($r = 0.24$) and in all other groups together ($r = 0.25$).

From virological studies it was inferred that in the MS group there is only an insignificant correlation between CSF measles and p. infl. type III antibody titer and serum antibody level, even lower than in the control groups. Nevertheless, measles and p. infl. type III serum and CSF HI antibody titer, and the frequency of positive CSF are significantly higher in the MS group than in controls, in contrast to low, very comparable p. infl. type I serum and CSF antibody response in the MS and control groups.

Table 4. The incidence of measles, parainfluenza type I, and type III specific Hl antibody in the serum of MS patients as compared to controls

		MS group	Control groups		
			Neurotics	CNS diseases with CSF protein <40 mg%	CNS diseases with CSF protein >40 mg%
No. sera tested		97	37	34	10
Measles antibody					
Number of positive sera	n (%)	94 (97%)	34 (92%)	32 (94%)	10 (100%)
	P_{MS}	not significant			
Geometric mean	GMT	4.425	3.636	3.878	3.090
	P_{MS}	–	$P_{MS} < 0.01$	$P < 0.1$	$P < 0.01$
	P_N	$P_N < 0.01$	–		not significant
Parainfluenza type III antibody					
Number of positive Sera	n (%)	92 (95%)	36 (97%)	31 (91%)	10 (100%)
	P_{MS}	not significant			
Geometric mean	GMT	2.377	1.405	1.766	1.900
	P_{MS}	–	$P_{MS} < 0.001$	$P_{MS} < 0.05$	not significant
	P_N	$P_N < 0.001$	–		not significant
Parainfluenza Type I antibody					
Number of positive sera	n (%)	69 (71%)	32 (86%)	25 (74%)	8 (80%)
	P_{MS}			not significant	
Geometric mean	GMT	1.119	0.781	0.600	1.250
	P_{MS}	–	not significant	$P_{MS} < 0.05$	not significant
	P_N	not significant	–		not significant

GMT = geometric mean titre expressed as $\lg_2$ (serum dilution^{-1}) for measles antibody of expressed as $\lg_2 \left(\frac{\text{serum dilution}^{-1}}{10}\right)$ for parainfluenza antibody

P_{MS} = level of statistical significance between MS group and other groups

P_N = level of statistical significance between neurotics group and other groups

Table 5. The incidence of measles, parainfluenza type I, and type III specific Hl antibodies in the CSF of MS patients as compared to controls

		MS group	Control groups			Neurosyphilis
			Neurotics	CNS diseases with CSF protein < 40 mg%	CNS diseases with CSF protein > 40 mg%	
Number of CSF tested		105	39	39	11	35
Measles antibody						
Number of positive CSF	n (%)	49 (47%)	4 (10%)	6 (15%)	1 (9%)	4 (11%
	P_{MS}	–	< 0.001	< 0.001	< 0.001	< 0.001
Geometric mean titre	GMT	1.122	0	0.333		0.750
	P_{MS}	–	$P_{MS} < 0.05$	$P_{MS} < 0.1$		not significant
	P_N	$P_N < 0.05$	–	not significant		$P_N < 0.05$
Parainfluenza type III antibody						
Number of positive CSF	n (%)	43 (41%)	10 (25%)	4 (10%)	3 (27%)	4 (11%)
	P_{MS}	–	< 0.02	< 0.001	< 0.02	< 0.001
Geometric mean titre	GMT	0.697	0.300	0.250	1.333	1.000
	P_{MS}	–	not significant	$P_{MS} < 0.1$		not significant
	P_N	not significant	–	not significant	$P_N < 0.01$	$P_N < 0.1$
Parainfluenza type I antibody						
Number of positive CSF	n (%)	13 (12%)	2 (5%)	2 (5%)	2 (18%)	2 (6%)
	P_{MS}					
Geometric mean titre	GMT	0.307	0.500	0	1.000	0.500
	P_{MS}	–		not significant	$P_{MS} < 0.1$	not significant
	P_N	not significant	–		not significant	

GMT = geometric mean titre expressed as Ig_2 (CSF dilution^{-1})
P_{MS} = level of statistical significance between ms group and other groups
P_N = level of statistical significance between neurotics group and other groups

Table 6. The relationship between oligoclonal CSF Ig pattern and measles-specific HI antibodies in the CSF and sera of MS patients

			MS group A KL/LL ratio abnormal $n=49$	MS group B KL/LL ratio normal $n=51$	Total number of MS cases $n=100$
1	CSF JgG mean level mg%	x ± SD	4.718 ± 3.046	4.169 ± 2.934	4.396 ± 3.021
		P	not significant		
2	CSF measles antibodies present	*n*%	30 (61.2%)	19 (37.2%)	49 (490%)
		P	$P<0.02$		
3	CSF antibody GMT	x ± SD	1.100 ± 0.831	1.158 ± 1.136	1.122 ± 0.961
		P	not significant		
4	CSF JgG mean level mg%	x ± SD	5.388 ± 3.387	5.715 ± 3.621	5.511 ± 3.479
		P	not significant		
5	Serum measles antibody GMT	x ± SD	4.608 ± 1.437	4.588 ± 1.286	4.600 ± 1.374
		P	not significant		
6	The ratio of serum/CSF antibody litre	x ± SD	3.435 ± 1.527	3.294 ± 1.362	3.375 ± 1.461
		P	not significant		
7	CSF measlies antibodies not present	*n*%	19 (38.8%)	32 (62.8%)	51 (51.0%)
		P	$P<0.02$		
8	CSF JgG mean level mg%	x ± SD	3.660 ± 1.998	3.299 ± 1.994	3.434 ± 2.003
		P	not significant		
9	Serum measles antibody GMT	x ± SD	4.428 ± 1.050	4.379 ± 1.243	4.400 ± 1.166
		P	not significant		
10	Statistical significance between JgG levels in line 4 vs 8	*P*	$P<0.1$	$P<0.01$	$P<0.001$
11	Statistical significance between GMT in line 5 vs 9	*P*	not significant	not significant	

GMT = geometric mean titre expressed as lg_2 (CSF or serum dilution^{-1})
P = the Level of Statistical significance
n = number of cases

Relationship Between the Oligoclonal CSF Ig Pattern Recognized from KL/LL Chain Ratio Derangement and CSF Measles and P. Infl. Type III HI Antibody Response in the MS Group

CSF measles antibody was evidenced in 30 (61%) CSF showing simultaneously the KL/LL ratio shift (MS subgroup A, Table 6), and in only 19 (37%) CSF with a normal KL/LL ratio (MS subgroup B, Table 6). This difference was statistically signifi-

Table 7. The relationship between oligoclonal CSF Jg pattern and parainfluenza type III specific HI antibodies in the CSF and sera of MS patients

			MS group A KL/LL ratio abnormal $n=49$	MS group B KL/LL ratio normal $n=51$	Total number of MS cases $n=100$
1	CSF JgG mean level mg%	x ± SD	4.718 ± 3.046	4.169 ± 2.934	4.396 ± 3.021
		P	not significant		
2	CSF parainfluenza, type III antibodies present	*n*%	24 (49.0%)	15 (29.4%)	39 (39.0%)
		P	$P<0.05$		
3	CSF antibody GMT	x ± SD	0.609 ± 0.417	1.000 ± 1.000	0.757 ± 0.819
		P	not significant		
4	CSF JgG mean level mg%	x ± SD	5.127 ± 2.527	6.176 ± 2.487	5.513 ± 2.562
		P	not significant		
5	Serum parainfluenza type III antibody	x ± SD	2.471 ± 1.036	2.923 ± 1.071	2.666 ± 1.258
		P	not significant		
6	The ratio of serum/CSF antibody titre	x ± SD	1.882 ± 1.182	1.846 ± 1.350	1.867 ± 1.258
		P	not significant		
7	CSF parainfluenza type III antibodies not present	*n*%	25 (51.0%)	36 (70.6%)	61 (61.0%)
		P	$P<0.05$		
8	CSF JgG mean level mg%	x ± SD	4.175 ± 3.655	3.666 ± 2.399	3.875 ± 2.989
		P	not significant		
9	Serum parainfluenza type III antibody GMT	x ± SD	2.000 ± 1.323	2.467 ± 1.477	2.259 ± 1.430
		P	not significant		
10	Statistical significance between JgG levels in line 4 vs 8	*P*	not significant	$P<0.01$	$P<0.001$
11	Statistical significance betwen GMT in line 5 vs 9	*P*	not significant		not significant

GMT = geometric mean titre expressed as Ig_2 (CSF dilution^{-1}) and Ig_2
P = level of statistical significance
n = number of cases

cant ($P<0.02$). In contrast, no significant difference was found in CSF antibody titer, CSF IgG level, serum antibody titer, and serum/CSF antibody titer ratio between MS subgroup A with KL/LL ratio derangement and MS subgroup B with normal KL/LL chain ratio. The differences between IgG level in measles antibody-positive versus measles antibody-negative CSF were statistically significant ($P<0.001$) for the total number of MS cases, at $P<0.01$ for MS subgroup B with normal KL/LL ratio, and only at $P<0.1$ for MS subgroup A with CSF KL/LL chain imbalance (Table 6, line 10).

Similar analysis of p. infl. type III antibody response in relation to KL/LL chain ratio and IgG level is depicted in Table 7. CSF p. infl. type III antibody was found in 24 (49%) CSF showing simultaneously the KL/LL ratio shift (MS subgroup A), and in only 15 (29%) CSF with a normal KL/LL ratio (MS subgroup B, Table 7). This difference was significant ($P < 0.05$). To the contrary, no significant difference was encountered between MS subgroup A (with KL/LL ratio derangement) versus MS subgroup B (with normal KL/LL ratio) in the CSF antibody titer, CSF IgG level, serum antibody titer, and serum/CSF antibody titer ratio. The differences between IgG mean level in p. infl. type III positive CSF versus p. infl. type III negative CSF were highly significant for the total number of MS cases ($P < 0.001$), significant at $P < 0.01$ for MS subgroup B, but not significant in MS subgroup A characterized by CSF KL/LL chain derangement (Table 7, line 10). There was also no significant difference between measles and p. infl. type III serum antibody level in MS cases with virus antibody positive CSF versus MS cases with virus antibody negative CSF (Tables 6, 7, line 11).

Conclusions

In our MS population:

1. Local, intrathecal production of oligoclonal Ig takes place in the majority of MS cases.
2. Serum of MS patients is characterized not only by increased titer of measles-specific HI antibody, but also by increased titer of p. infl. type III specific HI antibody.
3. CSF of MS patients is characterized by increased frequency of measles and p. infl. type III specific HI antibody. There is also a conspicious increase of these antibody titers in the CSF of MS patients.
4. There is only very superficial, insignificant positive correlation between CSF antibody response and serum antibody level in MS cases.
5. Strong positive correlation exists between CSF antibody response and CSF IgG level in MS cases with normal KL/LL chain ratio. By contrast, this correlation is insignificant in MS cases characterized by KL/LL chain ratio derangement.
6. Very significant positive correlation exists between the CSF oligoclonal Ig pattern recognized from KL/LL chain ratio shift and CSF measles and p. infl. type III specific HI antibody response. Oligoclonal CSF Ig accounts for CSF antibody response. However, the nature of the correlation between the occurrence of oligoclonal Ig and high measles and parainfluenza CSF antibody response remains to be explained [1, 5, 8, 9].

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Oligoclonal Bands in Cerebrospinal Fluid

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Considering the well-known importance of CSF IgG in laboratory tests for the diagnosis of MS, we have studied purified CSF IgG using the technique of isoelectric focusing (IEF) (Table 1) in 17 cases of MS and a control population of 22 Other Neurological Disease (OND) and 14 normal subjects (Table 2).

We have confirmed the frequent finding of oligoclonal bands with alkaline isoelectric points (pI) (pH 7.5–9.0) in the CSF of patients suffering from MS (Table 3). Their identity as IgG was verified using crossed immunodiffusion.

Among the MS patients, a correlation was found between CSF IgG $\geq$ 3 mg%, and the presence of an alkaline oligoclonal pattern (11 of 12 patients).

In the OND group, none of the cases with CSF IgG $\geq$ 3 mg%, (19 of 22) showed an oligoclonal pattern (Table 4). The comparison of all CSF with their respective sera excluded the presence of oligoclonality in the serum of all subjects studied. The oligoclonal pattern is a notable diagnostic aid for MS. In fact, in 70% of MS cases, the oligoclonal aspect can be demonstrated using electrophoresis, while of the remaining 30% negative at electrophoresis, three out of four can be shown to have oligoclonal bands using IEF. It is here that the practical application for MS lies.

Table 1. Methods

1. Preliminary purification of CSF IgG using ion-exchange chromatography (DEAE-cellulose)
2. Concentration (MINICON-B) of eluate to as close to 1 mg/ml IgG as possible (30–200 mg/100 ml)
3. Isoelectric focusing using thin-layer polyacrylamide gel (LKB 2117 Multiphor) as described by Westenberg (1973) with a pH range of 3.5–9.5
4. Crossed immunoelectrofocusing from polyacrylamide gel into Agar

Table 2. Patients studied

17 cases of MS
22 cases of Other Neurological Diseases (OND)
– 10 inflammatory
– 1 tumor
– 2 trauma
– 9 other
14 normal subjects

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Table 3. 17 Cases of MS

	Age	Sex	Duration of disease (years)	Clinical course	Clinical stage	Last exacerbation (with respect to lumbar puncture)	CSF IgG mg. %
MS 1	53	F	23	INT	STA	< 3 months	3.7
MS 2	50	M	5	INT	AGG	> 3 months	6
MS 3	46	M	9	PRO	STA	< 3 months	3
MS 4	64	F	7	PRO	STA	< 3 months	11.4
MS 5	50	F	17	INT	AGG	> 3 months	18
MS 6	36	F	21	INT	STA	< 3 months	6
MS 7	60	F	14	INT	AGG	> 3 months	4.5
MS 8	46	M	12	PRO	AGG	< 3 months	4.5
MS 9	34	F	9	INT	STA	> 3 months	12
MS 10	28	F	3	PRO	AGG	< 3 months	6
MS 11	21	M	1	PRO	AGG	> 3 months	2
MS 12	45	F	8	PRO	AGG	< 3 months	2
MS 13	49	F	4	PRO	AGG	< 3 months	7.5
MS 14	33	F	4	PRO	AGG	> 3 months	6.5
MS 15	21	M	2	PRO	AGG	< 3 months	< 3
MS 16	49	F	5	INT	STA	> 3 months	4
MS 17	–	M	16	PRO	AGG	< 3 months	< 1

INT = intermittent; STA = stationary; PRO = progressive; AGG = aggravation

Table 4. IgG oligoclonal aspect (OA) in CSF

		OA		
		Cases	Present	Absent
Cases of MS				
with CSF IgG	≧3 mg %	12	11	1
	< 3 mg %	5		5
Normal subjects		14		14
Other neurological diseases		22		22

We have begun a second phase of our research to investigate the antibody-functional characteristics of these bands. Experiments using immunodiffusion systems with antigens such as myelin basic protein and measles cell-pack antigen are currently underway.

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Quantitative Determination of IgG Subclasses in CSF and Serum of MS Patients: Comparison with Other Neurological Diseases and Normal Controls

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Introduction

Local IgG production has been shown to take place in the subarachnoid space of patients with chronic inflammatory diseases of the central nervous system (CNS) [2, 3, 5, 6, 7, 13]. Numerous investigators have further characterized the CSF IgG fraction with regard to different molecular properties [1, 4, 8, 9, 10, 14, 15].

As yet, only qualitative data on IgG subclasses in CSF and serum of MS patients are available [12, 14]. In our joint study, quantitative determinations of the four IgG subclasses were performed in the CSF of 62 patients and 25 control persons and in the sera of ten MS patients and seven normal controls.

Materials and Methods

Patients

Twenty-six cases of MS, grouped according to the course of the disease, eight cases of other inflammatory neurological diseases (OID), and 28 cases of noninflammatory diseases of the nervous system (NID) were compared with 25 controls (Table 1). The controls were selected out of a number of psychiatric patients and patients with lumbar disc protrusions guided by the criterion of normal routine CSF findings including IgG/albumin ratios [2]. MS patients with additional blood-brain barrier disturbances were excluded from the study [3]. CSF was obtained by lumbar puncture.

Quantitative Determinations of Total Protein, Albumin, Total IgG, and IgG Subclasses

In all CSF specimens, cell count, total protein, normomastix reaction, and the concentrations of albumin, IgG, IgA, and IgM were determined as described by Eickhoff and Heipertz [3]. The quantitative determination of IgG subclasses was performed according to Morell and Skvaril [11].

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Table 1. Grouping of the patients included in the study

Group	Course of disease/diagnosis	No. of patients
MS	Relapsing	10
$n=26$	Relapsing and progressive	10
	Chronic progressive	6
OID	Meningitis (viral)	1
$n=8$	Encephalitis	3
	Polyradiculitis	1
	Radiculomyelitis	2
	Neurosyphilis	1
NID	Epilepsy	1 (3.6%)
$n=28$	Brain Tumor	11 (39.3%)
	Tumor of the spinal cord	1 (3.6%)
	Subarachnoid hemorrhage	1 (3.6%)
	Polyneuropathy	5 (17.9%)
	Hydrocephalus	1 (3.6%)
	Brain infarction	5 (17.9%)
	Neurofibromatosis (von Recklinghausen's disease)	1 (3.6%)
	Palsy of the sixth cranial nerve	1 (3.6%)
	Myatrophic lateral sclerosis	1 (3.6%)

Statistical Calculations

Data are presented as mean values ± SD. The significance of differences was tested using Student's *t*-test.

Results

The preliminary outcome of our study is summarized in Tables 2 to 4.

Because of the heterogeneity of the NID group with regard to blood-brain barrier dysfunction, we did not test the differences between this group and the MS subgroups for significance.

Discussion

Our quantitative results confirm the qualitative findings of Vandvik et al. [14], who described a predominance of the IgG1 subclass in the CSF of MS patients as compared with the corresponding sera and with normal CSF. Oligoclonal IgG bands in the CSF of MS patients seem to belong mainly to the IgG1 subclass [14].

Table 2. Age and routine CSF laboratory parameters of the persons included in the study

	Age (years)	Cells/mm³	Total protein (mg/100 ml)	Albumin (mg/100 ml)	Total IgG (mg/100 ml)	$\frac{\text{IgG} \times 100}{\text{Albumin}} = Q_G$	$\frac{\text{IgG} \times 10}{\text{Total protein}}$
Normal controls $n=25$	46.8 ±15.0	4.20/3 ±3.64/3	36.40 ±13.41	17.60 ±7.42	2.13 ±1.15	12.24 ±5.45	0.59 ±0.22
MS $n=26$	33.9 ±9.5	43.65/3 ±60.82/3 * Control * OID	56.51 ±18.60 * Control * OID * NID	30.77 ±13.66 * Control * OID * NID	10.62 ±5.24 * Control * OID * NID	35.37 ±12.50 * Control * NID	1.85 ±0.66 * Control * NID
Other inflammatory neurological diseases (OID) $n=8$	45.6 ±13.7	319.25/3 ±652.79/3 * Control * MS * NID	196.63 ±292.87 * Control * MS	69.78 ±55.28 * Control * MS	37.35 ±63.29 * Control * MS	35.86 ±28.0 * Control * NID	1.55 ±0.55 * Control * NID
Noninflammatory neurological diseases (NID) $n=28$	50.9 ±16.5	43.0/3 ±97.28/3 * Control * OID	183.46 ±212.02 * Control * MS	117.73 ±110.16 * Control * MS	20.08 ±19.8 * Control * MS	19.54 ±12.0 * Control * MS * OID	1.14 ±0.59 * Control * MS * OID

* means: significantly different from ... (at $P< 0.05$)

Table 3. IgG subclasses in the CSF

	IgG1 (%)	IgG2 (%)	IgG3 (%)	IgG4 (%)
Normal controls $n=25$	52.36 ± 10.88	36.06 ± 10.52	7.05 ± 3.62	4.53 ± 2.56
MS (all subgroups) $n=26$	78.11 ± 8.77 * Control * OID * NID	16.71 ± 7.55 * Control * OID * NID	3.31 ± 1.44 * Control * OID * NID	1.76 ± 1.08 * Control * OID * NID
MS relapsing $n=10$	77.1 ± 8.0 * Control * OID	17.2 ± 6.6 * Control * OID	3.6 ± 1.9 * Control	2.1 ± 1.3 * Control * rel. and progr.
MS relapsing and progressive $n=10$	80.3 ± 6.7 * Control * OID	15.2 ± 6.0 * Control * OID	3.2 ± 1.0 * Control	1.1 ± 0.4 * Control * OID * relapsing * chron. progr.
MS chronic progressive $n=6$	76.2 ± 13.2 * Control * OID	18.5 ± 11.5 * Control * OID	3.1 ± 1.4 * Control	2.3 ± 1.0 * Control * rel. and progr.
OID $n=8$	50.98 ± 14.77 * MS	39.0 ± 14.24 * MS	4.90 ± 2.96 * MS	5.13 ± 5.47 * MS
NID $n=28$	46.47 ± 12.39 * MS * Control	44.31 ± 11.07 * MS * Control	5.84 ± 2.92 * MS	3.37 ± 3.16 * MS

* means: significantly different from... (at $P < 0.05$)

Table 4. Simultaneous determination of the IgG subclasses in CSF and serum

	IgG1 (%)		IgG2 (%)		IgG3 (%)		IgG4 (%)	
	CSF	Serum	CSF	Serum	CSF	Serum	CSF	Serum
Normal controls $n=7$	42.79 ± 6.60	51.87 ± 9.72	46.47 ± 7.10	38.13 ± 8.89	4.93 ± 3.27	6.14 ± 3.61	5.83 ± 2.29	3.86 ± 1.68
MS $n=10$	83.62 ± 8.77	63.65 ± 6.00	12.07 ± 7.90	29.08 ± 5.39	2.98 ± 0.83	4.94 ± 2.66	1.33 ± 0.56	2.33 ± 2.94
Significance of the difference between controls and MS	$P<0.0005$	$P<0.005$	$P<0.0005$	$P<0.01$	$P<0.05$	n.s.	$P<0.0005$	n.s

n.s. = not significant

Summary

Radioimmunological determinations of the IgG subclasses in the CSF of control persons and several groups of neurological patients as well as in the sera of MS patients and control persons were performed. We found that the relative proportion of IgG1 in the CSF was significantly higher in the MS group than in the other groups, whereas the percentages of the three other subclasses were significantly lower in the MS group. No major differences were found between the distributions of the IgG subclasses in the CSF of different groups of MS patients (grouped according to the course of the disease) and between the OID and NID groups. The predominance of IgG1 over the other IgG subclasses was less marked in the sera than in the CSF of MS patients.

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Assessment of Process Activity in Multiple Sclerosis and Changes in Spinal Fluid

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Introduction

The correct assessment of the activity of the pathological process in the CNS of MS patients has been impossible until now. All methods of scoring or ranking clinical findings in patients are hampered by the fact that an active and extensive demyelinating process may be present in a "silent" part of the CNS, and that small lesions may cause extensive signs and symptoms.

It is unknown in what way fleeting signs and symptoms in MS are caused and if these short bouts are related to the activity of the pathological process. Also, there is no certainty about the significance of presenting symptoms for the eventual course of the clinical situation.

By scoring the clinical course one can clearly discern a rather constant progressive course over the years in most MS patients [2, 3]. This led Jersild et al. [8] to use a rough scale of progression. Dividing a score of clinical signs and symptoms at a certain moment in the development of the disease by the number of years of duration of the disease, they found a quotient that indicates the number of scored signs added per year to the total score. It is called a progression rate (PR).

At present, an easily measurable biochemical or biophysical parameter indicating the activity of the pathological process in MS would be of utmost importance. In particular, changes in CSF have been studied in this respect. Gammaglobulin, total IgG content, serum/CSF IgG ratio, oligoclonal bands, and CSF cells have been correlated with clinical aspects, but without success [10]. In recent years, visually evoked response and somato-sensory-evoked response have also been studied, but have given no indication as to the activity of the pathological process in the CNS of MS patients. In this study we will undertake to correlate the progression rate with two recently developed parameters: "local" IgG levels in CSF, and histocompatibility typing.

Patients and Methods

The patients reported in this study are from a group of 86 definitive MS patients treated with intensive immunosuppression [6, 7]. All patients had been in a chronic progressive phase of the disease for at least 1 year. Examination and treatment had no relation to a recent exacerbation.

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The clinical signs of the patient were scored according to the Kurtzke scales: (1) Standard Neurologic Examination (SNE); (2) Functional Systems (FS); and (3) Disability Status Scale (DSS).

All patients were scored immediately before (I), immediately after (II), and 3 months after intensive immunosuppression (III), and in October 1976 (1–5 years after the treatment). The progression rate (PR) was calculated by dividing the total SNE score at I minus 20 by the number of years of duration of the disease.

At I, II and III, spinal fluid and serum were examined to determine albumin and IgG. From these values, the "local" IgG production was calculated according to Delpech and Lichtblau [1] and Ganrot and Laurell [4] as described by Lamers et al. [9].

Total spinal fluid IgG minus "local" IgG is called transudation IgG. Histocompatibility testing was done using 180 different sera from the 1977 Oxford Workshop [5].

Results

Total Spinal Fluid IgG

A correlation of the PR with spinal fluid parameters at I, II, and III was not found. However, the percent reduction of the PR between I and October 1976 showed a weak-positive correlation with total spinal fluid IgG at I ($n=44$; $0.05>P>.02$; $r=0.29$).

This indicates that as the total spinal fluid IgG at I is higher, the percentage decrease of PR by immunosuppressive treatment is higher.

Local and Transudation IgG in CSF

No correlation could be demonstrated between PR and absolute values of local and transudation IgG. No correlation was found between PR and local or transudation IgG at I, II, and III expressed as percentage of total IgG ($n=18$).

If in each patient local IgG at II and III is expressed as a percentage of local IgG at I, than a close correlation between the decrease of percentage at II and PR is found ($n=18$; $0.05>P>0.02$; $r=0.54$; Table 1).

No PR correlation was found with local IgG at III and with transudation IgG at II and III.

This indicates that decrease in local IgG by immunosuppressive treatment is higher, as the PR at the start of the treatment is higher.

Histocompatibility Typing

A correlation between DR_{w2} and PR was demonstrated. Not only did the DR_{w2}-positive group ($n=35$) show a higher progression rate than the DR_{w2}-negative group ($n=20$; Fig. 1; $0.05>P>0.02$), but also the number of homozygous DR_{w2}-positive patients with a PR above 10 is higher than the number of DR_{w2}-negative patients ($0.02>P>0.01$; Fig. 1).

Table 1. Local IgG of CSF at II and III as a percentage of I

Patient number	CSF local IgG in mg/l at I	% at II	% at III	PR	CSF total IgG in mg/l at I	at II	at III
53	110	40	30	18	123	66	47.5
56	30	53	79	57	79	57.5	60.3
57	29	0	0	29	46.5	37.5	13.7
58	92.4	30	43	38	149.3	46.0	73.0
59	57.4	62	61	12	85.2	60.6	66.9
60	25.4	59	56	8.5	53.9	33.0	37.2
61	81.4	35	36	2.5	134	77.5	65.0
62	31.4	71	86	3.2	58.1	49.7	46.6
63	13.6	113	91	3.3	46.8	31.5	31.9
64	112.2	69	56	16	181.0	127.0	111.8
65	65.6	84	76	4.5	90.5	75.7	74.9
66	16.9	154	151	4.0	68.1	53.7	67.3
67	13.2	136	165	13.5	60.8	46.8	58.0
68	77.3	86	64	7	135.5	159.6	114.2
69	41.4	67	41	18.6	55.7	38.7	32.5
70	78.2	49	60	20.3	114.2	61.0	71.0
72	55.5	65	77	2.3	87.1	56.7	81.3
73	42.2	49	66	22	69.8	36.8	45.1

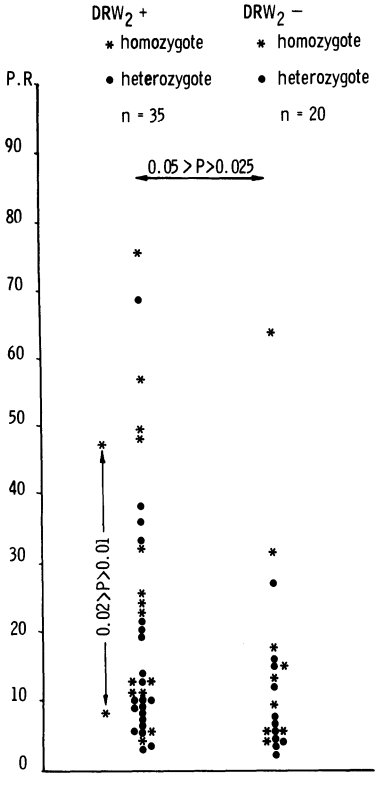

Fig. 1. PR values for DR_{w2}-positive and DR_{w2}-negative MS patients

Local IgG and Histocompatibility Typing

As DR_w typing is correlated with PR and PR with decrease in local IgG by immunosuppression, the relation between local IgG at I and DR_w typing was studied in 35 patients (Fig. 2). Indeed, a tendency for local IgG to be higher in DR_{w2}-positive homozygous patients is present, with lower values for PR in DR_{w2}-positive heterozygous, and still lower values in DR_{w2}-negative patients. However, the differences are not statistically significant.

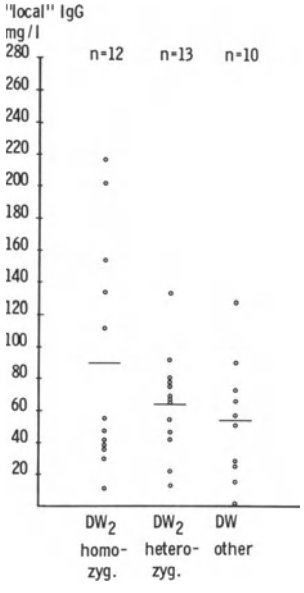

Fig. 2. Local IgG values in spinal fluid of MS patients with different DR_w typings

Summary and Conclusions

The decrease of progression rate by immunosuppressive treatment in MS patients shows a positive correlation with total IgG content of the spinal fluid before treatment.

The progression rate also shows a positive correlation with the decrease of local IgG in CSF induced by immunosuppression. There is a tendency for the PR to be high in DR_{w2}-positive and low in DR_{w2}-negative patients.

Thus progression rate, effect of immunosuppressive treatment, total and local IgG content of CSF, and histocompatibility typing are in some complex way correlated.

DR_{w2} is a lymphocyte-determined antigen that maps genetically to chromosome 6 close to the immune response locus. The DR_{w2} locus may be responsible for some genetically determined disturbance of the immune response system. This then is correlated with PR in our patients. A similar type of correlation was demonstrated for patients with LD-7a [8]. The progression rate also shows a correlation with ef-

fects of immunosuppressive treatment and with IgG fractions in the CSF, in such a way that as the PR is higher, the sensitivity of the "local" IgG-producing system in the CNS for immunosuppression is higher.

In our second paper in this volume it will be demonstrated that both IgG content and PR seem to correlate with effect of immunosuppressive treatment in the long term.

Therefore some arguments are presented here which consider the local IgG content of the CSF as an indicator of the activity of the disease process, not in its absolute value, but in its sensitivity to immunosuppression.

In this light, it would be of importance to study (in a larger group of patients) the relation between DR_w typing and local IgG synthesis in the CSF. If such a relation can be found, it would connect the genetic markers for severity of MS with the reactivity of the immune system in the CNS.

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Evaluation of IgG Synthesis in the Central Nervous System

H. REIBER [1]

Summary

The cerebrospinal fluid (CSF)/serum concentration ratios of IgG (y) and albumin (x) are used as measures to characterize increased IgG levels in CSF. From a group of control patients ($n = 334$) the normal range of these ratios was evaluated. The correlation coefficient ($r = 0.71$), the regression line ($y = 0.43\,x$), and the confidence interval of y for a given x ($\pm\, 2s_{yx} = \pm\, 0.001$) were calculated. The contribution of the pathological fraction IgG(P) synthesized in the CNS to the total IgG in CSF can be calculated with maximal significance using the following formula:

$$\mathrm{IgG(P)} = \mathrm{IgG(CSF)} - \left(0.43\,\frac{\mathrm{Alb\ (CSF)}}{\mathrm{Alb\ (S)}} + 0.001\right) \cdot \mathrm{IgG(S)}.$$

Introduction

The actual concentration of IgG in CSF is the sum of the IgG from serum which has passed the blood-CSF barrier plus the amount of IgG that may be synthesized in the CNS reduced by the amount continuously eliminated from the CSF. This pathological IgG elevation in CSF due to synthesis in CNS is of interest for the diagnosis of inflammatory neurological diseases.

The protein concentration in CSF depends on age, sex, and volume of CSF extracted.

Ganrot and Laurell [3], Eickhoff and Heipertz [1], and Link and Tibbling [4] have shown that the CSF/serum ratio of IgG and the CSF/serum ratio of albumin together allow a more significant characterization of deviations from the normal range than the absolute value in CSF or the alternatively used IgG/albumin ratio in CSF. The dependence of the CSF/serum ratio of a protein on its effective molecular volume has been described by Felgenhauer [2]. A first approach for a numerical evaluation of local IgG synthesis is given in Tourtellotte et al. [5].

The aim of this study is to characterize the control group in the diagram of Ganrot and Laurell [3] using the regression line and the corresponding confidence interval and to give a formula for the satisfactory calculation of the pathological CSF fraction of IgG synthesized in the CNS.

Methods

IgG and albumin concentrations in CSF and serum were determined using a radial immunodiffusion assay (Behring, Partigen plates). Statistical evaluation of the val-

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ues for y = CSF/S ratio IgG and x = CSF/S ratio albumin was performed by a linear regression. The confidence range of y for a given x is determined as $y \pm 2\, s_{yx}$ with $s_{yx} = \sqrt{(y_i - y_i')^2 / n-2}$ and $y_i' = a + b x_i$.

Results

For CSF and serum samples of control patients (n=334) (cell number $\leq 4 \cdot 10^6$/l, IgG $\leq$ 40 mg/l, albumin $\leq$ 340 mg/l, total protein $\leq$ 500 mg/l) the CSF/serum concentration ratios of IgG (y) and albumin (x) were calculated. The regression line was found to have the function $y = 0.00014 + 0.41\, x$ with a correlation coefficient of $r = 0.71$. The SD of the y values for a given x is $s_{yx} = 0.00049$. This function together with the confidence interval is shown in Figure 1.

For theoretical and statistical reasons it is plausible that the regression line goes through the origin, meaning that $y = \bar{y}/\bar{x} \cdot x = 0.43\, x$ is a better approach for the regression line. This function is valid in the range $\bar{x} \pm 2 s_x$ ($0.002 < x < 0.0074$).

From Figure 1 the validity of the following equation becomes obvious:

$$y_i = \frac{\text{IgG (CSF)}}{\text{IgG (S)}} = y_i' + 2\, s_{yx} + \frac{\text{IgG (P)}}{\text{IgG (S)}}$$

The data for $y_i' = 0.43\, x_i$ and $s_{yx} \approx 0.0005$ are inserted into this equation. By a rearrangement, one gets the final equation for the calculation of the pathological fraction IgG(P), synthesized in CNS:

$$\text{IgG(P)} = \text{IgG(CSF)} - \left(0.43 \frac{\text{Alb (CSF)}}{\text{Alb (S)}} + 0.001\right) \cdot \text{IgG(S)}.$$

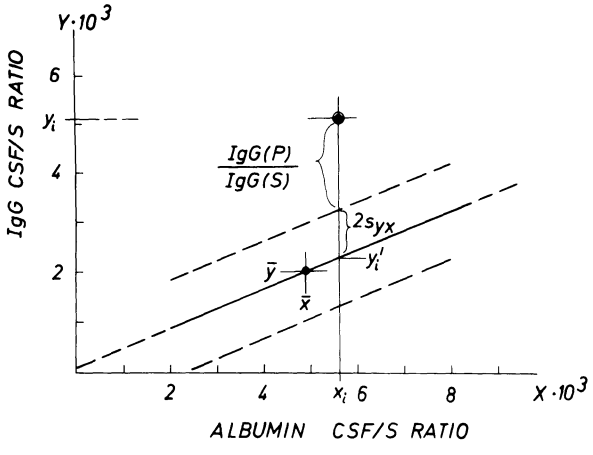

Fig. 1. Calculation procedure for the pathological, locally synthesized IgG fraction in CSF: IgG(P) = pathological contribution of CNS to IgG concentration; IgG(S) = total concentration of IgG in serum; y_i' = value of the regression line for a given x_i; ($y = 0.00014 + 0.41\, x$); y_i, x_i = values of patient i; $\bar{y}$, $\bar{x}$ = mean values of the total population of control patients (n = 334); s_{yx} = SD of the y value for a given x

Discussion

The equation given above for the IgG synthesis in CNS is derived from the observation that for a known albumin CSF/serum ratio, the SD of the normal IgG CSF/serum ratio is smaller (s_{yx} = 0.00049) than for the mean value of the total population of values (s_y = 0.00075). The formula given by Tourtellotte et al. [5] does not have this advantage, as it refers to the mean values ($\bar{x}$, $\bar{y}$) instead of to a regression line. This has the consequence of false-negative results for patients with a dense blood-CSF barrier. Link and Tibbling [3] characterize the increased IgG by the quotient of the two ratios (IgG CSF/serum and albumin CSF/serum). Using this method of evaluation, the slope of the line between the origin and the data point y_i, x_i is used. Above a slope of 0.43 (for the regression line) for the same slope, normal and increased IgG values can hold. This has the consequence of a tendency toward false-negative results for patients with a less dense (but nevertheless normal) blood-CSF barrier.

Using the method given above, we describe the statistically most significant method of evaluation: For a measured albumin ratio which characterizes the individual blood-CSF barrier condition of the patient, the maximal normal value of the IgG ratio is calculated and compared with the measured value of the patient.

Above x = 0.0074, where additional blood-brain barrier dysfunction occurs, the function of the regression line and the confidence interval are not yet established. The meaning of IgG(P) is that of a steady-state concentration. It cannot give a direct measure for the rate of IgG synthesis in CNS, since in this case, the rate of the IgG elimination must also be known.

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CSF Lipids in Demyelinating Diseases

D. SEIDEL [1], R. HEIPERTZ [2], and R. BUCK [2]

Introduction

Compared to other organs, the central nervous system (CNS), in particular the white matter, is rich in lipids, especially sphingolipids (approx. 35%) containing mainly long-chained fatty acids (C_{20}–C_{26}). In sphingomyelin, the proportion of long-chained fatty acids (FA) is approximately 65% [2].

In contrast, the total lipid content of cerebrospinal fluid (CSF) is low (approx. 10–20 mg/l), and compared with serum, only 1/300th the concentration.

The concentration of linoleic acid ($C_{18:2}$) in serum is approximately 25% of total FA or 2.5–4.0 µmol/ml. The concentration of linoleic acid in brain is much lower (only 1% of total FA) and in CSF, 4% of total FA or 1.6 nmol/ml.

Large demyelinating plaques in MS are often located near the ventricles and corresponding biochemical alterations of lipid metabolism and degradation lipids may be transmitted to the surrounding CSF.

Research Aums

The research described here was directed toward several questions:

1. Is it possible to draw conclusions about the origin of CSF sphingomyelin from its FA profile? Is the concentration of sphingomyelin with typical myelin FA pattern increased in demyelinating diseases?

2. Is the concentration of linoleic acid in CSF increased with disturbance of blood-brain-barrier (BBB) function, and, assuming its origin from serum, can CSF linoleic acid consequently be used as a serum marker similar to albumin? In MS there is often an overproportional CSF-IgG elevation related to CSF-albumin [1]. Can this overproportional CSF-IgG elevation also be demonstrated in relation to CSF linoleic acid?

Results

Total lipid extracts from pooled CSF (control and MS) were separated by thin layer chromatography (TLC) and the individual lipid classes quantitated by densitometry. Individual bands were scraped off the plate, eluted, and transmethylated and

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Table 1. Fatty acid composition of total lipids and individual fractions from pooled control CSF (in relative percent)

Fatty acid	Total lipids	Neutral lipids	Lecithin	Sphingo-myelin
C_n				
12 : 0	6.3	9.5	2.6	3.1
14 : 0	10.6	12.9	6.8	3.8
16 : 0	30.1	32.0	38.6	21.2
16 : 1	6.9	7.1	8.1	2.9
18 : 0	18.8	14.0	16.1	33.3
18 : 1	15.2	15.0	20.7	9.8
18 : 2 (linoleic)	3.8	3.0	6.7	1.9
18 : 3	2.5	3.1	tr.	0
20 : 0	0.2	0	0	0.8
22 : 0	1.3	0	0	7.9
20 : 4	2.6	3.4	0.4	0
24 : 0	1.0	0	0	8.1
24 : 1	0.7	0	0	7.2

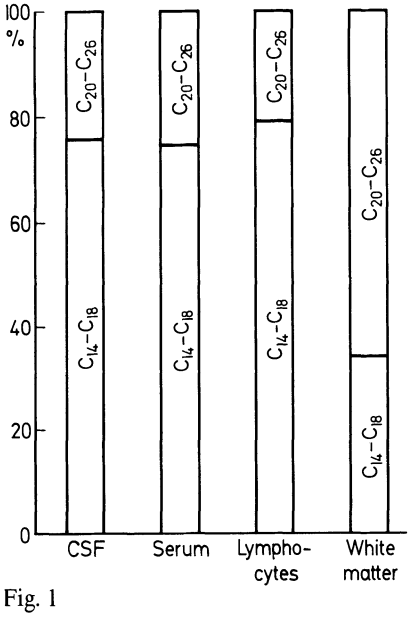

Fig. 1

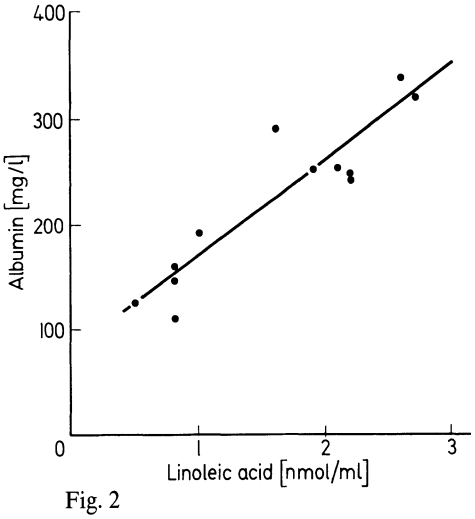

Fig. 2

Fig. 1. Fatty acid composition of sphingomyelin from CSF, serum, lymphocytes, and cerebral white matter

Fig. 2. Correlation between CSF linoleic acid and albumin in 12 control specimens

the FA composition analyzed using gas liquid chromatography (GLC). Serum and lymphocyte preparations were treated in a similar manner.

The FA composition of total lipids and individual fractions from control CSF can be seen in Table 1. A comparison of sphingomyelin FA profiles from CSF, serum, lymphocytes, and cerebral white matter is shown in Figure 1.

MS in acute exacerbation, commonly with pleocytosis, shows a slight increase of CSF sphingomyelin [6] compared to CSF lecithin without alteration of the FA composition.

Figure 2 shows the close correlation (r) between CSF linoleic acid and albumin in 12 control specimens of 1 ml each ($r = 0.92$) and in 10 MS specimens ($r = 0.95$), although here linoleic acid concentration has almost doubled (1.6 ± 0.8 nmol/ml in controls to 3.9 ± 3.7 nmol/ml in MS).

Similarly to the established Q_G quotient:

$$Q_G = \frac{\text{IgG (mg/l)} \times 100}{\text{albumin (mg/l)}} \text{ (upper normal limit 20)}$$

a Q_L quotient was calculated according to the following formula:

$$Q_L = \frac{\text{IgG (mg/l)}}{\text{linoleic acid (nMol/ml)}}.$$

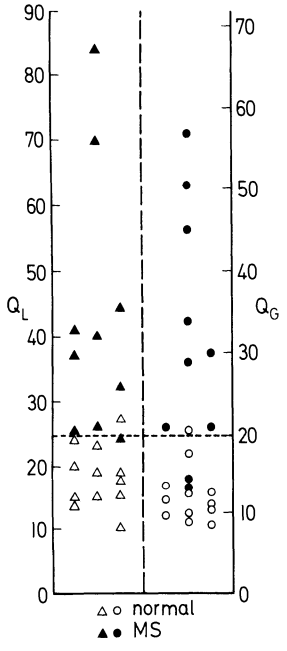

Fig. 3. Increased Q_G and Q_L quotients (see text) in MS as expression of the overproportional IgG elevation in CSF

In our control specimen, a Q_G of 12.2 ± 3.6 and a Q_L of 18.2 ± 4.9 was found. As might be expected, these quotients were increased in MS (Fig. 3) as an expression of the overproportional IgG increase in CSF, in relation to both albumin and linoleic acid as serum markers.

Discussion

A cerebral origin of CSF sphingomyelin seems unlikely considering its FA composition (Fig. 1). Glycolipids only present in CSF in trace amounts have R_F values of

glucocerebrosides which we also found in lymphocytes (approx. 8% of total lipids there); their FA composition was not typical of myelin, with a predominance of C_{16} and C_{18} FA.

The small increase of sphingolipids in CSF with pleocytosis from acute MS indicates a hematogenic origin of CSF sphingomyelin; decomposing cells are apparently the major source of CSF lipids. No correlation of CSF lipids to degradation lipids found in demyelinating plaques in the vicinity of ventricles is possible, as has been discussed in a previous report [4].

As previously reported [3, 5], CSF (normally low in linoleic acid content) shows a significant increase of unsaturated FA (particularly linoleic acid). We could demonstrate that linoleic acid in CSF is closely correlated to CSF albumin and that both function as serum markers. Also, in relation to CSF-IgG, both behave in an identical manner so that Q_G and Q_L may demonstrate an overproportional CSF-IgG concentration. One can assume that CSF linoleic acid content is dependent on BBB function (as is CSF albumin); it seems unlikely that it could be derived from the CNS (as has been postulated for CSF-IgG), because the concentration of linoleic acid in CNS tissue is very low.

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Diagnostic Value of Myelin Basic Protein in Cerebrospinal Fluid

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Introduction

Four years ago we described a chemical test relating to a component of the central nervous system (CNS) [3]. Our original aim was to provide a means for objective evaluation of demyelinating activity in multiple sclerosis (MS). A specific protein of myelin, the basic protein, was detected by radioimmunoassay in the cerebrospinal fluid (CSF) of MS patients undergoing exacerbation. Moreover, the levels of myelin basic protein (MBP) correlated with the clinical course of MS. In contrast, we could not detect any MBP in spinal fluid from inactive MS patients or patients with non-demyelinative neurologic disease. In this report, based on 500 additional samples from patients with a wide variety of neurological diseases, we confirm the usefulness of this test in evaluating activity in MS. In addition, radioimmunoassay of MBP in CSF may be a useful adjunct to the clinical assessment of other neurological diseases in which myelin breaks down acutely. These include leukodystrophies, severe anoxia, and the myelopathies and encephalopathies due to radiation therapy or chemotherapy.

Materials and Methods

Patients

All patients except some of those with optic neuritis were from Johns Hopkins Hospital. Approximately half of the optic neuritis spinal fluid samples came from Dr. Shirley Wray of the Massachusetts General Hospital.

We have divided our patients into the following categories based on clinical symptoms:

1. Classical MS – at least two attacks occurring in different parts of the nervous system more than 1 month apart and not explainable based on other disease processes. Age between 10 and 50 years. This category may be divided into several subcategories:

a) Active – within 1 week of the onset of new neurological symptoms.

b) Inactive (remission) – patients more than 2 weeks from the onset of any new neurologic symptoms or change in existing symptoms.

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c) Chronic MS – a progressive disease of more than 6 months duration affecting more than one area of the CNS (this may occur in the absence or presence of previous exacerbations and remissions). For the purposes of this study, typical remitting disease which has become progressive is included in this category.

2. Optic neuritis without evidence of other nervous system involvement.
3. Myelinopathy – diseases affecting myelin other than MS.
4. Nondemyelinating neurologic disease.

Assay of Cerebrospinal Fluid

All samples were obtained by lumbar puncture and stored at –10° C until the assay. The spinal fluid basic protein appeared to be stable, as identical values for this protein were obtained on a sample before and after incubation for 1 week at room temperature. However, because of the possibility of elevated protease activity in an occasional sample, we suggest freezing the sample until the assay.

For the assay, 0.05 ml of a 10-fold concentrated assay buffer (2 M Tris-acetate, pH 7.5, containing 10 mg of histone per ml) and antiserum at the appropriate concentration were added directly to 0.5 ml of spinal fluid. This mixture was incubated for 1 h at 37° C, 15,000 cpm of ^{125}I-labeled basic protein (specific activity, 10–20μCi/μg) were added, and the mixture was incubated for an additional 10–24 h at 4° C. The antibody-basic protein complex was then precipitated with cold ethanol, the pellet and supernatant fraction were separated by centrifugation, and each was assayed for radioactivity. The percentage of ^{125}I basic protein bound (i.e., in the pellet) was then determined [2]. Results are reported as negative (<4 ng/ml), weakly positive (4–8 ng/ml), or positive (> 9 ng/ml) [1].

These studies were done using two similar batches of basic protein antisera. Our experience has demonstrated that not all antisera react equally well with the CSF basic protein. However, in studies with different sera, elevated basic protein is consistently found in CSF from patients with active demyelinating diseases.

Results

Samples have been assayed from 793 patients with known neurologic disease (Fig. 1). These include 165 samples from MS patients, 9 from patients with other active demyelinating diseases, 597 from patients with nondemyelinating neurological diseases, and 22 with optic neuritis.

Multiple Sclerosis Patients

Forty-eight of 58 MS patients in acute exacerbation had spinal fluid basic protein levels greater than 8 ng; the remaining ten had levels between 4 and 8 ng/ml. The lumbar punctures on these ten patients were obtained between 5 and 15 days after the onset of the acute exacerbation. Thirty-four of 61 patients with the slowly progressive form of the disease had basic protein levels between 4 and 8 ng/ml. All but two patients with inactive disease had levels below 4 ng/ml (Fig. 1). These two pa-

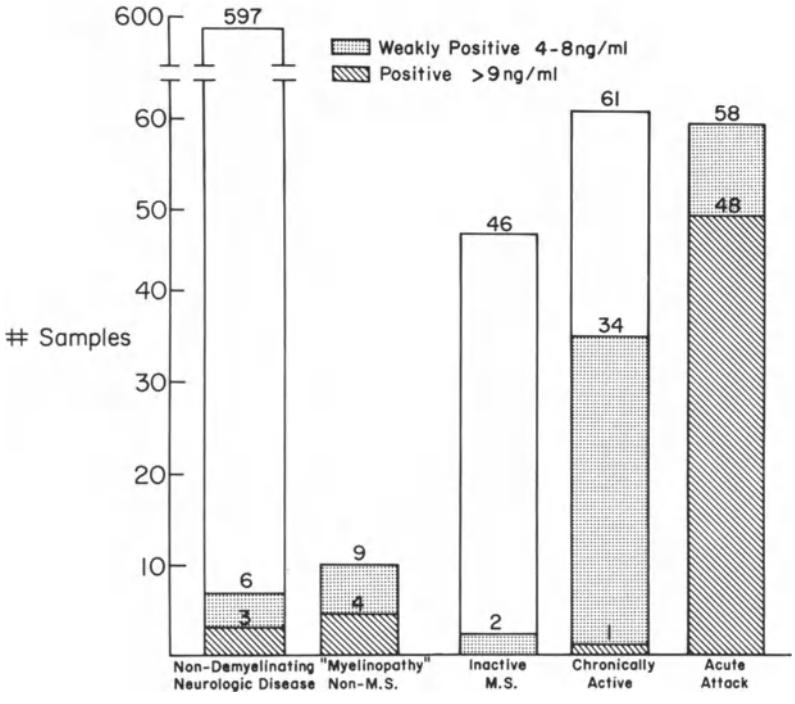

Fig. 1. Presence of MBP in CSF. – *Clear area* = less than 4 ng/ml; *stippled area* = between 5 and 8 ng/ml; *shaded area* = more than 9 ng/ml of CSF. The nondemyelinating neurologic diseases with no detectable CSF MBP included at least one sample from each of the following: Arteriovenous malformation, seizures, neuroblastoma, arthrogryposis, temporal arteritis, cerebellar degeneration, microcephaly, peripheral neuropathy, presenile dementia, subacute sclerosing panencephalitis, stroke, subarachnoid meningitis, hydrocephalus, migraine, labyrinthitis, Moya-Moya Syndrome, vascular headache, progressive supranuclear palsy, lacunar infarct, Guillain-Barre, spastic paraplegia, pseudotumor cerebri, senile dementia, progressive multifocal leukoencephalopathy, meningoencephalitis, sixth nerve palsy, fifth nerve palsy, trigeminal anesthesia, vasculitis, post anoxic encephalopathy, third nerve sarcoid, progressive spastic paraparesis, progressive external ophthalmoplegia, striatonigral degeneration, cervical degenerative arthritis and radiculopathy, polymyositis, trauma, motor neuron disease, Meniere's disease, vertebral basilar ischemia, herpes simplex encephalomyelitis, and neurosyphilis. The non-MS patients with positive CSF basic protein are listed in Table 1

tients were recovering from acute exacerbations that occurred 14–21 days prior to the lumbar puncture. In addition, five of the patients in this group presented the first clinical evidence of MS and their CSF revealed elevated MBP. These patients all had second acute attacks, confirming the diagnosis of MS.

Serial samples were obtained from patients before, during, and after attacks (Fig. 2). The CSF basic protein levels rose and fell with the exacerbation. Those with significant cerebrospinal basic protein 10–14 days after exacerbation still demonstrated some progression of their disease (Fig. 2, patients D. W. and E. C.), while those whose basic protein had returned to normal levels were recovering from the exacerbation (Fig. 2, patients R. W. and R. Q.). Thus, as the patient improved, the CSF MBP returned to normal levels. Those patients who went from acute attacks to

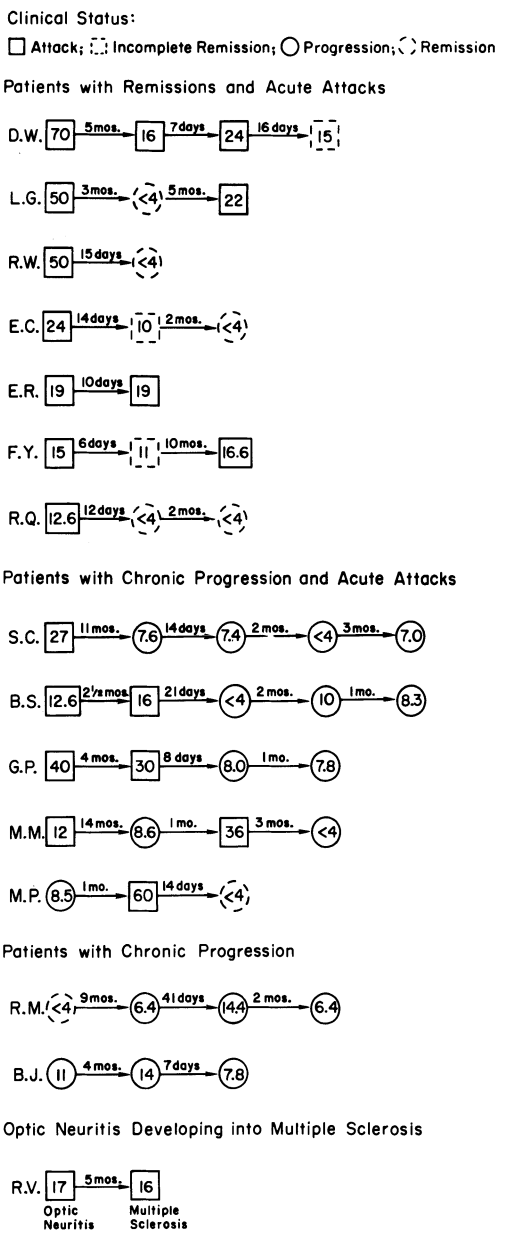

Fig. 2. Relationship of MBP to clinical course of MS. Each symbol represents the clinical status of the patient at the time of lumbar puncture: □ attack; ⬚ incomplete remission; ○ progression; ◌ remission. The number in each symbol is the CSF MBP value in ng/ml

slow progression usually showed low but significant levels of spinal fluid basic protein months after the attack (Fig. 2, patients S. C., B. S., G. P., and M. M.) Thus, of the slowly progressive group of patients, those with spinal fluid MBP (approximately half) are frequently the ones with exacerbations superimposed on their chronic progression.

Of 22 patients with optic neuritis, nine have had basic protein in the CSF. All these patients are being observed to determine the outcome of this first attack. One of these patients has subsequently developed MS (Fig. 2, patient R. V.).

Other Diseases of Myelin

The category of "myelinopathies" included patients with transverse myelitis, metachromatic leukodystrophy, central pontine myelinolysis, adrenal leukodystrophy, an undescribed hereditary leukodystrophy, methotrexate myelopathy, and Pelizaeus-Merzbacher Disease. These also had elevated CSF basic protein (Table 1). Two additional patients with Pelizaeus-Merzbacher Disease had no detectable CSF basic protein.

The patient with methotrexate myelopathy received a bone marrow transplant, whole body irradiation, and prolonged treatment with intrathecal methotrexate. He began to show photophobia, and 2 weeks later developed a transverse myelitis. The patient died 2 weeks after this and the spinal cord pathology revealed severe diffuse microvacuolization of the long tracts of the spinal cord. During this time he also had elevated spinal fluid basic protein. We are currently completing a study in conjunction with the Pediatric Oncology Branch of the National Cancer Institute on patients who have received intrathecal injections of methotrexate. Out of 100 patients, four had clinically detectable encephalopathies and these four also had elevated spinal fluid MBP.

Controls

All but six of the 597 controls had less than 4 ng of basic protein per ml of CSF. These six patients are listed in Table 1. They include two severe strokes on the sur-

Table 1. Levels of CSF MBP in patients without MS

	Basic protein (ng/ml)
Neurologic disease – nondemyelinative	
Lateral medullary infarction	60
Cerebellar infarction	56
Wernicke's disease	18
Anoxic encephalopathy	8
Microglioma	8
Encephalitis	13
Myelinopathies other than MS	
Leukodystrophies	
Hereditary leukodystrophy	23
Metachromatic leukodystrophy	12
Adrenal leukodystrophy, pt. 1	8
Adrenal leukodystrophy, pt. 2	10
Pelizaeus-Merzbacher, pt. 1	8
Pelizaeus-Merzbacher, pt. 2	8
Other demyelinating disorders	
Transverse myelitis and systemic lupus erythematosus	100
Central pontine myelinolysis	50
Methotrexate myelopathy	17

face of the brain, Wernicke's Disease, a microglioma, encephalitis, and severe anoxia following surgery. With respect to this last patient, it is interesting that Kohlschutter [5] has recently reported the application of the basic protein radioimmunoassay to the CSF from children. He found that 8 of 41 CSF samples were positive for MBP. These eight samples were from six children with severe hypoxia and one case of encephalitis with cardiac arrest. In five of six cases the brain damage led to death. Thus, this test may be useful in assessing brain tissue destruction in patients experiencing hypoxia.

Discussion

These studies indicate that MBP or fragments thereof are released into the CSF as part of the myelin breakdown process, thus confirming and extending our previously reported results [3]. MBP is present not only in the spinal fluid of patients with active MS, but also in patients with other active demyelinating conditions such as metachromatic leukodystrophy and central pontine myelinolysis. The few positive tests in other neurologic conditions clearly indicate that myelin breakdown products may also be released into the CSF as a result of damage by different processes such as hypoxia and necrosis due to radiation or chemotherapy.

Similar results have been obtained in the laboratories of Whitaker [7] and Trotter et al. [6] on patients undergoing acute demyelinative episodes, while Kohlschutter [5] has used a CSF basic protein assay to assess brain tissue destruction in hypoxic children. Carson et al. [1] have recently reported the presence of a large component (mol. wt., 50,000 daltons) in the CSF of patients with MS that cross-reacts with antibody to MBP.

In a given pathological condition in which myelin breaks down, several factors may determine how much MBP will be released, and whether it will appear in the lumbar CSF. These include (1) location of myelin breakdown and direction of CSF flow, and (2) the nature of the pathological process.

The location of myelin breakdown is important, as material released from superficial lesions has good access to CSF pathways. Thus the predilection of MS plaques for the periventricular white matter and the superficial white matter of the brain stem and spinal cord would be likely to result in the release of substantial amounts of basic protein into the CSF. Much of this MBP would then appear in the lumbar CSF.

The nature of the pathological process may also determine the properties of the basic protein that appears in CSF during an attack of MS. The CSF basic protein may be present as intact protein or peptide fragments. It may be whole myelin, or myelin fragments or cells with ingested myelin. Alternatively, this CSF basic protein may be in a lipid, protein, or nucleic acid complex. For example, the finding of peptide fragments in the CSF would suggest extracellular enzymatic degradation as the mechanism of demyelination, whereas the presence of whole myelin would indicate that the entire sheath was being removed by cellular attack. In the experimental demyelinating disease, Allergic Encephalomyelitis, which is clearly autoimmune, the basic protein released into the spinal fluid is bound to its antibody [4]. The presence

of antibody-bound basic protein has not been demonstrated in MS, although very low levels of antibody may be present. We are continuing our investigation of the properties of this CSF basic protein in MS.

It is important to distinguish between a test that is diagnostic and one that measures activity of the disease. CSF MBP levels rise and fall with the exacerbations and remissions that are typical of MS. This is consistently observed, although in individual patients there are considerable variations in the amount of spinal fluid basic protein at the height of the attack, probably reflecting the amount of tissue undergoing demyelination. In patients with inactive disease, no CSF basic protein is found, and thus the radioimmunoassay for MBP should not be regarded as a definitive diagnostic test for MS. However, it is clearly a useful indicator of active myelin degradation and as such, when used in conjunction with the clinical data, is a very useful adjunct in the diagnosis and management of MS. Currently, the test is being used in a prospective fashion to assess the efficacy of steroid treatment. We anticipate that the test will be used in a similar fashion as newer methods of MS therapy become available.

There are other situations in which the physician wishes to know if a patient is experiencing an acute demyelinating process. Thus, in the case of leukodystrophies, anoxia, and encephalopathy or myelopathy due to cancer, radiation, or chemotherapy, the presence of MBP in the CSF may be an indicator that myelin breakdown is actually occurring.

As our knowledge of neurochemistry increases, one can visualize specific tests for each of the cellular components of the nervous system. Using immunochemical methods, it may eventually be possible to identify in the CSF breakdown products of highly specific cell types and transmitter systems.

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Myelin Basic Protein in Cerebrospinal Fluid as an Indicator of MS Process Activity

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The concentration of myelin basic protein (MBP) was measured using a radioimmunoassay [2] in unconcentrated frozen CSF from MS and other patients. Here 800 µl of CSF were sufficient for duplicate analysis. The clinical diagnoses and the results of the measurements were kept coded throughout the study. The diagnoses were classified as active MS (acute exacerbations and chronic progressive forms), inactive

Table 1. Myelin basic protein (MBP) in 86 CSF specimens

Diagnosis	Numbers of CSF specimens		
	MBP positive >4 ng/ml	Questionable	Negative
MS, active	16	2	4
MS, inactive	0	2	15
MS, activity uncertain	2	2	1
Possible MS	2	3	6
Other diseases	7	4	20

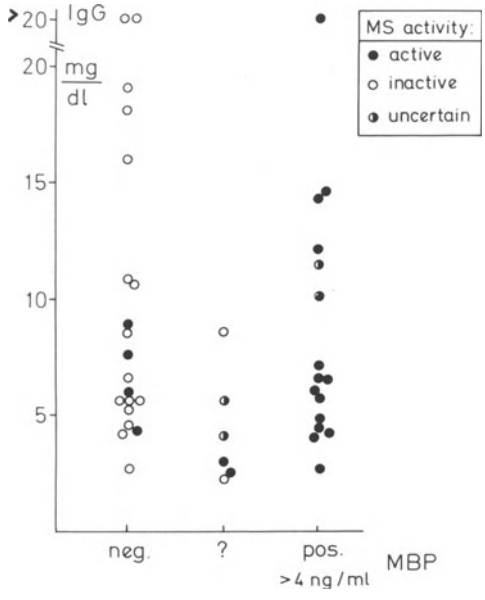

Fig. 1. IgG concentration and MBP content of 42 CSF samples from MS patients. Samples are grouped as MBP negative, questionable, and positive. Symbols represent process activity as indicated

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MS, possible MS, and other diseases. The CSF samples were classified according to their MBP content as positive (> 4 ng/ml), questionable (1–3 ng/ml), or negative.

The results (Table 1) show that out of 22 samples from patients with active MS, 16 were MBP positive, while out of 17 samples from inactive cases, none was positive and 15 were clearly negative. In the group of 31 other diseases there were seven positive samples. These included three cases of cerebrovascular accidents, one of them having the highest MBP concentration of the series (88 ng/ml). The other positive samples were from patients with syringomyelia, acute hydrocephalus, Behçet's disease, and an ill-defined myelopathy. In samples from MS patients there was no correlation between the concentrations of MBP and IgG (Fig. 1). The same was true for MBP and cell count.

These results suggest, as have other studies [1, 3, 4], that the testing of CSF is not diagnostic for MS but that it is a useful index of process activity in MS, particularly since the procedure has been simplified remarkably.

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Central and Peripheral Myelin Basic Protein in Cerebrospinal Fluid of Multiple Sclerosis and Other Neurological Disorders

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Introduction

Myelin contains two different basic proteins, the central myelin basic protein (MBP) (= A1) and the peripheral basic protein (= P2). The central MBP is present in the central nervous system (CNS) as well as in nerves, whereas peripheral basic protein is only present in the myelin sheet from nerves. Cohen et al. [3] have shown that in cerebrospinal fluid (CSF) from patients with demyelinating neurological disorders the level of central MBP is significantly increased. Patients with multiple sclerosis (MS) in acute exacerbation had high levels of this basic protein, whereas its concentration was low in remission. These results have been confirmed by others [6, 7].

Up until today nothing has been known about the level of peripheral MBP in spinal fluid. Therefore we have recently developed a radioimmunoassay for the determination of this protein. In this report we describe the results of investigations in which the level of both MBPs has been measured in spinal fluid from patients with neurological disorders.

Experimental Procedure

Peripheral MBP was isolated from bovine intradural roots. At first, basic proteins were extracted from roots at pH 3 as described by Deibler et al. [5]. From this extract, peripheral basic protein (purity > 95%) was isolated by column chromatography according to Brostoff et al. [2]. Iodinated peripheral MBP was prepared using the ^{125}J-labeled reagent of Bolton and Hunter [1]. Experiments to iodinate the protein by chloramine-T oxidation were unsuccessful. The labeled protein was separated from the reagent and purified by chromatography on a Sephadex G-100 column. Antibodies against this protein were prepared in rabbits. Six animals were injected sc with 1 mg of antigen emulsified in complete Freund's adjuvant (CFA) and absorbed by Al_2O_3. Five booster injections were given at 1–2 month intervals. None of the animals showed clinical signs of an experimental allergic neuritis.

Spinal fluid samples were stored until assay at – 30° C. The radioimmunoassay of both MBPs was performed basically as described by Day and Pitts [4]. Their procedure was based upon the precipitation of the antigen-antibody complex in 1.27 M Na_2SO_4, and our main variation was that we used Tris buffer (pH 7.2) to dissolve antigens and antibodies instead of phosphate-buffered saline.

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Results and Discussion

Figure 1 gives typical dose-response curves of the radioimmunoassays for central and peripheral MBP. The A1 assays were performed using 0.1 and 1 ml spinal fluid, respectively, to enlarge the range of the assay. From 2 to 100 ng of A1 protein could be measured per ml of spinal fluid. In the case of peripheral MBP (P2), no more than 0.1 ml spinal fluid was applied and from 5 to 50 ng basic protein could be determined per ml.

Central and peripheral MBP differ in their amino acid composition, sequence, and conformation. In radioimmunoassays we could not detect a significant cross-reaction between these two antigens (Fig. 2).

Table 1 lists the liquor data from 10 patients with MS. The number of cases was too small to divide them into different groups according to clinical symptoms; how-

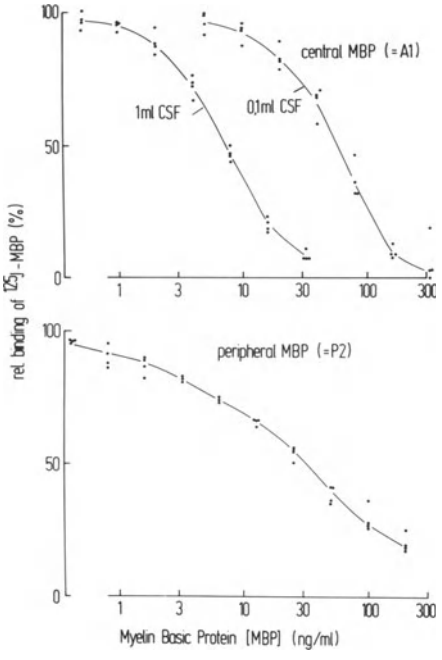

Fig. 1. Dose-response curves of the radioimmunoassay for central and peripheral MBP

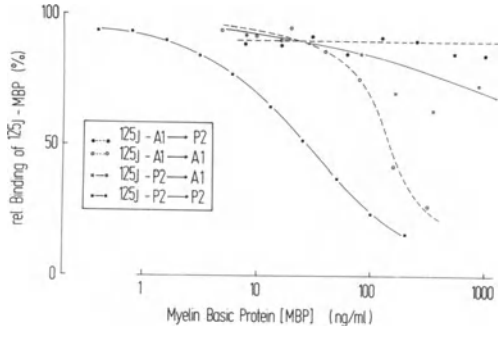

Fig. 2. Cross-reaction between central (A1) and peripheral (P2) MBP

Table 1. Central (A1) and peripheral (P2) basic protein in CSF of patients with MS

Age (a)	Total protein (mg/100 ml)	Alb IgG	Basic protein (ng/ml)		Leuco/ml $\times 10^{-3}$
			Al	P2	
20	47	–	150	n. d.	40/3
34	24	5.9	10.3	11.0	24/3
15	20	–	7.2	n. d.	21/3
39	50	2.2	7.0	6.2	28/3
45	33	0.7	6.5	n. d.	19/3
41	24	–	4.4	n. d.	8/3
45	36	–	4.0	n. d.	29/3
39	31	2.9	3.4	n. d.	8/3
37	42	1.3	3.3	n. d.	32/3
50	38	1.5	1.2	n. d.	9/3

n. d. = not detectable

Table 2. Central (A1) and peripheral (P2) basic protein in CSF of patients without MS

Disorder	Total protein (mg/100 ml)	Alb IgG	Basic protein (ng/ml)		Leuco/ml $\times 10^{-3}$
			A 1	P 2	
Basic proteins > 6 ng/ml					
Encephalitis	60	1.9	130	n. d.	111/3
after 20 days	60	1.5	5	–	42/3
Chron. Meningitis	52	6.0	66	13	35/3
after 28 days	59	6.8	15	–	73/3
Meningo-radiculit.	97	2.7	10	13	280/3
Meningo-radiculit.	56	5.0	9	12	250/3
Brain stem Infarct.	35	9.6	13	6	10/3
Intracran. tumor	80	–	8	–	56/3
Lymphoma Meningiosis	135	2.7	9	n. d.	108/3
Neurosyphilis	36	3.5	7	13	72/3
Basic proteins < 6 ng/ml					
Meningo-encephalitis (2), Virus meningitis (2), Tbc meningitis (2), Polyneuropathy (4), Polyradiculitis (1), Guillain-Barré (1), Intracranial tumor (4), Controls (13)					

Number of patients in brackets
n. d. = not detectable

ever, these data indicate that in most cases with elevation of the level of central MBP (> 4 ng), no peripheral basic protein was detectable in spinal fluid. Table 2 gives the data from patients with other neurological disorders. In general, the level of each basic protein in CSF was less than 6 ng/ml. In seven cases the A1 level was higher than 6 ng/ml. It is remarkable that in most of these cases the level of P2 was also elevated. The presence of P2 in CSF indicates that in these disorders intradural peripheral nerves are affected.

In the beginning, the first two patients in Table 2 showed an extremely high level of A1 in CSF. In both cases the A1 levels decreases rapidly as is already known from the investigation of Cohen et al. [3].

The radioimmunological determination of both MBPs in CSF may be suitable for a more precise biochemical localization of the neurological disorders related to MS.

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The Activity of a Cholesterol Ester Hydrolase in Human Cerebrospinal Fluid

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The cerebrospinal fluid (CSF) of patients afflicted with various neurological diseases was analyzed for its myelin-specific cholesterol ester hydrolase (EC 3.1.1.13, cholesteryl-oleate $\rightleftharpoons$ cholestereol + oleic acid) [1]. In a recently published study the activity of the microsomal species of the cholesterol ester hydrolase was measured in CSF. In this investigation the myelin-specific species could not be determined, since the detergent employed was unsuitable. We therefore used the method of Igarashi and Suzuki for the *enzyme assay*.

Taurocholate, phosphatidylserine, and 4-^{14}C-cholesteryl oleate were suspended in phosphate buffer, pH 7.2, by ultrasonication. Five-hundred µl of this suspension together with 100 µl of concentrated (5–10-fold) CSF were incubated at 37° C for 2 h. ^{14}C-cholesterol liberated by the enzymatic reaction was determined using the digitonin precipitation procedure. Blank tubes contained a heat-inactivated enzyme source. Lack of protein in the blank tubes increases the blank values. CSF samples (1–2 ml) were concentrated in centriflo-cones (Amicon, CF 25) for 10 min at 3000 *g* at 4° C.

In characterizing the enzyme, the measured activity depends on the amount of total protein in a nonlinear sigmoidal function. For a limited range, a linearization may be acceptable.

Regarding the stability of the enzyme, if fresh, centrifuged CSF was stored at room temperature, the specific activity of the enzyme decreased about 1% per hour. In each freeze-thawing process, its activity was decreased by about 15–20%.

Regarding the enzyme's solubility in CSF, 100% of the enzymatic activity is recovered in the supernatant after centrifugation for 1 h at 120,000 *g*.

The enzyme has several clinical aspects. Control CSF from 80 patients (normal protein levels, normal cell number, and normal immunglobulin fractions) had a cholesterol ester hydrolase activity of 4 nmol/mg protein/h. This is 1–2% of the maximal specific activity of the solubilized 300–500-fold purified enzyme preparation of Igarashi and Suzuki [2], or 10–50% of the activity in isolated myelin fractions (rat or ox, respectively).

Serum contains no detectable amount of the enzyme. As a consequence, blood-brain barrier dysfunction should not influence the amount of enzyme per volume CSF (80 pmol/100 µl CSF/h for 80 controls).

Due to these facts, it is of interest to study enzyme activity in the CSF of patients with various neurological diseases, especially with respect to demyelinating processes. First results with clinical relevance were reported recently [4].

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Clinical Relevance of the Determination of IgM in the Cerebrospinal Fluid with Special Reference to Multiple Sclerosis Patients

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The determination of plasma proteins in cerebrospinal fluid (CSF) enables us to assess the filtration process through the blood-brain barrier. Their increase generally indicates the existence of an inflammatory reaction in neurological tissues. Particular attention has been paid to IgG because of its possible local origin, and its determination is now fairly common. On a theoretical basis, the determination of IgM in CSF should be more useful than that of IgG. Because IgM is less liable to gain access to CSF by mere transudation on account of its large molecular size, an increase in IgM concentration should be a better index of the local immune response. Moreover, IgM should become detectable at earlier stages of the immune process, as it is generally the first antibody to be produced. However, in practive, the determination of IgM in CSF is not easy, as it requires very sensitive methods.

We report here the preliminary results of a study on the clinical relevance of IgM determination in the CSF of patients with various neurological disorders. IgM was determined by a novel method called the Particle Counting Immunoassay (PACIA). The basic principle of this immunoassay is the agglutination of antibody-coated particles (latex) by the antigen to be determined. This agglutination is measured by counting the residual, nonagglutinated particles using a device designed to count blood cells.

Materials and Methods

The PACIA system has been automated using a Technicon AutoAnalyzer with a special sampler and an AutoCounter (Technicon Instruments Corporation, Tarrytown, N. Y.). The height of the recorded peaks is directly proportional to the number of free particles.

Calibrated polystyrene particles 0.8 μ in diameter, a gift from Rhône-Poulenc (Courbevoie, France), were coated with antibody as described by Cambiaso et al. [1]. Briefly, IgG purified from goat anti-IgM antiserum was adsorbed on particles by simple mixing of the reagents.

Standard curves were obtained by making serial dilutions of a standard serum from Technicon. Samples of CSF had been stored frozen in the presence of sodium azide (0.1%). For the immunoassay, it was generally necessary to dilute the samples by a factor of at least four with 0.1 M glycine-HCl buffer, pH 9.2, containing 0.17 M

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NaCl, 0.1% bovine serum albumin, and 50 mM EDTA. To increase sensitivity, a solution of polyethylene glycol was added to the incubating tube until it reached a final concentration of 1.33%.

Patients

We considered four groups of patients:

1. Ten with non-neurological disorders (minor neurosis, or uveitis without neurological signs).
2. Seventy-three with various neurological disorders but normal CSF, i.e., a protein level below 40 mg/%, a number of cells below or equal to 5 per mm^3, and normal appearance in agar gel electrophoresis.
3. Forty-two with clinically definite multiple sclerosis (MS).
4. Eighteen with viral or bacterial (including tuberculous) meningoencephalitis.

Results and Discussion

In the group of ten patients with non-neurological disorders (Fig. 1), the level of IgM in the CSF ranged from 30 to 400 ng/ml with a normal logarithmic distribution and a median value of 117 ng/ml. Adding two SDs, the upper normal limit was set

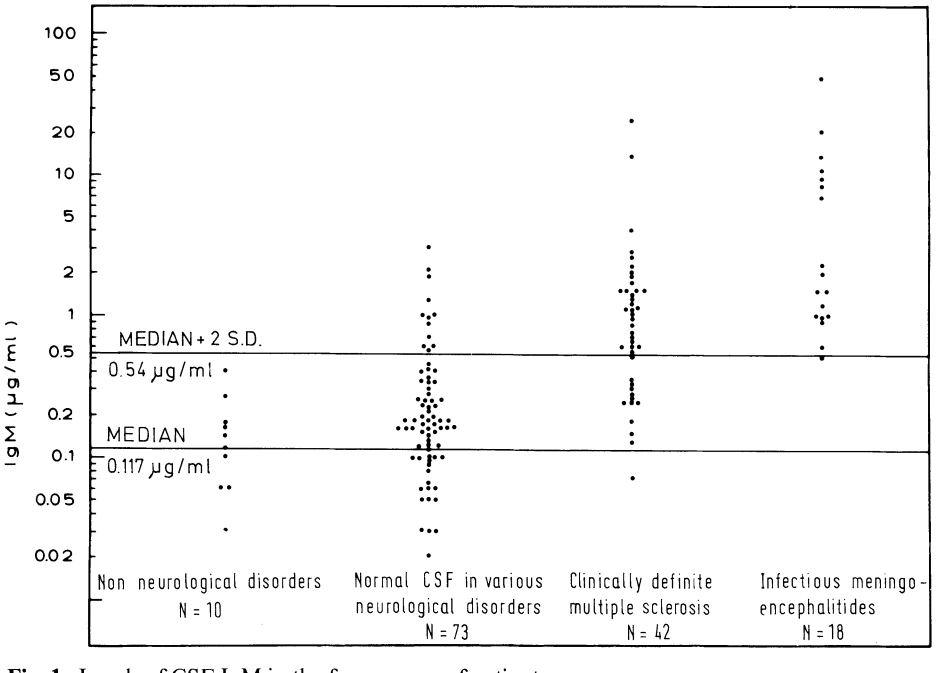

Fig. 1. Levels of CSF IgM in the four groups of patients

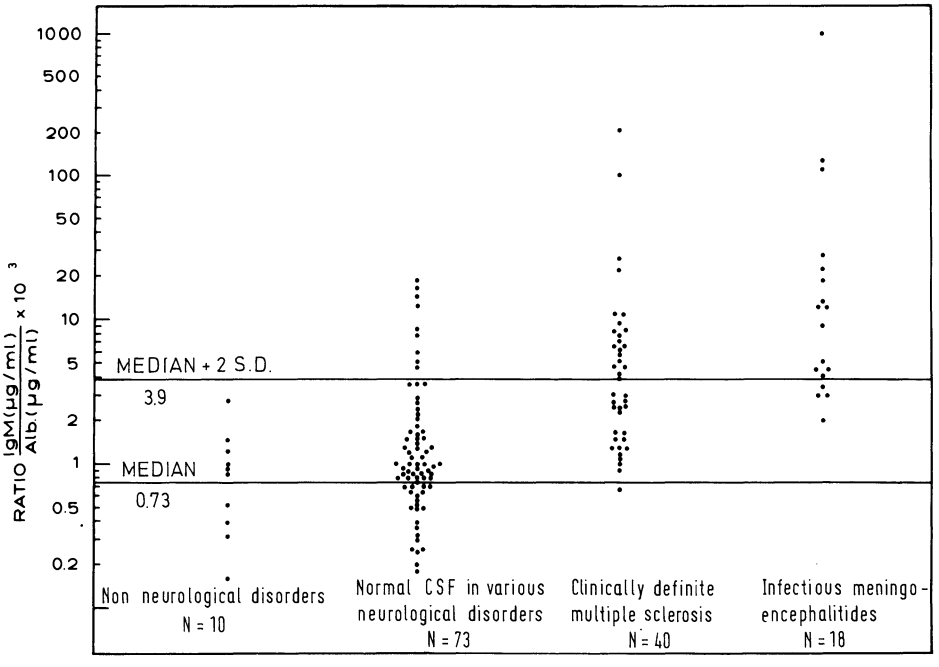

Fig. 2. Ratio IgM · 10^3/Alb in the four groups of patients

Table 1. Correlation between the occurrence of oligoclonal bands and IgM concentration in the CSF of patients with MS

Concentration of IgM in CSF (µg/ml)	Oligoclonal bands in CSF		
	Present	Absent	Total
More than 0.54 µg/ml	28	1	29
Less than 0.54 µg/ml	14	10	24
Total	42	11	53

$\chi^2 = 9.37$; $0.005 > P > 0.001$

at 540 ng/ml. Of the 73 patients with neurological disorders and normal CSF, 12 had abnormally high levels of CSF IgM. The level of IgM in the 42 patients with clinically definite MS exceeded the normal upper limit in 28 cases (66%), whereas in meningoencephalitis, all patients except for one gave abnormal IgM values.

To distinguish between passive transfer of IgM or local biosynthesis, we have studied the ratio between the levels of IgM and albumin $\left(\frac{\text{IgM (µg/ml)}}{\text{Alb (µg/ml)}} \cdot 10^3\right)$, the latter being determined by immunonephelometry (Fig. 2). In the group of non-neurological disorders, the ratio ranged from 0.16 to 2.86 (median: 0.73; median plus 2 SD: 3.9). In the group with neurological disorders and normal CSF, nine patients had a ratio exceeding 3.9 : two of these had peripheral neuropathies of unknown etiology, one had possible MS and normal agar gel electrophoresis, one had arteritis of the

central nervous system (CNS) of unknown etiology, one had idiopathic megaencephaly and a mental deficit, one had sciatica and three could not be definitely diagnosed.

In the group of clinically definite MS, 50% had an abnormally high ratio compatible with local production of IgM, whereas 16% had a normal ratio, despite an abnormally high concentration of IgM. In the meningoencephalitis group, high ratios were observed in most cases (14/18).

No correlation was found between the levels of IgM and IgG (determined by immunonephelometry) in the CSF of clinically definite MS ($r = 0.006$). However, a highly significant correlation was found between the presence of oligoclonal bands and a high concentration of IgM (Table 1).

The association of oligoclonal bands with high levels of IgM is reminiscent of what is seen in reconstitution experiments [2]. The immunoglobulin pattern in the sera of children with severe combined immunodeficiency is characterized, in the days following transplantation with bone marrow, by the appearance of homogeneous immunoglobulins and predominant IgM concentration [3].

Finally, when the number of cells in CSF of patients with definite MS was above $10/mm^3$, the level of IgM always exceeded the upper normal limit. However, abnormally high values of IgM were also detected in patients with normal cell counts.

Our results, by confirming and extending those of Schuller et al. [4] and Williams et al. [5], suggest that the IgM concentration in CSF might be a useful index of immunological disorders affecting the CNS.

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Discussion

Dr. Nagai: I would like to comment on the radioimmunoassay technique: you know that labeling of the protein with iodine sometimes produces loss of EAE-inducing activity. The molecular conformation is important, and in some cases this may be changed by iodination. Secondly, if you use rabbits for immunization, they sometimes produce a very different kind of antibody. With some antibodies, there is a variety in recognizing the basic protein. Thirdly, you know that some fragments in MS and EAE basic protein may be degraded in various ways. Maybe a different kind of fragment of MBP will be produced and some antibody can actually recognize such a fragment but another serum cannot recognize it. So it depends on the MBP fragments produced.

Dr. McKhann: I'd like to comment on Dr. Nagai's statement, because he is absolutely right. It is possible to make antiserum to basic protein in other species that will not react with human CSF. Despite the fact that with purified basic protein there appear to be various degrees of cross-reactivity, I don't know the actual explanation for that, except for this observed phenomenon. The second question you raise about the state of basic protein in spinal fluid is of interest because Dr. Peter Brown in Canada, using an antibody, has foud not only basic protein but some higher molecular weight protein which may be a dimer of basic protein in spinal fluid. So there is a possibility, and one hypothesis may be that after an acute attack, one might first get the release of free basic protein and then basic protein complexed in fragments. And one could determine that one might have some method of actually age dating what was going on. But this is speculation as to what one first has to figure out and what's in there.

Dr. Nagai: In my case, with tritium-labeled BP, it is much more safe compared with the iodine level. Such a tritium label using the borohydride-formaldehyde method yields very good (EAE) active labeled basic protein. In the future we will have to use the first fractionation of basic protein obtained by some means, for example, by disc electrophoresis or by other means. And then perform the test on the fractioned, basic protein fragment by the radioimmune assay.

Dr. Salk: I don't know whether I have missed Dr. McKhann's earlier remarks. Did you mention how you made the antibody that you used in your radioimmune assay? Because that's the all critical factor.

Dr. McKhann: I didn't mention it in this particular talk. But the studies have been reported here. The antibody is made in a rabbit and the source of the basic protein was calf. So it's a rabbit antibody to calf basic protein. Subsequently we have used other basic protein as the source of antigen. That has not given us trouble. What has given us trouble is going to different species for the source of the antibody. We went to the goat, thinking we would have a large animal with a continu-

ing source of antibody. And a goat antibody does not react with human CSF at the time that the rabbit antibody does.

Dr. McKhann: Let me make one comment. People have asked us on occasion because it comes up to whether or not we should be able to consider this as a relatively routine assay. I think that Dr. Nagai's comment is very appropriate in this regard. Our feeling at the present time is that the answer is no, that there are still too many technical problems with this system to think that you can go out and make any old antibody to any old form of basic protein and expect it to work. So I would caution people about that.

Question: I should like to ask Dr. McKhann about his controls. Did you try to make some match of controls regarding the activity of the disease? I think that the activity of MS has been related today to some immunological activity in CSF and even in peripheral blood. Nobody did the controls and measured them for activity of the disease, only the next to last speaker, made a comment on it. That in cerebrovascular accidents, in the active phase, you get higher results and I think you should make the controls about activity of disease.

Dr. McKhann: We have not looked at that in a systematic fashion. But being in an acute hospital I would guess that we have close to 100 patients in this group with cerebrovascular accidents, most of whom would be there within ten days after their cerebrovascular accident. Only three of those have been positive. All of them had cerebrovascular accidents in the posterior fossa. I don't have data specially correlating with disease activity in the controls, but I think, that this is a good point.

Comment: We've got results that it's accurately related to the acitivity of the disease. There have even been results with the two basic proteins in head injury, related to the severity of head injury.

Dr. Clausen: I have a question to Dr. McKhann concerning basic protein. We have found four years ago that the basic protein precipitates with the β-lipoprotein of serum. It makes a very sticky complex. And that may be the explanation that you do not find the basic protein in serum. Because if it is liberated from the brain it may immediately be complexed to the beta-lipoprotein.

Dr. McKhann: I think that may be correct. Originally we thought that it was coming down with the clot, so we moved to plasma. But plasma didn't solve that problem. So I would say, there is either a very rapid turnover, which actually has been studied, and it is rapid in primates, or there is complexing to something and thus it is not available.

Nucleic and Viral Antibodies in Serum and CSF of MS Patients

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Two years ago, we described [6] a new method of counter-immunoelectrophoresis which allows the determination of DNA and RNA [7] antibodies in serum and unconcentrated CSF. This type of IgG antibody is found in normal serum but in pathological CSF only.

A preceding work [8] showed a very probable correlation between oligoclonal aspect, local synthesis of viral (measles and rubella) antibodies (VAB), and local synthesis of nucleic antibodies (NAB) in some MS CSF.

Material and Methods

We present here results obtained in 101 MS, 12 subacute sclerosing panencephalitis (SSPE), and 30 control cases (other neurological diseases). For each patient, serum and CSF were investigated (1) using electrophoresis on cellulose acetate after standardized concentration [2]; (2) by electroimmunodiffusion for IgA, IgM, IgG, C3, C4, and CRP, as previously described [3, 4, 5]; (3) DNA and RNA antibodies were determined by counter-immunoelectrophoresis and expressed in absolute value (serological dilution) and percentage [6], while CSF patterns were analyzed following our classification into five types [8]; and (4) measles and rubella antibodies were determined using hemaglutination inhibition.

Clinical data (sex, age of the patient, duration of the disease, clinical stage, type of course, and disability grade using Kurtzke's scale) were collected for each patient after careful analysis of the criteria for MS diagnosis.

Results

Table 1 shows the increase of NAB in 58 sera and their abnormal presence in 31 CSF of MS patients without apparent correlation. In SSPE, NAB were increased in serum and present in CSF in all but one patient, in contrast to their rare variations in controls. The presence of NAB and VAB was analyzed according to CSF patterns (Tables 2, 3). They are clearly linked to a local synthesis of IgG (inflammatory and meningitis patterns only for NAB and even "normal" pattern for VAB) in MS and

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Table 1. NAB in serum and CSF

NAB % in serum	NAB in CSF					
	MS (101)		SSPE (12)		Controls (30)	
	Absence (70)	Presence (31)	Absence (1)	Presence (11)	Absence (21)	Presence (9)
Normal	31	12	0	2	16	5
Increased	39	19	1	9	5	4

Table 2. NAB and CSF immunological patterns

CSF patterns (143)	MS (101)		SSPE (12)		Controls (30)	
	Absence (70)	Presence (31)	Absence (1)	Presence (11)	Absence (21)	Presence (9)
Normal (34)	26	–	–	–	8	–
Inflammatory (69)	34	21	1	8	4	1
Non inflammatory transudate (19)	6	–	–	1	9	3
Inflammatory transudate (3)	–	–	–	1	–	2
Meningitis (18)	4	10	–	1	–	3

Table 3. VAB and CSF immunological patterns

CSF patterns (143)	MS (101)		SSPE (12)		Controls (30)	
	Absence (49)	Presence (52)	Absence (0)	Presence (12)	Absence (19)	Presence (11)
Normal (34)	19	7	–	–	8	–
Inflammatory (69)	24	31	–	9	2	3
Non inflammatory transudate (19)	3	3	–	1	6	6
Inflammatory transudate (3)	–	–	–	1	1	1
Meningitis (18)	3	11	–	1	2	1

SSPE patients. On the contrary, a transudation is generally present in controls with NAB and/or VAB in their CSF.

To analyze the eventual relations between NAB and VAB in the CSF of MS patients, we divided our MS population into three groups: the first without NAB and VAB ($n = 42$), the second with VAB only ($n = 28$), and the third with both ($n = 31$). Significant differences appear between the frequencies of CSF patterns in the three

Table 4. CSF immunological patterns in the three MS groups ($n=101$)

CSF patterns	Group I: Absence of AB in CSF ($n=42$)	Group II: VAB only in CSF ($n=28$)	Group III: VAB and NAB in CSF ($n=31$)
Normal (26)	19 [b]	7 [a]	– [b]
Inflammatory (55)	19	15	21
Non inflammatory transudate (6)	3	3	–
Meningitis (14)	1 [b]	3	10 [b]

[a] $P<0.05$
[b] $P<0.01$

Table 5. CSF lymphocytes in the three MS groups ($n=90$)

	Group I: Absence of AB in CSF ($n=38$)	Group II: VAB only in CSF ($n=24$)	Group III: VAB and NAB in CSF ($n=28$)
Lymphocyte mean (by mm^3)	2.7 ± 2.6 [a]	4.3 ± 4.9	6.9 ± 7.0 [a]

[a] $P<0.05$

Table 6. Oligoclonal aspect in the three MS groups ($n=99$)

O. A.	Group I: Absence of AB in CSF ($n=41$)	Group II: VAB only in CSF ($n=28$)	Group III: VAB and NAB in CSF ($n=30$)
Absent (42)	23	11	8
Present (57)	18 [a]	17	22 [a]

[a] $\chi^2=6.30$ ($P<0.05$)

Table 7. CSF immunoglobulins in the three MS groups

CSF immunoglobulins	Group I: Absence of AB in CSF ($n=42$)	Group II: VAB only in CSF ($n=28$)	Group III: VAB and NAB in CSF ($n=31$)
IgA (mg/l)	2.8 ± 2.0	3.2 ± 2.0	5.8 ± 9.0
IgG (mg/l)	58 ± 28 [a]	90 ± 101 [a]	151 ± 75 [a]
IgM (mg/l)	0.4 ± 1.0 [a]	0.6 ± 1.5 [a]	2.0 ± 3.4 [a]

[a] $P<0.05$ between III and I, III and II

Table 8. CSF IgM in the three MS groups ($n = 101$) [a]

CSF IgM	Group I: Absence of AB in CSF ($n = 42$)	Group II: VAB only in CSF ($n = 28$)	Group III: VAB and NAB in CSF ($n = 31$)
Absence (76)	35	23	18
Presence (25)	7	5	13

[a] $P < 0.02$ between I and II ($\chi^2 = 5.73$) $P < 0.05$ between II and III ($\chi^2 = 4.02$)

Table 9. CSF NAB and VAB in 101 MS patients [a]

Viral antibodies	Nucleic antibodies	
	Absence (70)	Presence (31)
Absence (49)	42	7
Presence (52)	28	24

[a] $\chi^2 = 12.04$ ($P < 0.001$)

Table 10. Serum immunoglobulins and CRP in the three MS groups

Proteins		Group I: Absence of AB in CSF ($n = 42$)	Group II: VAB only in CSF ($n = 28$)	Group III: VAB and NAB in CSF ($n = 31$)
IgA (mg/l)		1 974 ± 835 [a]	2 069 ± 816	2 346 ± 643 [a]
IgG (mg/l)		13 228 ± 2 754 [a]	13 374 ± 3 196 [a]	15 348 ± 2 995 [a]
IgM (mg/l)		883 ± 333	836 ± 247	1 036 ± 289
CRP (59)	Absence (44)	17	15	12
	Presence (15)	10 [a]	2 [a]	3

[a] Significant for $P < 0.05$

groups (Table 4). A clear predominance of normal pattern is seen in the first (contrasting with its absence in the third) and, conversely, obvious prevalence of meningitis pattern in the third. Some other significant correlations may be observed in CSF: a lymphocytic pleiocytosis in the third group, contrasting with normal mean in the first (Table 5); highest frequency of oligoclonal aspect in the third (73%), which is relatively rare in the first (Table 6); important increase of IgG in the third, with significant differences between the second and the first, with IgG mean only slightly elevated in this last (Table 7); and frequent presence of IgM in the third (42%), which is significantly higher than in the two other groups (17%) (Table 8). As previously proposed, an obvious correlation is found between simultaneous presence or absence of NAB and VAB (Table 9).

Table 11. Serum NAB and VAB in the three groups (serological dilution and percentage)

Antibodies [a]	Group I: Absence of AB in CSF ($n=42$)	Group II: VAB only in CSF ($n=28$)	Group III: VAB and NAB in CSF ($n=31$)
NAB			
DNA	109 ± 26 [c] (4.1 ± 0.8) [b]	112 ± 21 [b] (4.4 ± 0.9)	148 ± 44 [c] (4.8 ± 0.8) [b]
RNA	204 ± 46 [b] (7.9 ± 1.9)	220 ± 43 (8.8 ± 2.3)	248 ± 80 [b] (8.1 ± 2.2)
VAB			
Measles	46 ± 31 [b] (0.4 ± 0.3)	180 ± 142 (1.5 ± 1.3)	135 ± 100 [b] (0.9 ± 0.7)
Rubella	88 ± 114 [b] (0.8 ± 1.3)	179 ± 131 [b] (1.4 ± 1.1)	125 ± 87 (0.8 ± 0.6)

[a] For each antibody, the first line gives the reciprocal of serological dilution: the percentage is indicated in parenthesis
[b] $P<0.05$
[c] $P<0.01$

Table 12. Sex differences in the three MS groups [a]

Sex	Group I: Absence of AB in CSF ($n=42$)	Group II: VAB only in CSF ($n=28$)	Group III: VAB and NAB in CSF ($n=31$)
Men	22	11	8
Women	20	17	23

[a] $P<0.05$ between I and III (χ^2: 5.20); I and II + III (χ^2: 4.14); I + II and III (χ^2: 4.05)

Other interesting facts may be observed in the serum. IgA and IgG means are significantly elevated in the third group (Table 10) in comparison with the first, and unexpectedly, there is a frequent (and abnormal) presence of CRP in the blood of patients of the first group, contrary to others. Moreover, a significant increase of DNA antibodies (in absolute value and percentage) is found in the third group, contrasting with rather normal values in the other groups (Table 11). Measles and rubella antibodies were increased in groups II and III, in contrast to normal values in group I.

Clinical data show no differences between the three groups (especially with regard to the age of the patients, duration of the disease, clinical stage, and type of course), with an interesting exception concerning sex repartition (Table 12). A clear prevalence of women exists in the third group, contrasting with the slight dominance of men in the first, and a classical repartition (60% of women and 40% of men) in the second group. The mean disability grade is clearly the same in the three groups, and

Table 13. Disability grade and course according to the three MS groups

Course	Group I: Absence of AB in CSF ($n=42$)	Group II: VAB only in CSF ($n=28$)	Group III: VAB and NAB in CSF ($n=31$)
Intermittent (50)	2.6 ± 1.6 [b]	2.7 ± 1.8 [a]	2.2 ± 1.4 [b]
Progressive (51)	4.4 ± 1.8 [b]	5.0 ± 1.0 [a]	5.3 ± 2.2 [b]

[a] $P < 0.05$. SD in each group between intermittent and progressive course
[b] $P < 0.01$

is not influenced by sex, age of the patient, or duration of the disease, but only by the type of course (Table 13), as demonstrated previously by Fog and Linnemann [1].

Conclusion

Different hypothesis may be discussed based on the clinical, immunological, and virological data (Table 14). These three MS populations may represent three successive steps in the same disease. However, this hypothesis seems very improbable regarding the same duration of the disease and the same disability grade in the three groups. Another hypothesis suggests three events during the same disease, with an alternation between a silent phase (type I), a viral (type II), or viral and autoimmune reactions (type III). Two other hypothesis may also be proposed:

1. The possibility that there is a unique agent involved in three different immunogenetically determined processes in connection with the sex differences observed. Type I may be supported by an immunological defect, type II may be correlated with a pure viral process, and type III may be conditioned by an immunological hy-

Table 14. CSF, NAB and VAB in 101 MS patients: Summary of the data

	Group I: Absence of AB in CSF ($n=42$)	Group II: VAB only in CSF ($n=28$)	Group III: VAB and NAB in CSF ($n=31$)
Sex	Men: 52% Women: 48%	Men: 40% Women: 60%	Men: 26% Women: 74%
Viral immunity reaction	Normal	Increased in serum and present in CSF	Increased in serum and present in CSF
Nucleic immunity reaction	Normal	Normal	Increased in serum and present in CSF
Blood-CRP	Frequent	Rare	Rare

perreactivity (autoimmunity after viral persistance) as observed in SLE and in female NZB mice, which develop much more IgG anti-DNA than do males [9].

2. There may be an eventuality of different agents for the same disease, with immunosuppressing action in the first type and immunostimulating effect in the third. Obviously, the choice of an efficient therapy depends on a confirmed hypothesis.

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CSF Proteinase Inhibitors

M. L. CUZNER[1]

Introduction

Reports of myelin basic protein (MBP) fragments [2,11] and raised proteolytic activity [8] in the cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients in exacerbation are indicative of inflammatory demyelination. Basic protein is highly susceptible to digestion by the neutral proteinases of polymorphonuclear leucocytes (PMNL) and macrophages [1, 5]. During inflammation the major serum proteinase inhibitors, α2-macroglobulin (α2M) and α1-antitrypsin (α1AT) are associated with increased activity of proteolytic enzymes [9]. The CSF, which is an ultrafiltrate of serum and is in intimate contact with the brain, particularly with certain areas of myelin, contains measurable amounts of these two proteins. In order to assess the regulation of proteolysis by the CSF, we have measured the levels of α2M and α1AT and assayed the trypsin-binding and proteolytic inhibitory capacity of the CSF in a variety of neurological conditions.

Methods

Proteolytic enzyme activity was measured by previously described methods [48]. CSF levels of α2M, α1AT and transferrin were determined using the rocket electrophoresis technique of Laurell [7]. The methods adapted to measure trypsin-binding and total proteolytic inhibitory capacity are outlined in Figures 1 and 2.

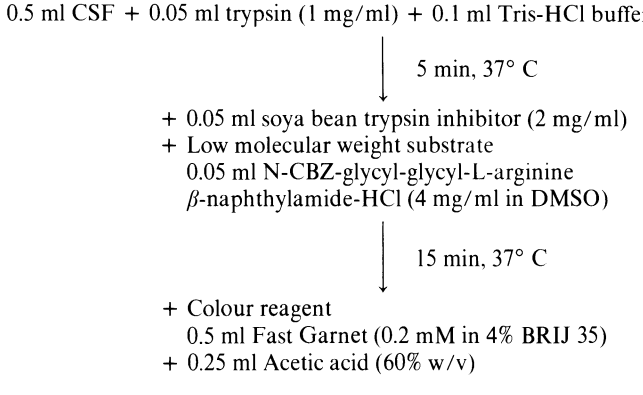

Fig. 1. Proteolytic inhibition by α2-macroglobulin [9]

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CSF or serum + 1 × 10^6 PMN cells (enzyme source) + 0.25 ml haemoglobin (4% w/v)
+ 0.167 M Tris-HCl buffer (pH 7.6), total assay volume – 0.75 ml

↓ 60 min, 37° C

+ 1.25 ml TCA

Centrifuge

Assay of Folin-positive, TCA-soluble products

Fig. 2. Total proteolytic inhibitory capacity

Results

CSF Proteolytic Activity

The neutral proteinase activity of CSF polymorphonuclear cells differed little from that of circulating cells (Table 1), but acid proteinase activity was reduced. This result reflects secretion of acid hydrolases by activated phagocytic cells. Significant proteolysis of haemoglobin at both neutral and acid pH was demonstrated in CSF supernatant, in contrast to serum. However, marked digestion of basic protein by CSF supernatant occurs only at acid pH.

CSF Proteins

The mean values of α2M, α1AT and transferrin in CSF of four groups of patients are shown in Table 2. Except in cases of meningitis, total protein values are similar. Levels of the individual three proteins measured appear to be lower in MS than in most other conditions; however, only the results for α1AT and transferrin differ significantly. When the samples are considered as simply MS or non-MS (omitting the meningitis group) the percentages by which α2M, α1AT and transferrin are depres-

Table 1. CSF proteolytic activity

	Neutral proteinase		Acid proteinase	
	Haemoglobin [a]	Basic protein [b]	Haemoglobin [a]	Basic protein [b]
PMN cells				
Blood	0.27	4.2	0.035	0
CSF	0.13	6.0	0.004	0
CSF supernatant	0.07	±	0.05	40

Proteolytic enzyme substrates:
[a] Haemoglobin; cells (nmol tyrosine/10^3 cells/h); CSF supernatant (μmol tyrosine/ml/h)
[b] Basic protein; cells (μg BP lost/10^3 cells/h); CSF subernatant (μg BP lost/ml/h)

Table 2. CSF proteins (mg/100 ml)

	Total protein	IgG	α_2-macro-globulin	α_1-anti-trypsin [c]	Transferrin
Multiple sclerosis ($n=31$)	42	6.8	0.42	1.46	1.57
Non-infectious neurological disease ($n=41$) [b]	38	3.7 [a]	0.47	2.13 [a]	2.11 [a]
Encephalitis ($n=3$)	46	5.1	0.46	1.89	2.22
Meningitis ($n=3$)	101 [a]	–	$\cong 2$	8.00 [a]	4.96 [a]

[a] Significantly different from MS group ($P<0.05$)
[b] BIH, MND, discs, tumours, spondylosis
[c] Serum level, MS and neurological controls = 212

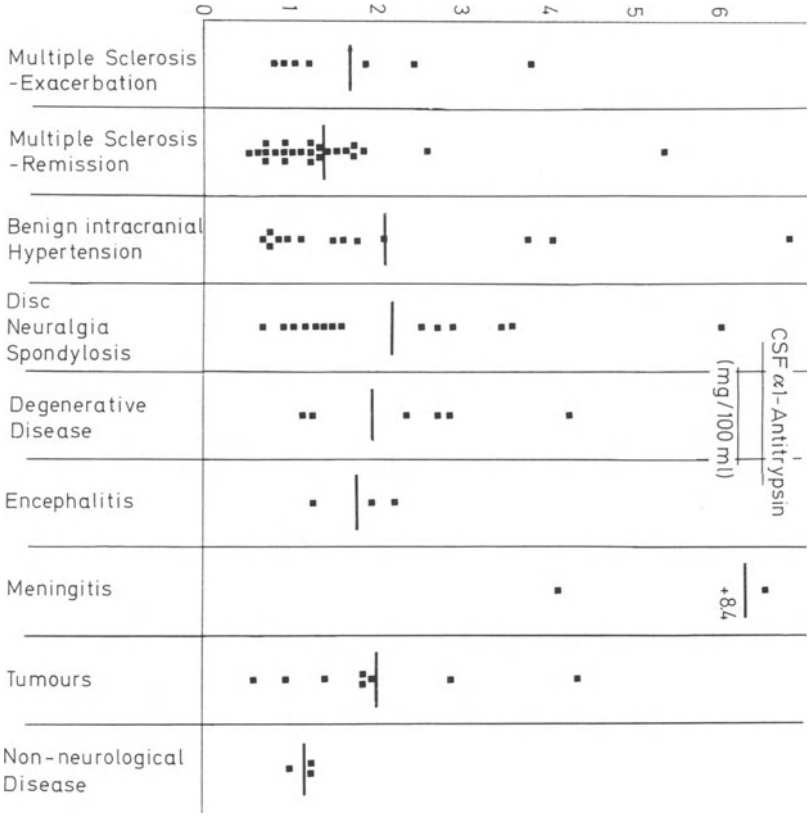

Fig. 3. CSF-alpha 1-antitrypsin (mg/100 ml) in patients with MS, other neurological diseases and normal controls

Table 3. Proteolytic inhibition by CSF α2-macroglobulin and α 1-antitrypsin

	Trypsin bound to α2 M (ng/0.5 ml CSF)	Binding efficiency	Vol (μl) to produce 50% inhibition of N. P. activity of 1×10^6 PMNL [b]
Multiple sclerosis	420 ($n=16$)	1.75	191 [a] ($n=10$)
Non-infectious neurological disease	567 ($n=15$)	1.72	165 ($n=8$)
Encephalitis	825 ($n=2$)	2.11	–
Meningitis	660 ($n=2$)	0.39	–

[a] Volume to produce 50% inhibition by MS and non-MS serum = 0.81
[b] Essentially an assay of α 1 AT activity

sed in MS are 20%, 36% and 31%, respectively. Even when the neurological controls are separated according to specific diagnostic criteria, the mean CSF value of α1AT in MS is lower, with the exception of one group of inadequately diagnosed patients (Fig. 3). Serum levels of α1AT were also measured and no significant differences were noted between normal controls, neurological controls and MS patients.

Proteinase Inhibitors

The activity of α2M in CSF can be measured by a trypsin-binding assay [6], as proteinases are bound to the inhibitor in a catalytically active form. However, α1AT is responsible for 90% of serum proteolytic inhibitory capacity and binds enzyme at the active site. Thus the activity of α1AT can be estimated by measuring the residual activity of serine proteinase after the enzyme has been incubated with a dilution of serum or CSF. The activity of α2M and α1AT in the CSF in the four categories of neurological disease is shown in Table 3. The inhibition of proteolytic activity by CSF of patients with MS, particularly those in the remission group, was lower than in all other groups, although these results did not reach statistical significance. The reduced activity appears to be a direct result of the decreased level of α2M and α1AT in the CSF in MS, as the binding efficiency of the inhibitors was the same as that of the neurological controls. The binding efficiency of α1AT in CSF was comparable to that of a standard pooled serum while the efficiency of α2M binding was increased.

Discussion

Proteolytic activity is increased and fragments of MBP appear in the CSF of MS patients at the time of an exacerbation of the disease [2, 8, 11]. Acid proteinase accounts for the greater part of the intrinsic proteolytic activity of the CSF and al-

though increased at the time of a relapse, is an unlikely candidate for the digestion of basic protein in the CSF, the pH of which is slightly alkaline. Acid hydrolases, produced locally in the white matter by astrocyte lysosomes, might account for the appearance in the CSF of the protein fragments, but there is also evidence of proteolysis of basic protein at physiological pH by macrophages and PMNL [1, 5]. Furthermore, the neutral proteinase of the cellular fraction of CSF is raised in acute MS [8].

The potent inhibitors of neutral proteinases, α2M and α1AT, present in serum, are also found in CSF, at a 200-fold dilution and in different proportions, due to selective filtration of smaller protein molecules [10]. The binding efficiency of α2M and α1AT to proteinases is similar in CSF and serum (Table 3) and we have found that the PMNL of a CSF sample with a count of 3000 cells per mm^3 had negligible neutral proteinase activity in the presence of the CSF supernatant (P. Price and M. L. Cuzner, unpublished observation). In acute inflammation, α1AT and α2M escape into the tissue and serum levels of α1AT, an acute phase protein, rise, as a result of increased synthesis in the liver [3].

In the synovial fluid of rheumatoid joints, α2M accumulates in substantial amounts, both free and complexed with proteinases [9]. Elevated levels of these two proteins in the CSF during the acute phase of MS would be supportive evidence of an inflammatory reaction. But we have observed the opposite result. When compared to a wide range of neurological controls, the CSF levels of α2M, α1AT as well as that of transferrin, were found to be lower in MS patients, whether in relapse or remission. The statistically significant reduction in the amount of α1AT, which, in serum, is responsible for 90% of the trypsin binding, was accompanied by a reduction in proteolytic inhibitory capacity.

There are two possible explanations for these results. The immunological evidence of raised CSF IgG concentration is suggestive of ongoing infection. If this is the case, the reduced levels of α1AT may result from steady consumption by locally released proteinases. Once formed, enzyme-inhibitor complexes are removed by reticuloendothelial cells. But the evidence, as follows, points more strongly to an alteration in the transport rate across the blood-brain barrier: (1) The CSF level of transferrin, which is not a proteinase inhibitor, is also significantly lower in MS, but serum levels of α2M, α1AT and transferrin in all patient groups were the same as those of normal controls. (2) Total protein in CSF is not increased in MS but the percentage of IgG increases from 9.5 to 16%. If the local production of IgG is not accompanied by the vascular permeability characteristic of the inflammatory reaction, as for example in meningitis, the increased concentration of IgG may reduce the transport rate of other proteins, in this case, α2M, α1AT and transferrin, from serum to CSF.

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The Occurrence of Immune Complexes in Patients with Multiple Sclerosis

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Abstract

Using a complement consumption test and a thrombocyte aggregation test, the occurrence of circulating immune complexes was investigated in serum samples from 53 patients with multiple sclerosis (MS). The results were correlated to the concentration of complement factors Clq, C4, C3, C5, and C9, total hemolytic complement activity in serum, and the presence of activation products of C4 and C3 in plasma.

A good correlation was found between the two tests for circulating immune complexes, which were detected in approximately 40% of the cases. The occurrence of immune complexes was well correlated to activation of the complement system via the classical pathway, estimated by presence of activation products of both C4 and C3. Fifty percent of the patients without detectable circulating immune complexes demonstrated a significantly lower concentration of serum C3 than did the rest of the patients. The presence of CIC seemed to be related to the clinical course of the disease.

Introduction

Although the precise pathogenetic mechanisms in MS remain unclear, it is now established that immunological factors are involved [3, 8]. Futhermore, circumstantial evidence of the presence of circulating immune complexes (CIC) [2, 7], and the demonstration of immunoglobulin G and complement component Clq in brain plaques [10], suggests a decisive role for immune complexes in the pathogenesis of this disease.

In the present investigation of 53 patients with MS, the presence of CIC has been substantiated by the determination of complement components and investigations of in vivo activation of the complement system.

Materials and Methods

Fresh frozen (– 80° C) serum and EDTA plasma samples were obtained from 53 patients with typical MS. No patients had signs of intercurrent infectious disease.

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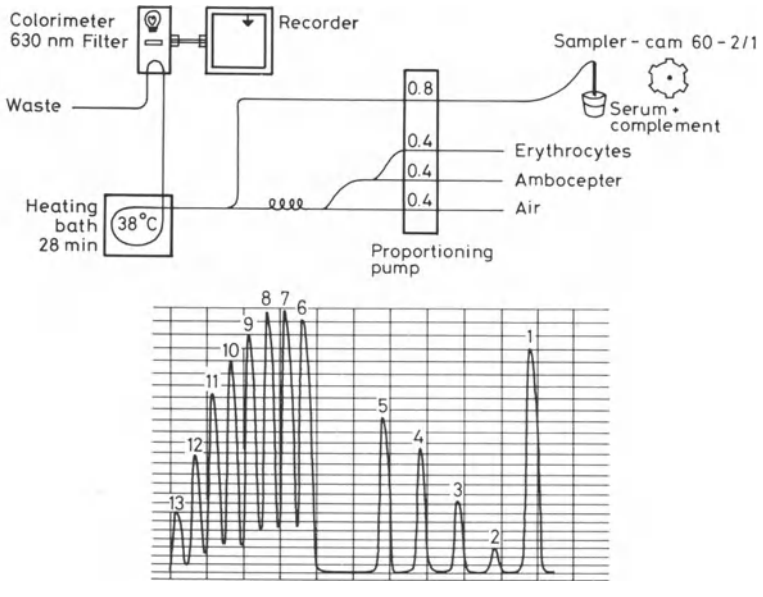

Fig. 1. Flow diagram and recordings of the CCT and CH 50 estimation procedures. Pumpline sizes are shown as flow rate in ml/min

The topography of the neurological lesions was defined according to Fog (personal communication).

CIC Detection

Two tests were used: (1) The thrombocyte aggregation test (TAT) [6], and (2) the complement consumption test (CCT), performed as a semiautomated method in a continuous flow system (Technicon Inc.) (Fig. 1), using a 3.4% suspension of sheep red blood cells (SRBC) optimally sensitized with rabbit hemolytic serum. The optimal lysis of SRBC in this system was achieved after 28 min of incubation at 38° C, according to methods previously described [9]. Barbital-buffered saline (BBS), containing .005 M magnesium and calcium, was used for the dilution and washing procedures. Heat-inactivated serum (56° C, 60 min) was diluted 1 : 6, 1 : 10, 1 : 18, and 1 : 34 and incubated with one CH 90 unit of guinea pig complement (Institut Pasteur) for 30 min at 37° C just before analysis. The reduction in degree of hemolysis in samples containing the various serum dilutions (curves 2, 3, 4, and 5) was expressed as a percentage of a control containing BBS instead of serum dilution (curve 1, Fig. 1).

Complement Analysis

Quantitation of serum complement components Clq, C4, C3, C5, and C9 was performed using a electroimmunoassay [5], using pooled serum from 100 healthy group-A blood donors as standards. The values were expressed as percentages of

the standards. Complement conversion products (split products) of C4 and C3 were identified in EDTA plasma [1], using conversed C4 and C3 from normal serum as controls. Monospecific antisera against Clq, C4, and C3 (Dako, Copenhagen) and C5 and C9 (Behringwerke AG, Marburg an der Lahn) were employed in the electrophoretic procedures.

The total hemolytic complement activity of serum was determined in the continuous flow system described above. Eight serum dilutions (1 : 41, 1 : 61, 1 : 81, 1 : 101, 1 : 121, 1 : 134, 1 : 151, and 1 : 167) were analyzed immediately after thawing. The recordings are shown in Figure 1 (curves 6–13). The CH 50 value was determined [4] and expressed as a percentage of the pooled standard.

The reference values for complement components and CH 50 were chosen as the mean ± 1.96 SD of the results from each of the 100 sera in the standard pool.

Calculations for Presence of CIC

The CCT was registered as positive if inhibition of hemolysis in every serum dilution was greater than the inhibition in the corresponding dilutions of any of the donor sera collected for the pooled standard previously mentioned. The upper normal range corresponded with heat aggregated IgG (63° C, 60 min) in a concentration of 5 μg/ml.

The TAT was registered as positive if thrombocyte aggregation appeared in serum dilutions greater than 1 : 8.

Results

Presence of CIC with simultaneous activation in vivo of complement was found in 22 patients (41.5%) (Fig. 2). In three cases, positive CCT or positive TAT were dis-

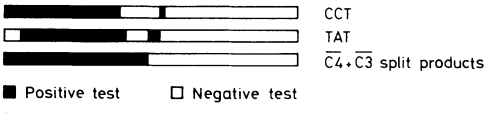

Fig. 2. Occurrence of positive tests for CIC in 53 patients with MS

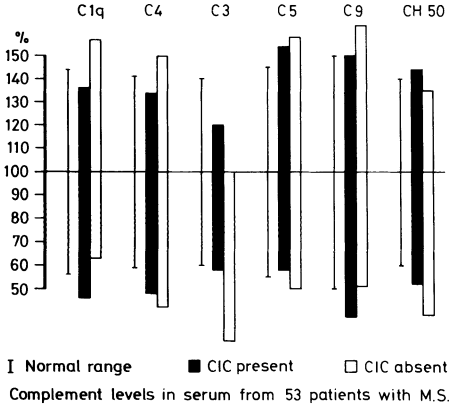

Fig. 3. Complement levels in serum from 53 patients with MS

closed without detectable complement activation. Signs of complement activation were noted in four patients without positive CCT or TAT. Split products of C4 or C3 always appeared simultaneously. The results of the complement components and the CH 50 estimations nearly showed normal values, as is shown in Figure 3. Notable deviations were found only in the C3 estimations, where levels below the reference value were registered in 15 of 31 patients (48%) without CIC. Only one patient with CIC demonstrated low C3. The total frequency of low C3 was 30%.

Discussion

In vivo, most immune complexes are probably able to activate the complement system, particularly via the classical pathway. Based on this hypothesis, and employing a sensitive CCT and TAT, we have firmly established the occurrence of CIC in 22 sera (41.5%) from 53 patients with MS. Previously, the Raji-cell assay, which is at least partly complement dependent, has disclosed CIC in 49% of a patient group [7]. Only a small deviation between the two studies was noted. This discrepancy might easily be explained by differences in the sensitivity of the tests. Another possible reason might be presence of non-complement-binding CIC in the sera, but only two patients in our study revealed a positive TAT without signs of complement activation.

Four patients with negative TAT and CCT revealed signs of complement activation. These patients had no further common characteristics. In our study, 16 patients (30%) had significantly low C3 levels. This is in accordance with the 31.8% found by Trouillas and Beutel [8], who further suggested a genetic background for this feature. Surprisingly, 15 of the 16 patients with low C3 levels in our study belonged to the group without CIC.

The significance of these results remains to be shown. Neither the results of Tachovsky et al. [7] nor our own have proved a strict correlation between the presence of CIC and the clinical status of the patients. Our results, however, indicate a slight correlation between the clinically estimated topography of the plaques and the serological findings, as shown in Table 1.

Table 1. Topography of attacks and occurrence of CIC

	CIC present	CIC absent
Brain-stem cerebellar	7	0
Pyramidal spinal	10	15
Disseminated	5	8
Dyscoordinative hemiphlegic cerebral	0	8
	22 (41.5%)	31

Acknowledgments. This study was supported by grants from the Danish Multiple Sclerosis Foundation, the Kong Christian den Tiendes Foundation, and the Danish League Against Rheumatism. Send reprint requests to: C. Jersild, Regional Blood Transfusion Center, P. O. Box 561, DK-9100 Aalborg, Denmark.

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Immune Complexes in Cerebrospinal Fluid and Serum of Patients with Multiple Sclerosis

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Introduction

During recent years, circulating immune complexes have been described in an increasing number of diseases [21]. In some chronic inflammatory disorders, evidence for immune complex-induced tissue damage has been convincingly documented [22]. In other disease states, e.g., in malignant tumors, the presence of immune complexes may simply indicate prolonged antigenic stimulation without direct relation to tissue lesions [13].

In multiple sclerosis (MS), circulating immune complexes were first described by Tachovsky et al. [17], and more recently by two other groups [7, 8] using three different methods. A decrease of complement components in the sera of MS patients during the acute phases of their disease has been noted by Kuwert et al. [9, 10]. These authors also described decreased complement component levels in cerebrospinal fluid (CSF) in MS [10], thus indicating possible consumption of complement in the process of an immunologically induced central nervous system (CNS) tissue lesions. Evidence for the presence of immune complexes in CSF in MS has not been presented to-date.

Materials and Methods

Patients, Sera, and Cerebrospinal Fluid Samples

Altogether, 123 MS patients were studied. The diagnosis of MS was established by U. P. and P. H. according to clinical and laboratory criteria using a standardized documentation scheme [11]. Blood samples, drawn from all patients regardless of disease activity or type of treatment, were allowed to clot at room temperature for 2 hours; they were immediately tested or kept frozen at –70° C. CSF samples were obtained at the time of the patient's first hospital admission, or during an exacerbation period when re-evaluation of the clinical status was found necessary. They were tested immediately or frozen at –70° C.

IgG and albumin concentrations were measured by radial immunodiffusion using Tri-Partigen and LC-Partigen plates (Behring-Werke AG, Marburg/Lahn,

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Germany). Evidence for local IgG production in the CNS was sought by calculating the CSF-IgG index as described by Delpech and Lichtblau [5], namely,

$$\frac{(\text{CSF IgG} \times 10^3)/(\text{Serum IgG})}{(\text{CSF albumin} \times 10^3)/(\text{serum albumin})} = \text{CSF IgG index.}$$

Immune Complex (IC) Determination

Immune complexes were assayed using the C1q binding test (C1q BT) as described by Zubler and Lambert [20]. A pool of 100 normal sera, frozen at –70° C and giving consistently negative results in the C1q BT, was used as a control. CSF samples were mixed with 1 vol of frozen normal serum negative in the C1q BT to obtain the appropriate protein concentration for PEG precipitation. In control experiments using heat-aggregated IgG as an IC model, the addition of 1 vol of C1q BT-negative frozen normal serum was shown not to influence C1q binding save for the dilution effect.

Since in our experience, the standardization of the C1q BT using heat-aggregated IgG showed considerable batch-to-batch variation, a simple normalization procedure based on the percent binding values of normal control and patients' sera and CSF samples was employed. In each test series, 3–4 normal C1q BT-negative sera were included as negative controls. By dividing the percent binding values of the patient's serum or CSF sample by the arithmetical mean percent binding of the negative controls and multiplying the obtained values by ten, an estimate of the amount of IC based on a comparison with the normal controls was obtained and expressed as "IC units" (ICU):

$$\frac{\text{patient's sample (\% binding)}}{\bar{x}\ \text{normal controls (\% binding)}} \times 10 = \text{number of ICU.}$$

The normal range ($\bar{x} \pm 2\, s_D$) for healthy control sera (n = 118) was calculated as 10.1 ± 1.6 ICU. ICU values above 11.7 were considered as evidence for the presence of IC in patients' sera or CSF. By comparison, heat-aggregated IgG at a concentration of 1 mg/ml gave a mean value of 23.3 ICU by this procedure ($n = 30$).

Statistical analysis was done using student's t-test for unclassified samples.

Results

As shown in Figure 1, IC were detected in 26 of 98 MS patient's sera (= 26.53%). Only 3 out of 118 healthy control sera showed marginally positive IC values. Positive results were also obtained in 6/42 sera from patients with miscellaneous neurological disorders. Among the latter, the highest value (17.1 ICU) was found in the serum of one patient with systemic lupus erythematodes and CNS involvement. Other negative controls included encephalitis and myasthenia cases. Results obtained with CSF samples are depicted in Figure 2. Since normal control CSF was not available, the group of normal sera from Figure 1 served as a control. As mentioned in the methods section, CSF samples were diluted with 1 vol of normal serum. Im-

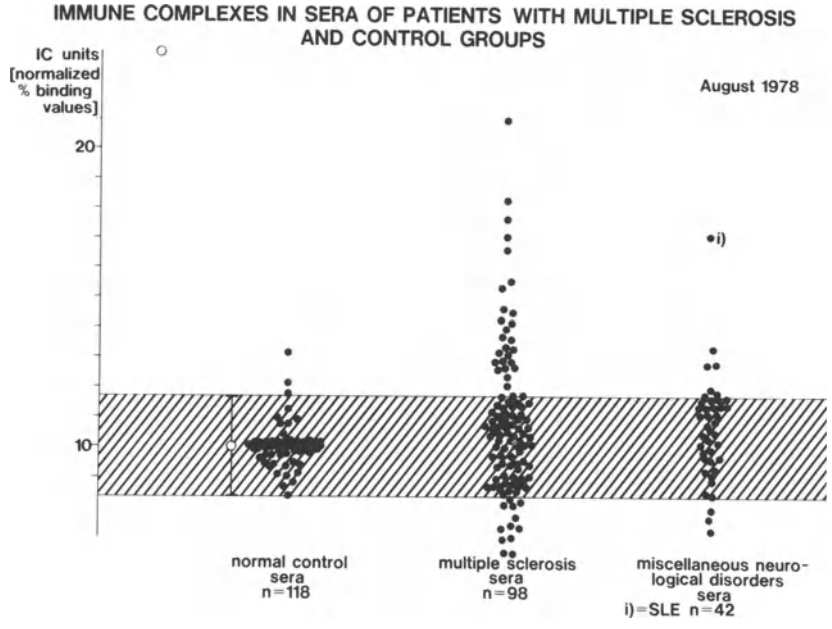

Fig. 1. Results of C1q binding tests in sera of MS patients, compared to a normal group and a group of miscellaneous neurological diseases

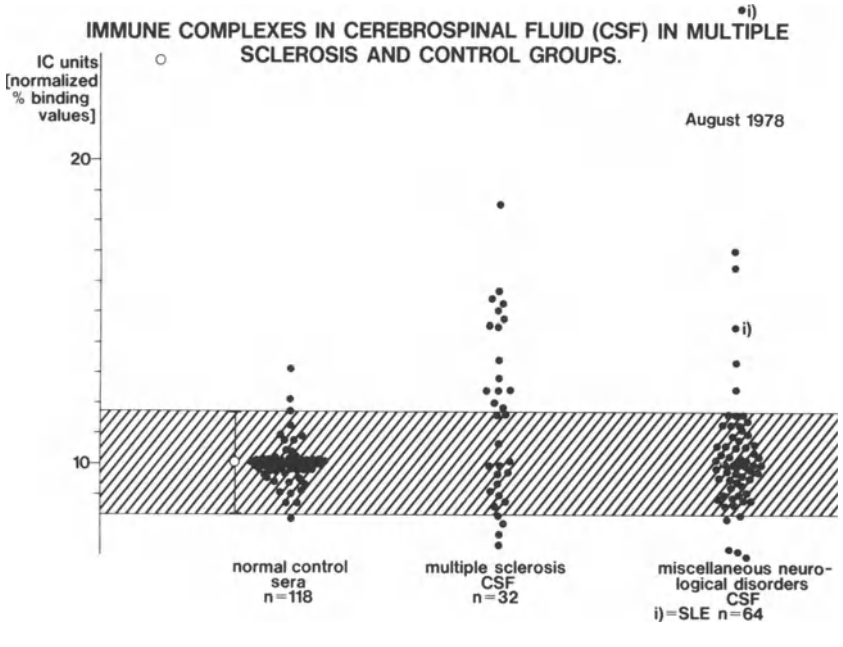

Fig. 2. Results of C1q binding tests in CSF of MS patients, compared with a control group of patients with miscellaneous neurological diseases. The normal control group consists of 118 normal sera (see Methods section). Note the presence of immune complexes in CSF of two patients with systemic lupus erythematosus and CNS

Table 1. The relationship between CSF-IgG and the presence or absence of CSF immune complexes in MS patients ($n=30$, all with a MS bout)

C1q test	positive	negative
n	14	16
CSF-IgG mg/100 ml	7.69 ± 4.49	4.55 ± 2.61
	$2P > 0.9$ [a]	

[a] Difference not significant

Table 2. The relationship between CSF-IgG index and the presence or absence of CSF immune complexes in MS patients ($n=30$, all with a MS bout)

C1q test	positive	negative
n	14	16
CSF-IgG index	1.07 ± 0.93	0.76 ± 0.41
	$2P > 0.9$ [a]	

[a] Difference not significant

Table 3. The relationship between CSF cell count and the presence or absence of CSF IC in MS patients ($n=30$, all with a MS bout)

C1q test	positive	negative
n	14	16
x/3 cells/mm^3	39.9 ± 52.5	14.8 ± 18.0
	$0.01 > 2P > 0.005$	

mune complexes were detected in 15 out of 32 CSF samples from MS patients, i.e., in 46.9%. Within the control group of 64 CSF samples from patients with miscellaneous neurological disorders, six (= 9.4%) showed positive C1q binding. Again two patients with CNS-SLE gave positive results. In four other control CSF, a relationship between the clinical diagnosis and C1q binding of the CSF sample was not apparent.

The relationship between the presence of IC in CSF and several characteristic features of CSF in MS was also investigated. Although CSF samples with IC contained more IgG then those negative for IC, the difference was not statistically significant due to the marked variation in individual values (Table 1). Similarly, although the mean CSF-IgG index in the IC-positive group indicated intracerebral IgG synthesis (CSF-IgG index > 0.85 according to Delpech and Lichtblau [5]), whereas this

index was distinctly lower in the IC-negative group, the difference again did not reach statistical significance (Table 2). The group of patients with positive evidence for CSF-IC displayed a significantly higher cell count if compared to the IC-negative group ($P < 0.01$) (Table 3).

Finally, in a limited number of cases, serum and CSF samples were tested simultaneously (Table 4). Whereas concordant results were obtained in 5/14 cases, discordant ones were seen in the remaining nine cases. If conclusions can be drawn from this limited investigation, it may be noted that CSF-IC values were more often

Table 4. Simultaneous immune complex determinations in 14 MS patients

n	CSF	Serum
2	positive	positive
7	positive	negative
2	negative	positive
3	negative	negative

positive during exacerbation periods (all CSF samples were obtained during clinical bouts) than serum IC determinations. No certain correlations were found between the presence of serum or CSF IC and duration or severity of the clinical MS picture. The only indication of a possible relation between the clinical status of the patients and IC in the serum was obtained from a total of 88 patients whose complete clinical data were available from the data bank: Out of 26 patients with serum IC, 17 (= 65.4%) had had a clinical exacerbation within the last three months, whereas among the 62 IC-negative patients, only 18 (= 34.4%) had experienced a similar bout.

Discussion

MS is a chronic demyelinating disease with a presumed allergic etiology. Antibodies reacting with myelin protein but also with other non-myelin CNS structures, and displaying demyelinating activity in tissue culture, have been found by several groups [1, 2, 6, 12, 15, 18, 19]. Complement-dependent neuroelectric blocking activity of MS serum IgG has also been noted [14, 15]. Other groups have described cellular hypersensitivity reactions leading to the in vitro release of lymphokines in the presence of myelin basic protein (MBP) [16]. However, the precise role of these immunologic features in the pathogenesis of the MS tissue lesion is still not proven [15]. Goust et al. [7], describing the decrease of a particular lymphoid subpopulation in MS together with the presence of serum IC, interpreted these results as possible evidence for a defect in the regulation of the immune response in these patients, leading to a prolonged chronic course of the disease.

IC have so far only been described in MS serum [7, 8, 17], with no certain relation to the clinical status. We have confirmed these results and have demonstrated

the presence of IC in CSF in one-half of MS patients during an acute bout of their disease (Figs. 1, 2). Whereas no relation to CSF IgG levels could be observed, we found a significant correlation to the number of CSF cells. Clear-cut evidence for a relationship to disease activity has so far not been observed; the increased CSF index in IC-positive CSF samples as well as the fact that IC were detected more often in patients with recent exacerbations of their disease would support the notion that humoral immune reactions may be directly involved in the pathogenesis of demyelinization. Tavolato's demonstration of IgG and complement deposits in brain areas adjacent to MS plaques can also be interpreted in this way. Decreased complement levels in MS serum and CSF [9, 10] may thus be interpreted as direct indication of an antibody-dependent tissue-damaging mechanism in MS [6, 15, 18]; soluble IC particularly in CSF may be regarded as further evidence for such a process.

Alternatively, antibodies reacting with tissue components may be a consequence rather then the cause of the demyelinating process, i.e., IC may just indicate a functioning clearing process for antigenic material released in the course of tissue destruction caused by another mechanism, e.g., a chronic viral infection. At any rate, the presence of IC in plasma and CSF indicates continuous antigenic stimulation as well as long-term antibody formation, which also takes place within the CNS [4] (Table 2). A decisive step toward a better understanding of the true role of tissue reactive antibodies as well as of the role of IC will be the identification of the antigen(s) involved in the formation of IC in MS.

Summary

Applying the C1q binding test (C1q BT), immune complexes were detected in 26/98 sera of MS patients in different stages of the disease with or without treatment. Positive C1q BT were also observed in 14.3% of 42 control sera from patients with miscellaneous neurological disorders, whereas only 2.5% of 118 normal control sera revealed marginally positive results. In 15/32 (46.9%) of CSF samples from MS patients, immune complexes were also found. Positive results were also obtained in 6/64 control CSF including different inflammatory neurological diseases. Whereas no correlation was found to CSF-IgG or to the CSF-IgG index, presence of CSF immune complexes showed a significant correlation to CSF cell count ($0.01 > P > 0.005$). No definite connections between duration or severity of diseases, disease activity, and presence or absence of immune complexes have become apparent, although IC positive patients displayed a higher frequency of recent MS bouts.

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Viral-Induced Immunological Incompetence as an Etiologic Factor in Multiple Sclerosis

L. P. WEINER and S. A. STOHLMAN [1]

There are two concepts which are generally accepted by workers in the field of multiple sclerosis (MS). First, early in life, the MS patient is probably exposed to a common environmental factor, most likely a virus; second, the pathology of demyelination in MS is most consistent with an immunopathologic process. There have been frequent, mostly unsuccessful attempts to isolate virus from the brains of MS patients; however, no virus has been demonstrated in the CNS of MS patients in any consistent fashion. If a virus is involved in MS, it may no longer be present in the CNS. In that case, how does one tie the early role of virus in this disease to the immunopathology and chronic course of the disease? A reasonable series of hypotheses can be proposed. Perhaps MS is a residual effect of a CNS infection in which specific brain antigens have inadvertently become targets for immunologic attack. To explain the periodic attack on self antigens, one might postulate selective destruction of the regulatory cells of the immune response by persistent extraneural viral infection.

In this paper, we give experimental evidence for the hypothesis that viral infection of immune cells contributes both to the persistence of virus and to an immune incompetent state. Finally, we will present evidence suggesting that the persistence of virus in immune cells might lead to an autoimmune immunopathologic process. At this point it is important to state that immunopathologic processes and immunosuppressive states are two sides of the same coin. Immune incompetence can lead to immunopathology. It is well known that if one suppresses a specific population of immune cells, the regulation of other immune cells might lead to an overly zealous response and produce an autoimmune pathologic state. Thus, viral-induced immune incompetence could conceivably lead to immunopathology by the selective destruction of populations of immune cells.

Although one need not postulate that the virus persists in cells of the immune system, it certainly makes sense that in a disease in which periodic exacerbations and remissions occur, an ongoing viral infection may exist somewhere in the body. The persistence of virus can be dependent on multiple factors: the pathogenicity of the virus, interferon induction, and modulation by the host of the antigenic nature of the virus, as in "antigenic shifts" [12]. However, it is also possible for viral persistence to be a result of a direct interaction with the immune response. The ideal condition for the persistence of virus is for the virus be nonantigenic. Thus the virus is not recognized by the immune response and therefore does not evoke the host's defense. This is apparently the case in infections caused by unconventional agents, such as Kuru, Creutzfeldt-Jakob syndrome, or scrapie. The next best thing, from an

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evolutionary point of view, is for the virus to replicate in cells of the immune system, the very system designed to destroy the agent. It is interesting that viruses known to produce immunosuppression also have been shown to persist in both experimental animals and man.

Viruses affecting the immune response consist of enveloped and naked RNA and DNA viruses. Viruses have been found in the thymus, lymph nodes, spleen, bone marrow, bursa of Fabricius, stem cells, plasma cells, lymphocytes, and macrophages [13]. The specific effect of viruses on the immune system appears to depend on which cell is involved.

The concept that virus can affect the host immune response dates back to the studies of Von Pirquet in 1908. He described a measles infection during which there was a loss of the delayed hypersensitivity to a PPD skin test during measles virus infection. Since then, we have discovered that all types of immune respones, both humoral and cellular, can be interfered with by viruses. In their 1970 paper Notkins et al. [13] hypothesized that viruses induce changes in the immune response by interfering with the uptake and processing of antigens, by causing depression of cellular protein synthesis (antibody), by destroying antibody-producing cells or their precursors, by inducing transformation of antibody-producing cells into neoplastic cells, by altering thymic function, and by accelerating immunoglobulin metabolism and antigenic competition. Thus, viruses interfere with T- and B-cell function, macrophage processing, and phagocytosis as well as biological amplification systems such as the complement pathway and the alternate pathway. Finally, they interfere with the production and activity of lymphokines.

To better understand the role of virus in the induction of immunosuppression, it might be important to review the immune system briefly. T lymphocytes have antigen receptors which induce proliferation and differentiation into immune competent effector cells. T cells are the effector cells in delayed hypersensitivity, graft rejection, and tumor immunity, as well as resistance and recovery from both bacterial and viral infections. T cells also regulate humoral antibody production. It is the antigen-activated T cells which amplify the inflammatory response by secretion of soluble factors termed lymphokines which in turn recruit nonspecific cells and produce blood vessel changes in the inflammatory reaction. Humoral antibody production is also influenced by T cells [27]. Antibody production to many, although not all antigens, requires cooperation between antigen-responsive T and B lymphocytes. The secretion of antibody and the switch from IgM to IgG synthesis seem to be influenced by helper T cells. T cells can also actively suppress antibody production, and these are called suppressor T cells. A wide variety of viruses can depress T-cell function. This is particularly interesting because T-cell function appears to be essential for recovery from most viral infections. Measles, mumps, and influenza are all known to produce transient depression of the PPD skin hypersensitivity. Measles, which is perhaps the best studied of these agents, has been shown by McFarland [9] to selectively affect T lymphocytes. Live attenuated measles vaccine, polio vaccine, and yellow fever virus vaccine all have been associated with decreased tuberculin reactions. Both children with congenital rubella [15] and adults with rubella [6] have negative delayed hypersensitivity to skin test antigens, and decreased mitogenic reactivity to the T-cell specific mitogen phytohemagglutinin (PHA). In addition, patients with infectious hepatitis and influenza A infections also show a diminished re-

activity to optimal concentrations of PHA [3, 26]. Suppression of delayed hypersensitivity in children infected with wild or attenuated measles virus parallels the inability of lymphocytes to respond to specific antigen in vitro.

Graft rejection, another function associated with T-cell activity, has been shown to be delayed by experimental infection. Lactic dehydrogenase virus (LDV) [19], Gross murine leukemia virus [4], and Marek's disease (a herpes virus) all inhibit efficient graft rejection [18].

Dent [4] and Mortensen et al. [11] have also shown that both oncogenic and nononcogenic viruses can interfere with lymphokine production. Monocyte chemotaxis, a phenomenon known to correlate with depression of cell-mediated immunity, has been shown by Kleinerman et al. [7] to be inhibited by both herpes simplex and influenza viruses.

The depression of humoral immunity by viruses has also been well studied, and a multitude of viruses have been implicated. Epstein-Barr virus (EBV) selectively transforms B cells [22]; Friend mouse leukemia (FLV) virus suppresses antibody formation to sheep red blood cells (RBC), salmonella lipopolysaccharide, coxsackievirus antigen, and influenza [4]. Friedman [5] has shown that the effect of FLV is only detected when infection occurs prior to the antigenic challenge. Suppression appears to be by the virus and not by the leukemic process.

Macrophage function may be the most important alteration of the immune system during viral persistance. Infection with LDV, ectomelia, or mouse hepatitis virus (MHV), all of which replicate in macrophages, depress carbon particle clearance. In vitro infection of polymorphonuclear leukocytes by mumps, influenza, or coxsackieviruses decreases their ability to engulf bacteria. In addition to impairment in phagocytosis, there are alterations in the ability of macrophages to break down antigens and release leukotoxic substances. Interferon produced by infected macrophages appears to have no effect on other macrophages [10]. Certain virus-antibody complexes are taken up by macrophages and degraded; however, reovirus appears to be ingested as an antigen-antibody complex and remains infectious in the macrophage. Allison [1] has further commented that many viruses which replicate in macrophages but produce no cytopathologic effect have also been associated with chronic infection. These include LDV, equine infectious anemia virus, lymphocytic choriomeningitis virus (LCM), MHV, and Aleutian mink disease [23]. The persistence of these viral agents in the host thus appears to depend on their ability to escape destruction by macrophages. The enhancing effects of macrophages on the clearance of viral infection may also be altered by virus. Recent studies of polio have shown that uninfected macrophages enhance the response of lymphocytes to PHA; however, this response was inhibited by infecting the culture with polio [20]. In this model, lymphocytes do not support polio replication, therefore, one can assume that the polio inhibits lymphocyte responses by suppressing macrophage function. Thus, macrophage infection appears to be important in immunosuppression by altering antigen processing, phagocytosis, interferon production and action, and, finally, enhancement of T- and B-cell response.

There are a number of well-studied experimental and human models in which the immune response has been affected by virus. These include the LCM virus, the mouse leukemia viruses, and measles virus. The LDV virus is of particular interest because it produces some interesting immunological abnormalities in NZB mice.

The NZB strain of mice is known to have an immunopathology occurring as an interstitial immune complex nephritis. LDV is an RNA-enveloped virus which produces a prolonged viremia and replicates in peritoneal macrophages [19]. Mahy [8] has shown that the elevated enzyme levels from which the virus derives its name is due to the failure of the infected macrophages to clear these proteins. During LDV infection, there is an exaggerated humoral response to some antigens and depression to other antigens [14]. The graft-versus-host reaction, induction of tolerance, and phagocytosis are depressed [13]. In LDV-injected NZB mice with severe immune complex nephritis, mortality is reduced.

Rowson and Mahy [19] have postulated that the prolonged viremia in LDV-infected mice may be related to incomplete neutralizing antibody activity. They suggest that this may be due to blocking factors such as antigen-antibody complexes or to an inhibitory immunoglobulin. Allison [1], however, has suggested a failure of macrophage function, since an enhanced IgG response has been described in LDV-infected animals. Enhanced antibody levels may be due to destruction of suppressor T cells or to an adjuvant effect of the persistent viral infection. White [24] has reviewed adjuvant effects of microbial products and stressed the importance of macrophages in the enhancement of the immune response.

There is also evidence suggesting that T cells are destroyed by LDV. Notkins et al. [13] have shown that within the first 24 h, LDV can cause lymphoid depletion and necrosis of thymus-dependent areas in affected mice. Snodgrass [21] has found viral particles only in reticular cells. The budding virus particles can be seen at the macrophage membrane, and they are in close approximation to the surface membrane of lymphocytes. Woodruff and Woodruff [27] have suggested that death of the T cells does not appear to be related to viral replication in these cells but to other factors. They postulated a toxic factor released from macrophages, or a mechanism involving virus on the macrophage membrane interacting with determinants on the surface of T cells. The T-cell destruction in LDV-infected animals coupled with an increase in the number of germinal centers and plasma cells in thymus-dependent areas of spleen and lymph nodes may account for the Ig enhancement and for the defects in cell-mediated immunity. The findings in LDV are not unlike certain autoimmune processes in which increased IgG levels are found in conjunction with defects in cell mediated immunity.

In humans, nature as usual has performed a number of interesting experiments with immune responses. One such experiment is seen in Bruton syndrome (X-linked agammaglobulinemia) which occurs as an inherited process. In this condition, there are no B cells detectable in the peripheral blood. IgG is remarkably reduced and IgA and IgM are less than 1% of normal. Antigenic stimulation fails to provoke an antibody response; however, T-cell numbers and function appear normal, and the thymus is intact. Children with this disease can sustain most childhood exanthems and produce low levels of antibody to most viruses suggesting that circulating immunoglobulin in such patients is not inert. A complication of some importance in this disease resembles a collagen vascular disease, dermatomyositis [16]. This is characterized by a rash and severe involvement of muscles. The muscles, and often the CNS, show lymphorrhages around small blood vessels. Recently Wilfert et al. [25] reported five patients with agammaglobulinemia who had persistent and prolonged ECHO virus infections of the nervous system. Echovirus was isolated from the CSF

for up to 3 years. However, there were few signs of acute CNS infection. Three of these five patients had a dermatomyositis-like disease [2], and echovirus type 24 was isolated from the muscle of such a patient. Pathologically, the cases described had perivascular round cells in all the major viscera, and the meninges were thickened with lymphocyte infiltration. The lesions, however, were predominantly in the white matter, consisting of perivascular cuffs, microglial nodules, and gliosis. It was of further interest that three of the patients with persistent echovirus had histocompatibility antigen HLAB7, while seven other X-linked agammaglobulinemics without echovirus persistence did not. The etiologic relationship to this collagen vascular-like disease cannot be clearly established. However, one must keep in mind that the echovirus is a naked RNA virus which does not bud from membranes, and therefore its antigen is not exposed on the surface of infected cells. The virus is released by lysis of cells. Perhaps the viral infection has resulted in the alteration of a host antigen.

There are no clear experimental models of a virus-induced autoimmune process; however, we have outlined experimental evidence which shows that viruses can interfere with immune responses. They can, in a selective fashion, involve subsets of immune cells, both lymphoid and nonlymphoid. One can postulate that this alteration of immune regulation releases an autoaggressive type of response to the host's own proteins, a host antigen cross-reacting with a virus antigen, or an altered host antigen derived from a previous CNS viral infection. One therefore does not have to postulate a continuous CNS infection to say that MS is the result of a viral infection. In fact, we suggest that MS is a disease in which a CNS viral infection in childhood or adolescence may have been accompanied by impairment of immune regulatory mechanisms resulting in an autoimmune disease.

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Discussion

H. Deicher: I should like just to mention that in connection with Dr. Jerslid's paper, we have so far examined 32 CSF samples from MS patients and have found evidence for the presence of immune complexes in CSF using the C19 binding method in 50% of these patients in the active phase of MS.

A. W. Ellison: Dr. Cuzner's paper brings up a very important theoretical point for neuroimmunologists doing tests of cell-mediated immunity against basic protein. For example, in the leukocyte migration inhibition assay there are large numbers of polymorphonuclear leucocytes. Do these eat up or destroy the basic protein before it can act as an antigen and does that explain why we have difficulty detecting cell-mediated immunity to basic protein in some tests and in some patients? Macrophages are also known before they change, as monocytes may be able to do the same thing. The serum present or absent in the medium might also influence the level of basic protein.

V. ter Meulen: The question I have is, is it feasable to analyze the complexes and give an answer about the antibodies or maybe the antigens? Because that would be the important question. Because once you have determined that they are immune complexes, it would be of great importance to find out what these immune complexes consist of, what's antigen and what's antibody.

C. Jersild: Sure, but I think there are many difficulties in analyzing the circulating immune complexes, because several antigens are able to bind to complexes which are not true antigen antibody.

W. A. Sibley: I was very interested in Dr. Cuzner's paper. We have been in the process of trying to measure plasminogen activator levels in CSF in MS. Dr. James Corregan, a pediatric hematologist at our institution, has been doing most of this work. As you know, plasmin is not only a fibrinolytic enzyme but it is also myelinolytic. Dr. Kemmer and Blume and Norton at Albert Einstein have recently shown that it selectively digests MBP and does not attack other myelin proteins. Dr. Adams showed several years ago that the earliest lesion at the margins of MS plaques is an attack on MBP. He showed this using histochemical methods. Not only that, but plasminogen activator occurs in the maximal concentration in venules in the brain, about which the earliest lesions of MS often develop. And the fourth reason for measuring this substance in spinal fluid is that plasminogen activator inhibitors such as epsilon-aminocaproic acid have been shown first by Prof. Wüthrich and his collaborators Rieder and Ritzel in 1963 to modify allergic encephalomylitis in rabbits. Recently we have confirmed in our own laboratories that in Lewis rats one can either drastically prevent EAE with epsilon-aminocaproic acid or treat the disease by giving epsilon-aminocaproic acid as late as the eleventh day after induction of EAE. So we think that it is pertinent to measure plasminogen activator in spinal fluid in MS, and we have made some early attempts in this regard. We find it difficult. We are using an unheated fibrin plate method. So far, we are convinced that many patients

with MS, however, have increased levels, especially those with chronic progressive MS. We have also found increased levels in many patients with a demyelinating neuropathy. It is elevated in other patients of course as well. I just call your attention to what I think is a promising new avenue of investigation in MS.

Dr. Wiesniewski: I think that it is a very intriguing idea, because our attempts to recover the virus are, as we all know, failing us. So anything like the mechanism you are talking about can be accepted, and we all believe that the epidemiology indeed indicates that the role of the virus is there and the age factor is very important, that in zero to 12 or 14 years it is of critical importance. And again, judging on the basis of experiments, we think that the age factor is of critical importance as to when and how we are exposed to the antigen, which in the future may play such an important role.

E. Schuller: Some people say that C-reactive protein is a lymphokine secreted by T-suppressor cells in the direction of macrophages. Do you have any experience? We found C-reactive protein in the blood of patients with little or no immunological reaction in the CSF. And this is my question.

Dr. Weiner: I have no personal experience with it. But I think that Peter Dowling is using it as a marker for depressed immunity in patients with autoimmune disease and particularly Guillain-Barré I think in MS too.

H. J. Bauer: I would like to say a word concerning virus research and MS from another viewpoint. Most of the studies have been carried out with the aim of finding a virus which is responsible, but I think it could be a very good thing if we could all pool the information on various viruses. We all know about measles, herpesvirus, influenza, vaccinia, and mumps. We have one case whose CSF measles titer went up from less than 1 : 4 to 1 : 32, and the next time she came with a bout she had a mumps titer. And things like that are happening, as I hear from my other colleagues. This might shed some light on this question whether one or more viruses are involved and get us into perspective with this problem.

H. Koprowski: I should like to make one short comment because I can add myself to your list of nine points. One other is that there would be a nice combination of effect of viruses on the CNS and through that on immune mechanisms for which I don't know as yet what the link is. And this is with street viruses of rabbits. If you inject street virus intracerebrally in mice, you suppress all mediated immunity completely. In other words, these mice can be skin transplants, and so when my colleague Dr. Victor informed me that it occurred, I thought, that we should maybe recommend it for kidney transplantation in man. The patient will die from rabies but will have his kidneys intact. But since rabies virus does not replicate, does not invade, it cannot infect any cells of the immune system. It is purely and only limited to a certain group of neurons of the CNS. Here you have a very interesting possibility that from the neurons some information or lack of information is delivered to the immune system so that it wipes out the cell-mediated immunity without effecting the humoral immunity, which would probably mean the destruction of T cells.

L. P. Weiner: That's very interesting. I didn't go into that, because there was just an indication that with polio in such a situation, there is no real evidence that there is anything more than one cycle of replication of polio in macrophages in that study. So it may not even be necessary for the virus to persist; it could be just an abortive infection and result in impairment of the cellular immunity. Just one further

comment, and that is on this idea of the viruses. The reason I got all these viruses is to indicate that it may be just an age and genetic susceptibility to any number of agents. The specific agent is not really important.

H. Wiesniewski: One thing, if we accept that the active disease in MS is only during the first 2 to 3, or a maximum of 5 years, then we are dealing pretty much with a burnt out phenomenon of autosensitization. Very importantly, when we are trying to isolate, we probably should concentrate on cases of very early MS. Because in the late ones we may not find anything like basic protein in relapsed EAE and this is opening the gate and the antigen probably has very little to do with the disease. Other antigens are of critical importance.

H. Koprowski: I think we should try to isolate virus before MS occurs. And I think that will be probably the ideal time.

Approaches to Understanding the Role of Antibody in Multiple Sclerosis

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Introduction

The first suggestion that antibody might play a role in multiple sclerosis (MS) was the discovery of a selective increase in cerebrospinal fluid (CSF) gamma globulin in MS patients, as demonstrated by Tiselius moving-boundary electrophoresis [15]. This abnormality is not limited to MS; elevation of CSF gamma globulin, and the more recently described oligoclonal pattern in the gamma globulin region of MS CSF also occur in a variety of acute and chronic infections of the central nervous system (CNS).

In 1963, following earlier studies using serum from animals with experimental allergic encephalomyelitis (EAE), Bornstein [3] observed that serum from 60% of patients with acute bouts of MS demyelinated CNS tissue cultures, an observation later confirmed in other laboratories. CSF and brain extracts from MS patients induced similar CNS tissue culture demyelination [16], suggesting that the demyelination is caused by some component(s) found in both MS spinal fluid and brain, as well as in serum. However the demyelinating activity of serum seems to lack specificity. For example, sera from some patients with amyotrophic lateral sclerosis (ALS) also induce tissue culture demyelination.

Antimyelin Activity in Multiple Sclerosis

Tissue Culture Studies

The nature of the component(s) in MS serum which is responsible for tissue culture demyelination is not yet clear. Demyelinating activity was poorly absorbed and removed by purified myelin, but was absorbed with a nonmyelin central nervous system (CNS) tissue pellet. This pellet contained oligodendrocytes and other neural components [27]. Further, removal of over 90% of MS serum IgG did not remove tissue culture demyelinating activity [12]. Only minimal demyelinating activity was found in the isolated IgG fraction, and its destruction by heating was only partially restored with human complement [13]. These immunologic studies using the organotypic culture assay system may lead to the conclusion that immunoglobulins are responsible for only part of the demyelination of cultures by serum.

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Immunofluorescence Studies

The difficulties of the organotypic culture assay system, and the need to confirm the possible presence of antibody to myelin or oligodendrocytes led to the use of the immunofluorescence technique to detect antigen-antibody binding. In this way, serum antibody which bound myelin in CNS tissue sections was found [8]; titers were higher in patients with clinically active MS than in normals or patients with inactive MS [18]. However, this abnormality was not restricted to MS patients; patients with Guillian Barre Syndrome, and ALS, and some normals also had elevated antimyelin gamma globulin titers. Similar immunofluorescence studies showed binding of serum gamma globulin from most MS patients to isolated bovine oligodendrocytes. Similarly diluted serum from patients with other neurologic diseases and from normals showed the same phenomenon in only a very few instances [1].

The Nature of Experimental Demyelinating Antibodies

The evidence suggesting a pathogenetic role for antibody to lipid haptens derives from studies employing in vitro and in vivo animal model systems. Serum from rabbits with whole nervous system-induced experimental allergic encephalomyelitis (EAE) readily produced demyelination of myelinated organotypic cultures [4]. Serum from guinea pigs with EAE induced by myelin basic protein (MBP) alone did not induce culture demyelination [17, 24]. Studies of inhibition of primary myelination (Myelination inhibition) in cultures provide a more sensitive and objective assay of serum antimyelin activity [5], although the factors inducing myelination inhibition may not be identical to those which induce demyelination. Extensive studies using this assay to compare the effects of serum from whole tissue-induced EAE animals with serum from several species of animals with myelin basic protein (MBP)-induced EAE have demonstrated that antibodies to MBP also fail to induce myelination inhibition in vitro [15, 25], while sera from whole tissue-induced EAE animals have readily demonstrable myelination inhibition activity.

The evidence that an antigen(s) other than MBP induces serum demyelination and myelination inhibition activity in animals with EAE led to testing of specific lipid haptens. Antibodies induced in rabbits against galactocerebroside, a prominent myelin lipid, demyelinated cultures [7] and produced myelination inhibition [9].

Table 1. Serum-induced demyelination

	CNS culture	PNS culture	In vivo sciatic nerve
Whole CNS EAE	+	+	+
MBP EAE	–	–	–
Antigalactocerebroside	+	+	+
Antioligodendrocyte	+	–	–
MS	+	–	–

Development of a sensitive liposome assay for quantitation of antilipid hapten antibodies [10] permitted studies which demonstrated that the minimum effective concentration of rabbit anti-CNS tissue antisera required to induce myelination inhibition correlated with titers of antibody to galactocerebroside [6]. Thus it appears that antibodies to galactocerebroside play an important role in the capacity of experimental antisera to induce tissue culture demyelination or myelination inhibition.

However, it seems that other factors must be involved as well, as suggested by the specificity of certain sera for CNS versus peripheral nervous system (PNS) (see Table 1). Since rabbit antisera to isolated bovine oligodendrocytes, which do not contain significant antibody titers to galactocerebroside, demyelinated CNS but not PNS cultures, we must consider the possibility that reactivity to another antigen is involved in producing this specificity [20].

Local Transfer of Demyelination by Intraneural Injection of Serum

Direct injection of antisera into the sciatic nerve of the rat has made it possible to detect the presence of humoral demyelinating factors in vivo [19, 21, 22]. Rabbit antisera to PNS or to CNS tissue, or to galactocerebroside, induced a rapid sequence of abnormalities starting with changes in the Schwann cells prior to invasion by cellular elements, followed by demyelination and invasion of monocytes and macrophages [21, 22]. The demyelinating activity of the sera was destroyed by heating it at 56° C for 30 min, and removed by absorption with CNS or PNS myelin, but not with liver. Thus the in vivo demyelinating activity is organ specific and likely to be complement dependent. These studies demonstrate that humoral antibody alone can induce cytolytic effects in the nervous system if the antibody gains access to the appropriate target cells. Neither serum from MS patients nor from rabbits immunized with oligodendrocytes has induced demyelination in the rat sciatic nerve, but this may reflect CNS versus PNS specificity or species specificity rather than a lack of in vivo demyelinating capacity. The capacity to induce demyelination in vivo again correlated with measured levels of antigalactocerebroside activity in the sera tested.

Galactocerebroside-Induced EAN

The biological importance of the demonstrated immune reactivity to galactocerebroside is enhanced by the observation that 13 of 31 rabbits repeatedly immunized with pure galactocerebroside in bovine serum albumin and complete Freund's adjuvant have developed demyelination of their spinal dorsal root ganglia and peripheral nerves, without the prominent presence of the perivenular lymphocytic infiltration seen in EAN or EAE [23]. A possible explanation for this localization of demyelination is the evidence that the blood-nerve barrier is leaky around the spinal ganglia and nerve roots [26].

Summary and Perspective

Antibody to at least one myelin lipid component, galactocerebroside, is capable of inducing CNS and PNS demyelination and myelination inhibition in vitro and PNS demyelination in vivo, both spontaneously and by direct intraneural injection. Other serum factors in CNS tissue and purified oligodendrocyte-induced antisera probably confer the CNS versus PNS specificity observed in vitro and in vivo. Some components of MS serum, CSF, and brain extract possess antimyelin activity in vitro. Serum gamma globulin binding to oligodendrocytes occurs to a greater degree in sera of MS patients than in controls. While there are clear differences between the experimental antisera and MS serum, CSF, or brain extracts, their similarities suggest that the excessive synthesis of immunoglobulin within the neuraxis of the MS patient may include production of antibodies which play a role in the production of demyelination or in inhibiting remyelination. The levels of responsible antibodies might be very low and yet be capable of exerting an effect by their continued presence within the CNS. The essential role of cell-mediated immunity in the induction of EAE and EAN is well documented [2, 11]. Demyelinating antibodies may act independently or cooperate with cell-mediated responses in the pathogenesis of both experimental and human demyelinating disorders. These thoughts go beyond the direct evidence available and await further exploration.

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Partial Purification of MS-Specific Antigens

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Summary

The present study was devoted to an immunochemical elucidation of antigenic similarities and differences between cytoplasmic and microsomal fractions of six multiple sclerosis (MS) and seven non-MS brain autopsy specimens. The antigenic composition of the samples studied was traced by crossed immunoelectrophoresis using antibodies made by immunization of rabbits with the corresponding fraction. The following data were obtained: (1) An antigen present in measles-infected Vero cells and two specific antigens have been purified more than 3000-fold from MS brains using molecular filtration and DEAE cellulose chromatography; (2) all three antigens have a molecular weight between 10^5 and 10^6 daltons and isoelectric points between 3.5 and 6.0; and (3) measles antigen has been also found in three out of seven non-MS brains; however, it did not stimulate antibody formation in rabbits, in contrast to measles antigen of MS brain.

The significance of the above-mentioned data is discussed in view of the immunological abnormalities previously found in MS patients. The antigens found may represent one or more viral antigens.

Introduction

MS is a neurological disease of unknown etiology, but epidemiological studies have revealed a statistically significant increased serum measles antibody titer in MS patients [1, 4, 7]. Thus the demyelination process in MS may be due to a chronic (viral) infection or to immunological abnormalities. A virus infection in the central nervous system (CNS) may cause formation of new (viral) antigens. However, even an autoimmune process may lead to lysosomal activation and conformational abnormalities in CNS antigens.

Recently, we demonstrated a "measles antigen" and a specific antigen in MS brain cytosole fraction not present in non-MS brain homogenates [8, 15]. In this paper a method for partial purification of these antigens and new findings concerning "MS specific antigens" are described.

Materials and Methods

Chemicals. Agarose A45 was from l'Industrie Biologique, France, and Freund's complete adjuvant was from Difco Labs., USA. Polyacrylamide gel electrophoresis

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Table 1 A. MS brains

Brain no.	Subject	Sex	Age	Diagnosis	Duration (years)	Autopsy diagnosis beside MS	CNS pathology	Remarks
1	A.C.H.	F	51	MS	26	Carcinoma of urinary bladder Bronchopneumonia	Plaques periventricularily	Attacks with progression
2	E.P.	F	44	MS	30	Bronchopneumonia, urinary infection, decubitus	Plaques periventricularily	Severe chronic
3	B.B.	F	65	MS	38	Bronchopneumonia, pyelonephritis, endocarditis	Plaques in medulla spinalis periventricular plaques in right hemisphere	Progressive
4	G.H.	F	52	MS	18	Cancer of the stomach	Plaques distributed in whole CNS	Slowly progress
5	N.H.	M	62	MS	25	Bronchopneumonia, bronchogenic carcinoma, arteriosclerosis	Periventricular plaques	Very slow progressive
6	V.P.	F	35	MS	12	Bronchopneumonia, pyelonephritis	Periventricular plaques	Acute MS

Table 1 B: Non-MS brains

Brain no.	Subject	Sex	Age	Diagnosis	Autopsy diagnosis
7	E.K.J.	F	76	Diabetes, pneumonia	Pneumonia
8	F.H.	M	65	Generalized arteriosclerosis, thrombosis of the heart	Thrombosis of the heart
9	S.L.	M	75	Generalized arteriosclerosis, thrombosis in pulmonary artery	Thrombosis of pulmonary artery
10	C.C.B.	F	82	Generalized arteriosclerosis, thrombosis of basilar artery, hypertension	Thrombosis of basilar artery
11	C.H.S.	F	28	Pneumonia	Pneumonia
12	G.P.	F	62	arteriosclerosis	Thrombosis of the heart
13	M.L.	F	78	Cancer with metastasis	Thrombosis of the heart

(PAGE) plates containing ampholine for isoelectric focusing were from LKB Products, Sweden (cat. no. 1804-101). DEAE-cellulose, acrylamide, and sodium dodecylsulfate (SDS) were from Sigma Co., USA. Millipore pellicone membranes (different types) were from Millipore Corporation, USA. All other chemicals were of the highest purity obtainable from E. Merck, FRG.

"Measles antigen" was prepared from infected Vero cells [13]. In studies tracing "measles antigen", an antigen solution of uninfected Vero cells, prepared similarly to the "measles antigen" was used as a control. Human myelin basic proteins (MBP) from MS and Non-MS brains were prepared according to Eylar et al. [10].

Control and MS Brains. Brains from six MS patients and seven non-MS patients (Table 1) were used in the present investigations. Brains were collected 16 h after death and twelve of them were immediately frozen (–70° C). MS brain no. 6 was, however, immediately processed. Macroscopic inspection of all six MS brains revealed plaques mainly in the periventricular area (Table 1). Classical microscopic examination revealed demyelination in these areas. When alive, all the patients were clinically diagnosed as having "certain MS" [16]. No plaques were found in the non-MS brains.

Cerebrospinal fluid (CSF) and serum from "certain MS" patients was used for the search of specific antigens or antibodies. CSF was concentrated by ultrafiltration using Sartorius Membrane Filters (SM 13200).

Immunization of rabbits and preparation of antisera has been described before [15]. Details on the injection schemes are described in Table 2. First the rabbits received sc injection of 0.2 ml of Freund's complete adjuvant together with the antigen solution. Antibodies used were concentrated threefold by precipitating them from antiserum with 2.0 M ammonium sulphate, pH 6.8. The precipitate was dissolved in 0.005 M sodium Na-phosphate buffer (pH 8.2) and dialyzed against the same buffer.

Table 2. Immunization of rabbits [a]

Injection MS	Non MS	Dose μg protein	No. of patients involved	No. of rabbits injected	Antibodies to specific antigens	Measles anti-bodies	Injection period
Brain microsomes		900	4	5	–	+	Over a period of 2 years
	Brain microsomes	900	4	5	–	–	Over a period of 2 years
Brain cytosole		900	5	10	+	+	Over a period of 2 years
	Brain cytosole	900	7	5	–	–	Over a period of 2 years
Serum		0.2 ml	6	5	–	–	1 year
CSF		400	Pooled	5	–	–	1 year
Lymphocytes		400	Pooled	2	Not checked	Not checked	3 months
	Lymphocytes	400	Pooled	2	Not checked	Not checked	3 months

[a] One control rabbit (cytosole) and one control rabbit (microsomes) died after paralysis in legs. None of the animals died in the other groups

Brain cytosole and microsomes were prepared as described previously [15].

Fractionation of Cytosole. MS and non MS-brain cytosole were fractionated stepwise using a Millipore Hi-flux molecular filtration system. Millipore Pellicone membranes, which retain particles over a definite size (mol. wt.) were used for filtration. The scheme indicated in Figure 1 was used.

DEAE-Cellulose Column Chromatography. Ten to fifteen mg protein in 1 ml fraction C (material retained on filter PTHK, Fig. 1) were loaded on a DEAE-cellulose

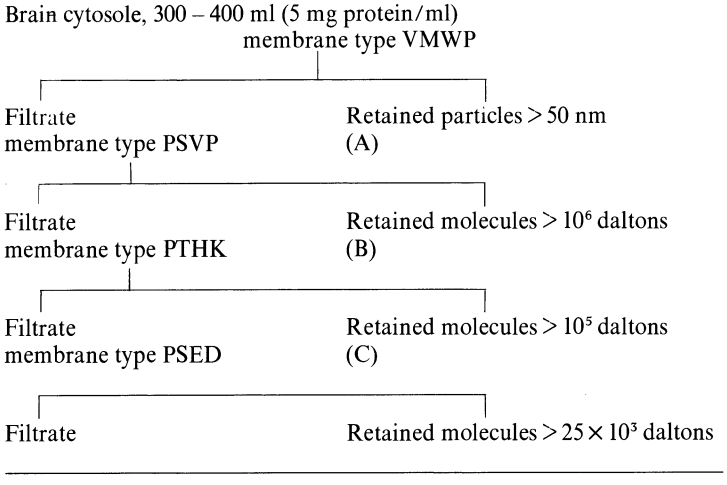

Fig. 1. Scheme for molecular filtration of cytosoles (for experimental details see the text)

(pH adjusted to 7.2 – 7.4 after washing) column (22 cm × 0.8 cm^2). Discontinous elution was performed with 50-ml batches of distilled water, 0.25 M, 0.5 M, 0.75 M, 1 M, 2 M, and 3 M sodium chloride solutions (22° C). Then 3.6 ml fractions were collected on a LKB fraction collector (Ultrarac 7000) equipped with UVICORD 8303A, LKB Ltd., Stockholm, Sweden). The optical density of each fraction was determined at 280 nm. The pooled distilled water eluates (see Results) were concentrated by freeze drying. The fractions eluted in 0.25 M NaCl were concentrated by ultrafiltration through a SM 13200 membrane (Sartorius Membranes, Göttingen. W. Germany).

Crossed immunoelectrophoresis (IE) in 1% agarose was performed as described previously [15], except that the buffer was replaced by 0.05 M sodium barbital buffer, pH 8.5. Unless otherwise mentioned, electrophoresis in the first direction was carried out for 3 h at 250 V (10 – 12° C).
To determine the specificity of MS-specific antigens (cf. below):

1. MS antigen was electrophoresed against MS and non-MS antibodies and vice versa.
2. MS and non-MS antigens were run on the same gel (Tandem-crossed IE).
3. Intermediate gel-crossed IE [6] was run with MS antigen against MS and non-MS antibody containing gels.
4. MS antigen was electrophoresed against MS antibodies preabsorbed with various amounts of non-MS antigen [15] and vice versa.

Isoelectricfocusing on commercial PAG plates (pH range 3.5 – 9.5) was performed using a LKB Multiphor no. 2117 apparatus and a LKB power supply no. 2103. Power supply settings at 10° were, p = 30 W, U = 1200 V, and I = 50 mA. Variable amounts of proteins (MS and non-MS fraction C, MS and non-MS peak dl) in 15 µl were analyzed for 1.5 h. The anode electrode solution was 1 M H_3PO_4 and 1 M NaOH was used as a cathode solution. After electrophoresis fixation of proteins (by means of trichloroacetic acid and sulfosalicylic acid in 33% methanol, 22° C), staining with Coomassie Brilliant Blue R-250 (in methanol-acetic acid water 25 : 8 : 67, 60° C) and destaining with ethanol-acetic acid-water was carried out as described in the standard method provided with the gels.

SDS-PAGE. The sample containing about 4 mg of protein in 1 ml was mixed with equal volume of sample buffer (1 ml 25 mM Na-phosphate buffer pH 7.0; 60 mg SDS; 30 mg dithiothreitol; 140 mg sucrose; and 5 µl of saturated Bromophenol Blue solution). Electrophoresis was carried out as described by Agrawal et al. [2] in glass tubes with an internal diameter of 5 mm and a length of 100 mm. The gels were stained with Coomassive Brillant Blue R-250 and destained by diffusion [3].

Protein was determined using the method of Lowry et al. [11].

Results

Crossed IE of measles antigen revealed presence of measles antibodies in all antisera isolated from rabbits injected with MS cytosole and MS microsomes (Fig. 2 a). The anti-MS antisera did not precipitate control Vero cell antigen. However, none

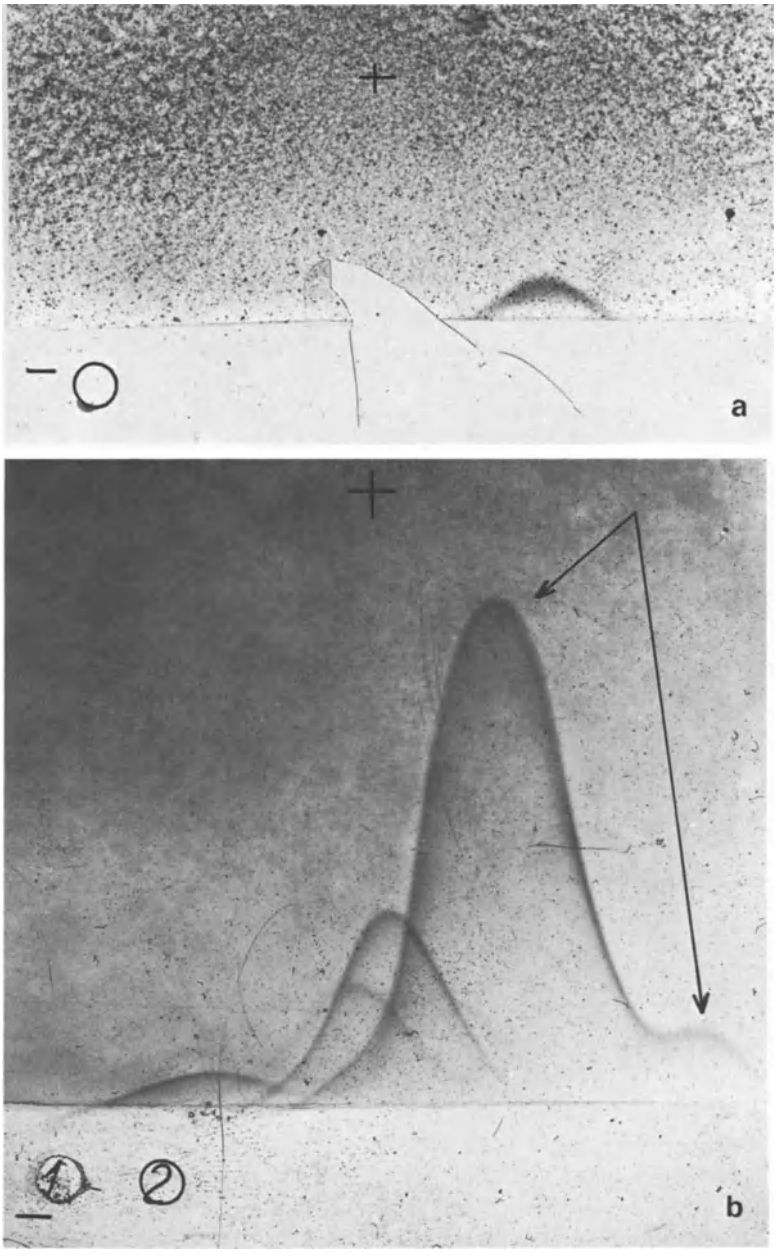

Fig. 2. a Crossed immunoelectrophoresis (CIE) of measles antigen applied in the hole below to the left. Primary migration from left to the right. Secondary migration into the gel containing antibody: from below upward. *Antigen* – measles antigen i.e. an extract of measles infected Vero cells (400 µg protein); *Antibody* – anti-MS cytosole (45 µl/cm³ gel) **b** CIE-Tandem technique. For experimental details see legend to Figure 2 a. *Antigen* – hole 1: MS fraction C (250 µg protein), hole 2: Measles antigen (400 µg protein); *Antibody* – Anti MS cytosole (45 µl/cm³ gel). The two fused immunoprecipitation arcs (*arrows*) represent the measles antigen from holes 1 and 2

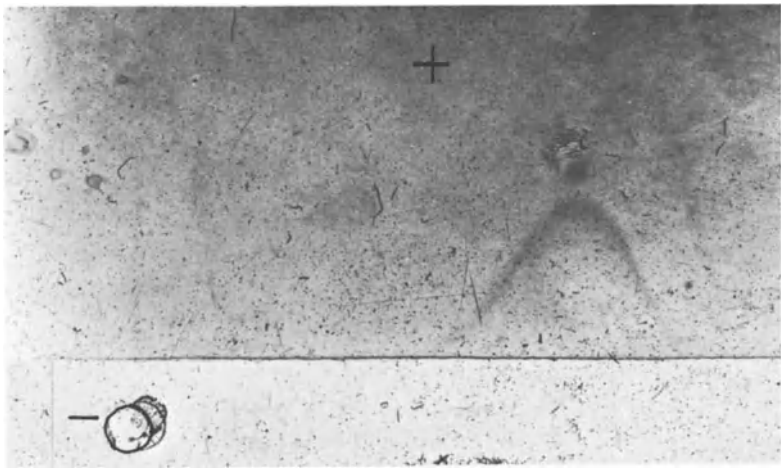

Fig. 3. CIE of MS fraction C employing anti-MS cytosole neutralized with non-MS fraction C. For experimental details see legends to Figure 2 a. *Antigen* – MS fraction C (250 µg protein); *Antibody* – Anti-MS cytosole (45 µl/cm³ gel) preabsorbed with non-MS (brain 13) fraction C (1.1 mg protein/600 µl antiserum) followed by centrifugation at 10.000 *g* for 10 min

of the antisera prepared from rabbits injected with non-MS cytosole or non-MS microsomes were found to contain measles antibodies.

Fractionation of cytosole by Millipore membranes followed by Tandem-crossed IE of fractions revealed that MS fraction C (Fig. 1, molecules between 10^5 and 10^6 daltons) contained an antigen present in measles infected vero cells (Fig. 2 b). However, when anti-MS cytosole was absorbed with fraction C of non-MS brain no. 12 prior to use, "measles antigen" did not precipitate in the gel.

Thus the absorption of measles antibodies by the non-MS brain fraction revealed that measles antigen may be present in some of the non-MS brains. Re-examination of all non-MS brains (by means of crossed IE of fraction C versus anti-MS cytosole) revealed that three non-MS brains out of seven (nos. 10, 12, and 13) contained the measles antigen.

Besides measles antigen, crossed IE of MS fraction C versus anti-MS cytosole revealed two extra immunoprecipitation arcs compared with crossed IE of the same versus anti-non-MS cytosole. Crossed IE of fraction C from all seven non-MS brains versus anti-MS cytosole did not reveal any of these two immunoprecipitation arcs. Intermediate gel-crossed IE, in which MS fraction C was electrophoresed against gels containing anti-MS cytosole and anti-non-MS cytosole (in the intermediate gel) also revealed presence of two extra immunoprecipitates only in the gel containing anti-MS cytsosole. However, crossed IE of non-MS brain fraction C versus anti-MS/anti-non-MS cytosole absorbed with various concentrations of MS/non-MS fraction C did not reveal any immunoprecipitation arcs. Thus, the two immunoprecipitation arcs shown by MS fraction C (Fig. 3) may represent two antigens present in MS brains only.

An attempt to further purify these antigens from fraction C using Sephadex gel filtration failed. Discontinuous elution of fraction C on a DEAE-cellulose column

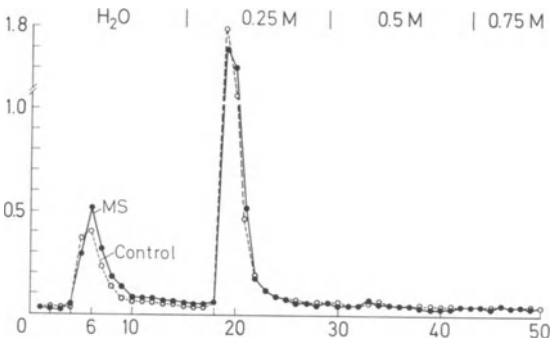

Fig. 4. Elution pattern for DEAE-cellulose column chromatography of fraction B (for details see text); *Ordinate:* O. D. at 280 nm; *Abscissa:* Fraction no. (3.6 ml). The concentration of NaCl used for elution is indicated on the top

with 0 – 3 M NaCl revealed two major peaks (Fig. 4): one eluted in distilled water (peak 1) and one eluted in 0.25 M sodium choloride (peak 2). Further elution with increasing concentrations up to 3 M NaCl eluted only trace amounts of proteins. The elution patterns of MS and non-MS fraction C on the DEAE-cellulose column were identical (Fig. 4). Peak 1 from both MS and non-MS material contained MBP (judged by SDS-PAGE and isoelectricfocusing, IP > 10) together with some other proteins.

Analysis of peak 2 by crossed IE using crosswise absorbed antibodies with or without the Tandem technique revealed that peak 2 contained measles antigen (all MS and three controls) and the two MS specific antigens were only present in peak 2 of MS brains (Fig. 5).

Isoelectric focussing of peak 2 revealed that isoelectric point of proteins in this fraction were between 3.5 and 6. SDS-PAGE revealed that the peak 2 may contain only traces of MBP (Fig. 6).

As the preparation is impure, we have not analyzed it for constituents other than protein. Total recovery of protein in peak 2 was thus found to be approximately

Fig. 5. CIE (for details see legend to Figure 2 a) of MS peak 2 employing anti-MS cytosole neutralized with non-MS peak 2. *Antigen* – MS peak 2 (150 µg protein); *Antibody* – anti MS cytosole (45 cm^3/gel preabsorbed with non-MS peak 2 (brain 12) (1.5 mg protein/600 µl antiserum. Electrophoresis period first direction: 1.5 h

0.3 mg protein/g wet brain (both MS and non-MS). Thus, measles antigen as well as specific antigens from MS brains are purified more than 3000-fold starting from the brain.

We have also been able to prepare peak 2 directly from the supernatant of brain homogenate centrifuged at 1000 *g* for 25 min. The supernatant was then filtered through Millipore membrane type GSVP (which retains particles over 220 nm). The filtrate was then filtered through membrane type VMWP (50 nm) and further purified as was the case with the cytosole.

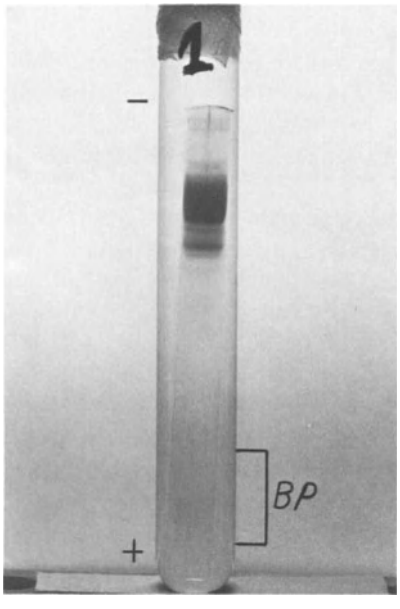

Fig. 6. SDS-PAGE of MS peak 2 (40 µg). For experimental details see the text. Basic protein region is shown in the figure

Crossed IE of serum and CSF of MS patients versus anti-MS cytosole or crossed IE of peak 2 versus anti-MS serum or anti-MS CSF did not indicate the presence of either measles or specific antigens in MS serum or MS CSF.

Discussion

Results of this investigation demonstrate that a measles antigen was present in all six MS brains studied. The presence of measles antigen in three out of seven non-MS brains studied indicates that measles antigen may not be a primary cause of MS. This is in agreement with Ammitzbøll et al. [5], who showed that the oligoclonal IgG bands of cerebrospinal fluid (CSF) occurred significantly related to increased CSF measles antibody titer but were not specific for MS.

The CNS may be looked upon as a closed compartment with limited immunological capacities. This may cause measles virus to be activated during different pathological processes, similar to the in vitro isolation of measles virus of lymph node cultures of humans previously infected with measles [9].

Though measles antigen was present in three of the non-MS brains, none of these gave rise to antibody formation in rabbits. Thus, the antigenic site may still be masked in these brains. In vivo production of antibody by measles antigen may depend on the unmasking of antigenic sites, which in turn may be achieved by a stimulus due to other infection(s) or to physiological, immunochemical, or genetic abnormalities.

In this investigation, two specific antigens were found only in MS brains. These findings confirm our previous reports of the presence of a specific antigen in MS brains and a measles antigen. The demonstration of two specific antigens was only possible considering the following points: (1) That one of the antigens caused antibody formation in experimental animals after several months of immunization; (2) that the antibodies used in the present investigation were concentrated threefold; and (3) that the antigen used (fraction C or peak 2) is a rather purified (enriched) material compared to cytosole.

As indicated in the results, the two specific antigens have not yet been traced in CSF or serum of MS patients.

The peak 2 isolated by DEAE-cellulose column chromatography of MS fraction C contained measles antigen as well as the two specific antigens. The isolation of all three antigens in the same fraction at more than 3000-fold purification may indicate close physicochemical properties, e.g., similar isoelectric points of these antigens. The nature of these antigens has not yet been defined, because their preparation is still crude. However, they may be: (1) Microbiological antigens (e.g., viral glycoproteins or RNA) or their degradation products; (2) complexes of microbiological agent and host antigens; or (3) brain cell proteins with changed conformation state due to immunological abnormalities in the brain (lysosomal activation).

We have ruled out the possibility that the specific antigen is MBP, because there was a lack of fusion of immunoprecipitates and by absorption experiments. However, it is not certain whether the specific antigens represent conformationally changed complexes of other brain proteins with MBP, degraded basic protein, or modified basic protein.

In a recent report [12], an infectious agent has been isolated from MS bone marrow cells and was reported to produce cytopathogenic effects similar to paramyxoviruses. The size of this infectious agent has been found to be approximately 220 nm. We could not isolate any specific antigen in the MS cytosole fraction larger than 50 nm. Thus, if such an agent was present in the brain of MS patients, it may have been detectable using the immunological methods described in this report.

The peak 2 isolated by MS brains has been found to be stimulating AER-forming lymphocytes from MS patients only [14].

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Discussion

E. J. Field: I think that a very good control brain to use would be one in which there was already hypertrophy of astrocytes. Such brains are kuru brains or Creutzfeldt-Jacob brains. It is quite conceivable that these will share antigens with MS brains, since there is so much hypertrophy of the astrocyts in MS brains.

J. Clausen: Yes, we have discussed with Link and several other people what to use as control brains and of course that is a very tricky question. I don't like to work with Jacob-Creutzfeldt brains. This is one thing. The other is, we don't have subacute sclerosing panencephalitis brains in our laboratory. So this has hampered our study and we try to do it in this way.

E. Norrby: I wondered about what additional controls you have made to assure yourself that you are dealing with a measles-specific antigen. Firstly, have you checked that the sera which you raised in rabbits, if you absorb them with noninfected tissue culture cells, do not remove the specific precipitate? And have you used any other serological techniques than the one described here to detect measles-specific antibodies?

J. Clausen: We have used the same antigen for our studies of measle antibodies precipitating in gels and we have found a correspondence between the commercial measle antigen and our antigen. We have defined it by content of RNA and protein.

Dr. Wiesniewski: Don, you mention that the antibody injected in the sciatic nerve induces demyelination without the presence of the cells. That's what you said, correct? Because in my feeling trauma by definition is bringing cells. So what you are observing is antibody-dependant cell-mediated demyelination of the type which we observe in the rabbit eye. And that is the cleanest model. Because the moment you touch any tissue you bring cells. That's just the very moment you bring cells. So that in my feeling, it is antibody dependent and cell mediated. Indeed, you need the antibody. But that's the mechanism.

D. H. Silberberg: We see changes before we see the cells, by several hours.

V. ter Meulen: Dr. Clausen, I wanted to expand the question raised by Dr. Norrby. And you probably are aware of the fact that measles virus is highly cell associated. It is very difficult to purify measles virus in such a way that you eliminate host protein. Could you make sure in your assay that the immune precipitation that you observe is not an antibody cross-reacting with an antigen which you carry along by your measles virus purification?

J. Clausen: No, we are working on that question at the moment. We are even trying now to separate out the different glycoproteins of the measles. To try to study if one of these is the antigen we have traced. But I have to add one thing more, namely that, as will be discussed later today, we have isolated those fractions and used them to stimulate lymphocytes from MS patients by the active E rosette technique

and I can tell you now, that the fractions we have isolated are specific fractions which stimulate the MS lymphocytes.

E. K. Kuwert: I would like to ask a question to Dr. Silberberg. Did I understand you correctly, that you were able to induce an EAE-like disease in those white rabbits just by immunizing those animals with cerebroside and complete Freund's adjuvant?

D. H. Silberberg: That's an EAE-like experimental allergic neuritis affecting the proximal nerve roots.

E. K. Kuwert: Did you make sure that your antigen was just cerebroside? Some auxiliary lipids perhaps? And was not contaminated by other material? Because when we did similar studies in EAE, they were published in 1965 in the Annals of the New York Academy, we were not able to induce any disease by active immunization of rabbits with cerebroside and cholesterin and lecithin. We were not able to find any pronounced histological signs, neither by active nor by passive immunization with huge amounts of anticerebroside EAE antibodies. Now, the further question is then, how often did you inject your animals?

E. Koprowski: May I now intervene? I think this is really a technical question. So we could rather discuss this during the coffee break.

E. K. Kuwert: Yes, but the principal thing is then, you know, the actual way of thinking, that MBP induces the disease, and not cerebroside. So I am somehow surprised that those animals came down with the paralysis.

D. H. Silberberg: I didn't catch the last part of that. But the galactocerebroside that we have used migrates as a single spot in thin-layer chromatography – it appears to us to be pure. Of course the animals are also being injected with complete Freund's adjuvant. So it's not just galactocerebroside, but CFA animals do not develop disease.

C. S. Raine: We are very much interested to see Dr. Silberberg's results on the presence of oligodendrocyte staining by MS serum and the reported almost total lack of staining by control serum. We did a very detailed repeat study hoping to confirm Dr. Silberberg's study. There are three other groups around the world which I know of, and there are probably more. We have all come to the same results, and that is, that MS serum does have an affinity. In our hand it's 63% of all MS tested, and we tested 54 specimens, and 45% of all controls – and we have tested almost 100 control sera, which also have the same staining pattern. I won't go into it because it is technical. But we did every control imaginable and we could prove that the staining is not an antioligodendrocyte antibody by absorption, etc. We did *Fab* fragmentation of the MS-positive serum, the control positive serum, and a very well-characterized antioligodendrocyte serum. The MS-positive serum, the control-positive serum, and the *Fab* fragments do not stain. The antioligoantiserum *Fab* fragment does stain. We conclude from this that the staining is not oligospecific or MS specific; the staining is probably due to nonspecific binding to *Fc* receptors.

D. H. Silberberg: The reply to this is, I believe, very technical. What we have to do is see that we are doing exactly the same thing. We have had enough confidence in the difference between MS and controls that we have gone on to the next step which is to begin to try to identify the antigens responsible for the antibody affinity.

And we see a clear difference consistently. We have to sit down and go through step by step.

E. J. Field: Dr. Clausen is very understandably reluctant to work with Kuru or Jacob-Creutzfeldt brains. Then he may with safety work with scrapie – scrapie mouse brain – because, as the antigenic stimulation from hypertrophied astrocytes or even from the astrocytes induced by cuprazone treatment show scrapie is completely nonspecific and will work with human brains. If you are frightened of scrapie, don't worry. You eat plenty of scrapie. You eat it in "wurst", and, very commonly, every time you have mutton.

Dr. Schipper, Göttingen: I would like to comment once more on Dr. Silberberg's work. We have almost completed an extensive study on the demyelinating factor in MS serum and CSF. And we agree with you that the degree of demyelination is much less in MS specimens than in EAE specimens. But what is also coming out of our work is that a demyelinating factor in MS is not an antibody, in contrast to EAE. We have done this – we have isolated antibody in different ways and we have never found a demyelinating activity in these antibodies.

Y. Nagai: My comment is to Dr. Silberberg. Dr. Silberberg could show that the immunization of rabbit with galactocerebroside in complete Freund's adjuvant caused paralysis. To my experience, two years ago I published a paper in which ganglioside immunization was shown to acctually cause such paralysis. But this is only limited to a certain strain of rabbit, that is, a Japanese white, but the regular type of New Zealand white does not develop such paralysis. And also, a histopathological examination gave a negative result. But also, we have many times tried immunization with galactocerebroside, and not only in the New Zealand white but also in the Japanese white rabbit. No paralysis developed. Yesterday I talked about some cases.

Studies of Immune Reactions in Multiple Sclerosis by Active T-Rosette Test, Immunofixation, and Myelinotoxicity of Lymphocytes in the Rabbit Eye

H. LINK, S. KAM-HANSEN, K. KRISTENSSON, M. LAURENZI, A. FRYDÉN, and B. ROSTRÖM [1]

The rapid development of new or improved techniques in immunology has, during the last few years, considerably increased our understanding of the basis of immune reactions and broadened our knowledge regarding the importance and influence of these reactions in human pathology. However, application of new techniques to functional studies of peripheral blood lymphocytes (PBL) in MS have hitherto not given any conclusive evidence indicating a derangement in cell-mediated immunity outside the CNS. Efforts to study the function of lymphocytes within the CNS have been hampered by the highly cell-consuming techniques in relation to the low lymphocyte numbers obtainable from CSF and by the increased fragility of these cells. The application of technical modifications and microtechniques has, however, enabled studies of CSF lymphocytes compared with PBL regarding B and T lymphocyte distribution and response on mitogen stimulation. Decreased B and elevated T lymphocyte values in CSF compared with blood have thus been reported [11, 26], and CSF lymphocytes have been shown to be poor or nonreactive to the mitogens phytohaemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM), while PBL proliferate normally on mitogen stimulation [12, 15]. These deviations in CSF lymphocyte function are similar to those reported in aseptic meningitis [3, 4], but stimulation with mumps virus antigen in mumps meningitis revealed stronger proliferation of CSF compared with blood lymphocytes [6], indicating that the CSF lymphocytes are immunologically competent despite their low response on mitogen stimulation, and probably of importance for the development and course of this acute and benign CNS disease.

Active T Cells in Blood and CSF in MS, Optic Neuritis and Aseptic Meningitis

For further evaluation of the cellular immunocompetence with special reference to CSF lymphocytes, a modification [10] of the active T-rosette test of Wybran and Fudenberg [34] has been used. The test was adopted to 50×10^3 lymphocytes [10]. A sheep red blood cell (SRBC) to lymphocyte ratio of 30 : 1 was used.

Figure 1 shows that CSF lymphocytes from MS and optic neuritis (ON) patients gave significantly ($P < 0.001$; t-test) lower mean percentages of active T cell rosettes compared with PBL (Kam-Hansen, unpublished data). In contrast, patients with aseptic meningitis displayed significantly ($P < 0.001$) higher active T cell values in

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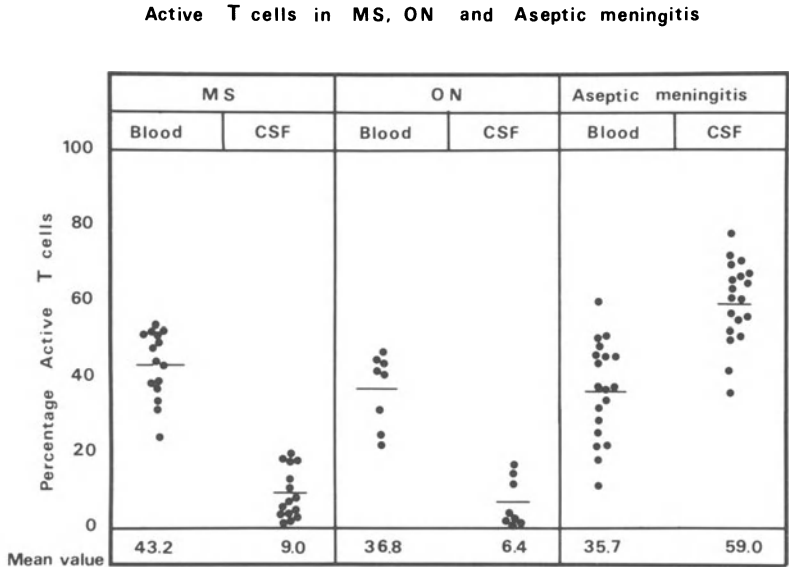

Fig. 1. Percentage of active T cells in simultaneously obtained blood and CSF specimens from patients with multiple sclerosis (MS), optic neuritis (ON), and aseptic meningitis

CSF compared with blood. The mean percentage of active T cell rosettes in blood in the three groups did not differ significantly. In eight healthy controls hitherto investigated, the mean value of active T cells in blood was 44.4% (range 28.0–60.5%).

The consistently low active T cell rosettes obtained with MS CSF lymphocytes using a SRBC to lymphocyte ratio of 30 : 1 agree with previous findings with a SRBC to lymphocyte ratio of 175 : 1 [10]. It is not known whether this abnormality is secondary to an accumulation of active T cells within and around plaques, or a consequence of a primary defect in cellular immunity. The elevated active T cell count in CSF in aseptic meningitis may reflect the adequate immune response against viral antigen within the meninges.

Normal numbers of active T cells in MS patients' blood have been described previously [7, 8, 10]. The possible derangement in cell-mediated immunocompetence as reflected by low numbers of active T cells in MS and ON seems therefore also to be confined to the CNS.

Low active T cell numbers in CSF have also been found in one case of neurosyphilis and one of Mollaret's chronic meningitis [10]. Determination of active T cells in CSF can therefore not be used as a specific MS test. However, a discrimination seems possible between MS and acute aseptic meningitis and probably also other acute CNS infections, which is of importance, since such discrimination has as yet not been feasible using tests for the humoral immune response within the CNS as measured by Ig values or the oligoclonal immune reaction [22].

Studies on Myelinotoxicity of CSF Lymphocytes and PBL in MS in the Rabbit Eye

An experimental model is needed to enable investigations of whether CSF, brain, and blood constituents in MS have myelinotoxic or demyelinating activity. Previous experimental models implying observations on myelinated organotypic cultures of mammalian central nervous tissue [1] have not been suitable for the study of CSF, since myelin lesions could only be induced by highly concentrated CSF involving pooled samples [14]. In addition, in vitro systems as a whole cannot be expected to reflect the dynamics of immune responses of the host. Recently, Tabira et al. [29, 30] have shown that unconcentrated CSF from MS patients may cause myelin lesions in the optic nerves of tadpoles (*Xenopus laevis*). These findings have been confirmed and extended to patients with optic neuritis (ON) with oligoclonal IgG present in CSF [27]. CSF from ON patients without oligoclonal CSF IgG produced myelin lesions in the same low number as did CSF from control patients and physiological saline. Thus the induction of myelin lesions may be coupled to the presence of oligoclonal IgG, and experiments are underway to confirm this.

In the rabbit eye there are two large bundles of myelinated nerve fibers visible to the naked eye. These fibers are separated from the vitrous only by a basement membrane and a sheath of glial cell processes through which proteins injected into the vitrous can penetrate [16]. The rabbit eye has therefore proved to be a convenient model for testing the effect on myelin of sera and PBL in various experimental conditions [2, 28].

We have investigated the effect of PBL from 16 MS patients, two Guillain-Barré syndrome (GBS) patients, and 16 healthy controls in the rabbit eye model [13]. PBL (10×10^5) suspended in 0.2 ml saline were injected into the vitrous under direct ophthalmoscopic observation. PBL suspension from a patient with MS or GBS was injected into the right eye, while the left eye was used for injection of PBL suspension from a healthy control. One or two rabbits were used for testing PBL from each patient. Seven days after the PBL injections, the retina with the optic disc and the epiretinal myelinated bundles were dissected and examined by light and electron microscopy.

PBL from five of the 16 MS patients, both GBS patients, and two of the healthy controls (one of them a technician who had worked with basic protein) evoked infiltration of inflammatory cells (lymphocytes, plasma cells, and macrophages) which was strikingly localized to the myelinated strips and did not involve the surrounding retina [13]. The results were read blind on coded specimens, and the infiltrations were graded as moderate to strong in the five MS patients, extensive in both GBS patients, and slight to moderate in the two healthy controls. The inflammatory cells surrounded the vessels above and within the strips, and penetrated the limiting membrane into the strip. In rabbits where a stronger inflammatory reaction was seen, deep parts of the strips were also affected. Macrophages penetrated into the myelin sheaths, and, ultrastructurally, macrophage cell processes could be seen to separate otherwise intact myelin lamellae. Myelin degradation products in macrophages were scanty and only a few completely denuded axons were found.

These data indicate that no specific discrimination in myelinotoxicity can be demonstrated in the rabbit eye using PBL only from MS patients and healthy controls.

The quantitative differences observed must be proven using a larger sample. The extensive inflammatory response after injection of PBL from both GBS patients may reflect sensitization of PBL against myelin constituents.

In preliminary investigations in the rabbit eye model using only 10×10^4 CSF lymphocytes and the same number of PBL, a moderate infiltration of inflammatory cells in the myelinated nerve fiber bundles was obtained with CSF lymphocytes from one of five MS patients, while the corresponding PBL did not give any inflammation in the strips.

Further studies are underway to document the myelinotoxic effect of PBL, CSF lymphocytes, serum, and CSF from MS patients and controls in the rabbit eye model. When myelinotoxicity or demyelination is obtained, the model could be useful to test the effects of various types of immunomanipulation.

Studies on Immunoglobulin Class and Light Chain Type of Oligoclonal CSF Bands

The occurrence of oligoclonal immunoglobulins (Ig) in CSF due to Ig synthesis within the CNS is a well-known phenomenon in MS. Agar-gel or agarose-gel electrophoresis [17, 18, 20], and isoelectric focusing [17], yielded similar high frequencies (about 90%) of oligoclonal Ig appearing as extra bands in the gamma globulin region in MS. Due to its higher resolution, isoelectric focusing also revealed oligoclonal Ig in serum from about 40% of MS patients [17], probably due to Ig diffusion from CSF. The oligoclonal Ig in MS CSF has been identified as IgG [18] and oligoclonal IgG is also present in MS brain [19]. Additional evidence for the oligoclonal character of IgG in MS CSF has been obtained by demonstrating a predominance of kappa light chains [21, 22, 24] and γ_1 heavy chains [33].

The antibody character and function of the oligoclonal IgG in MS is still unknown, and further immunological characterization of this IgG is warranted. The introduction of immunofixation [25] has improved our possibilities to confirm the presence of oligoclonal Ig and enabled the characterization of oligoclonal bands with regard to Ig class, light chain type, and presence of free light chains [5, 21].

We have investigated CSF and serum from 39 consecutive patients with clinically definite MS [21] and from ten patients with aseptic meningitis [5] by agarose gel electrophoresis and subsequent immunofixation. Preformed agarose gels (Panagel slide, Millipore Biomedica, Acton, Massachusetts, USA) and a panagel electrophoresis apparatus (Millipore) were used as described previously [9, 21]. For immunofixation, cellulose acetate strips (Sepraphore III, Gelman Instrument Company, Ann Arbor, Michigan, USA) were dipped in specific antisera against IgG, IgA, IgM, and kappa and lambda chains (Dakopatts, Copenhagen) and applied on the gel [5]. After incubation for 1 h, the strips were removed, and the protein not bound to antiserum was absorbed and washed away. The gel was then dried, stained, and destained, and the band patterns obtained were compared with that found at electrophoresis only (Fig. 2).

All 39 MS patients had IgG bands, one patient also had one IgA band, and another patient had one IgM band (Table 1). The oligoclonal CSF IgG formed 1–10

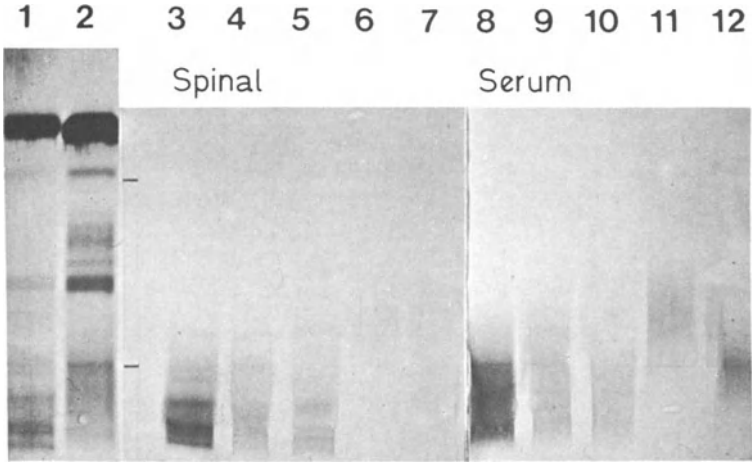

Fig. 2. Agarose-gel electrophoresis of CSF (1) and serum (2) from one MS patient, and patterns obtained after immunofixation of CSF (3–7) and serum (8–12) from the same patient with antisera against IgG (3,8), Kappa (4,9), Lambda (5,10), IgA (6,11) and IgM (7,12). CSF revealed six IgG bands, five of them with kappa and lambda chains, one with lambda chains only. No bands were found in serum. *Arrows* denote application slits

Table 1. Ig class and light chain type in MS and aseptic meningitis

Oligoclonal CSF bands	MS ($n = 39$)	Aseptic meningitis ($n = 10$)
IgG bands	39	10
IgA bands	1 (lambda)	0
IgM bands	1 (lambda)	2 (1 kappa, 1 lambda)
Kappa chain bands	0	0
Lambda chain bands	7	2

bands (mean 5 bands). No patient had bands consisting of free kappa chains, but seven of the MS patients had bands consisting of free lambda chains. Bands migrating on the cathodic side of the application slit, were found in all 39 CSF. Sixteen also had 1–2 bands migrating on the anodic side of the slit. Oligoclonal IgA and IgM migrated on the anodic side. The findings in aseptic meningitis are similar (Table 1). CSF bands consisting of free light chains have previously been described in MS and CNS infections [32] using crossed immunoelectrophoresis. However, this method is complicated and less reproducable, at least in our experience.

Determination of the light chain type revealed IgG bands exclusively of the kappa type in ten of the MS patients and a predominance of IgG bands of the kappa type in an additional 20 patients (Table 2). Only five MS patients had a predominance of IgG bands of the lambda type, and none had IgG bands of the lambda type only. One or more IgG bands with kappa and lambda present in the same band were found in 27 of the MS patients. This is most probably due to microheterogeneity within single bands, which is corroborated by the observation of addi-

Table 2. Relation between light chain type of oligoclonal CSF IgG bands, CSF kappa/lambda ratio, and CSF IgG index in 39 MS patients

Oligoclonal IgG	CSF kappa/lambda ratio			CSF IgG index [b]	
	>1.7 ($n=14$)	0.7–1.7 ($n=20$)	<0.7 ($n=2$)	>0.70 ($n=35$)	≦0.70 ($n=4$)
Kappa only ($n=10$)	5	5	0	8	2
Lambda only (0)	–	–	–	–	–
Predominance of kappa ($n=20$)	9	9	1 [a]	19	1
Predominance of lambda ($n=5$)	0	3	1 [a]	4	1
Same number of bands with kappa and lambda ($n=4$)	0	3	0	4	0

[a] Band containing free lambda chains present
[b] CSF IgG index, equal to (CSF IgG/serum IgG):(CSF albumin/serum albumin) [31]

tional bands of oligoclonal IgG when MS CSF is investigated by using isoelectric focusing [17]. No correlation was observed between the total number of oligoclonal bands or their light chain type, and the electrophoretic mobility of the bands.

The kappa/lambda ratio was determined in 36 of the 39 MS CSF specimens. Abnormally high ratios were found only in patients with oligoclonal IgG of the kappa type or a predominance of IgG bands of the kappa type (Table 2). Only two patients displayed abnormally low CSF kappa/lambda ratios, and in both of them bands consisting of free lambda chains were found. This contrasts with the situation in aseptic meningitis [5] and also with our findings in GBS [23], where most oligoclonal IgG is of the lambda type. These observations await confirmation on larger patient materials. If they are correct, a selection of the oligoclonal immune response within the CNS can be proposed with regard to the etiologic agent or acuteness of disease.

Our experience with agarose gel electrophoresis and subsequent immunofixation allows the conclusion that this rather simple technique is useful for the confirmation of the presence of oligoclonal Ig, for demonstration of free light chain migration as bands, and for definition of class, subclass, and light chain type of oligoclonal Ig.

Concluding Remarks

Our knowledge of the cellular immune response within the CNS in MS is fragmentary, due to among other things, the low number of cells available in contrast to the highly cell-consuming techniques. It is not known whether lymphocytes normally present in CSF differ functionally from PBL. Functional studies of CSF lymphocytes in MS become more meaningful if compared with similar studies carried out in well-defined CNS disorders. Table 3 summarizes some of our data regarding the immune response within the CNS in MS and acute aseptic meningitis. The main

Table 3. Characteristics of the immune response within the CNS as reflected in CSF compared with blood, in MS and acute aseptic meningitis

Type of immune response	MS	Aseptic meningitis
Humoral		
Ig synthesis within the CNS	Present	Present
Oligoclonal Ig response	Present (Mainly kappa)	Present (Mainly lambda)
Cell-mediated		
B lymphocytes	Decreased	Decreased
T lymphocytes	Increased	Increased
Active T cells	Decreased	Increased
Response to mitogens	No–low response	Low–good response
Response to specific antigen	Unknown	Increased

differences appear to be an oligoclonal Ig reaction mainly of the kappa type in MS compared with lambda type in aseptic meningitis, and decreased numbers of active T cells in MS compared with aseptic meningitis. Further studies along these lines are warranted to elucidate abnormalities of the immune response in MS.

Summary

Using a modification of the active rosette test of Wybran and Fudenberg [34], significantly lower percentages of active T cells were found in CSF compared with blood in MS and optic neuritis, while the reverse was observed in acute aseptic meningitis. Normal values of active T cell rosettes were found in blood from the three groups. The active T cell count of CSF may reflect involvement of cellular immunity in CNS disorders.

Myelinotoxicity after injection of lymphocytes was studied in the rabbit eye. Blood lymphocytes from 5/16 MS patients, 2/2 Guillain-Barré syndrome patients, and 3/16 healthy controls evoked infiltration of inflammatory cells, which was strikingly localized to myelinated nerve strips and which was graded as moderate to strong in MS, extensive in Guillain-Barré syndrome, and slight to moderate in healthy controls. No specific discrimination could be demonstrated in the rabbit eye using blood lymphocytes from MS patients and healthy controls.

Agarose gel electrophoresis and subsequent immunofixation carried out on CSF from 39 MS patients revealed oligoclonal IgG in all of them, and oligoclonal IgA and IgM in one each. The predominance of synthesis within the CNS of IgG type kappa was confirmed by the finding of only IgG kappa bands in ten patients and of a majority of IgG kappa bands in a further 20 patients. Only five of the MS patients had a predominance of IgG lambda bands. This contrasts to the observations in aseptic meningitis and Guillain-Barré syndrome, where most oligoclonal CSF IgG is of the lambda type. A selection of the oligoclonal immune response with respect

to etiology may therefore the proposed. Bands consisting of free lambda chains only were found in CSF in seven of the 39 MS patients. Twenty-seven MS patients also had IgG bands with kappa and lambda present in the same band, probably due to microheterogeneity. Immunofixation can be recommended as a method for confirmation of the presence of oligoclonal Ig, demonstration of free light chains migrating as bands, and definition of class, subclass, and light chain type of oligoclonal Ig.

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Antibody-Dependent Cytotoxicity of Lymphocytes Against Basic Protein of Myelin in Multiple Sclerosis

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Antibody-mediated lymphocyte cytotoxicity can be demonstrated in a number of diseases: tuberculosis, virus infections, tumors, graft rejections, and autoimmune diseases, e. g. Hashimoto thyroiditis. Antibody-dependent cytotoxic reactions have been described in experimental allergic encephalomyelitis (EAE) by Koprowski and Fernandes [6] and by Gipps et al. [5]. In multiple sclerosis (MS), various humoral and cellular immunoreactions against myelin basic protein (MBP) are found, and for this reason investigations are needed to find out whether an antibody-dependent cytotoxicity of lymphocytes against MBP also occurs.

The method used in this study, in accordance with the instructions given by Perlmann and Holm [7], was the release of ^{51}Cr from chicken erythrocytes coated with MBP, which served as target cells. Normal human and murine lymphocytes were the effector cells. The culture batch (total volume, 1.5 ml) contained 2.5×10^6 effector cells and 1×10^5 target cells, corresponding to a ratio of 25 : 1, and serum at a final dilution of 1 : 25 and 1 : 50. Triplicate cultures were incubated in a water-saturated 5% CO_2 atmosphere for 22 h at 37° C. Serum dilutions, the ratio of effector to target cells, the dose of antigen (10 g/ml), and the time of incubation were as had been determined as optimal in preliminary experiments [3].

The antibodies were determined to be IgG using column chromatography (Fig. 1); no addition of complement is necessary for IgG action. Serum and cerebrospinal fluid (CSF) were inactivated before use by heating to 56° C for 30 min. The antibody is highly specific: all of the tests with the basic proteins protamine sulphate and histone as control antigens were negative. Absorption experiments showed only a slight cross-reaction between MBP and the control antigens. The antibody-dependent cytotoxicity of lymphocytes against MBP is suppressed by normal IgG, and for this reason the antibody can only be found in dilute solutions; IgM and albumin do not have this effect. Actinomycin D and puromycin had a very slight inhibitory effect. EDTA practically eliminated the cytotoxicity. According to the results of the inhibition experiments, our results fulfill the criteria for antibody-dependent cytotoxicity of lymphocytes.

We investigated 170 MS patients, 175 patients with various neurological disorders, and 30 healthy persons. The results in the 170 MS patients are presented in Table 1 according to the type and stage of the process. Of these cases, 136 were positive. The frequency of positive findings correlates with the stage of the disease, amounting to 94% when the disease is active, i. e., deteriorates, and 54% when the disease is inactive, i. e., in a stable state. These findings have no relation to the type of MS, whether it be relapsing or chronic progressive.

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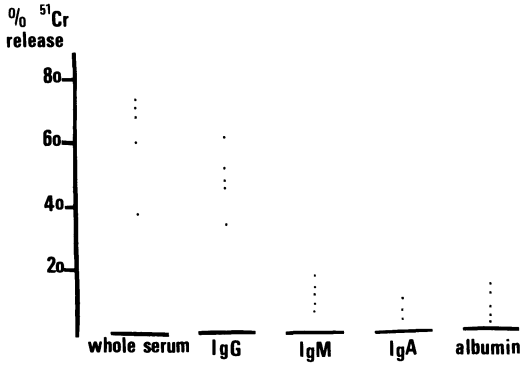

Fig. 1. Antibody activity in whole serum and serum fractions

Table 1. Antibody-dependent lymphocyte cytotoxicity against MBP in MS (positive reaction = ^{51}Cr release > 20%)

Course of MS	Total	Positive	Negative	% Positive
Relapsing				
Active	47	44	3	94
Inactive	37	22	15	59
Chronic-progressive				
Active	60	56	4	93
Inactive	26	14	12	45
All cases of MS	170	136	34	81

The antibody-dependent cytotoxicity of lymphocytes against MBP is very specific for MS. Control studies in 30 healthy persons were negative and in 165 patients with various neurological complaints, cerebrovascular disorders, brain tumors, cerebral atrophy, and inflammatory nervous disease of varied etiology were also negative. Ten cases, one of myasthenia gravis, one of facial palsy of unknown etiology, and eight recent subarachnoid haemorrhages were positive. The specificity of the immunological reaction for MS may be about 95%.

We examined 24 cases of neuritis of the optic nerve: 16 were positive (about 60%), and probably should be considered as incipient MS.

Because of its high specificity and the frequency of positive findings, a diagnostic significance can be attributed to the antibody-dependent cytotoxicity of lymphocytes against MBP. Its high specificity differentiates it from other cellular immunoreactions against MBP, lymphocyte transformation, or inhibition tests for macrophages and leukocytes. Using these methods positive results have been found not only in MS but also in a high percentage of other organic diseases of the nervous system [2, 4]. Demyelinating antibodies also show a comparable unspecificity which Bornstein [1] in particular has reported on.

Because of the close correlation between frequency and stage of progression of MS, the reaction is suitable for monitoring the course of the disease. Also the effect of therapeutic measures, e.g., an immunosuppressive treatment, can be supervised. Forty-eight MS patients were observed 5–30 months after onset of the disease (average of 18 months) and the antibody-dependent lymphocyte cytotoxicity against

MBP was investigated several times at various intervals. Thirty patients had a relapsing form, four active and eleven inactive courses remained clinically unchanged, and the cytotoxic reactions against MBP showed no change. Eleven active types became inactive, i.e., the MS came to a standstill. All patients had a positive cytotoxic reaction against MBP, which was markedly reduced in ten cases and became negative in one. Four inactive cases became active, i.e., the MS deteriorated. Three cases which had been negative became positive and one remained unchanged positive. Furthermore, we observed that in the acute attack in ten patients who had already shown a positive reaction previously, the intensity of cytotoxicity was reduced in six cases to 40–60%, and to 20% of initial cytotoxicity in three cases. One case remained unchanged. In 18 patients we found the chronic progressive type. In 13 cases the clinical course remained unchanged and the positive cytotoxic reactions also remained equally intense. Four active cases became inactive, i.e., the MS did not deteriorate further: two cases showed a marked reduction of the cytotoxic reaction against MBP. Two cases became negative. One case became clinically worse to a very marked degree and the cytotoxic reaction became quite considerably intensified.

To clear up the significance of the antibody-dependent cytotoxicity of lymphocytes against MBP, a series of transference experiments were carried out: spleen lymphocytes from Lewis rats which were incubated with antibody-containing serum and in vitro showed a marked cytotoxicity, and produced no neurological symptoms in isogenetic recipient animals; normal findings were also obtained histologically in the brain and spinal cord. Peripheral lymphocytes from rabbits were isolated, and, after incubation with antibody-containing serum, injected intraocularly in the region of the papilla. Neuritis of the optic nerve was established ophthalmologically and histologically three times, and also in the control investigations using lymphocytes which had been treated with normal serum. From these experiments it may be deduced that the antibody-dependent lymphocyte cytotoxicity against MBP is not a primary factor for the genesis of autoimmune diseases of the nervous system.

The pathogenetic significance of the antibody-dependent cytotoxicity of lymphocytes against MBP for MS is still unexplained. In case it is a secondary phenomenon, with regard to the specifity of the immunological reaction, an effect on the course of the disease cannot be excluded. When we examined serum and CSF of 22 MS patients, the cytotoxicity in serum and CSF was practically the same. The CSF was undiluted or diluted 1 : 2. These findings indicate that the antibody which produces the cytotoxicity of the lymphocytes against MBP in the CSF originates intrathecally and is not transferred from the serum to the CSF. No correlation between the intensity of the cytotoxicity in the CSF and the IgG content could be established. The CSF findings indicate that antibody-dependent lymphocyte cytotoxicity against MBP is important for the pathogenesis of the demyelination process in MS.

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New Aspects of Lymphocyte Reactivity and Lymphokine Activity in Multiple Sclerosis

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Three of the central problems of cell-mediated immunity in multiple sclerosis (MS) are of special interest:

1. The reactivity of lymphocytes to different organotypical antigens (this may be influenced by endogenic and exogenic factors).
2. The characterization of lymphokine activity and the kinetics of production of lymphokines.
3. The relation of lymphokine activities in vitro and in vivo.

The elucidation of these problems requires very sensitive methods. According to our experience this criterion is met by the electrophoretic mobility (EM) test of Field and Caspary [2, 6, 17].

In *principle* lymphokine determination using the EM test depends upon the interaction of specifically sensitized lymphocytes with the adequate antigen in vitro, resulting in the production of a slowing factor (e.g., macrophage slowing factor), which alters the electrophoretic mobility of indicator particles (macrophages or tanned sheep red blood cells).

Applying the EM test it has been shown that an exact analysis of charge-changing products of antigen-reactive lymphocytes (CPAL) is possible. In the past, several reports have been given by our group concerning the biological fundamentals and the kinetics of activities of CPAL as well as the modifications of the test system [12, 17, 19].

Lymphocyte Reactivity to Organotypical Antigens in MS

For estimation of lymphocyte sensitization, we studied reactivity to a structural protein, myelin basic protein (MBP – encephalitogenic factor) and a membrane-associated protein of normal brain, termed normal tissue antigen (NTA).

MBP was prepared following the procedure recommended by Dunkley and Carnegie [4]. The membrane-associated NTA could be isolated by hypertonic 3 M KCl extraction according to Meltzer et al. [14] as modified by Werner, analogous to the preparation of tumor-associated antigens (TAA). Purified protein derivative of tuberculin (PPD) was applied as a control antigen.

In all cases of MS, sensitization of peripheral lymphocytes to MBP was found by means of CPAL analysis (Table 1). However, similar positive results were detected

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Table 1. Sensitization of lymphocytes to MBP in various neurological diseases, detected as CPAL in the EM test (% slowing of indicator cells)

Groups	n	$\bar{M}$ %	σ
1. Multiple sclerosis	32	17.3	± 3.6
2. Brain tumors			
primary	41	15.1	± 5.8
secondary	9	17.8	± 5.1
3. Meningoencephalitis			
virus-dependent (partial chronic)	11	14.7	± 8.8
bacterial (partial chronic)	4	13.4	± 10.9
4. Degenerative diseases	12	9.2	± 9.8
5. Vascular diseases	12	7.6	± 6.5
6. Normal subjects	20	1.6	± 1.2

Table 2. Sensitization of lymphocytes to normal tissue antigen of brain (3 M KCl extract) in various neurological diseases, detected as CPAL in the EM-test (% slowing of indicator cells)

Groups	n	$\bar{M}$ %	σ
1. Multiple sclerosis	27	16.1	±4.0
2. Brain tumors			
primary	18	1.6	±2.0
secondary	5	1.3	±1.7
3. Meningoencephalitis	5	9.0	±7.9
(chronic)	3	14.5	±3.9
4. Other neurological diseases (vascular, degenerative)	5	3.3	±3.0
5. Normal subjects	5	1.2	±0.7

in brain tumors. The findings in meningoencephalitis were quite different: it could be proved that the reactivity was more dependent on the course and the stage of disease than on the etiology (i.e., chronic courses produced higher values). Lower reactivity was stated in degenerative and vascular neurological diseases. Patients with a long time and severe tissue damage (e.g., brain atrophies, apoplexies) exhibited a somewhat higher sensitization to MBP, in contrast to those with slightly destructive processes.

Using membrane-associated NTA, different results were obtained (Table 2). Lymphocytes of all MS cases showed a sensitization to NTA, independent of stage and course of disease. In contrast, this could not be stated in patients with brain tumors. On the other hand, employing various TAA – extracted by 3 M KCl – in neoplasms of CNS a characteristic reaction profile was observed (Jenssen et al., Meyer-Rienecker et al., to be published). Negative results using NTA were established in other neurological diseases, with the exception of chronic meningoencephalitis.

Lymphokine Activity in Vivo

Most of the methods for detecting lymphokines utilize in vitro systems. Recently, we have shown that some CPAL activity is also directly detectable in several body fluids, e.g. cerebrospinal fluid (CSF) [11, 18]. Studies directly assaying CPAL in CSF are summarized in Table 3. In MS, a high level of lymphokine activity in CSF was found. Similar activity was noted in patients with lues cerebrospinalis and in some meningoencephalitis, particularly in virus-dependent cases with chronic develop-

Table 3. Results of direct analysis of CPAL in cerebrospinal fluid of various neurological diseases using the EM-test (% slowing of indicator cells)

Groups	n	$\bar{M}$ %	σ
1. Multiple sclerosis	20	17.3	±3.9
2. Meningoencephalitis	11	12.4	±5.4
3. Lues cerebrospinalis	3	16.7	±5.3
4. Polyneuritis, polyneuropathies	6	2.6	±2.8
5. Vascular diseases	10	4.1	±3.1
6. Brain tumors	5	9.5	±5.5
7. Degenerative diseases	5	5.4	±3.2
8. Psychiatric diseases	5	6.8	±4.5
9. Nucleus pulposus prolaps and peripheral paresis	12	2.9	±2.5

ment. In brain tumors, CPAL values were mostly subnormal. In other neurological diseases, especially in the peripheral paresis, essential activities in CSF could not be detected.

Summarizing the values of CPAL activity in supernatants of lymphocytes stimulated by antigens and those obtained directly in CSF of MS in comparison (Tables 1–3), no significant differences between MBP and NTA-stimulation are evident. Conclusions about some differences of CPAL in CSF with respect to stages and courses of the disease cannot be drawn because of the relatively small amount of data presented here.

Differentiation of Lymphokine Activities

For further characterization of CPAL activities, several analyses were performed. Supernatants of lymphocytes cultured with antigen were separated by gel filtration (Sephadex G-75). Thereafter, lymphokine activities in fractions were assayed using different methods of cell mediated immunity (Fig. 1): the electrophoretic mobility test, the macrophage migration inhibition test, the spreading inhibition test, and the chemotactic assay. The results of these investigations show that lymphokines are detectable in different molecular weight regions.

The appearance of CPAL activities in the fractions was time dependent. In supernatants of cultures of lymphocytes stimulated by antigen, different patterns of

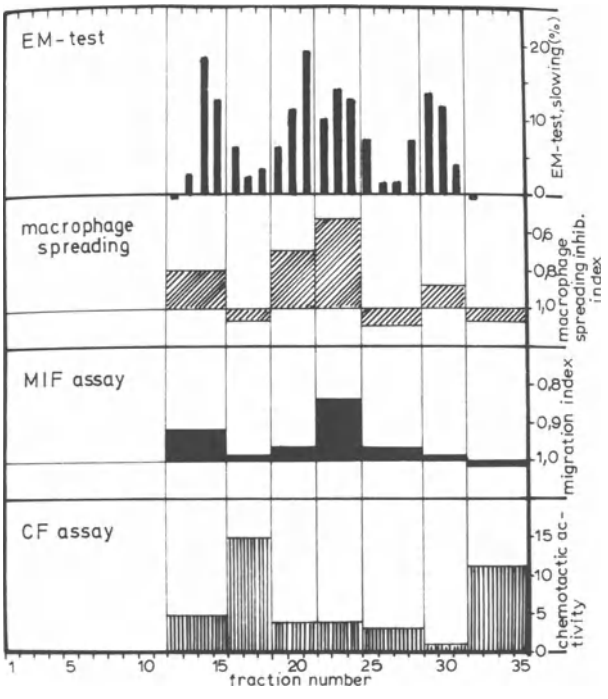

Fig. 1. Distribution pattern of lymphokine activities in supernatants of lymphocytes (cultured with MBP = human encephalitogenic protein (HEP)) assayed using several methods of cell-mediated immunity: the macrophage electrophoretic mobility test (EM test), the spreading inhibition test (macrophages), the macrophage migration inhibition test (MIF), and the chemotactic assay (CF); separation of lymphokine activities performed by gel filtration (Sephadex G-75); for marking of fractions and molecular weight of samples (i.e., region I–III) see text and Fig. 2

CPAL activity were evident for up to 10 h of incubation [12]. Activities could be demonstrated in three molecular weight regions: about 13,000, 40–60,000, and > 100,000 daltons (see regions III, II, and I in Fig. 2). Our results establish that CPAL activity in the usual incubation procedure (90 min) is connected with a molecular weight of about 13,000 daltons.

Correlation of Lymphokine Activities In Vitro and In Vivo

Comparative investigations of the CPAL activities in vitro and in vivo in MS are most interesting. Therefore the activities of fractions separated gelchromatographically were examined in EM test using samples of (1) supernatants of lymphocytes stimulated by antigens, (2) the CSF, and (3) the serum (Fig. 2). Surprisingly, an identical distribution profile of CPAL activity was found in supernatants, CSF, and serum. The occurrence of relatively high CPAL activities in CSF, supernatants of

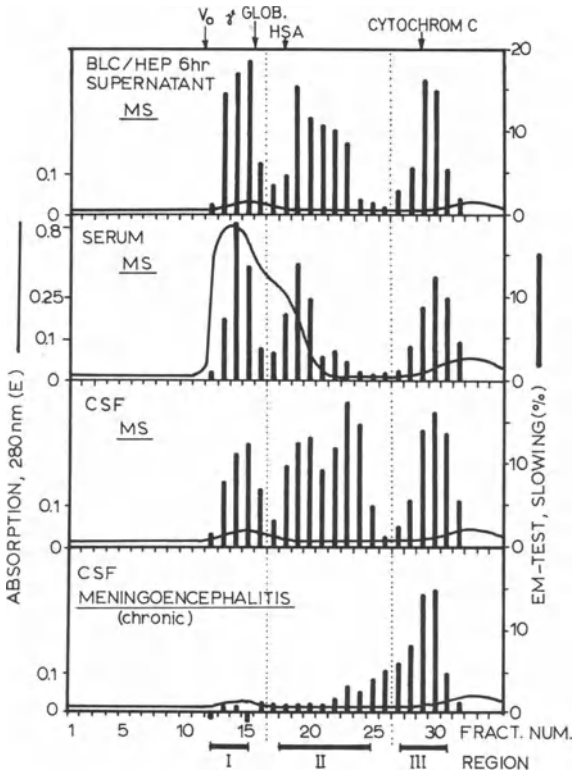

Fig. 2. Distribution pattern of CPAL activities (EM test) fractionated by gel filtration (Sephadex G-75) in supernatants of blood lymphocytes (BLC, cultured with MBP = HEP), in serum and cerebrospinal fluid (CSF) of a patient with MS, compared with CSF of a chronic meningoencephalitis patient (mol. wt. of region I = > 100,000, II = 40,000–60,000, III = > 13,000 daltons)

lymphocyte cultures, and serum of MS patients is remarkable. Similar findings were established in other chronical inflammatory processes of CNS. In this respect the following additional studies are of interest.

Lymphokine Pattern in CSF of MS

CSF of the diseases mentioned, fractionated under the same technical conditions, gave similar distribution patterns of charge changing effects in the active samples. Fundamental differences in quality did not exist, but quantitative alterations were found (Fig. 2). CSF of MS patients showed marked and distinct CPAL activities in all molecular weight regions. However, in chronic meningoencephalitis, the fractions with the lower molecular weight (region III) seem to be dominant. They may correspond to lymphokines in short-time cultures – and therefore probably to the early phase of stimulation. The demonstrated results were reproducible. Neverthe-

less, these studies must be extended to a series of other neurological diseases (to allow more definitive conclusions).

Conclusions

Beside pathogenetic aspects, one of the aims of our former and present investigations is to develop a diagnostic scheme grounded on immune reactions relatively specific for MS [16, 18]. In connection with the conventional methods, a preliminary diagnostic regime (Table 4) can be offered. Most of the procedures concerning the examination of CSF in particular are not efficiently specific [13, 15, 20]. Several techniques assaying cell-mediated immunity, particularly migration inhibition tests, present difficulties [3]. Some procedures, for instance linoleic acid depression (LAD) test, probably indicate alterated membrane properties of reacting lymphocytes in MS [7, 10, 18]. Other distinct methods (e.g., antibody-dependent cytotoxicity, genetic typing of lymphocytes, mixed lymphocyte reaction) may soon become relevant [1, 8, 9], especially those with a simple technical practice [5].

Our results lead to some new points of view; however, critical comments are necessary. In this regard the investigations using the EM technique to assay lymphocyte reactivity and lymphokine activities should be summarized:

1. Sensitization to MBP can be established in all organic processes of the CNS, depending on the duration of antigenic influence and the intensity of parenchymal destruction. (a) The most evident degree of sensitization is noticeable in cases of MS, more than in brain tumors. (b) A moderate degree of sensitization can be observed in meningoencephalitis. The other neurological diseases (especially those of

Table 4. Diagnostic program for MS based on examination of CSF and blood cells

Step Program	*Cerebrospinal fluid* Cells	Proteins
I. minimum	1. Cell count	1. Total protein
	2. Cytogram	2. Electrophoretic fractionation
	3. Different cytological reactions	3. Subfractionation in different media
II. limited characteristic	4. Lymphokines, direct	4. Antibodies to endogenic or exogenic antigens
	5. Fractionation pattern of lymphokine activities	5. Immunglobulin changes, e.g., oligocl. IgG, kappa/lambda ratio
Special program	*Blood cells* Lymphocytes	(Erythrocytes)
III. relative specific	− Sensitization to MBP	
	+ Linoleic acid depression	(Eufa-depression)
	+ Sensitization to NTA	
	± Genetic typing, e.g., HLA, Dw; MLR	
	+ Cytotoxicity, antibody-dependent	

inflammatory, vascular, or degenerative genesis) show (c) a lower degree of reactivity. The latter is of limited pathogenic and diagnostic value.

2. A sensitization to membrane-associated NTA of normal brain proved to be relatively specific in patients with MS, but this could also be stated in some chronic meningoencephalitis. In addition, the application of several TAA allows the supplementary differentiation of brain tumors.

3. A relatively high level of lymphokine activity was demonstrable using in vivo determination in CSF of MS. The same distinct CPAL activity in chronic meningoencephalitis also seems to be generated by mononuclear infiltrating cells. In consequence of the "in vivo incubation" the liberated lymphokines penetrate into the subarachnoidal space.

4. A time-dependent pattern of activity is noted for CPAL. Comparing lymphokines in vitro and in vivo, a relatively similar distribution pattern could be detected in supernatants, CSF, and serum. A typical complete distribution pattern for the whole spectrum of lymphokine activities is determinable in CSF of MS patients (in contrast to an incomplete pattern in the higher molecular regions of lymphokine in chronic meningoencephalitis).

5. Estimation of CPAL in supernatants of lymphocyte cultures (stimulated by different antigens, especially NTA) and in CSF can be combined with other cellular and humoral methods to set up a diagnostic program (see Table 4) for clinical use in MS.

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Viral Antibody Responses in the Central Nervous System of Patients with Multiple Sclerosis

B. VANDVIK [1] and E. NORRBY [2]

In recent years it has been established that a local synthesis of virus antibodies in the central nervous system (CNS) takes place in many multiple sclerosis (MS) patients. The following is an attempt to focus attention on some aspects of the locally synthesized virus antibodies in MS and some infections of the nervous system, their relation to locally synthesized IgG, and their possible biological significance. A more detailed review of virus antibodies in MS has recently been published elsewhere [16].

Local Synthesis of IgG in the CNS

Kabat et al. [6] were the first to demonstrate a selective increase of the γ-globulin fraction of the CSF in patients with MS and neurosyphilis. The association between this increase and a local synthesis of IgG within the CNS in MS was established by Frick and Scheid-Seydel [3]. With the advent of high-resolution electrophoretic methods, such as agar- or agarose-gel electrophoresis, it has been established that local IgG synthesis is reflected in the occurrence of oligoclonal IgG in the CSF in a variety of CNS infections and in 80–90% of MS patients [8, 10, 12].

Local Synthesis of Viral Antibodies in CNS Infections

Subacute sclerosing panencephalitis (SSPE), a disease of children and adolescents caused by a measles virus infection of the brain, is regularly associated with a local synthesis of oligoclonal IgG [8, 12]. Several lines of evidence indicate that a local synthesis of measles antibodies takes place in this disease [21, 23]. Results from serological studies of IgG separated by electrofocusing [7], preparative agarose electrophoresis [17, 26], and immunoelectrophoresis [24] indicate that locally synthesized measles antibodies in SSPE are associated with the fractions of oligoclonal IgG in the CSF. Final proof of this association has been provided by specific absorption of the oligoclonal IgG of brain extract and CSF samples by measles virus antigens, and by the subsequent recovery of corresponding oligoclonal measles antibodies by acid elution of the antigen-antibody complexes formed by the absorption [27]. These

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data indicate that the occurrence of oligoclonal IgG in the CSF of patients with SSPE reflects a specific antibody response to the presence of measles virus antigens in the CNS.

Evidence for an association of oligoclonal IgG with virus-specific antibody activities has also been reported in chronic progressive rubellavirus panencephalitis [29] and mumps meningitis [30]. Recent work in patients with neurosyphilis has shown that the bulk of the oligoclonal IgG of the CSF represents antibody to *Treponema pallidum* (Vandvik, unpublished data). Thus, in these infectious diseases the bulk of the oligoclonal IgG synthesized locally in the CNS may also be explained as a specific antibody response to the causal agent.

Local Synthesis of Viral Antibodies in MS

Raised levels of measles antibodies in serum and CSF of patients with MS were first reported by Adams and Imigawa [1]. The unraveling of the role of measles virus in SSPE gave a boost to theories concerning measles virus as an etiological agent in MS. Evidence for a local synthesis in the CNS of measles antibodies in this disease was first provided by Salmi et al. [21]. Their work was based on the demonstration of serum/CSF quotients of measles antibodies which were significantly lower than corresponding quotients for other antibodies. Work along the same lines has since confirmed these findings [2, 5, 18, 19, 22, 25]. A local measles antibody synthesis in the CNS takes place in approximately 60% of MS patients [18, 19] and in up to 30% of patients with optic neuritis [11, 13].

In additional studies, analyses were made of antibodies to several different viruses in serum and CSF samples from 150 Scandinavian MS patients [19]. A local synthesis of measles antibodies was found in 57%, of rubella antibodies in 19%, of mumps antibodies in 15%, of antibodies to herpes simplex type 1 virus in 11%, and of antibodies to parainfluenza virus type 1 (Sendai) in 3%. A local production of antibodies to any one of the viruses was found in 71% of the patients. This included 48, 16, and 7% of patients who had a local synthesis in the CNS of antibodies to one, two, and three or more viruses, respectively.

Low serum/CSF quotients for antibodies to rubellavirus in MS have been reported in other studies [9, 22] and indications of a local synthesis of herpes simplex antibodies in one study [5].

The occurrence of low measles antibody quotients is correlated with the occurrence of oligoclonal IgG in the CSF of patients with MS [18, 19, 25] and optic neuritis [10]. Indications of an electrophoretic restriction of the locally synthesized measles antibodies have been reported based on serological studies of IgG separated by preparative electrophoresis [17] and by immunoelectrophoresis [25]. More recently, absorption-elution experiments have shown that the locally synthesized measles antibodies in MS are indeed of an oligoclonal nature [27]. However, these experiments showed that the locally synthesized measles antibodies were not associated with the major fractions of oligoclonal IgG in the CSF demonstrable by agarose electrophoresis.

Previously available methods have not allowed the investigation of an association of the oligoclonal IgG of the CSF in individual MS patients with antibody ac-

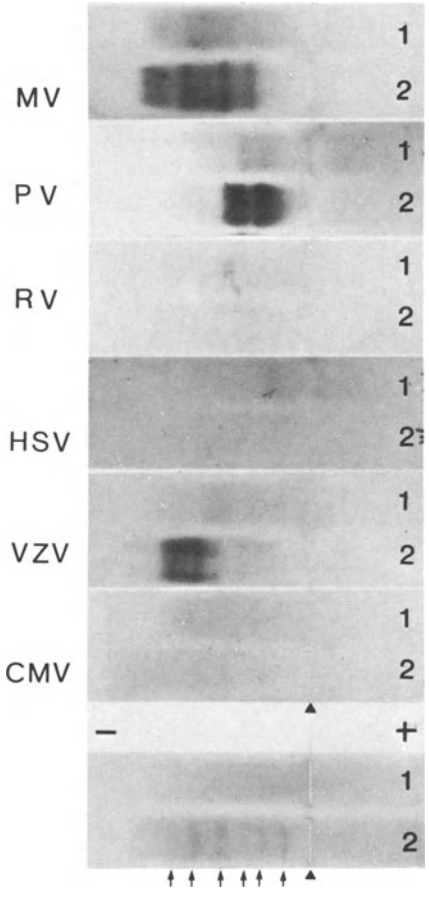

Fig. 1. Viral antibody patterns of serum (*1*) and CSF (*2*) from a patient with MS, obtained using IEIF. The serum and CSF samples were examined at an IgG concentration of 5 g/l. The antibody imprints were developed with I^{125}-labeled antiserum to human Ig. Comparisons between the serum and CSF antibody patterns in this patient indicates a local CNS synthesis of oligoclonal antibodies to measles, mumps, and varicella-zoster viruses. The antibody patterns should be compared with the Coomassie stained reference electrophoresis shown at the bottom. Explanation of symbols: *MV:* measles virus. *PV:* mumps (parotitis) virus. *RV:* rubella virus. *HSV:* herpes simplex type 1 virus. *VZV:* varicella-zoster virus. *CMV:* cytomegalo virus. *Arrows* indicate positions of oligoclonal IgG in the CSF electrophoresis. *Dark triangles* indicate sample application slit

tivities to different viruses. Recently, however, an "imprint electroimmunofixation" (IEIF) method has been developed for this purpose [14]. In this technique, serum and concentrated CSF samples are adjusted to the same concentration of IgG and separated by agarose electrophoresis. A gel plate containing virus or control antigens is incubated in direct contact with the electrophoresis gel containing the separated antibodies. The antigen plate is then washed to remove unbound antibodies. Imprints of bound antibodies are demonstrated by incubation with I^{125}-labeled rabbit anti-human Ig antibody and autoradiography. The occurrence of electrophoretically restricted fractions of antibody in the CSF without detectable or clearly weaker counterparts in the patient's serum is taken as evidence of a local synthesis of this antibody in the CNS [14].

Studies using the IEIF method have provided evidence for a local synthesis of electrophoretically restricted antibodies to one or more of four types of virus (measles, mumps, rubella, herpes simplex) in nine out of ten MS patients [15], and in five out of ten patients with acute monosymptomatic optic neuritis [30]. No local virus antibody synthesis could be demonstrated in ten patients with noninfectious and nondemyelinating neurological disease [14]. These observations have been confirmed

in an additional series of 12 MS patients and 12 age- and sex matched controls [31]. In the latter study, the virus antigen panel was extended to include varicella-zoster and cytomegalovirus antigens. Interestingly, a local synthesis of antibodies to varicella-zoster occurred more frequently (10/12) than to measles (6/12), and a local synthesis of antibodies to cytomegalovirus could not be demonstrated in any of the MS patients.

An example of the virus antibody patterns obtained by IEIF of serum and CSF in MS patients is shown in Figure 1. The electrophoretically restricted antibodies can be shown to differ in their $\varkappa$ and λ light chain determinants (Fig. 2), indicating that they are truly oligoclonal antibodies. The IEIF studies show, however, that the locally synthesized oligoclonal antibodies are not associated with the fractions of oligoclonal IgG of the CSF which are demonstrable by protein staining of the agarose electrophoresis. The lack of correlation between locally synthesized oligoclonal anti-

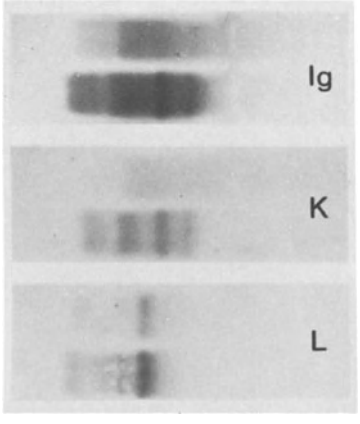

Fig. 2. Measles antibody patterns of serum and CSF from the same patient shown in Figure 1, developed with labeled antiserum to human Ig and with labeled antisera to human $\varkappa$ (*K*) and λ (*L*) light chain determinants

bodies and oligoclonal IgG is further illustrated by the finding of locally synthesized oligoclonal antibodies in electrophoretically normal CSF samples in patients with MS [15] and optic neuritis [30].

The IEIF method does not, in its present form, allow any quantitative estimation of the amount of locally synthesized antibody-specific IgG in CSF. However, the results indicate that the locally synthesized viral antibodies account for only a minor fraction of the locally synthesized IgG in the CSF of patients with MS and optic neuritis [15, 30].

Concluding Remarks

Today there is much evidence to show that a local synthesis of oligoclonal viral antibodies takes place in the CNS of the majority of MS patients and in some patients with optic neuritis. In many patients, antibodies to two, three, or four separate viruses are synthesized locally. Judging from the observations made with the IEIF meth-

od, antibodies to varicella-zoster and measles viruses are more commonly associated with a local CNS synthesis than antibodies to mumps, rubella and herpes simplex viruses. However, no relationship has been established between the locally synthesized antibodies identified so far, and the major fractions of oligoclonal IgG demonstrable by agarose electrophoresis of the CSF.

The significance of the local viral antibody response in MS is not clear. It seems to differ basically from that seen in SSPE and other CNS infections so far studied, in which the bulk of the oligoclonal IgG appears to represent antibody directed against the causal agent. In MS, therefore, the local synthesis of antibodies to measles and other viruses does not allow the conclusion that it reflects the presence in the CNS of the respective viruses or their antigens.

One explanation of the puzzling data in MS is that the disease in some way is associated with a nonspecific, i.e., "nonimmunogenic", activation of cell clones producing antibodies against a variety of agents to which the host has previously been sensitized. Whether this activation is a general one, or confined to clones deriving from local CNS antibody responses mounted by the host during previous CNS infections, is not clear. In this respect, it is of interest that an oligoclonal antibody response occurs very early after onset of mumps meningitis and persists for long periods [4, 28]. One may speculate that antibody-producing cell clones developed during this and other CNS infections ("memory" B cells?) might be reactivated at a later stage if the individual develops MS.

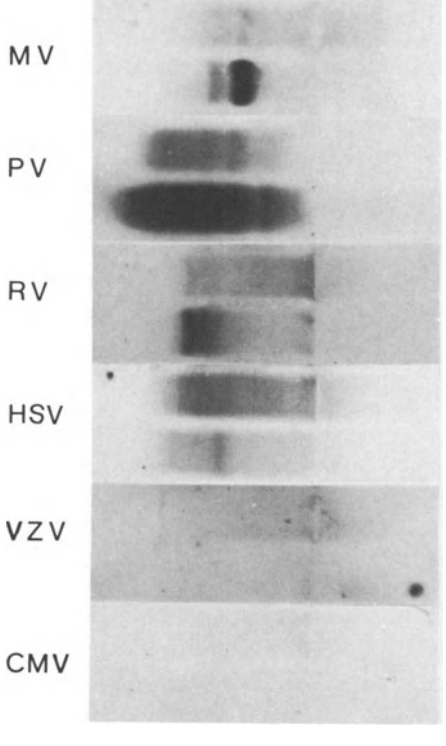

Fig. 3. Viral antibody patterns of serum and CSF from a 9-year-old girl with mumps meningoencephalitis. The samples were examined at an IgG concentration of 2.5 g/l. Experimental conditions were otherwise as in Figure 1. For explanation of symbols, see Figure 1. Note the prominent local antibody response to mumps virus and the less pronounced local synthesis of antibodies to measles and rubella viruses

A nonspecific activation might explain all locally synthesized oligoclonal IgG in the CSF of patients with MS. There remains, however, another possibility. It is known that specific antibody production against one antigen may be accompanied by production of antibodies against completely unrelated antigens [20]. Thus, the specific immunization caused by an infection could be associated with an activation of a variety of antibody-producing cells. In MS, it is still conceivable that the bulk of the oligoclonal IgG represents antibody directed against an as yet unknown infectious agent or agents. The small amounts of locally synthesized oligoclonal virus antibodies identified so far might then represent products of nonspecificially activated clones.

Since infectious CNS diseases represent the only major group of neurological disorders associated with a local synthesis of oligoclonal IgG besides MS, further studies of CNS infections should proceed along with those concerning MS. In particular, the question of whether a nonspecific coactivation of cells producing antibodies to other agents than the causal one takes place should be explored. Preliminary studies of a series of patients with SSPE, mumps meningitis, neurosyphilis, and meningoencephalitis of unknown etiology indicate that a local synthesis of small amounts of oligoclonal antibodies, similar to that observed in MS, takes place in a few patients (B. Vandvik unpublished data). In the patients with unknown disease etiology, this synthesis occurred along with the dominating antibody response to the causal agent. An example is shown in Figure 3.

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Measles Reactivity in Patients with Multiple Sclerosis

H. VALDIMARSSON [1] and G. AGNARSDOTTIR [2]

Summary

Patients with active multiple sclerosis (MS) showed significantly less skin reactivity to measles virus than did MS patients with stable disease or healthy adults. This defect was selective to measles as judged by reactivity to common environmental antigens such as streptokinase-streptodornase and *Candida albicans*.

Introduction

Epidemiological and immunological findings suggest that MS may have an infectious etiology, and measles virus has been implicated [2, 3]. Intact cellular immune mechanisms are known to be of critical importance for controlling infections by this virus in man [1]. Therefore, if MS is in any way connected with activation of measles virus, patients might be expected to show impaired cell-mediated immunity to this virus. Moreover, by analogy with chronic infections such as leprosy, MS patients with active or rapidly progressing disease might be expected to have a more pronounced cellular immune defect to measles than MS patients with stable or slowly progressing disease.

Unfortunately, there are at present no satisfactory in vitro tests available to assess specific T-lymphocyte reactivity to measles virus. The virus has been shown to suppress responses in lymphocyte transformation assays [4]. Moreover, the majority of human peripheral blood lymphocytes, mostly T cells, have been shown to carry receptor for measles virus [5]. This has hampered assays for testing leukocyte migration inhibition and lymphocyte-mediated cytotoxicity. Specific measles reactivity in these assays is thus probably to a large extent masked by the nonspecific association of the virus with human lymphocytes.

We therefore decided to test the delayed hypersensitivity responses to measles virus in a group of MS patients and compare the reactivity of patients with active and stable disease.

Materials and Methods

Fifty-one MS patients and 13 patients with other neurological diseases were skin tested. The MS patients were divided into two groups according to the activity of their

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disease. Patients who were experiencing an exacerbation of their disease at the time of skin testing or had suffered at least one episode of deterioration during the previous 6 months were considered to have active MS. Those who had experienced no change in their condition during the last 2 years were considered as patients with stable MS.

The antigens used were inactivated measles vaccine (Mevilin, Evans Medicals Ltd) and a control material for measles, similarly prepared. Control antigens were *Candida albicans* (1% Bencard) and streptokinase-streptodornase (SK/SD) (Lederle). A 0.1 ml volume of the antigens was injected intradermally into four different sites of the volar surfaces of both forearms and the responses recorded 24 h later. Injection and reading of skin tests was done blindly with regard to diagnosis and disease activity in the MS patients. An induration of 2 mm diameter or more was considered positive for measles. Approximately 5% of the subjects, who also responded to the measles control material, were considered measles negative.

Three-hundred and fifty-three adult, healthy volunteers, aged 18–50 years, had previously been skin tested with the measles antigen. All had experienced measles infection in the past. This group gives an idea of the level of response in the general population to the measles antigen used.

Results

Delayed hypersensitivity responses to measles antigen are shown in Table 1.

Sixty-two percent of patient controls and 46% of the healthy adults were positive. Considerably fewer MS patients (33%) showed positive measles reactivity.

Table 1. Skin testing with measles antigen

	No. tested	Negative	Positive	
			(2 – 4 mm)	(≧ 5 mm)
MS (active)	25	80%	16%	4%
MS (stable)	26	54%	15%	31%
Patient controls	13	38%	8%	54%
Healthy adults	353	54%	23%	23%

Table 2. Skin testing with SK/SD antigen

	No. tested	Negative	Positive	
			(5 – 14 mm)	(≧ 15 mm)
MS (active)	25	4%	68%	28%
MS (stable)	26	27%	54%	19%
Patient controls	13	8%	62%	31%

Moreover, only four of the 25 patients with active disease were positive compared with 12 of the 26 patients with stable MS. This difference is significant ($P < 0.05$).

Patients with active MS showed normal responses to *Candida* and SK/SD, when compared with stable MS patients and patient controls (Table 2).

Discussion

We have made the observation that patients with active MS show a significant measles skin test anergy compared with patients with stable MS and other neurological diseases. This anergy was restricted to measles in as much as patients with active MS responded normally to *Candida* and SK/SD antigens. Thus our observation indicates that during episodes of clinical exacerbations in MS there is a selective defect in cell-mediated immunity to measles virus. We have also recently found impaired release of infectious measles virus from MS lymphocytes during an in vitro infection, and various other findings have linked measles virus to MS in the past.

These findings may reflect an impaired handling by MS patients not only of measles virus, but of enveloped RNA viruses in general. Therefore, investigations on the cellular immune responses to other enveloped viruses in MS patients might help to elucidate further the pathogenetic mechanisms in MS.

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Measles Virus Infection of Human Lymphocytes

G. AGNARSDOTTIR [1] and H. VALDIMARSSON [2]

Summary

During an in vitro measles infection, virus-induced killing of lymphocytes was found to be similar in normal controls, patients with subacute sclerosing panencephalitis (SSPE), and patients with multiple sclerosis (MS). However, release of infectious virus from lymphocytes of MS patients was significantly impaired as compared with normal controls. In contrast, SSPE lymphocytes showed a virus release similar to that of normals.

Introduction

A fatal, persistent infection of the brain, SSPE, is caused by measles or a measles-like virus [1]. MS, another chronic neurological disease, possibly also of infectious etiology, has been associated with measles [4]. In SSPE, pathological changes are seen in the brain only, but immune complexes containing measles antigen have been detected in organs other than the brain [2]. Moreover, virus isolation has been reported from a lymph node of an SSPE patient [3]. Human lymphoid cells have been shown to be susceptible to measles virus infection, both in vivo and in vitro [6, 8], and may be functionally impaired during and after clinical measles [7]. Furthermore, it has been shown that human T lymphocytes have a non-specific receptor for measles virus [9], while in vitro experiments have indicated that both T and B lymphocytes can be infected with the virus [5].

If lymphocytes carried measles virus in a disease like SSPE, this might compromise an effective immune response against infected brain cells and might also provide a continuous source of virus. From experience with persistently infected, non-lymphoid cells, such virus carrier lymphocyte populations might be expected to be refractory to a superinfection with the same virus [10]. We therefore decided to compare the ability of normal lymphocytes, on the one hand, with those of SSPE and MS patients, on the other, to support replication of measles virus in vitro.

Materials and Methods

Hypaque-Ficoll separated lymphocytes from defibrinated, venous blood were washed three times in Hepes-buffered TC199 medium, containing 2% fetal calf serum (FCS). They were then infected with measles virus (Edmonston) at a low multiplici-

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ty of infection (0.001) by allowing the virus to adsorb in a volume of 0.5–1 ml at 37° C during continuous rotation for 2 h. The lymphocytes were then resuspended in TC199 medium containing 10% FCS to a concentration of 5×10^5/ml and Phytohaemagglutinin (PHA) (Burroughs Wellcome) added to a final concentration of 1 μg/ml. After 2 h at 37° C the PHA was removed by three washes, the lymphocytes resuspended in medium without PHA to the same concentration as above, and placed stationary in a CO_2 incubator at 37° C in 10-ml aliquots of TC199 medium, containing 5% bicarbonate, 10% FCS, and antibiotics. One ml aliquots were harvested daily for 6 or 7 days. Viability of cells was assessed by trypan blue exclusion and cytocentrifuge preparations made. Cells were stained with May-Grünewald Giemsa to detect the appearance of giant cells. Acetone-fixed cells were used for detecting intracellular measles antigens by the indirect fluorescent antibody test (FAT). Measles antigens on lymphocyte membranes were tested for on unfixed cells, in an indirect FAT. SSPE serum and fluorescein-conjugated sheep anti-human IgG (Burroughs Wellcome) were used for intracellular and membrane measles antigen detection. Culture supernatants were harvested daily and stored at -70° C until titrated for infectious virus. All supernatants from one individual culture were tested simultaneously. A plaque assay [5] used for detecting infectious measles virus was slightly modified.

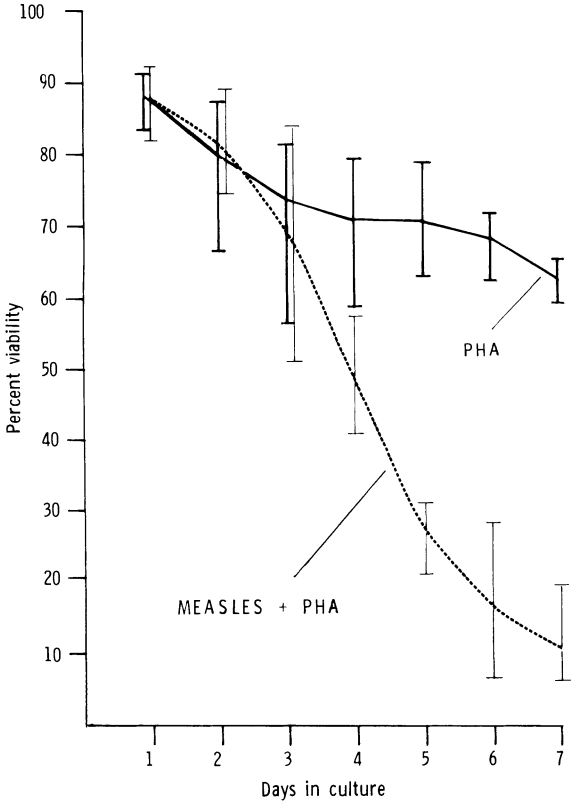

Fig. 1. Viability of PHA-stimulated lymphocytes in culture with or without measles. (Range of seven normal controls)

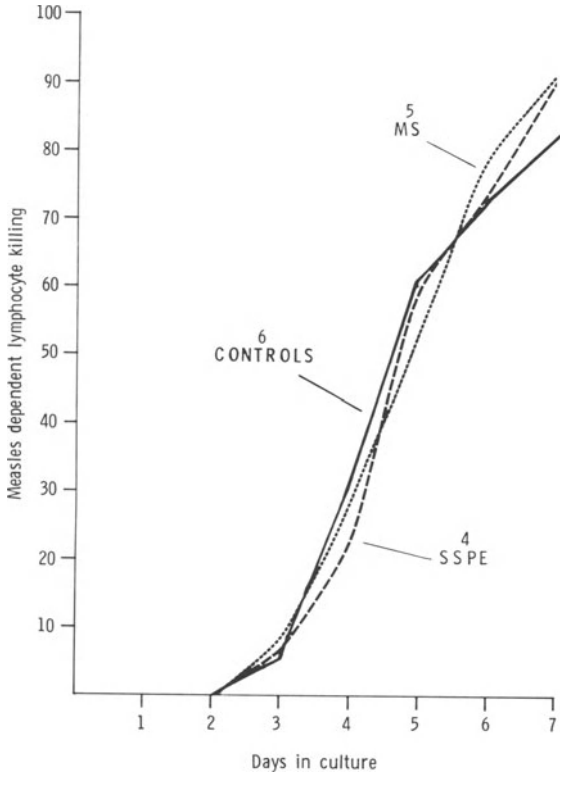

Fig. 2. Measles-induced killing of PHA-stimulated lymphocytes was calculated, using the following formula:

$$100 - \frac{\text{Percent viability of infected lymphocytes}}{\text{Percent viability of uninfected lymphocytes}} \times 100.$$

Results

Viability of PHA-Stimulated Lymphocytes with or Without Measles

After 7 days in culture, about 60% of PHA-stimulated lymphocytes were viable as judged by trypan blue exclusion. However, where measles virus had been added, the viability fell to about 10% (Fig. 1).

The virus-induced killing was found to be similar in six normal controls, four patients with SSPE, and in five patients with MS (Fig. 2).

Evidence for the Presence of Measles Virus in Infected Lymphocytes

Numerous giant cells were seen in the infected lymphocyte cultures. These were usually maximal on the third or the fourth day after infection. Measles antigen was

Table 1. Measles virus titer in PFU/ml × 10^5

		Day 4 [a]	Day 5
6 MS patients	B	0.05	0.01
	C	0.75	0.15
	H	3.50	6.50
	J	15.00	23.00
	S	0.10	1.00
	W	0.00	0.01
4 SSPE patients	F	35.0	7.0
	G	95.0	6.0
	P	2.0	5.0
	S	21.5	10.5
7 normal controls	Ch	24.5	5.0
	C	10.0	5.5
	D	16.5	4.5
	F	20.0	1.0
	M	46.0	8.0
	Sa	34.0	10.0
	Si	15.0	10.0

[a] Virus release from infected lymphocytes was maximal on the fourth and fifth days after infection. A comparison was made between individual results from MS and SSPE patients and those of normal controls

seen to increase gradually from the second day onwards in the cytoplasm of infected cells. Viral membrane antigens were maximal on the fourth to the fifth day after infection, when at least 60–70% of lymphocytes showed positive measles-specific fluorescence.

Release of Infectious Measles Virus from Infected Lymphocytes

Virus release was detectable from the second day onward and was maximal 4 days after infection in seven normal controls and four SSPE patients, when it reached a mean titer of 2.5×10^6 and 4×10^6 PFU/ml, respectively. Virus release from all MS patients tested, except one, was much lower at all times during the 7 days. Using a two-sample Wilcoxon test for all six MS patients and the seven normal controls, a significant difference was demonstrated in virus release on the fourth day between the two groups ($P < 0.01$) (Table 1).

Discussion

The findings from this preliminary study are somewhat unexpected. They show that lymphocytes from SSPE patients react to measles infection in a way similar to that of normal controls, whereas lymphocytes from MS patients handle measles virus abnormally. SSPE patients, who are known to be infected, at least in their brain, by

measles or a measles-like virus, might have been expected to show abnormal handling of this virus. On the other hand, the association of measles virus with MS has been very tenuous and indirect and there is no obvious explanation for the impaired measles virus release observed.

It is not known whether the same number of virus particles are being adsorbed and taken up by MS lymphocytes as in normals, but the fact that MS lymphocytes are killed by the virus in the same way suggests that it is unlikely that they are refractory to infection. Similarly, if the impaired release was due to interference because the lymphocytes were already infected by measles or another virus, one might expect a degree of protection from superinfection with measles, but this is not seen.

A possible explanation may be that MS lymphocytes have a tendency to produce defective, noninfectious measles virus particles, which would not be detected in the infectivity assay used. It is also possible that this impaired handling of measles virus by MS lymphocytes is not specific for that virus and would apply to other enveloped RNA viruses.

Further work is needed to define the mechanisms of this impaired handling of measles virus. Moreover, the ability of MS lymphocytes to handle infections with other viruses, especially enveloped RNA viruses, should also be tested.

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Kinetics of Measles Virus Antibodies in Multiple Sclerosis Patients in Correlation to the Clinical Course of the Disease

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Introduction

Elevated levels of antibodies against measles virus in serum and cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients have been described by a number of investigators since the first reports [1, 13]. Only few data are available about the kinetics of these antibodies and their correlation to the clinical course of the disease. In a recent report, Reunanen et al. [14] failed to demonstrate a correlation between the antibody titers against measles virus and against rubellavirus in 20 patients with regard to relapse or remission of the disease. The purpose of our study was (1) to look for antibodies in MS patients at different stages of the clinical course of their disease, and (2) to follow up the antibody titers in individuals in a longitudinal study during the time of hospitalization.

The viral antigens employed in the study included measles virus and subunits thereof as well as parainfluenza virus type I, which is thought to be a possible agent in the etiology of MS [18]. Special attention was directed to the monitoring of the respective antibody titers during a bout.

Materials and Methods

The study population consisted of 145 patients of the University hospital of Göttingen and Essen, and 12 patients of the MS-Clinic Hachen. The diagnosis had been confirmed by at least two neurologists independently and corresponded to criteria as reported by McAlpine [8] for probable MS. Both groups of patients had been characterized by clinical parameters according to the course of their disease. Seventeen of 145 patients presented primary onset of the disease, 36 revealed a chronic progression, 20 were in remission for 2–6 months, 12 were in remission for more than 6 months, and 60 patients had an acute bout (Table 1); 32 of 145 patients received immunsuppressive therapy. Their antibody profile in serum and CSF did not differ from those patients without immunsuppressive therapy.

We were able to get serum and CSF samples from these 145 patients at the same time. Twelve patients from the Hachen clinic had an acute phase of their disease. Serum samples were collected at the time of admission, 21 days thereafter and at the time of discharge from the hospital. As antigen for the measles virus hemagglutina-

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tion inhibition test (HIT) we used Tween-Ether-split measles virus (Edmonston strain) according to Norrby [9]. Measles nucleoprotein (NP) antigen and hemolysin antigen (HL) were prepared as described by Norrby and Hammarskjöld [10]. Sendai virus partly purified by differential centrifugation served as source of the parainfluenza type I antigen. Hemagglutinin, NP, and HL were prepared according to Hosaka [6, 7]. All samples were analyzed against one antigen at a time in the same assay.

Virus-specific antibodies were determined against the subunits of measles and parainfluenza type I virus. MS-nonspecific antibodies against poliovirus type I were assayed using the neutralization enhancement test (NT) [2]. Detailed descriptions of serological assays such as the complement fixation test (CFT), HIT, the hemolysin inhibition (HLI) test, and NT may be found elsewhere [5].

The statistical evaluation of the results included geometric mean titers, variance analysis, a *t*-test, as well as titer distribution according to the Information statistics test [15].

Results

The highest mean serum antibody titer against hemagglutinin (HA) of measles virus as detected in the HIT was found immediately after clinical manifestation of MS (Table 1, Figure 1). Patients with the progressive form of the disease and patients suffering from an acute bout had significantly lower titer values.

The highest antibody titer against measles virus HL was detected in patients who had suffered a bout of at least 2 weeks duration. The highest mean titer against the NP of measles virus was found in patients who had suffered a bout of at least 8 weeks. Comparison of antibody mean values against mealses HL 2 weeks and 4 weeks after bouts showed a significant difference ($P < 0{,}05$). The comparison of

Table 1. Total number, positive reactants, and mean values (m) of antibodies against antigens of measles virus and SDS in MS-patients with MS at the beginning, in the bout, in the stationary phase, and with the chronic progressive form. Determination of antibody in serum with HIT, CFT (NP), and HLI-test mean values of titers is given (reciprocals and logarithmic SDS)

Phase of MS	Total number	Measles hemagglutinin			Measles nucleoprotein			Measles hemolysin		
		Pos	m	SD	Pos	m	SD	Pos	m	SD
Acute MS	17	17	226	0.691	15	56	0.438	17	184	0.439
Bout (1 week)	11	11	87	0.812	11	42	0.209	11	210	0.467
Bout (2 weeks)	11	11	136	0.778	9	26	0.158	11	372	0.411
Bout (4 weeks)	19	19	169	0.489	15	32	0.288	19	152	0.648
Bout (8 weeks)	19	19	68	0.776	13	88	0.384	19	136	0.205
Bout (2–6 months)	20	20	107	0.729	19	48	0.345	20	230	0.471
Bout (> 6 months)	12	12	80	0.632	9	52	0.305	10	180	0.495
Chronic progressive form	36	36	80	0.630	29	52	0.373	36	236	0.500

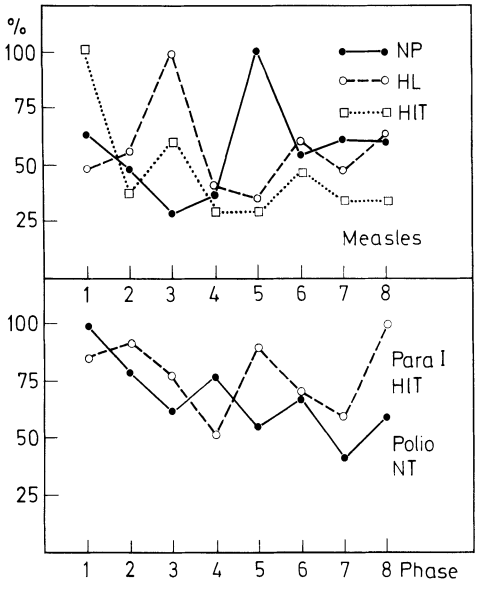

Fig. 1. Serum antibodies against NP measles virus antigen assayed using the CFT, HL assayed using the HLI test, and HA assayed using the HIT test as well as serum antibodies against parainfluenza type I virus assayed using the HIT and neutralizing antibodies against polio type I in MS-patients during different phases of their disease. The highest mean titer value was taken as 100% when patient groups were compared (see Table 1). Phases of disease: 1 = acute MS ($n = 17$); 2 = bout of 1 week ($n = 11$); 3 = bout of 2 weeks ($n = 11$); 4 = bout of 4 weeks ($n = 19$); 5 = bout of 8 weeks ($n = 20$); 6 = bout of 2–6 months ($n = 20$); 7 = bout more than 6 months ago ($n = 12$); and 8 = chronic progressive form ($n = 36$)

NP-antibody mean values 4 and 8 weeks after bouts also showed significant differences ($P < 0{,}05$).

The antibodies against the NP and HL of the measles virus at the onset of the disease (acute MS) in its nonactive phase, and in its primary chronic progressive form had identical titers. The HI antibody titer remained unchanged during subsequent bouts, even in the chronic progressive course. No significant differences between measles antibody concentration assayed in the HIT, the CFT, and the HLI test could be demonstrated in CSF of the patients (Table 2, Fig. 2). However, the highest percentage of measles antibody-positive individuals was found in the group of MS patients in the acute phase of the disease (acute MS, bout, chronic progres-

Table 2. Total number, positive reactants, and the mean value of measles and polio type I antibody in CSF in MS patients with MS at the beginning, in the bout, in the stationary phase, and with chronic progression of their disease (c.f. Table 1)

Phase of MS	Total number	Measles hemagglutinin			Measles nucleoprotein			Measles hemolysin		
		Pos	m	SD	Pos	m .	SD	Pos	m	SD
Acute MS	17	10	9.8	0.568	5	3.1	0.268	12	2.1	0.200
Bout (1 week)	11	5	4.6	0.328	6	1.6	0.245	4	3.7	0.450
Bout (2 weeks)	11	5	2.6	0.164	4	4.0	0.425	3	2.5	0.173
Bout (4 weeks)	19	5	3.0	0.268	4	2.0	0.245	2	5.7	0.637
Bout (8 weeks)	19	5	7.0	0.446	10	3.5	0.396	10	3.0	0.245
Bout (2 – 6 months)	20	7	5.4	0.549	11	3.0	0.300	5	2.3	0.251
Bout (> 6 months)	12	3	0.0	0.520	3	3.2	0.346	5	1.8	0.251
Chronic progressive form	36	12	5.3	0.414	19	2.6	0.286	22	2.1	0.352

sion). The highest percentage of hemagglutination inhibition HI titers (60%) was found in patients suffering from acute MS, while lower percentages of titers were found in patients in later stages of the disease or suffering from acute bouts. An increase or decrease of HLI antibodies was found to be followed by an increase and decrease of NP antibodies of the same kinetic and showed the same tendency in the CSF of MS patients suffering from acute bouts (Fig. 2). Neutralizing antibodies against poliovirus type I in serum and CSF remained unchanged during all phases of the disease. Based on the titer distributions and geometric mean values in the different study groups, HI and NP antibodies against parainfluenza I did not undergo significant quantitative changes. Moreover, the number of individuals with antibodies against NP of parainfluenza I virus was low in all groups. Antibody against poliovirus type I detected in the NT remained quantitatively unchanged during severe bouts and in other stages of MS. Determination of serum IgG and IgM concentrations revealed no quantitative difference in the groups under study.

We conclude that antibodies against structural and functional units of measles virus obviously follow different patterns at various times and depend on the acute phase (bout). Antibodies against measles virus (HL and NP) reach their peak activity approximately 1–3 weeks after the onset of a bout. The highest values of HI-antibodies were found at the onset of the disease. This is of particular interest, since antibodies against parainfluenza type I virus, poliovirus type I, and the content of IgM and IgG remained unchanged.

To substantiate our observations we took serum samples from hospitalized individuals to determine their individual antibody kinetic. This study included 12 patients from the Hachen clinic. The comparison of antibody titers against HA, HL, and NP of measles virus was of special interest. Figure 3 shows the antibody titer kinetic of each patient suffering from an acute phase. A decrease of antibodies against HL could be demonstrated in ten of 12 patients. One patient had an increase, and in one case the titer remained unchanged. Seven individuals showed a significant (four fold) increase of antibodies against measles NP.

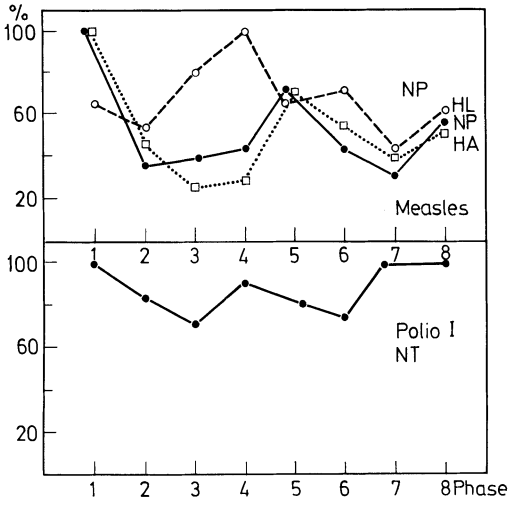

Fig. 2. Antibodies against antigens of measles and poliovirus type I in CSF of MS patients depending on the stage of the disease (cf. Fig. 1)

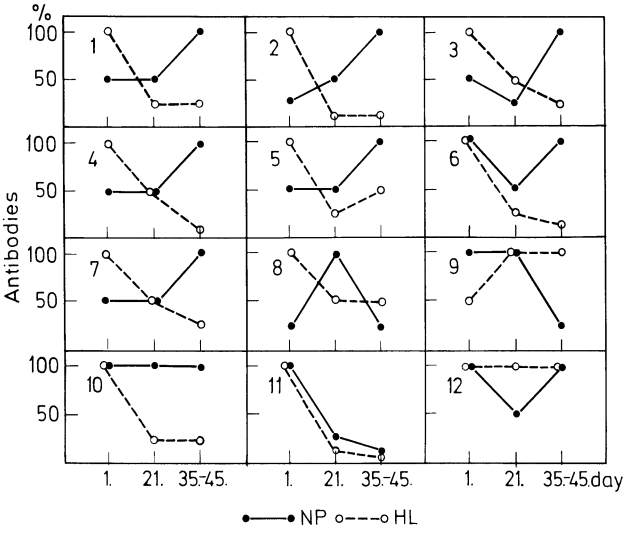

Fig. 3. Relative course of titer in 12 MS patients in the acute phase of their disease over 6–7 weeks. Antibodies against HL and NP of measles virus were assayed in three serum samples from each patient. The highest antibody titer was taken as 100%

A consecutive increase and decrease (one patient) and a decrease only (one patient) was observed. Antibody titer remained unchanged in three patients. With regard to the serological data of these patients, the time elapsed from the onset of the bout to admission to the hospital must be considered as an important factor. Antibodies to poliovirus type I, parainfluenza type I (HA, NP, and HL) as well as to measles virus HA and the content of IgG and IgM remained unchanged in all 12 patients during the observation period.

Discussion

The validity of virus-serological investigations in MS patients depends on the exact clinical classification of the disease. Each patient in our study had an exactly defined case and course of MS. Data concerning antibodies against subunits of measles virus in serum and CSF have been published by Norrby et al. [11, 12] and Salmi et al. [16, 17]. Our study is addressed to the following questions:

1. Do viral antibodies in MS patients reveal a selective affinity to measles virus subunits as compared with parainfluenza I or poliovirus type I?
2. Is there a regular relationship between antibodies against different subunits of measles virus in different courses of the disease and during acute phases? Recently, Reunanen et al. [14] have failed to demonstrate such a correlation.

Besides NP and HI antibodies, we have emphasized HLI antibodies because they most probably interfere with adsorption and mediate neutralization. HL antigen is thought to exercise a special function in virus-induced cytolysis. To our knowl-

edge this antigen has not been structurally identified as yet. Very likely it is identical with the glycoprotein isolated by Hall and Martin [3, 4]. This antigen has been functionally characterized by specific antibody-mediated inhibition of its biological activity against african green monkey red blood cells.

The successive appearence of IgG antibodies against structural components of measles virus, including NP, could be demonstrated in a number of MS sera. (Figs. 1, 3), and this observation that cannot be interpreted conclusively for the time being. However, it seems conceivable that with reactivation of a chronic latent infection, single gene expression of measles virus becomes phenotypically manifest and the antigenic gene products become immunologically active. Our observations should be confirmed by further studies on patients with acute measles virus infection. Our study clearly shows that differences between antibodies against parainfluenza type I antigens are not associated with the various forms of MS, including the acute phase.

Summary

Serum and CSF samples of 145 MS patients were investigated serologically with regard to their content of virus antibodies. The aims of this study were (1) to prove the selectivity of measles antibody formation when compared to parainfluenza type I and poliovirus type I antibodies, and (2) to elucidate the time relationship between measles virus subunit antibody response and the disease process.

The patients were assigned to groups according their stage of MS. Particular attention was given to the time course of the acute phase and to the onset of the disease.

Antibodies against subunits of measles and parainfluenza type I and against poliovirus type I were determined. No changes of parainfluenza- and poliovirus-specific antibody titers could be demonstrated. Antibodies to HL and NP of measles virus reached their peak titers within 1–3 weeks after onset of the acute phase. The highest titers of HI antibodies were found at the beginning of the disease (first bout). In another 12 patients in the acute phase, the antibody profile was followed longitudinally during their hospitalization. In ten of these the consecutive appearance of HL and NP antibodies was detected within 1–3 weeks.

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Longitudinal Studies of Viral Antibody Synthesis in MS Patients

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Introduction

It has been shown that immunoglobulins are produced intrathecally in multiple sclerosis (MS) patients [8, 11]. This is a good diagnostic sign for the diagnosis of MS. The antibody specificity of the bulk of immunoglobulins produced locally in the central nervous system (CNS) has been and remains an enigma. It is not known whether these immunoglobulins have any correlation to the etiology or pathogenesis of MS.

However, there is evidence that antibodies against a few viruses are produced intrathecally in at least some MS patients. Although studies have shown that antibodies to measles virus are commonly produced, later studies have demonstrated that antibody production against viruses is a more general phenomenon including many of the common enveloped viruses [7, 14, 21].

If the intrathecally produced antibodies are related to the pathogenesis of MS, one might expect changes in antibody production to be related to the fluctuations in the clinical disease. This longitudinal aspect of antibody production in MS patients has been studied in an only few instances and the techniques used have not been accurate enough to detect small quantities of antibodies or small fluctuations in antibody levels [15, 19].

We have started longitudinal studies on MS patients in order to find out whether any changes in antibody synthesis occur intrathecally or generally in patients' bodies, and whether possible changes can be correlated with the clinical course in these patients.

The present results are from a preliminary study, part of which has been published [1].

Specimens and Methods

Serum and Cerebrospinal Fluid Specimens

Twenty patients with a confirmed diagnosis of MS were studied longitudinally for fluctuations in intrathecal antibody synthesis against measles, rubella, and respiratory syncytial virus antigens. Serum and cerebrospinal fluid (CSF) specimen pairs were taken between 1971 and 1976 from patients who had visited the outpatient clin-

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ic or had been admitted to the Department of Neurology of the University of Oulu.

Additional longitudinal series of serum specimens from two MS patients were collected at the Department of Internal Medicine, Turku City Hospital. One of the patients had a chronic progressive course of 30 years' duration and the other had a fulminant disease course of 2 years' duration. The last two specimens from the second patient were taken 1 week before death and at autopsy, respectively. Twenty-four serum specimens taken at monthly intervals were available from these two patients.

Viral Antigens

Preparation of the virus antigens for radioimmunoassay has been described in detail elsewhere [2, 12, 24] except for measles virus nucleocapsid antigens, which were purified as follows: Measles-infected Vero cells in PBS were disrupted in a glass homogenizer. Trypsin was added to a final concentration of 0.25%. After 1 h at room temperature the debris was centrifuged at 2000 *g* for 20 min, and the supernatant pelleted using a step gradient consisting of 25%, 30%, and 40% CsCl. The banded nucleocapsid in the 30% layer was dialyzed against PBS and treated with 0.05% trypsin for 30 min at room temperature. The material was banded again as described above. Finally, the nucleocapsid was centrifuged to equilibrium in a third CsCl gradient. After dialysis against PBS it was stored at – 20° C until used.

Radioimmunoassay (RIA) for Viral Antibodies

Radioimmunoassay methods have been described in detail elsewhere [2, 12]. The principle of the methodology is as follows: Viral antigen is adsorbed on polystyrene beads 6.4 mm in diameter. The beads are then incubated in dilutions of the serum and cerebrospinal fluid (CSF) specimens. The specific antibodies adsorbed to the antigen on the solid phase are detected and quantitated by iodinated antibodies to the gamma chain of human IgG produced in pigs.

Only one dilution of each specimen was tested in triplicate, and a dilution series of a standard reference serum pool was included in every test. The antibody quantity was expressed as relative titer measured from the distance between the sample's mean cpm value and the linear part of the dilution curve of the standard serum. The CSF/serum ratio was calculated from the relative titer values. The serial serum and CSF specimens from each patient were tested simultaneously. Details of this procedure have been described previously [1, 10].

Preparative Agarose Gel Electrophoresis and Analysis of Antibody Activity in the Fractions

The details of this method will be published separately (in preparation). The principle of the method is as follows: Serum specimens were electrophoresed in agarose

gels. The IgG region of the gel was cut into 1-mm slices which were eluted overnight into 0.4-ml phosphate-buffered saline (PBS) at + 4 °C. The eluted IgG was tested in duplicate for virus antibody activity using a radioimmunoassay. The cpm values were converted into titers by comparing them to standard serum dilutions tested simultaneously. The results were expressed as the percentage of antibody activity in every fraction calculated from the total amount found in all the fractions.

Results

Fluctuations in the Intrathecal Synthesis of Antiviral Antibodies in MS Patients

Our earlier studies suggest that measles and rubella antibodies are synthesized in the central nervous system (CNS) of many MS patients [14, 20, 22], and therefore these viruses were selected for these studies. Because respiratory syncytial virus is not included in the group of viruses commonly causing infections of the CNS, it was selected as a control virus for these studies.

The CSF/serum ratios of albumin were normal [17] in these patients, indicating an intact blood-brain barrier (BBB). Calculations of the IgG/albumin indexes [4] showed local IgG synthesis in the CNS of all the patients. When the CSF/serum antibody ratios of 1/80 or more were taken to indicate local synthesis of antibodies in the CNS, 15/20 of the patients produced antibodies locally against one or more of the viruses; six patients against rubella *or* measles, five against rubella *and* measles, and four patients against all three viruses studied.

Table 1. Number of patients having significant fluctuations in CSF/serum antibody ratios against the viruses studied

Significant fluctuations in intrathecal antibody synthesis against	No. of patients
Measles virus	5/20
Rubellavirus	1/20
Measles, and rubellavirus	1/20
Measles, rubella and respiratory syncytial viruses	1/20

To study the fluctuations of intrathecal antibody synthesis, a comparison of each patient's serial CSF/serum antibody ratio was made. The CSF/serum ratios of different antibodies were also compared to each other. The accuracy of the antibody ratio determination in our RIA system was found to be high, the SD deviation being less than 7% of the ratio. The 95% confidence limits were calculated for the CSF/serum ratio calculations. Based on this, it was established that eight of these patients had fluctuations in the intrathecal antibody synthesis. Most of these eight patients had fluctuating levels of measles antibody synthesis, as shown in Table 1.

An example of the fluctuations is shown in Fig. 1. Specific synthesis in the CNS of rubella antibodies is suggested by the high CSF/serum rubella antibody ratios in all three of the specimens which were taken. The increase in the second CSF-serum pair is significant.

When changes in intrathecal antibody synthesis were compared to the changes in clinical condition of these patients, no correlation was found.

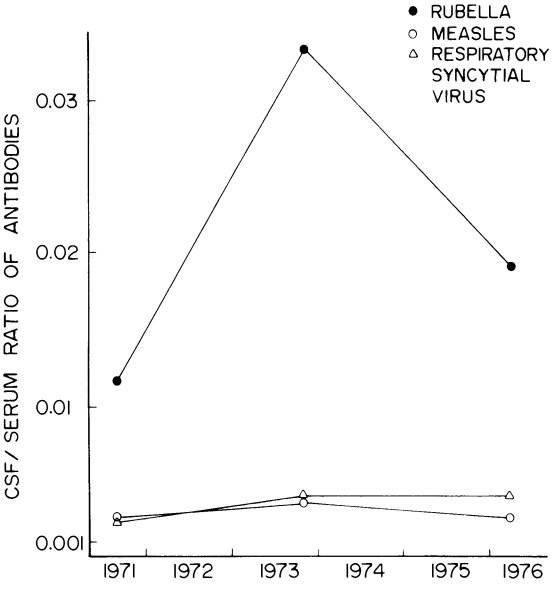

Fig. 1. CSF/serum viral antibody ratios versus time of three specimens from one MS patient showing elevation and significant fluctuation of CSF/serum ratios for rubella antibodies

Changes in Antibody Synthesis Against Measles Virus Nucleocapsid Antigens in MS Patients

Two serial serum specimens from two MS patients were fractionated in preparative agarose electrophoresis. The IgG in each fraction was tested for antibody activity against purified measles virus nucleocapsids by RIA. There were no changes in the antibody distribution found in serial specimens from the patient with a chronic progressive disease course of 30 years' duration. On the other hand, significant changes in antibody distribution were seen in the rapidly progressing patient. This was most clearly seen in specimens taken just before or after death as seen in Figure 2. No similar changes were observed when the same fractions were tested for antibody activity against mumps virus.

Discussion

The longitudinal aspect of antibody synthesis is not well known in MS patients. In some studies, fluctuations in the antibody synthesis against measles virus have been

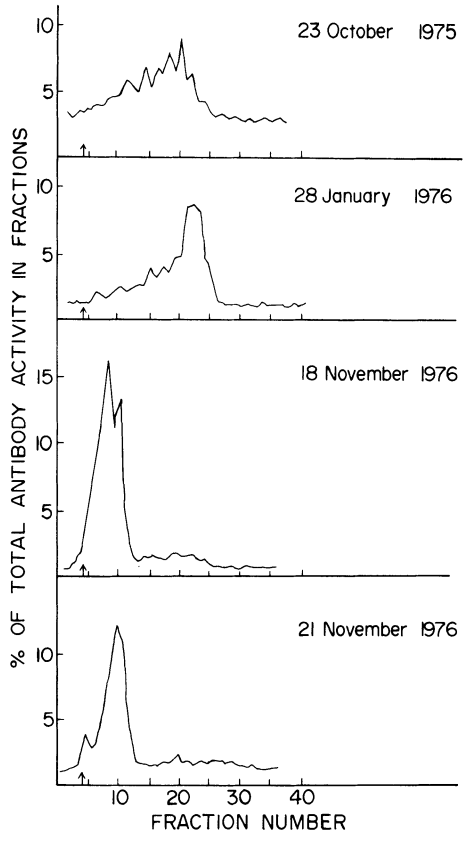

Fig. 2. Antibody activity against measles virus nucleocapsid antigen observed in IgG fractions of serial serum specimens from one MS patient (expressed as % of total antibody activity in fractions) showing activation of new clones in the latter two specimens and waning of former ones

observed in serum or CSF specimens [9, 15, 18]. But the techniques used have been rather insensitive and the accuracy of the methods has not allowed analysis of small changes in antibody levels.

Antibody synthesis and its fluctuations in the CNS is more difficult to study, because small amounts of antibodies are always found in CSF if sensitive enough techniques are used [13]. Serum antibody levels should therefore be taken into account. By comparing serum and CSF levels of antibodies, synthesis in the CNS could be studied more specifically [21]. This approach was used in this study and the CSF/serum antibody ratios were compared longitudinally in the same patient. Significant fluctuations in antibody synthesis against all three viruses were seen, but most often in antibody synthesis against measles virus.

It is not known whether fluctuations in antibody synthesis against specific antigens occur in normal individuals. Natural cyclic variations in the activity of the immune system have been observed [5]. The effect of stress on the immune system has also been recognized [6]. Therefore any major change in the balance of the homeostasis of the body might have an effect on the immune system and its functions. It is reasonable to assume that at least small variations in antibody synthesis may be seen in normal individuals. Before this normal variation is known in detail, no final conclusions can be drawn based on the small but significant changes in antibody production in the MS patients.

This distinctive feature of the intrathecal antibody synthesis in these patients who were followed longitudinally was that *specific* changes in antibody synthesis occurred in some of the patients. These changes were not limited to only one virus but occurred in some patients simultaneously in two or three of the viruses studied. These changes could be explained by at least three mechanism: (1) There is one or more of the viruses or virus-related antigens present simultaneously in the CNS of the patients; (2) There is antigen-specific regulation of the antibody synthesis in the CNS of these patients; or (3) The polyclonal activation of antibody production is not of similar strength on all of the different plasma cells. There is not enough experimental evidence for or against any of these possible explanations of the findings.

No correlation between the fluctuations in the intrathecal antibody synthesis and the clinical course of these patients was found. This agrees with earlier reports in which the synthesis of IgG is not clearly correlated with the clinical course of the patients [16]. If this observation holds true in more detailed analysis, the bulk of IgG and antibody synthesis should be considered as an epiphenomenon without any etiological or pathogenetic implications.

The waning of some clones and activation of other ones with the same specificity against purified measles virus nucleocapsid was observed in one MS patient. Such changes have evidently not been described in healthy human beings. However, in animal models, antibody-producing clones may senesce and new clones with the same specificity but with different physicochemical properties can be seen after new antigenic stimulation [3, 23]. If antigens are needed for the emergence of new antibody-producing clones, our observations suggest that measles virus nucleocapsid or a cross-reacting antigen was present in the body of our patient with a rapidly progressing course of MS. On the other hand, the major qualitative changes observed might only be reflections of the immunological changes at the terminal stage of the MS patient. The specific changes seen in the antibody production could have happened randomly at such a stage. Therefore, no generalizations could be drawn from this patient. Because this finding might have important implications in the etiology and pathogenesis of MS, similar studies on larger number of specimens from MS patients and control patients are warranted.

Summary

Series of serum and CSF specimens taken from 20 MS patients were tested using a RIA for antibodies against measles, rubella, and respiratory syncytial virus antigens. Calculations of the CSF/serum antibody ratios revealed significant changes in intrathecal synthesis of antibodies against one or more of the viruses during the disease course of eight of the patients. No correlation of these changes with the clinical stage of the patients was observed. Qualitative changes in the antibodies against measles virus nucleocapsids were observed in serial serum specimens from a rapidly progressing MS patient.

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Discussion

E. J. Field: Nature's experiments which we call diseases are often pure and unsophisticated, especially in children. And, can I have the first two slides please? Here you see what happens when an English child which has not been immunized against measles, catches the disease. This is clinical measles, and the spots of sensitization against measles antigen are indicated by the crosses, and this is again the encephalitogenic factor. And in fact on the days before the appearance of Koplik spots, you arrange to get blood from another little child in the family when one has got it. You can show already in the simple diagram that there is a rise not only in sensitization to measles, of lymphocytes to measles, but in the sensitization of lymphocytes to the encephalitogenic factor. This is simply a more sophisticated slide showing the control values along the bottom. The individual arrows point to the same child and again you will see that in the incubating stage there is already a beginning sensitization to measles followed by sensitization to the encephalitogenic factor, which dies away after about 7 weeks to well below 5%, which is normal. Whereas with measles antibody, the measles lymphocyte sensitization in this macrophage electrophoretic mobility (MEM) test falls to around 11% and remains as such. If now you want to be very sophisticated, you go to the laboratory and you inject animals, and then of course you get into trouble. If you inject measles, you get a magnificent response; if you inject influenza, you get a magnificent response to influenza, and you get quite a response with respect to the encephalitogenic factor. If you inject measles, you get a very good response of lymphocyte sensitization to measles. But at the same time you get a very sizeable response to the encephalitogenic factor and the same applies to a lesser degree perhaps to pertussis. And the lesson to be drawn from this is that measles shares antigenic determinants with the encephalitogenic factor. In other words, if you have a breakdown of brain from any cause, you will secondarily get sensitization of lymphocytes to the encephalitogenic factor. And this must be borne in mind in all interpretations of measles studies in MS and indeed in other diseases.

A. Wajgt: Now a short contribution to the first, second and third reports. In my group of one-hundred MS patients I found a highly statistically significant positive correlation of oligoclonal patterns recognized from multiple bands in an electrophoretic run and also of the kappa and lambda light polypeptide chain arrangement. And in the titer of measles and parainfluenza type 3 to the same extent as measles antibody, there was cerebrospinal fluid (CSF) response. In contrast to negative results with parainfluenza type 1 response in the CSF, this response also correlated strongly with measles and parainfluenza type 3 antibody-specific response in CSF. It also correlated strongly with IgG level, not only with respect to oligoclones, but independently of this, with IgG CSF level. But there was no positive correlation between antibody titer in CSF and in the corresponding sera, despite the fact that the measles and parainfluenza type 3 antibody titer was also increased in the sera of MS patients.

E. Norrby: Maybe I could just make a brief response to E. J. Field's remark. You say that you interpret this data to mean that there is a cross-reaction between basic protein and measles antigens? By the same token there could also be a cross-reaction between influenza antigens and basic protein and pertussis antigen and basic protein. I think we have to distinguish here between the possibility of a cross-reaction and the possibility that the measles infection as such activates an autoimmune response. And it is known that this occurs not only in terms of possible antibodies to basic protein but also in terms of antibody to actin. So there is a trenchant IgM SMA response in patients with regular measles.

E. J. Field: I could reply to that, but it would take a considerable number of slides. In principle, if you take measles antibody and you stick it on to Sepharose gel beads and then you pass down the MS lymphocytes, then nothing is taken out. And a whole lot of well-controlled experiments were published about 4 or 5 years ago – too long ago to put them before this audience at this point.

Y. Kuroiwa: I am very much impressed by Dr. Norrby and Dr. Vandvik's study. But I was just asking them: you fix the amount of IgG to a certain level, 5 mg/ml. But if you make a dilution, you may get a more quantitative estimation or comparison with MS and other disease. We did this using another measles antibody that we diluted, and we found no difference.

B. Vandvik: Yes, well it gives me an opportunity to say that of course this method is purely qualitative and we don't really know very well how to make it quantitative. Your suggestion, of course, we have tried. We tried to run samples in serial dilutions. Now the problem is that we certainly can give an endpoint at which staining is lost, but we cannot maintain a really reliable electrophoretic pattern through these dilutions in agarose electrophoresis. So that will not be a satisfactory method. Whether we could develop methods that could allow counting of the activity in the individual fractions is another question, and we are working on it.

E. J. Field: May I just quickly say that if you cut out a little bit of Sepharose gel eluted off your stuff and tested it against circulating lymphocytes you would be able to tell which is which.

Question: Has anybody done studies like Dr. Salmi and continued the antibody titer over years and in connection with the course of MS? I think that is very important. Is there anybody here who can say anything?

Dr. Thomssen: We only can contribute one case of such a longitudinal study. In this case we determined the antibody ratio for measles, for rubella, and for coxsackie before. And in this case we observed and increase of the ratio not in the acute stage of the disease but several weeks after the acute stage. Have you any answer to this observation? And I may ask you another question. Have you ever observed a change from IgG antibody to IgM antibody in your studies?

G. W. Ellison: We have done longitudinal studies on the serum of only MS patients, and against measles and a host of other viruses. This work has been done by Dr. James Cherry and also by Roger Deedle in collaboration with John Summer's lab. And we are not able to report any change in the serum titers to various antigens over an approximately 5-year period. And we do not have ratio data. And I think that these may be much more critical than just looking at the serum.

B. Vandvik: We have studied the serum and spinal fluid of four MS patients over a 4-year period. We have absorbed and eluted antibodies from the samples and

looked at the patterns of some IgG. And we have also done the same now with the imprint technique. We found absolutely no changes in the pattern of antibodies. But that does not necessarily conflict with Salmi's data, because he is studying a single specificity, while we have been studying a broad range of antibody specificities, but at least in our studies the antibody pattern is similar. Also there was a question about IgM. We have again been using the imprint technique, trying to demonstrate responses of the IgM class. But so far we have not succeeded in demonstrating that.

A. Salmi: Yes, a couple of comments. One question was about IgM antibodies, for example. We had been studying very carefully measles IgM antibodies in SSPE and MS. We occasionally find some real measles IgM in SSPE. But using five different types of technique applied to quite a big number of MS sera and CSF, we never found any measles-specific IgM. The other comment which I would like to make is about the antibody synthesis in the central nervous system against viruses. We had been studying, using the ratio method and sensitive and accurate radioimmunological methods, a limited number of MS patients and trying to find out against which viral antigens antibodies were being made. And I would just like to keep the list as it is now, indicating which antibodies are produced according to our experience. There are many viruses and bacterial antigens which are still lacking. But we are hoping to complete the series in a year or so. Right now we have measles, rubella, parainfluenza 2, parainfluenza 3, influenza A, influenza B, mumps, two coronaviruses, OC 43, 229E, and occasionally vaccinia respiratory syncytial virus.

We practically never have adenovirus, rarely herpes viruses, and bacterial antigens. What is important here is that it looks as if every patient has a characteristic pattern of two or more antibodies being produced intracerebrally, although the patients simultaneously produce antibodies outside of the brain against all the other viruses which we have been studying. So there is some kind of individual selectivity. I think it is a very essential to find out what the rules of that this are and what this really means. This certainly does not suggest any single agent as an etiological agent for MS.

Suppressor Lymphocyte Function During Attacks of Multiple Sclerosis

J. P. ANTEL and B. W. ARNASON [1]

Multiple sclerosis (MS) is characterized by a relapsing and remitting course, particularly in the early phases of the disease. Tests of immune function conducted both on peripheral blood and spinal fluid strongly suggest that aberrant immune function occurs in MS patients. These aberrancies appear to be most striking during periods of active disease, although they may at times remain detectable during periods of clinical quiescence. For example, in studies of sensitivity of peripheral blood or cerebrospinal fluid (CSF) lymphocytes to myelin antigens, the most striking changes have been reported in patients with active disease. Similarly, changes in the percent of B and/or T cells in peripheral blood have been most evident when disease is active. Oger et al. [14] in contrast, have found that depressed numbers of avid T cells, probably representing a T-cell subset, are observed both when disease is active and when it is quiescent. Noronha et al. [13] have presented evidence that CSF lymphocytes of MS patients remain activated as measured by RNA/DNA content, even during seemingly quiescent disease. The possibility that MS is seldom "burned out" must be seriously entertained.

The investigation of immune function in MS has expanded with our increasing knowledge of how the immune system is organized. As is well known, immune function is mediated through lymphocytes and cells of the macrophage-monocyte series. Each of these cell types can be subdivided into subsets based on functional properties, surface properties, and antigenic specificity. Lymphocytes have classically been divided into T and B subclasses (responsible for mediating cellular and humoral immunity respectively); although lymphocytes without the properties of either B or T cells exist (null cells).

Subsets of cells exist within each major lymphocyte subclass. T-lymphocyte subsets can be divided into effector cells and into regulator cells. Effector cells are responsible for such functions as production of lymphokines and cytotoxicity. Regulator cells can be divided into those which can amplify the immune response, the so-called helper cells, and those which inhibit the immune response, the so-called suppressor cells.

In animals, the suppressor and helper subsets appear to be distinct cell lines. For example, by using antisera against several cell surface antigens of the mouse (Thy-1, Ly) one can demonstrate differences in the surface antigens of helper cells which distinguish them from suppressor cells. In humans, helper and suppressor T cells have been segregated by their ability to bind either immunoglobulin M(IgM) or IgG containing immune complexes [12], by their differential sensitivity to radiation, and by density gradient centrifugation [16]. Separate subsets of suppressor cells are like-

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ly to blunt the responses of B and T effector cells. In addition, at least some suppressor cells can be shown to be antigen specific. The net magnitude of any given immune response is believed to depend on the relative influences of the different classes of regulator lymphocytes as well as of regulator macrophages on effector cells.

The mechanism whereby regulator T cells interact with effector cells has been studied extensively. The suppressor cell appears to act via release of a soluble substance which interacts with the effector cell. In an antigen-specific suppressor model, this substance has been shown to be produced by gene products of the *I–J* region of the major histocompatibility complex (MHC) of the mouse and for optimal effect requires histocompatibility between suppressor and effector cells [19]. The mechanism for activation of suppressor cells appears to involve a priming cell population, termed pro-suppressor, which, once activated, stimulates the suppressor cell. That DNA synthesis by suppressor cells is required before they can act on effector cells is suggested but not established. Multiple factors, including antigen itself, macrophages, and immune response products such as antibody or antigen-antibody complexes have all been suggested as the trigger for suppressor activation. Similar initiating factors for helper cells have also been proposed, although DNA synthesis does not appear to be a prerequisite.

Regulator cell dysfunction and, in particular, suppressor cell deficiency have been shown to play an integral part in some autoimmune diseases. In NZB/NZW mice, an autoallergic syndrome resembling human systemic lupus erythomatosus (SLE) develops along with the loss of suppressor T-cell function. Replacement therapy with suppressor substance prevents development of the disease [11].

In humans, aberrations of regulator cell function have been demonstrated or suggested in a number of disease states. Augmented suppressor function is suggested in some cases of common variable hypogammaglobulinemia [18, 20]. Recently, there have been reports of suppressor cell leukemia [7]. In SLE, subnormal levels of suppressor activity have been reported, with some correlation between suppressor cell defect and disease activity [1, 6, 10]. In vitro exposure of lymphocytes to the thymic extract thymosin corrected the deficit.

A number of operational methods have been developed to study suppressor cell function in man, but whether each measures the same cell subset remains conjectural. Waldman et al. [20], utilized the relative inhibitory effect of purified T cells on Ig production by allogeneic and autologous pokeweed-stimulated B cells as a measure of suppressor activity. A method utilized by several groups, including our own, involves preactivation of suppressor cells with antigen or mitogen [5, 17]. At present, the most readily applicable method of suppressor-cell activation involves the use of polyclonal (predominately T cell) mitogens, usually Concanavalin A (Con A). Although this mitogen activates multiple T-cell subsets including helper and effector cells as well as suppressors, conditions of mitogen dose and duration of incubation can be selected to optimize the suppressor effect.

Since suppressor cells are thought to act via the release of soluble substances, several groups, including our own, have utilized supernatants of mitogen-activated cells as a source of inhibitory substance [2, 3, 9, 21]. Such inhibitory substances can be shown to block dividing nonlymphoid cell lines (proliferation inhibitory substance) [9] and lymphoid reactivity (e.g., mixed lymphocyte reactions) [21]. Supernatants of dividing lymphoid cell lines can also be shown to contain inhibitory sub-

stances. These supernatant substances derived from human lymphoid cells are akin to the soluble immune response suppressor substance (SIRS) studied in animals.

To explore the relation of suppressor cell function and the clinical course of MS, we have utilized the method of in vitro Con A activation of suppressor cells and measured the effect of both the cells on the mitogenic reactivity of autologous lymphocytes and the cell supernatants on dividing cell lines. This methodology is particularly applicable to a disease such as MS, in which a putative antigen has not been identified.

Previous work in our laboratory indicates that the suppressor effect we measure in vitro depends on the amount of suppressor substance produced by the Con A-activated cells as well as on the sensitivity of the autologous responder cells to suppressor influence [2, 3]. We have found that the level of suppression induced by activated suppressor cells on autologous lymphocytes is increased in elderly donors compared with young adults; however, the level of inhibition induced by the supernatants of activated cells of the elderly on a replicating cell line (mouse L cells) is less than that induced by cells of young adults. These findings indicate that one must be cautious in extrapolating from in vitro experiments on suppressor cell function to the in vivo situation.

Our methodology for activating suppressor (S) cells, collecting their supernatants, and testing the effects of cells on autologous responder cells and of supernatants on a proliferating cell line, usually mouse L cells, has been reported in detail elsewhere [2–5].

Initially, we compared the effects of S cells of MS patients of different ages to each other and to age-matched controls [5]. Elderly MS patients (age > 50) showed similar levels of suppressor activity in the S-cell assay to age-matched controls and significantly higher levels when compared to young clinically stable MS patients and young adult controls (Table 1). Mitogenic reactivity was also reduced in elderly compared to the younger groups of donors. It remains speculative whether these changes in immune function contribute to the evolution of the clinical course of MS into a more slowly progressive one, a change frequently occurring with age.

In the younger adult MS population, we have found that both suppressor cell function and mitogenic reactivity vary with disease activity [3]. In our studies, patients have been separated into three clinical subgroups – those whose disease had

Table 1. Mitogenic reactivity and suppressor cell function in MS

Donor groups	Number	Mitogenic reactivity		Suppressor cell activity	Number	Inhibition by supernatants
		cpm ± SEM [a]	S. I	% ± SEM		% ± SEM
Control – 50–70	7	8 566 ± 1 376	44 ± 13	69 ± 5	15	14 ± 4.0
MS – 50–70	5	21 015 ± 2 488	26 ± 6	64 ± 8	4	7 ± 6
Control < 45	19	31 560 ± 4 196	109 ± 18	40 ± 5	18	21 ± 3
MS < 45					11	7.0 ± 3
Stable	10	16 268 ± 2 014	51 ± 13	30 ± 8	8	3 ± 3
Active	6	41 059 ± 6 407	110 ± 17	3 ± 8	3	17 ± 4
Recovering	7	27 259 ± 7 621	127 ± 45	62 ± 5		

[a] Counts per minute (cpm) ± standard error of mean (SEM)

been quiescent for at least 6 months, and usually several years (stable); those who had new exacerbations or whose disease was actively progressing (active) and those who were recovering from exacerbations (recovering). Patients were classified prior to study; MS patients who could not be classified into one of the three categories were excluded from the study, as were patients on corticosteroids or those with overt systemic infections.

Representative data are shown in Table 1. Patients with stable disease showed borderline low suppressor cell activity and reduced mitogenic reactivity compared with age-matched controls. A recent study in our laboratory suggests that this reduced mitogenic reactivity reflects a defect in a monocyte-dependent lymphocyte subset [15]. Whether the patients in this group with the lowest suppressor activity are at greatest risk for a disease flareup is yet unknown.

The patients recovering from disease flareups demonstrated increased mitogenic reactivity compared with stable patients and increased suppressor cell activity compared with stable patients and controls. In a follow-up study of one patient who had remained in remission for 6 months, suppressor activity had returned to the control value range.

The active disease group showed increased mitogenic reactivity compared with stable patients and reduced suppressor cell activity. One patient studied sequentially showed a recovery of suppressor activity coincident with clinical improvement.

To determine whether the reduced suppressor activity found in the stable and active MS patients depended on the amount of suppressor substance produced and/or on the sensitivity of responder cells to suppressor influence, we measured the effect of the supernatants derived from Con A-activated cells on a proliferating cell line (L cells). The percent inhibition induced by the supernatants of cells from the MS group was significantly less than in the controls. Within the MS group, however, active patients with the lowest S-cell activity did not necessarily show the lowest supernatant effect. These results suggest that reduced suppressor effect in active MS may reflect both a reduced amount of suppressor substance and a resistance of the responder cells to this effect.

Whether the aberrant immunoregulation we have found in MS patients represents an intrinsic immunogenetic defect or is secondary to the effect of an acquired serum factor is unresolved. Goust et al. [8], found both elevated immune complexes and reduced suppressor activity as measured by finding a reduced number of T lymphocytes which rosette with and inhibit IgM production by a lymphoblastoid line. In our active MS group, three of four patients showed elevated immune complex levels in a Raji cell assay, compared to zero of five stable patients. Whether the reduced suppressor activity permits immune complex formation or is the result of immune complex influence on the regulator cells is unresolved. The interrelation between suppressor activity and other factors such as lymphototoxic antibodies also remains to be explored.

Findings of aberrant or fluctuating immunoregulatory activity in MS raise the potential for immunopharmacologic therapy. Levamisole, for example, reportedly augments suppressor activity of in vitro human splenic lymphocytes, although we did not detect a significant effect by this drug in our assay [2]. Hopefully, further insight into the activity of both effector and regulator immunocytes and the nature of the putative antigen in MS will lead to rational and effective forms of therapy.

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Discussion

E. Schuller: Do you find any correlation between HLA markers in response to Con A, like Petit, for example?

B. G. W. Arnason: Yes, our data are not totally persuasive; we think there is more likely to be a brisk response by HLA-B7. This is controversial again within the literature. But several people have found this kind of thing.

C. S. Raine: Dr. Arnason, can you match any increase corresponding to the decrease in suppressor cells which was shown here with active disease? Is there any match in increase in any effector cell population? Or do you think this is the only cell line which is affected? The other thing is, to which population of lymphocytes do you think suppressor cells belong? Some people even say they are null cells, which you shouldn't be measuring here.

B. G. W. Aranson: Right, what you are asking me is what I am doing currently – that I have not talked about. The point of course is that suppressor cells are only one of the regulator cell populations, that there are helper cells as well, that no one has studied helper cell function in MS, and that one would like to fractionate lymphocytes into T-mu, T gamma, null cells, and so on and see which of these have suppressor or helper functions. And of course we are doing all these things, but we don't have any data that I could present here at this point.

V. ter Meulen: I have a question. Did you investigate further the nature of the inhibitory material you have in your supernatant from your lymphocytes when you treat your cells and prevent thymidine uptake or cell division?

B. G. W. Arnason: We ourselves have not done so. Others are looking into this.

V. ter Meulen: Have you any hint about what it could be?

B. G. W. Arnason: Yes, but it is rather awkward, for it relates to unpublished work of other people. I really am not sure whether I should talk about it.

(Maybe, you can tell us at lunch time.)

H. J. Bauer: I may have missed this. Did you say anything about therapy during these active stages of MS? Were these patients getting any immunosuppressive substances?

B. G. W. Arnason: No, we deliberately set aside any patient who was on steroid or any form of therapy whatsoever. This has actually been a problem in terms of the design of this experiment, because, as you will have noticed, there is a 4-day interval between the first bleed and the second bleed. And it is very difficult to persuade clinicians to wait 4 days before starting steroids. We are now beginning to assemble data on patients who are on steroids. And I don't really have anything to report on that at this point. I can say that at least initially it doesn't seem to have basically altered things very much.

E. J. Field: Regarding steroids, a reasonable course of steroids lasting a month doesn't seem to have any effect on the macrophage electrophoretic test (MEM) for

lymphocyte sensitization. Second, you did not mention the lymphocyte depressing factor. There is an alpha-2 macroglobulin and a small protein going with it circulating in the blood, a lymphocyte depressing factor, and, in every condition in which lymphocytes become sensitized, an accompanying increase in the lymphocyte-depressing factor occurs in the blood. Remarkably, this goes up to a titer of 1 : 60,000 in ataxia telangiectasia. But in normal people it runs to about 1 : 60 and in MS it goes up to something like 1 : 240.

B. G. W Arnason: Yes, the lymphocytotoxic antibodies and so on are something one has to think about. These cultures were grown of course in fetal calf serum rather than in the patients' own serum. And we have not excluded that as a factor, but rather doubt that is a basis of what we are seeing.

E. Schuller: Do you have the same results with PHA?

B. G. W. Arnason: Yes, and we don't find it with pokeweed.

H. Koprowski: I thought I could discuss for one second what Cedric Raine asked namely, the nature of the killer cells. That is not an easy question to answer. In the paper which has been listed there, and for which Dr. Moretta made his disappearance rather than appearance, he'll probably talk about T-gamma cells, those cells which have receptors, C receptors, and IgG and which are the suppressor cells in the immune response, and which I think are those to which Dr. Arnason referred to as fluctuating and as of importance in MS. Now these cells have a cytotoxic activity, so in a certain way they are also killer cells, but apart from these cells, when you remove cells by rosetting them in the presence of IgG you still have a population of spontaneous killer cells left. So probably the T-mu cells don't have killer activity. So we have probably two populations of cells, one cytotoxic, and which is defined as the T-gamma fraction, and the other killer cells which do not, and which are left out when you rosette out T-gamma cells.

H. H. Peter: It appears to me that there was a discrepancy between your data showing that you have a prevalence of increase of low affinity (E-) rosetting cells in MS. And the data presented by the preceding speaker, who found high numbers of avid or active E-rosettes, particularly in those preparations where he added homogenate of MS.

B. G. W. Arnason: Yes, discrepancy between speakers is not new to the field of MS research.

J. Clausen: You see, we expressed the ratio of the active rosettes. So, it was not the absolute number of rosettes.

Question: What was the absolute number?

J. Clausen: We didn't count them.

Purification and Characterization of the T-Cell Antigen Receptor Recognizing the Basic Encephalitogenic Protein

J. EDER [1], B.-U. v. SPECHT [2], P. BARTMANN [2], D. TEITELBAUM [3], and W. BRENDEL [2]

Introduction

The molecular identity of the receptor molecule that enables T lymphocytes to recognize an antigen is unknown thus far. Binz and Wigzell [2] and Krawinkel and Rajewsky [9] have presented evidence that T cells express an antigen-recognizing receptor which seems to carry markers of the variable part of an immunoglobulin molecule; no evidence, however, has been found for the presence of the constant Ig domain. Hapten-binding material could be enriched from hapten-sensitized T-cells using an affinity method originally developed by Kiefer [8].

Experimental allergic encephalomyelitis (EAE) is studied both as a useful model of human demyelinating diseases and organ-specific T-cell dependent autoimmune diseases. It can be induced in laboratory animals by injection of myelin basic protein (MBP) in complete Freund's adjuvant (CFA) and results in characteristic lesions restricted to the central nervous system (CNS).

Bacteriophages modified by covalent attachment of antigens and haptens are inactivated by antibodies against the attached antigen [3–6] as well as by other antigen-recognizing proteins [1, 9]. We report on the development of such a viroimmunoassay as a probe for MBP-specific binding sites and its application to the enrichment of MBP binding material from spleen and lymph node cell suspensions of guinea pigs (strain 13), which had been sensitized with the basic encephalitogenic protein. This purification was performed in an attempt to produce an antiserum against the MBP-specific T-cell receptor, which may induce tolerance to EAE by eliminating the appropriate T-cell clones. In addition, evidence is presented concerning the molecular structure of the receptor and its role in cellular immune response.

Experimental Procedures

Inbred guinea pigs (strain 2 and strain 13, 400–650 g) were a gift from the Institut für Biologisch-Medizinische Forschung, Füllinsdorf, Switzerland. Nylon mesh (obtained from Schweiz. Seidengazefabrik AG) was partially hydrolyzed with HCl, succinylated, and activated by formation of a N-hydroxysuccinimide ester. Binding of the basic protein to the activated nylon mesh was performed in 0.2 M borate buffer, pH 8.2, and controlled by using ^{131}J-modified MBP. Discs 2.5 cm in diameter

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and having a central hole of 2 mm were cut out of the nylon mesh and stacked on a shaft with 3-mm teflon spacers. This was placed into a 50-ml glass column where it could be rotated at 5 rpm.

Basic protein was extracted from bovine spinal cord as described by Hirshfeld et al. [7]. Strain 13 guinea pigs were immunized by injecting 10 μg of MBP in PBS/CFA 1 : 2 into each foot pad. The animals were sacrificed after 12 days, spleen and draining lymph nodes isolated, and cell suspensions prepared in RPMI 1640. These were then incubated with the MBP-coupled nylon mesh for 1 h at 4 °C under gentle stirring. Nonbinding cells were removed by elution and washing with cold medium. Antigen-specific cells were released from the nylon mesh by shifting the temperature to 25 °C and were thus collected for further experiments. After washing the nylon mesh three times with PBS, antigen-binding material was eluted with 3.5 M KSCN in PBS.

Rabbits were immunized by an injection of 1 mg MBP and 1 mg phosvitin in CFA at multiple intradermal sites. A booster injection with the same antigen mixture was administered after 10 days, and intradermal reinjections were given in monthly intervals. Weekly bleeding from the marginal ear vein was started 6 weeks after the first injection. The T4-MBP conjugate was tested using an antiserum from a bleeding made 6 weeks after the primary injection.

Bacteriophages and the corresponding host bacterium *Escherichia coli B* were prepared and handled as described previously [4]. MBP was coupled to bacteriophage T4 using glutaraldehyde as a bifunctional reagent, by means of a procedure similar to that described by Haimovich et al. [6]. MBP was dissolved (40 mg/ml) in 0.05 M sodium phosphate buffer, pH 6.8. To a mixture of 50 μl of MBP solution and 50 μl of bacteriophage T4 suspension (10^{13} PFU/ml), were added 20 μl of glutaraldehyde (0.03%, v/v). The reaction mixture was left at room temperature for 2 h, and diluted to a final volume of 5 ml by addition of 0.05 M sodium phosphate buffer, pH 6.8. Any precipitate was removed by centrifugation for 5 min at 7000 *g*. The MBP-T4 conjugate was separated from unreacted protein by centrifugation for 50 min at 20,000 *g*. The pellet was washed with gelatin-containing buffer, covered with 1 ml of the buffer, and kept undisturbed at 4 °C for 12 h, after which it was resuspended by gentle shaking and diluted again to 5 ml with gelatin-containing buffer. This procedure of low speed centrifugation, high speed centrifugation, washing, and slow resuspension was repeated twice. After a final centrifugation for 5 min at 7000 *g*, the number of phages surviving the coupling process was determined by plating 50-fold serial dilutions of the preparation. It was found to be about 0.1% of the initial number of phages. The MBP-coated bacteriophages were kept in gelatin-containing buffer at 4 °C. The inactivation of MBP bacteriophage conjugate by anti-MBP antiserum and by antigen-binding material from lymphocytes, as well as the inhibition of the above inactivation with free MBP was carried out as described previously [4].

Results and Discussion

Basic protein from bovine spinal cord was covalently coupled to bacteriophage T4 with glutaraldehyde as a bifunctional agent. Antibodies raised in rabbits against

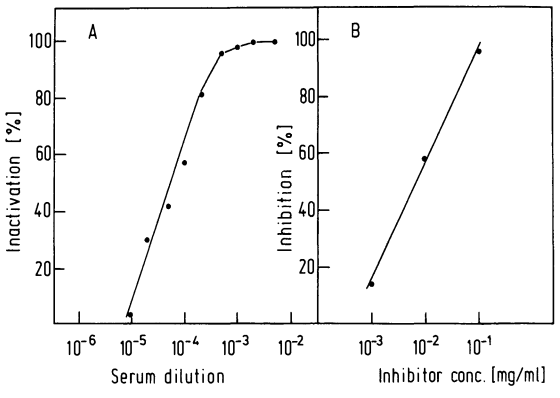

Fig. 1. **A** Inactivation of MBP bacteriophage T4 conjugate by rabbit anti-MBP antiserum. **B** Inhibition with free MBP of the inactivation of MBP-bacteriophage T4 conjugate by rabbit anti-MBP antiserum

MBP completely inactivate this MBP-T4 conjugate (Fig. 1 A). This immunospecific inactivation reaction displayed considerable sensitivity, as a 5×10^{-5}-fold antiserum dilution resulted in 50% inactivation. No inactivation of the modified phages was obtained with preimmune sera. Upon preincubation of anti-MBP antibodies with free MBP, prior to the incubation with the modified phage, an inhibition of the inactivation of the MBP bacteriophage conjugate takes place (Fig. 1 B), thus showing the specificity of this viroimmunoassay.

Figure 2 shows the analysis of material isolated from MBP-modified nylon mesh by the phage inactivation method. A typical inactivation pattern is observed which is comparable to that obtained with rabbit anti-MBP antiserum. Phage-inactivating material was obtained from lymph node cells as well as from spleen cells. To demonstrate that this material is not identical with humoral antibodies produced against the basic protein, absorption with cross-linked anti-guinea pig Ig was performed. No difference in phage inactivation capacity could be found after the absorption step, indicating that the inactivating material is not identical with common immunoglobulin molecules. Moreover, no inactivation capacity was found in the serum pool of the immunized animals. To exclude the possibility of nonspecific phage inactivation, ten guinea pigs were immunized with 10 µg of egg albumin and the whole procedure repeated as described for the MBP-immunized animals. No inactivation of MBP-modified T4 coliphage could be obtained, thus ruling out nonspecific interactions.

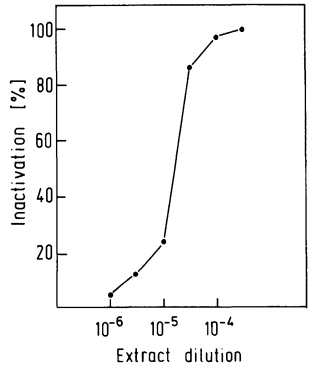

Fig. 2. Inactivation of MBP bacteriophage T4 conjugate by antigen-binding material from MBP-sensitized lymphocytes from guinea pigs (strain 13)

To analyze whether the phage inactivating material is produced by the modified nylon adherent cells, this cell fraction was kept in culture for 2 days. Repeated absorption of these cells to the affinity column again revealed – after the appropriate washing steps – phage inactivating material. This demonstrates that the cells can produce the antigen-recognizing factor under culture conditions.

To answer the question of a T-cell origin of the inactivating material, the cells were analyzed by immunofluorescence and cytotoxicity tests. More than 97% of the specifically bound cells were identified as T cells. These data demonstrate that T lymphocytes from guinea pigs immunized with MBP produce a specific antigen-recognizing protein; and that the technique of chemically modified bacteriophages can successfully be applied as an assay for this receptor material. It was shown that the material isolated is not identical with common immunoglobulin.

Acknowledgments. We are indebted to the Institut für Biologisch-Medizinische Forschung, Füllinsdorf, Switzerland, for a generous gift of guinea pigs of strain 2 and strain 13 and to Miss M. Waltenberger for excellent technical assistance. We thank Prof. H. Rembold, Max-Planck-Institut für Biochemie, Martinsried and Dr. E. Holler, Universität Regensburg, for providing research facilities. This work was supported by grant SFB 37 from the Deutsche Forschungsgemeinschaft.

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Immunological Cell Surface Marker Analysis in Multiple Sclerosis

U. PATZOLD [1], P. HALLER [1], and H. H. PETER [2]

The participation of immunological mechanisms in the pathogenesis of multiple sclerosis (MS) is a widely accepted although not undisputed concept [5]. The purpose of our study was to supplement a randomized azathioprine trial [9] with an analysis of immunological cell surface markers in azathioprine- and placebo-treated MS patients and in age- and sex-matched healthy controls.

Materials and Methods

Peripheral blood lymphocytes from MS patients and controls were isolated using the Ficoll-Metrizoate technique. Surface membrane immunoglobulins (SIg) were detected by direct membrane fluorescence using a 1 : 4 dilution of goat anti-human IgG, IgA, IgM antiserum (Hyland Laboratories). At least 200 cells were examined under a Leitz Orthoplan fluorescent microscope. T cells were identified by their capacity to form spontaneous rosettes with neuraminidase-treated sheep erythrocytes (E rosettes) at low temperatures [3]. After 12 h of incubation one drop of 0.2% acridine orange was added to the cell mixture and 200 cells were evaluated under a fluorescent microscope. Fc receptor-bearing cells were detected by the binding of human erythrocytes sensitized with subagglutinating concentrations of Anti M+N (Behring) IgG antibodies (EA rosettes). Incubation and evaluation was done as for E rosettes. These techniques have been described previously [6].

Results

Figure 1 summarizes the lymphocyte analysis in MS patients treated for 6 months or longer using azathioprine or placebo. No significant difference was noted for any of the known cell surface markers. Similarly, controls and MS patients showed no significant difference in their lymphocyte subpopulations (Table 1). In MS patients presenting an acute exacerbation during the 3 months preceding lymphocyte analysis, a slight, although not significant decrease in T cells and a concomitant increase in B cells was observed (Fig. 2).

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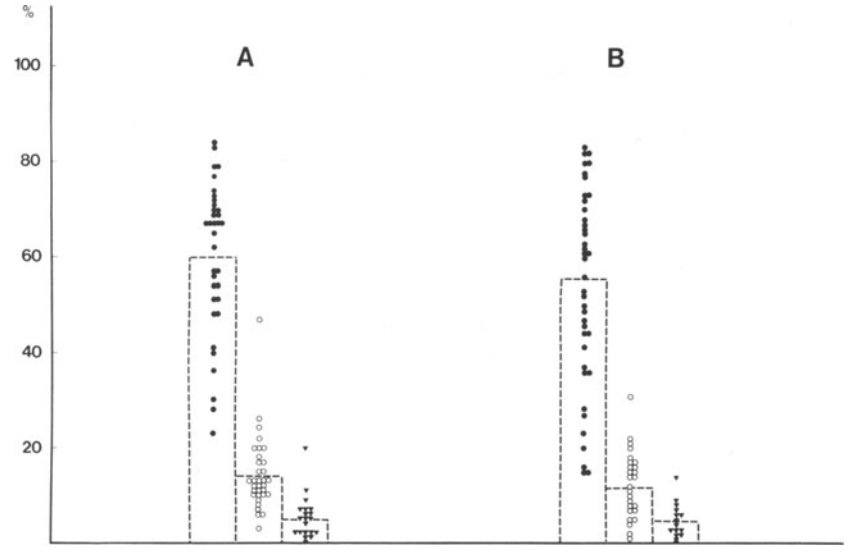

Fig. 1. Percentages of lymphocyte subpopulations in patients treated with azathioprine (**A**) and patients receiving no immunosuppressive therapy (**B**). ● E-rosette forming cells, ○ EA-rosette forming cells, ▼ cells with positive immunofluorescence

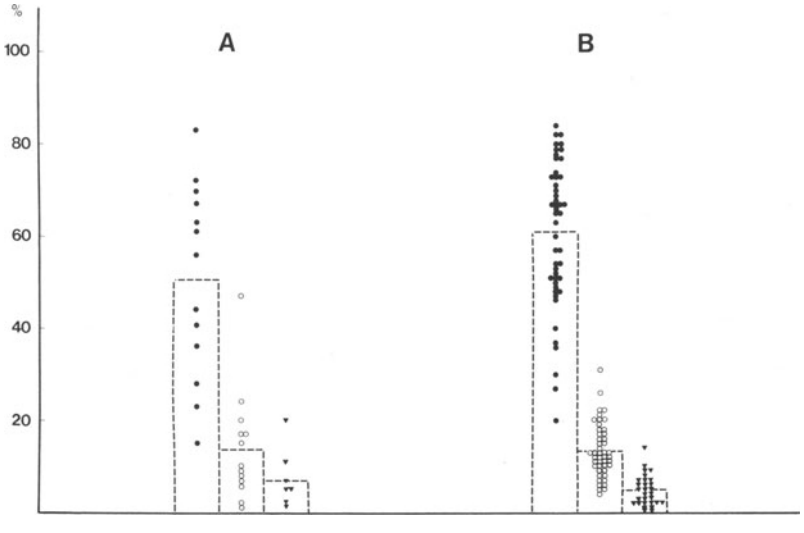

Fig. 2. Percentages of lymphocyte subpopulations in patients with a relapse during three months before investigations (**A**) and patients with no relapse during this period (**B**). ● E-rosette forming cells, ○ EA-rosette forming cells, ▼ cells with positive immunofluorescence

Table 1. Lymphocyte surface markers in patients with MS and healthy control persons

	Multiple sclerosis patients			Controls		
	n	*x*	SD	*n*	*x*	SD
E	62	59	17 [a]	21	57	11
EA	59	14	7	21	18	6
SIg	36	5	4	13	4	2

[a] Percentage established from the evaluation of 200 cells

Discussion

Reports on lymphocyte subsets in blood samples of MS patients have been rare and to some extent controversial. While stable patients have generally been found to exhibit normal levels of T cells [6, 8, 11], modest decreases in mean circulating T cells have been reported during exacerbation of the disease [6, 10]. Our findings agree with these data; in addition, they show that azathioprine treatment did not significantly alter the mean levels of circulating T-B and Fc receptor cells. The heterogeneity of the latter group of cells is well established. Besides the T_G subset [2], null lymphocytes [4], immature B cells, monocytes, and granulocytes may carry this marker [10]. A recent publication by Santoli et al. [12] reports an increase in the T_G subpopulation for MS patients.

Oger et al. [8] found a decrease in T cells with high affinity for sheep erythrocytes; West et al. [13] have shown that this T-cell subset does not carry Fc receptors. However, there is an increase of active rosettes after incubation with MS brain extract [7]. Apparently more work is required to clarify the distribution and possible diagnostic value of lymphocyte subpopulations in MS.

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The Enhancing Effect of Multiple Sclerosis Brain Homogenates on Active E-Rosette Forming Lymphocytes in Neurological Disorders. Relationship Between Rosette Formation and Clinical Parameters

H OFFNER [1], T. FOG [2], S. C. RASTOGI [1], G. KONAT [1], and J. CLAUSEN [1]

Introduction

Our recent studies [3] have described the enhancing effect of multiple sclerosis (MS) brain homogenates on the active E-rosette forming lymphocytes from MS patients. In this assay, antigen-sensitive T cells are measured by in vitro exposure of lymphocytes to brain homogenates prepared from MS and control brains. Since the increase in active E rosettes after antigenic stimulation is indicative of the sensitivity to the antigen [1], our data revealed MS lymphocytes to be sensitive to MS brain homogenates.

To establish whether the AER test may be used as a diagnostic aid in MS, we extended the test to coded samples of peripherial blood from 105 neurological patients, including 27 MS cases.

Materials and Methods

Materials

Nineteen patients meeting McAlpine et al.'s [2] criteria for definite MS, two with probable and six with possible MS, were studied. Their mean age was 43.1 years (range 22 to 65). All but one were clinically stable and none were receiving immunosuppressive therapy at the time they were studied.

Senventy-eight patients with a mean age of 54.8 years (range 15 to 80) with diagnosed neurological diseases were used as controls. All blood samples were delivered coded to the laboratory. Among all patients, 20 ml of peripheral blood taken by venipuncture of a cubital vein, were used as a source of peripheral blood lymphocytes. All samples were taken between 8:00 and 10:00 a.m. The patients were not fasting. The diagnosis of the control patients is shown in Table 1.

Methods

Preparation of homogenate of MS and non-MS brains and active E-rosette tests were performed as described previously [3]. All samples were run coded with the same batch of control and MS brain homogenates.

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Table 1. Diagnosis and number of non-MS control patients

Cerebrovascular diseases	29	Vertigo	3
Epilepsia	11	Chronic alcoholism	4
Mono- and polyneuropathy	8	Brain injury	3
Tumor cerebri	4	Neurosis	2
Disc prolapse	4	Parkinson's disease	2
Encephalopathies	3	Guillain-Barré syndrome	1
Cephalgia	3	Dementia	1

Table 2. Ag-AER/AER ratios [a]

Subjects	Ag-AER/AER with MS brain homogenate	Positive response	Ag-AER/AER with non MS brain homogenate	Positive response
MS definite (19)	1.40 ± 0.20	16 (84.2%)	1.07 ± 0.21	3 (16.7%)
MS probable (2)	1.26	1 (50%)	1.08	0 (0%)
MS possible (6)	1.31 ± 0.15	6 (100%)	1.05 ± 0.1	1 (16.6%)
Other neurological diseases (78)	1.11 ± 0.39	6 (7.7%)	1.10 ± 0.42	8 (10.2%)

[a] Lymphocytes from patients with MS and with other neurological disorders were incubated for 15 min at 22 °C with 2.3 μg protein/0.25 ml of MS and OND brain homogenates and assayed for AER. The ratio is calculated from the Ag-AER/AER formula and represents the mean ± SD

Table 3. Ag-AER/AER ratios [a]

	Lymphocytes from MS patients		Lymphocytes from OND	
Brain homogenate used for lymphocyte stimulation	MS Ag	Non-MS Ag	MS Ag	Non-MS Ag
Median	1.24	1.03	1.02	1.01
90% decile	1.57	1.21	1.12	1.13
10% decile	1.06	0.91	0.83	0.75

[a] Median and decile values were obtained using results of the AER test

The results are expressed as ratios of the average antigen-stimulated active E rosettes (Ag-AER) over nonstimulated lymphocytes from the same patients (AER). The antigenic stimulation of MS and other neurological diseases (OND) lymphocytes was performed with MS and with non-MS homogenate [3].

Changes in AER greater than 15% of the control value after antigenic stimulation were considered positive. These data are presented in Table 2 and 3.

Resuspension Apparatus. To overcome the technical problem of standardized resuspension of lymphocyte E rosettes, we designed a simple apparatus (Fig. 1). The apparatus was built on horizontal and vertical plates (20 × 20 cm) glued to-

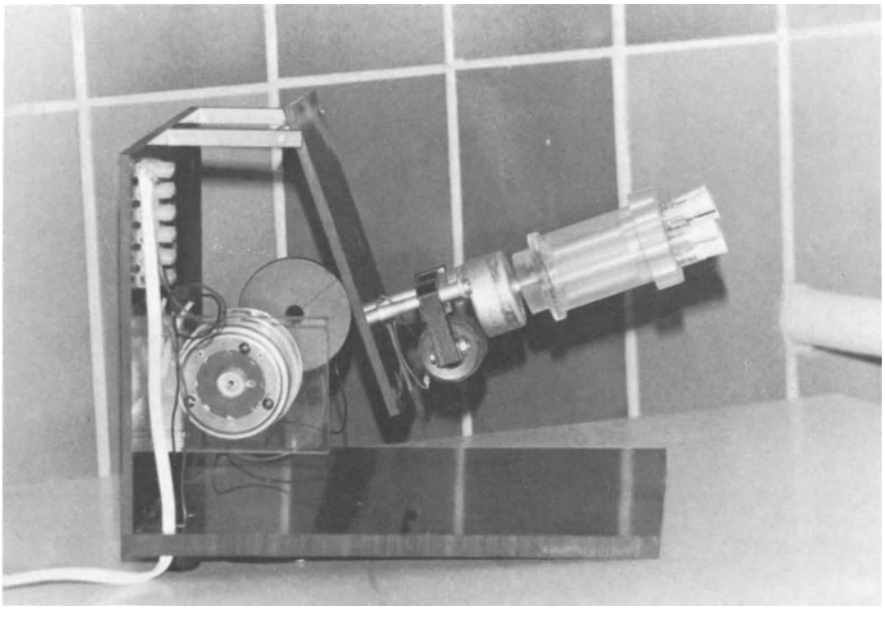

Fig. 1

gether at right angles at one of their edges. A cylindrical tube holder (4 × 6.7 cm) made of acrylplastic with six holes for AER sample test tubes) was subjected to a combined swinging and rotating motion by a system of two synchronous electric motors (220 V AC, 50 periods/min, type 6910 SSL, AEG, Telefunken Ltd., Copenhagen). Motor no. 1 was placed on the vertical plate and its axis was connected acentrically 1.1 cm from the other periphery of a circular plastic plate (diameter = 5.5 cm). A moveable 11 × 16 cm vertical plate was hung from a frame linked by two 5 × 0.5 × 0.5 cm brass sticks to the upper part of the vertical plate at a position so that the assymetrical rotating plate pushed to the moveable plate 5 cm from its lower edge. The rotation of motor no. 1 thus causes the vertical moveable plate to rock up and down 22° from its vertical position. Motor no. 2 is placed at the lower edge of the moveable plate and connected through its axis to the rotating plastic holder with six holes for AER tubes. Motor no. 2 rotates the six samples one full rotation per min. The holder also swings vertically up and down in the plane of its axis from a horizontal position to about 22° 12 times per min. This design permits a standardized double motion of the holder. A resuspension time of 3 min was found to be optimal.

Statistical Evaluation. Statistical evaluation was performed using parametric and nonparametric assays. Differences between groups were evaluated using Student's *t*-test to determine the difference between means and using a "chi-square" test, where median, 10%, and 90% decile values were presented.

The sensitivity, i.e., the percent of positive (true) data, specificity, i.e., the percent of negative (false) data, and the validity of the AER method was calculated as described by Zielhuis and Verberk [4].

Results

Findings in Multiple Sclerosis

Stimulation with MS Brain Homogenate. Of 19 patients with definite diagnosis of MS, 16 showed an Ag-AER/AER ratio higher than 1.15 when their lymphocytes were incubated with MS brain homogenate (mean 1.40 ± 0.20) (Table 2). Three MS patients gave negative results.

One case was a 64-year-old female who had had opticospinal MS since 1939 and now has spastic paresis of the upper left extremities and severe spastic paraparalysis. This may be a "burnout case".

The second case was a 48-year-old male suffering from opticospinal MS with bilateral retrobulbar neuritis as an initial symptom. He was neurotic and difficult to evaluate, but had pyramidal symptoms. He had been treated with prednisone for a long time, but not during the preceding 9 months.

The third case was one of classical MS, a 35-year-old female showing a progressive course since 1969, with spinocerebellar and pyramidal symptoms. The present assay was made during the initial phase of a severe mesencephalic attack, 6 weeks after the termination of intensive prednisone treatment that had lasted 6 months. A possible influence of prednisone upon the immune system might be considered.

Of two probable MS cases, one was positive in the AER test (mean 1.26). The negative case was a 54-year-old male diagnosed as having a myelopathy that had begun 17 years earlier and who had suffered an acute attack of retinitis in 1966. He had no brain stem or cerebral signs and symptoms, but a delay in visual and sensory evoked potentials occurred as in MS. In his spinal fluid a sharp band in the γ-3 area was found both in 1972 and 1978, together with an increase of gamma globulins.

Of six possible MS, six were positive (mean 1.3 ± 0.15). One of these cases is difficult to place in the diagnostic categories. She was a 38-year-old female admitted to the hospital in December 1977. In 1969 left visual disturbances occurred. In 1972 a left hemiplegia developed, especially in the leg, where a spastic paresis persisted and progressed over the years. In 1972 the spinal fluid showed one cell and 52 mg protein/100 ml, but an oligoclonal gamma-3 band was found using agar-gel electrophoresis. In 1975 no cells were found, protein was 28 mg%, and there were no sharp bands. The gamma area was diffusely stained, but no increase was found. In October 1973, the patient had a grand mal seizure. A right carotid angiography showed several small angioma-like changes in the right hemisphere, similar to but not typical of Moya-Moya. During the following years, a progressive dementia developed. In 1975 a computer tomography (CT) scan revealed a large low absorption area in the right frontotemporal region, which was infarct-like or a hematoma, and diffuse cortical atrophy. In 1977 a new CT scan showed that this area was unchanged; however, another large low absorption area was detected in the posterior part of the same hemisphere, close to the midline over the ventricular system. Cortical atrophy was found in the left hemisphere, but apart from this, no changes were seen.

Stimulation with Non-MS Brain Homogenates. Four MS patients also responded to non-MS brain homogenate (Table 2), but the response was lower than that to MS brain (mean 1.10 ± 0.38). The difference between Ag-AER/AER of MS lymphocytes and MS and control homogenate was significant ($P < 0.001$).

Findings in Patients with Other Neurological Diseases

The Ag-AER/AER ratio for neurological controls was 1.11 ± 0.39 and 1.10 ± 0.42 with MS and control brain, respectively (Table 2). Of 78 neurological patients, six were responding to MS antigen and eight to control antigen in the AER test. The response of four OND patients was higher with MS brain than with control brain. Four positive cases were more or less severe alcoholics, one with a possible liver damage and visual disturbances of unknown nature. One case showed cerebral disease and in one case a subacute polyneuropathy developed. One had had a dilantin intoxication 1½ year before examination and a halothan hepatitis 1 year before examination. In these cases, liver damage may have had some influence upon the immune system.

In two positive cases the diagnosis was unknown. One of these cases was a 63-year-old female. During the 2½ years prior to diagnosis intractable pains in the right leg, possibly of a spinal type, and numbness in the right foot were her only complaints, except for a psychogenic neurosis due to personal problems. No other neurological anomalies were found – apart from an increase in the spinal gamma globulins and a sharp monoclonal band in the gamma-3 area found in agar-gel electrophoresis. No cells were traced and the spinal protein was 36 mg%. The pains were intractable, even after physiotherapy and psychiatric treatment.

The other case was a 60-year-old female who complained of intermittent pain in the right lower jaw. Nine years before admission to the hospital she had had a 3-month period of atypical headache, and an EEG revealed slight left temporal anomalies. There was a complete remission, but 3 months before admission the same symptoms appeared and the focal EEG anomalies were more pronounced. A left carotid angiography was normal and her complaints disappeared. Spinal fluid studies were not performed.

The diagnosis in these two cases is unknown. Due to the age of the patients, MS seems unlikely, even if difficult to exclude.

The difference between the Ag-AER/AER ratios of MS and OND patients with MS antigen is significant ($P < 0.001$).

The results of non-parametric assay are presented in Table 3. The median value of the Ag-AER/AER-ratio of the whole group of MS patients stimulated with MS homogenate was significantly higher than the control ratio. Thus the frequency of positive response of lymphocytes from MS and OND patients compared with MS antigen is significant at the level $P < 0.001$ as measured by a chi-square test. However, there is no significant difference between lymphocytes from MS and OND patients with control antigen.

The AER method has a sensitivity of 0.84 and a specificity of 0.93 (Table 3). Thus the validity, i.e., the specificity plus sensitivity is 1.77, and thus above 1.00. This shows a positive correlation between the AER/Ag-AER ratio and the diagnosis of MS.

Discussion

The present blind study confirms our previous results regarding the existence of antigens in MS brains to which lymphocytes of MS patients responded in the AER test. This assay requires a minimal amount of material and blood and no sophisticated laboratory equipment. The test is quick and can be completed in 1 day. The apparatus for resuspension of E rosettes represents a standardized and reproducible method and reduces the sources of error in the test.

Although the present immunological test is not a "specific MS reaction", the sensitivity of 0.84 and the specificity of 0.93 shows the method to be highly specific for MS. The majority of MS cases are positive, and thus this test, together with other laboratory and clinical methods of diagnosis (spinal fluid; cell count, and content of oligoclonal immunoglobulin fraction; periphlebitis retinae, evoked potentials), may be an important diagnostic aid.

Further cases of neuroimmunological diseases, lupus erythematosus disease, and Guillain-Barré-syndrome will be studied as well as the possible influence of corticoids or other immunosuppressive drugs.

The nature of the antigen is under investigation.

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Recent Methods of in Vitro Diagnosis of Multiple Sclerosis

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Tests so far available for multiple sclerosis (MS) comprise:

1. The MEM-LAD (Macrophage Electrophoretic Mobility-Linoleic Acid Depression) test [17, 28, 36] – a test demanding healthy guinea pigs unexposed to infection (especially influenza antigens) and hence fraught with pitfalls [12, 15, 33, 35, 43, 45].
2. The E-UFA (Erythrocyte-Unsaturated Fatty Acid) test [15] (Seaman, et al., personal communication).
3. The PGE_2 (Prostaglandins) test [13].
4. The TEEM (Tanned Erythrocyte Electrophoretic Mobility) test [25, Field et al. 1978, unpublished].

Unsaturated fatty acids of the linoleic (LA) and arachidonic (AA) type make up an important part of the surface membrane of all cells in the body, and the methods depend upon the demonstration of an anomalous makeup of such surface membranes in the case of lymphocytes and red blood cells (RBC).

The MEM-LAD Test

The MEM-LAD test depends upon the observation that the interaction between lymphocytes and antigen (to which they are sensitized) is depressed by linoleic and arachidonic acids (LA and AA) to a very much greater degree in MS than in other neurological (destructive) diseases (OND) or normal subjects. For MS patients 90–100%; for OND, about 47%; and for normal subjects, about 56%. With practised technique, the OND and normal groups can be separated [26, 27], but the test demands much attention to detail, especially in providing healthy, noninadvertently immunized guinea pigs. Inadequate animals husbandry, or the substitution of SPF animals, leads to grotesque results. Recently the macrophage as indicator cell has been replaced by the tanned sheep RBC (TEEM-LAD test, see 4).

The MEM test itself depends upon the liberation of a lymphokine [different from macrophage inhibitor factor (MIF)] when sensitized lymphocytes come into contact with appropriate antigenic determinants. This lymphokine was called macrophage-slowing factor (MSF), though it is now known to act on a variety of cells. The degree of slowing measures the degree of lymphocyte-antigen interaction, and hence lymphocyte sensitization [11].

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A full account of the MEM-LAD test has been given by Field et al. [17], and the controversy it generated is reported by Mertin et al. [35], Shenton [42, 43], and Field and Shenton [15]. The method has, however, been independently confirmed by Jenssen et al. [28], meeting the "double-double blind" requirement of Mertin et al. [36].

The MEM-LAD test indicates that the surface membrane of lymphocytes in MS is different from that in OND and in normal subjects. The same conclusion may be drawn from a study of mixed lymphocyte reactions (MLR) between unrelated MS patients as compared with OND or normal patients. Källen et al. [29, 30], using transformation, and Field et al. [18] using the microelectrophoresis method, showed that the MLR between MS patients is much less than between the other groups.

The Erythrocyte-UFA (E-UFA) Test

The method starts out from the attractive hypothesis put forward by Thompson [46] that MS develops against an inborn background of mishandling of phospholipids ("Thompson's anomaly"). This would be expected to affect the cell surface membrane of all cells in the body of MS sufferers, including that of the readily accessible red and white blood cells. There is indeed some indication that the RBC membrane is unusual in MS [39, 41] (large average size of RBC as compared with OND patients and normal subjects; increased osmotic fragility) [5, 32]. Moreover, the surface of lymphocytes in MS as shown by the MEM-LAD test [17] and the MLR [18, 30] is unusual. Most recently, direct chemical evidence for abnormal phospholipid content of MS lymphocytes has been advanced [48]. Full experimental details of the E-UFA test are given by Field, et al. [20], together with the strict conditions to be complied with for success. This method has been fully confirmed by Bisaccia et al. [4] and (for LA) by Seaman et al. [41A] Portland, Oregon (personal communication).

Results

An abbreviated list of typical results is given in Table 1. RBC of MS patients travel more slowly in the presence of 0.08 mg/ml LA or AA (arachidonic acid), while those from OND or normal subjects travel more rapidly ($P < 0.001$).

Many more relatives of MS patients have now been studied. The great majority of anomalous results (i.e., RBC slow with LA and fast with AA) are among females. In every case the mother of an MS subject is what we call a "red circle", i.e., her RBC travel more slowly with LA and rapidly with AA as compared with control value. Something over 40% of near relatives show this anomaly, which is preponderant in females. The results of lowering the dose in normal, MS, and "red circle" subjects are presented in Table 2. Note the exceptional behavior of "red circles" (or "squares" – which are rare). "Red squares" are nearly always found when two closely related MS subjects occur in a family. (Circle signifies female; square signifies male, in accordance with international usage.)

Table 1. Effect of 0.08 mg/ml LA and AA upon the absolute mobility of RBC (μ/s/V/cm). Note slowing by both acids in MS and increase in speed by both acids in OND and normal subjects

Name	Sex	Age	Con	LA	AA	% change		
						LA	AA	
MS subjects								
PP	F	24	1.174 ± 0.034	1.123 ± 0.030	1.128 ± 0.027	− 4.34	− 3.92	Active 2 years
AC	F	24	1.090 ± 0.044	1.022 ± 0.041	1.004 ± 0.048	− 6.24	− 7.89	Active 1 year
BP	M	41	1.213 ± 0.037	1.155 ± 0.033	1.147 ± 0.036	− 4.78	− 5.44	Mod. active 15 years
OND subjects								
RH	M	22	1.109 ± 0.038	1.189 ± 0.044	1.184 ± 0.040	+ 7.21	+ 6.76	Old cerebral injury
TS	M	30	0.945 ± 0.040	0.965 ± 0.044	0.969 ± 0.042	+ 2.12	+ 2.54	Ac. diss. Encephalomyelitis 3 months ago. No virus isolated.
JMcI	M	53	1.166 ± 0.038	1.204 ± 0.037	1.228 ± 0.038	+ 3.26	+ 5.32	Neurosyphilis – WR; long standing.
SD	F	83	1.090 ± 0.047	1.204 ± 0.042	1.187 ± 0.029	+ 10.46	+ 8.90	Stroke 6 months ago.
Normal Subjects								
EF	M	61	1.084 ± 0.034	1.104 ± 0.040	1.132 ± 0.041	+ 1.85	+ 4.43	
GJ	M	45	1.068 ± 0.039	1.090 ± 0.029	1.105 ± 0.030	+ 2.12	+ 3.46	
ES	M	43	1.158 ± 0.036	1.205 ± 0.031	1.230 ± 0.036	+ 4.06	+ 6.22	

Table 2. Note the difference produced by altering the concentration of LA or AA from 0.08 mg/ml to 0.02 mg/ml; in particular, the unexpected reactivity in the case of "red circles" or "red squares" [a]

	LA		AA	
	0.08 mg/ml	0.02 mg/ml	0.08 mg/ml	0.02 mg/ml
MS	S	F	S	F
OND	F	S	F	S
Normal	F	S	F	S
"Red circle or square" (i.e. female or male)	S	S	F	S

[a] F = fast ($P < .001$); S = slow ($P < .001$)

The Prostaglandin (PGE_2) Test

Since in the body LA is converted into gamma linolenate (the active principle of Naudicelle capsules), and then into arachidonic acid and PGE_2 series, the effect of PGE_2 on MS, OND, and normal RBC mobility was studied, the effects on lymphocytes having previously been reported [43, 44]. An extensive protocol has been set out by Field and Joyce [13], and Table 3a is a small illustration of this. For ease of recognition, the table is reproduced as Table 3b, in which F means speeding up of RBC ($P < 0.001$) and S means slowing ($P < 0.001$). It will be seen that MS erythrocytes are not altered in speed by 1.0 μg/ml PGE_2 (an enormous dosage in prostaglandin terms) while normal and OND subjects' RBC travel more rapidly. On the other hand, at 31.25 pg/ml, MS RBC travel more slowly, while normal and OND RBC travel more speedily. At 62.5 pg/ml, MS travel more rapidly, while normal and OND give normal speeds.

It has recently been found [14] that the RBC of MS subjects who have been ingesting gamma linolenate for 2 years or more show great sensitivity to PGE_2 in that speeding up is extended down to 1.9 pg/ml and (on one or two occasions) even to 0.95 pg/ml.

The PGE_2 test does not slavishly follow the E-UFA test. The latter takes some 5–8 months to become positive when six capsules of Naudicelle (equivalent to 413.4 mg gamma linolenate and 2.664 g LA) are ingested daily, but the PGE_2 extension of range at the lower end takes much longer – between 21 and 24 months. This raises problems of PG receptors and their generation about which next to nothing is known with sufficient certainty to make speculation profitable (personal communication from Dr. J. Scanner, Searle Labs., Chicago).

The TEEM-LAD Test

Here tanned sheep erythrocytes have been used instead of macrophages in the original MEM-LAD test [25, 40]. The TEEM-LAD test has all the potential applications

Table 3 a. Influence of varying concentrations of PGE_2 in μg/ml on the absolute mobility of red blood cells. Note in MS there is no significant change at 1.0 μg/ml and a slowing at 31.25 pg/ml. In OND and normal subjects 1.0 μg/ml produces a speeding up, as does 31.25 pg/ml

Name	Concentration of PGE_2 (μg/ml)				
	CON	1.0	0.01	0.005	0.0025
MS subjects					
LR		CON: 1.004 ± 0.027			
F (51)	1.038 ± 0.024	0.992 ± 0.030	1.137 ± 0.032	1.030 ± 0.035	1.121 ± 0.032
FL		CON: 0.998 ± 0.028			
F (55)	1.037 ± 0.025	1.003 ± 0.027	1.126 ± 0.032	1.130 ± 0.036	1.132 ± 0.036
OND subjects					
MJ					
F (18)	1.041 ± 0.025	1.131 ± 0.028	1.123 ± 0.029	1.136 ± 0.028	1.034 ± 0.023
WE					
F (54)	1.041 ± 0.022	1.155 ± 0.032	1.138 ± 0.027	1.131 ± 0.029	1.047 ± 0.026
Normal subjects					
			CON:	CON:	CON:
GJ			1.023 ± 0.030	1.023 ± 0.030	1.023 ± 0.030
F (45)	1.041 ± 0.024	1.115 ± 0.030	1.112 ± 0.027	1.108 ± 0.026	1.026 ± 0.021
			CON:	CON:	CON:
BS			1.003 ± 0.027	1.003 ± 0.027	1.003 ± 0.027
M (24)	1.036 ± 0.026	1.127 ± 0.037	1.082 ± 0.027	1.090 ± 0.035	1.017 ± 0.030

Table 3 b. Simplified version of Table 3 a showing speeding (F) of RBC and slowing (S) of RBC. ($P < .001$) [a]

	Concentration of PGE_2 (μg/ml)								
	1.0	0.01	0.005	0.0025	0.0005	0.00025	0.00125	0.0000625	0.00003125
Normal M (61)	F	F	F	F	ns	ns	–	ns	F
OND F (48)	F	F	–	F	ns	ns	ns	ns	F
MS F (68)	ns	F	F	F	F	F	F	F	S

[a] F = faster migration speed in the presence of PGE_2 ($P < 0.001$ – based on 40 pairs of readings for each specimen); S = slow ($P < 0.001$; as above); *Note:* (a) Failure of MS RBC to respond to 1.0 μg/ml PGE_2, while non-MS cells show marked increase in speed; (b) Prolonged range of dilutions of PGE_2 at which slowing is effective in MS; (c) Abrupt change to slowing at about 3125 pg/ml PGE_2 in the case of MS RBC

0.0005	0.00025	0.000125	0.0000625	0.00003125	0.000015625
	CON: 1.004 ± 0.027	CON: 1.004 ± 0.027	CON: 1.004 ± 0.027		
1.124 ± 0.035	1.053 ± 0.032	1.060 ± 0.037	1.077 ± 0.023	1.001 ± 0.030	1.037 ± 0.023
	CON: 1.000 ± 0.028	CON: 1.000 ± 0.028			
1.130 ± 0.030	1.060 ± 0.037	1.051 ± 0.037	1.124 ± 0.024	0.999 ± 0.028	1.033 ± 0.020
1.045 ± 0.025	1.037 ± 0.021	1.040 ± 0.025	1.038 ± 0.029	1.143 ± 0.044	1.126 ± 0.027
1.046 ± 0.027	1.042 ± 0.032	1.040 ± 0.030	1.040 ± 0.025	1.130 ± 0.028	
	CON: 1.023 ± 0.030				
1.099 ± 0.029	1.026 ± 0.019	1.031 ± 0.027	1.037 ± 0.027	1.091 ± 0.040	
CON: 1.003 ± 0.027	CON: 1.003 ± 0.027				
1.015 ± 0.029	1.011 ± 0.025	1.033 ± 0.025	1.042 ± 0.025	1.096 ± 0.040	

of the original MEM-LAD test but obviates the need for a colony of healthy guinea pigs, which many workers have found difficulty in achieving [33], and so makes the method available to hospitals not equipped with an independent animal house. In particular, it enables sensitization to encephalitogenic factor (EF) to be determined (thus establishing the presence or absence of parenchymatous brain destruction). A few typical results are shown in Table 4. A remarkable feature of the TEEM-LAD test is that it does not pick out "anomalous" (halfway) relatives but only full MS subjects, while LA causes practically 100% reduction.

Discussion

The study of LA and AA activity in these tests arose from the report by Millar et al. [38] of the beneficial effect of sunflower seed oil (active principle LA) in reducing the number and severity of attacks of MS over a 2-year double blind trial. It was thought at first that LA and AA might act simply as immunosuppressives, but it is now known that this is not so [10, 34, 37]. When gamma linolenate administration is prolonged beyond the point (usually 7–9 months) at which the positive MS result

Table 4. A few examples of the use of the TEEM-LAD Test

	Sex	Age	% EF	% Thyroid	% PPD	% BSA	% LA +THY	% LA +PPD	% LA +THY	% LA +PPD
MS patients										
I. N. (Dutch)	F	33	24.07	26.13	22.33	−0.20	1.62	1.52	93.80	93.19
D. L.	M	18	14.60	18.23	17.53	0.74	1.05	0.07	94.24	99.60
L. L.	F	51	15.99	12.71	17.15	−0.10	−1.77	−1.68	113.93	109.80
D. J. W.	M	32	19.12	22.70	21.10	−3.27	1.43	0.83	93.70	96.07
J. H.	M	45		17.98	17.27	0.97	−0.20	0.63	101.11	96.35
H. B.	F	2	10.22	19.21	17.80	0.60	−0.29	1.09	101.51	93.88
ONDs										
S. K.	F	46		21.07	24.46	1.08	8.56	10.31	59.37	57.85
D. McI.	M	36	18.60	18.31	16.88	0.66	8.26	8.21	54.89	51.36
C. H.	F	60		18.78	19.84	0.37	7.60	8.17	59.53	58.82
G. D.	M	41	19.48	16.54	14.03	1.02	8.20	7.71	50.42	45.05
B. D.	M	41	20.48	18.12	16.33	0.71	8.64	8.45	52.32	48.25
M. G.	F	50		17.58	18.07	1.16	7.56	7.14	57.00	60.49
Normals										
D. N. (Dutch)	M	5	21.99	21.90	0.63	9.94	10.38	54.80	52.60	
J. J.	F	52	19.19	16.17	2.75	11.78	10.70	38.61	33.83	
E. J. F.	M	62	22.81	21.79	0.77	12.31	12.33	46.03	43.41	
E. G.	F	10	22.12	19.43	0.00	10.53	10.63	52.40	45.29	
D. D.	M	47	19.79	19.08	0.36	9.15	8.32	53.76	56.39	
G. J.	F	46	21.80	18.47	−0.71	8.67	8.92	60.23	51.71	

gives way to a negative (normal) one, the TEEM-LAD test shows that LA and AA now produce the same result (50–60% suppression) as in normal and OND patients, and no longer the 90–100% suppression which characterizes MS. Gamma linolenate came into consideration after it was shown that its effect in vitro in suppressing lymphocyte-antigen interaction was greater (on a weight for weight basis) than that of LA [16]. *If* it does in the body what it does in the test tube, it should therefore be more effective, as well as pleasanter to take, and more easily controlled than sunflower seed oil with respect to dosage of active material. Final proof must, of course, await clinical trial on a double blind basis of active ambulant cases of MS showing recurring episodes.

When gamma linolenate is administered beyond 6–8 months, the typical slowing with LA or AA is reversed, first with AA and then, a month or so later, with LA [10, 14] and the normal responses are thereafter maintained (Table 5). After about 21 months (and, curiously, *not* before) the PGE_2 reaction of MS is converted into the normal response. If medication is discontinued, the PGE_2 reaction is maintained many more months than that of gamma linolenate. A curious finding is that when gamma linolenate is continued for 2–3 years or more, the RBC become extremely sensitive to PGE_2 travelling faster in the presence of even 1.95 pg/ml (and occasionally even 0.975 pg/ml) – a phenomenon which might incidentally be developed as a very sensitive assay for PGE_2 [Field and Joyce 1978, unpublished]. Very

Table 5. Effect of gamma linolenate administration

Date	CON	SD	LA	SD	AA	SD	% change	
							LA	AA
G. H. (female) age 29								
24. 4. 76	1.113	±0.037	1.073	±0.036	1.073	±0.034	−3.64	−3.63
4. 5. 76	1.101	±0.034	1.058	±0.035	1.068	±0.032	−3.86	−3.02
18. 5. 76	1.108	±0.036	1.074	±0.040	1.075	±0.035	−3.04	−2.95
8. 6. 76	1.172	±0.035	1.117	±0.037	1.166	±0.030	−4.65	−0.52
28. 6. 76	1.144	±0.030	1.091	±0.029	1.162	±0.029	−4.64	+1.59
26. 7. 76	0.902	±0.024	0.879	±0.030	0.970	±0.041	−2.65	+7.45
6. 9. 76	0.980	±0.031	0.994	±0.034	1.024	±0.032	+1.41	+4.50
4. 10. 76	0.970	±0.041	1.036	±0.043	1.043	±0.037	+6.78	+7.49
8. 12. 76	1.002	±0.024	1.042	±0.035	1.047	±0.035	+4.03	+4.47
5. 1. 77 [a]	1.018	±0.026	1.072	±0.027	1.120	±0.043	+5.33	+10.03
5. 5. 77	0.998	±0.027	1.063	±0.022	1.062	±0.021	+6.52	+6.39
P. P. (female) age 24								
24. 6. 76	1.174	±0.034	1.124	±0.030	1.128	±0.027	−4.28	−3.88
28. 7. 76	0.935	±0.024	0.900	±0.025	0.909	±0.025	−3.80	−2.77
15. 9. 76	0.983	±0.023	0.951	±0.023	1.004	±0.034	−3.31	+2.15
25. 10. 76	0.978	±0.030	0.951	±0.040	0.991	±0.037	−2.76	+1.31
29. 11. 76	1.004	±0.031	1.031	±0.034	1.025	±0.028	+2.65	+2.12
10. 1. 77	1.025	±0.023	1.079	±0.037	1.095	±0.030	+5.27	+6.83
10. 2. 77	1.071	±0.032	1.117	±0.028	1.130	±0.020	+4.33	+5.49
30. 2. 77	0.987	±0.031	1.085	±0.029	1.111	±0.030	+10.00	+12.64
M. D. (female) age 29								
27. 9. 76	0.987	±0.035	0.950	±0.035	0.942	±0.030	−3.7	−4.56
15. 11. 76	0.996	±0.035	0.944	±0.038	0.919	±0.033	−5.22	−7.7
20. 12. 76	1.168	±0.044	1.125	±0.041	1.176	±0.045	−3.68	+0.6
7. 2. 77	1.019	±0.027	0.979	±0.027	1.079	±0.030	−3.9	+5.88
29. 3. 77	0.976	±0.030	1.043	±0.031	1.050	±0.028	+6.86	+7.58
20. 5. 77	1.020	±0.017	1.072	±0.016	1.083	±0.019	+5.09	+6.18
J. G. (female) age 30								
7. 7. 76	1.201	±0.043	1.161	±0.036	1.163	±0.032	−3.29	−3.16
19. 8. 76	0.976	±0.031	0.922	±0.023	0.952	±0.026	−5.46	−2.37
27. 9. 76	0.982	±0.024	0.965	±0.026	0.988	±0.029	−1.77	+0.66
14. 11. 76	0.998	±0.025	0.936	±0.031	0.994	±0.030	−6.20	−0.37
20. 12. 76	1.144	±0.029	1.195	±0.029	1.228	±0.027	+4.47	+7.31
7. 2. 77	1.026	±0.022	1.074	±0.024	1.101	±0.025	+4.65	+7.27
28. 3. 77	0.977	±0.030	1.056	±0.034	1.079	±0.027	+8.04	+10.40
20. 5. 77	1.023	±0.021	1.075	±0.020	1.079	±0.020	+5.13	+5.52

[a] Note the retardation produced by 0.08 mg/ml LA and AA. After some months' treatment with γ-linolenate all begin to show a reversion to the normal type of response (i.e. increased speed with these acids) beginning first with AA and followed a month or two later by LA so that ultimately they give full normal response. All figures are significant at $p < 0.001$, except around about the transition stage, for example, the AA reading for G. H. on 8. 6. 76 or the AA reading for M. D. on 20. 12. 76
On 5. 1. 77 capsules reduced to 1 tds

few people (some three out of several hundreds tested) have been refractory in some degree to the usual action of gamma linolenate in altering MS response to LA and AA, taking 1 year, 20 months and, on one occasion, more than 2 years to "convert" in the E-UFA test. The mechanism of this phenomenon remain to be studied, but the practical consequence is that we now test all "MS" subjects before they begin therapy (partly to assure diagnosis) and again at 6–8 months to make sure they are responders. Clearly, nonresponders should not go into a clinical trial. Moreover, if a patient presents, as is now unfortunately not uncommon, some 12 months or so after beginning to take Naudicelle (and sometimes with a dubious diagnosis in the first place), it is still possible despite the normal E-UFA result, to establish the original diagnosis because the PGE_2 still remains that of an MS subject.

It would appear that LA or gamma linolenate alter the abnormal constitution of all cell surface membranes in MS (e.g., lymphocytes RBC) to normality. If this alteration is a general phenomenon, then it would also affect the oligodendrocytes. Direct chemical evidence for the lymphocyte is provided by Tsang et al. [48], and indirectly by the MLR [18, 29, 30]. If Thompson's suggestion [46] indeed extends to *all* cells in the body, and all cell surface membranes in MS are abnormally constituted – either biochemically or biophysically (if the distinction can indeed be made in the complexity of the membrane structure), then the oligodendrocyte surface, too, would be included. And it is from this surface that the myelin sheath is made (with the addition of protein – probably secreted by the parent nerve cell). Hence myelin produced by a child with such an inborn anomaly (up to the age of 16 years, with slow turnover thereafter) might be expected to differ from normal and this, indeed, was found years ago [2, 21, 24]. Apparently normal myelin away from MS lesions is deficient in UFA. There are criticisms of these findings, chiefly on the grounds (very true) that it is most difficult (especially before formalin fixation) to recognize the limitations of lesions (and many are microscopic). However, the balance [47] would appear to suggest that the difference is real.

Thompson's suggestion that MS develops against a background of an inborn mishandling of UFA is borne out by MS family studies with the MEM-LAD and E-UFA tests [17, 20, 28]. The partial anomaly (LA – slow; AA – fast) is predominantly found in females of MS families, which serves to underline that *it* and it alone, is not sufficient to produce MS. Nor do we indeed know that the *full* "Thompson's anomaly", leading to slow LA and slow AA in the E-UFA test, is *in itself* sufficient to lead inevitably to MS. Probably it represents a prepared soil on which MS may develop, and several possibilities spring to mind:

1. The abnormally constituted myelin may simply not stand up to "wear and tear", i.e., may undergo patchy, perivascular "abiotrophy". It is fascinating to speculate that the absence of certain reactive sites (as shown by the low MLR) on an oligodendrocyte surface may lead to failure of the wrapped surface which makes up myelin to bind securely (zip fastener effect), and indeed in the early stages of myelin breakdown, as seen electron microscopically, there is a loosening of myelin lamellae.
2. It may be more susceptible to the experimental allergic encephalomyelitis (EAE) process – an idea supported by the work of Clausen and Møller [6] on the susceptibility of young rats to EAE when LA in the diet is altered. The neonatal rat

brain is very immature, so that feeding with UFA can alter its myelin composition.

3. It may constitute a more suitable substratum for the establishment of a "slow" infection by a banal virus (like measles), either *ab initio* or as a sequel to an attack, or (less likely) by some specific MS virus.
4. It may be more easily damaged as an innocent bystander when *any* allergic process goes on in the nervous system [22, 50].

Forward Program

The above argument provides important pointers for the prophylactic handling of MS – the real function of a National Health (rather than Sickness) Service. We know [16] that the composition of brain lipids may be altered (in young rodents) by feeding the appropriate UFA. If the same holds good for the human, and we convert the oligodendrocyte cell surface to normal, then *normal* myelin of a type *non-susceptible to the MS process* will be laid down, whatever its cause(s) may ultimately turn out to be. Fortunately, we can determine the inborn mishandling of UFA in very young children, and it would seem that screening of all children at risk (those born into MS families where clinical incidence in near relatives is 5–20 times that in the general population) should be carried out. We have already gathered full MS results in three children in this country, out of 130 examined, and others in the GDR [20]. A properly planned campaign and wise spending of money may well lead to the virtual elimination of MS in a generation, before its cause(s) is known – a state of affairs not unknown in other branches of medicine.

The geographic distribution of MS with its preponderance in 40–60° N and S latitudes is a long established "fact" in MS, though its simplicity may be deceptive [31]. Many correlates with the distribution of MS have been established, and some are difficult to accord biological significance. However, among the putative geographical predisposing factors (GPF) has been the consumption of milk [1] and this immediately links with the LA and AA considerations outlined above. Twomey [49] dismisses a correlation with milk consumption on the grounds that "MS is extraordinarily rare among Africans living on their own continent, though milk is an important food in many parts of Africa". Dean [8], like Twomey [49], fails to appreciate the critical importance of adequate linoleic acid intake by the child *at the critical time when myelination is most intense*, i.e., up to about 5 years of age and thereafter more slowly to 16 years of age and even beyond [50]. It is immaterial that the "Afrikanders of South Africa ... eat ... a diet ... which has a quite unusually high level of saturated fat – mutton three times a day ... and yet they don't get or very seldom get multiple sclerosis" [8]. Breast milk is especially rich in fatty acids of the long C chain (which are "essential"). Thus Bentivoglio [3] shows (Table 1, p 246) that linoleic acid (which he considers "really indispensible") makes up 8.3% (6.8–10.4%) of human milk fat against 1.6% (0.5–2.8%) cow's milk fat. Linoleic acid is also richer in human milk. In a fuller discussion of the EFA content of human milk, Insull and Ahrens [23] conclude that it is approximately seven times that found in cow's milk. The question is, how long does linoleic acid-rich breast feeding

go on among those groups which appear to have a low incidence of MS and for how long is it maintained in our "advanced" societies? In tropical areas in general and amongst "primitive" people, breast feeding tends to be prolonged sometimes for years. This will ensure an adequate linoleic acid supply over the critical period and hence good laying down of myelin. Thereafter, the intake of saturated animal fat may well be immaterial. It is precisely at the time when myelination is going on most vigorously, and it needs it most, that the 40–60° latitude child is deprived of adequate breast milk. If the child is borne with "Thompson's anomaly", then conditions must be especially adverse to the laying down of normal myelin. Once the crucial period is over, consumption of cow's milk becomes much less important. In some ways there may be a resemblance to the critical protein needs for proper brain development in the neonate, worked out by Dobbing.

Furthermore, the content of polyunsaturated fatty acids (PUFA) in the infant's food is a direct reflection of that of the mother's intake. There is also some evidence (Cash and Berger 1969) that cow's milk may contain specific inhibitors of the conversion of linoleic to arachidonic acid.

The naturally useful cis forms of EFA may well be converted into trans-forms in certain types of milk food processing. Clearly, as Dick [9] points out, the whole question of infant feeding in relation to propensity to develop MS needs prospective study.

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Discussion

C. S. Raine: Dr. Ute Traugott in my group, New York, has done a study on early T-cell rosettes longitudinally in 23 MS patients. Generally, the level of early T-cell rosettes (if this means anything) is lower throughout the course of MS, whether it is stable, in remission, or acute, than in the control population. The only really interesting thing which came out of this study was that during exacerbations there was a great statistically significant decrease in the level of early T cells. At the same time, we tested for MBP sensitization. If this means anything, this increased during exacerbations. I just thought, you might be interested in these results.

Distribution of Lymphocyte Subpopulations in the Peripheral Blood of MS Patients Under Corticotrophin Therapy

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Introduction

After Fog [10 a] and Alexander et al. [1] had published a number of papers on their clinical studies of corticotrophin (ACTH) action in multiple sclerosis (MS) patients, this therapy was widely applied in the USA and Western Europe. Numerous authors modified the original proposals and worked out dosage schedules of their own. In 1970 the results of a carefully planned multicentric investigation on the therapeutic value of ACTH in MS were published [24], which largely confirmed the existence of a beneficial effect of the drug. Discouraging results were obtained in studies of long-term treatment with corticotrophin [19] and in groups of patients in a chronic stage of the disease [4, 6, 23]. What remained was the rather uncontroversial conviction that ACTH is of considerable value in the treatment of acute exacerbations of MS [4, 6, 20, 23] as well as in the treatment of acute retrobulbar neuritis [22].

As cell-mediated immunity is supposed to play a major role in the pathogenesis of MS, we felt we should try to find out what happened immunologically in our MS patients with relapsing or relapsing and progressive courses of the disease who were admitted to the hospital in acute bouts and treated with corticotrophin according to the dosage schedule described below. As a first approach, we decided to follow up the distribution of the lymphocyte subpopulations in the peripheral blood of these patients before and during therapy, which, to our knowledge, had not been done previously in a similar group of MS patients.

Materials and Methods

Patients and Treatment

Twenty-two patients (17 women and 5 men) with clinically definite or probable MS (criteria given by Poser [21]) were included in the study. Three of them had had

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Table 1. Clinical and laboratory findings of the patients concerned

No.	Age	Sex	Duration of illness (years)	Adrenocortical response to ACTH [b]	Typical T-lymphocyte reaction	Clinical improvement during therapy [d]
1	56	F	12 9/12	14.1/46.5	+	–
2	30	M	9 3/12	11.8/33.0	+	–
3	21	F	1/12 [a]	8.2/14.5	+	+
4	24	M	2 4/12	16.8/45.6	+	+
5	24	M	1 8/12	20.1/56.1	+	–
6	33	F	1 2/12	14.4/44.0	+	+
7	46	F	5 11/12	19.2/75.2	+	–
8	21	F	1/12 [a]	30.0/61.6 [c]	+	+
9	25	F	3/12	11.6/22.4	+	+
10	23	F	2/12	30.4/56.0 [c]	+	+
11	30	F	9/12	9.4/28.0	+	+
12	39	F	6 4/12	not tested	+	+
13	20	F	3 5/12	18.4/53.6	+	+
14	29	M	8 10/12	13.1/48.6	+	–
15	39	M	6 5/12	32.0/59.0 [c]	+	+
16	38	F	16 8/12	4.8/34.4	+	+
17	28	F	1/12 [a]	33.6/86.4 [c]	+	+
18	20	F	2/12	10.7/40.9	+	+
19	53	F	5 9/12	12.3/41.1	+	–
20	38	F	8 3/12	9.0/24.2	+	–
21	34	F	13 4/12	21.6/21.8	–	–
22	33	F	7/12	37.2/70.4 [c]	–	+

[a] Patients with first bouts
[b] Plasma cortisol levels (μg/100 ml) before and after stimulation
[c] Cases with elevated plasma cortisol levels before stimulation
[d] Judged by clinical impression of the physician caring for the patient

their first bouts and were probable MS cases (Table 1). After an acute bout had been diagnosed based on our definition [21], the adrenocortical response of the patient to an i.v. dose of 0.25 mg ACTH (Synacthen, Ciba-Geigy) was tested as described below. Then we started the ACTH treatment which regularly consisted of daily i.v. injections of 1.0 mg Synacthen for a period of 28 days. The whole dose was administered between 8:00 and 8:30 a.m.

Hormone Determinations

Quantitative determinations of plasma cortisol were done applying a protein binding method described by Köbberling and Mühlen [17]. To test the adrenocortical response of the patients, plasma cortisol concentrations were determined before and 2 h after i.v. injection of 0.25 mg of ACTH between 8:00 and 8:30 a.m. Normal values for the plasma cortisol concentration at 8:00 a.m. ranged from 6.5 to 23.3 μg/100 ml. An increase of at least 10 μg/100 ml within 2 h after stimulation was considered to be normal [Köbberling, personal communication].

Counting, Separation, and Differentiation of White Blood Cells

Before and during therapy, 15-ml blood samples were taken from the patients at defined intervals by means of heparinized disposable syringes. To rule out a possible influence of food intake and circadian variations on some of the cell populations, all blood samples were taken before the patients had breakfast (at about 7:30 a.m.). Total leukocyte counts were made in a Fuchs-Rosenthal counting chamber. The percentage of mononuclear cells was determined on 200 white cells of a differential smear. The major part of each blood sample was used for the isolation of the lymphocyte fraction on a Ficoll-Ronpacon gradient according to Bøyum [5]. T-lymphocytes were counted as E-rosettes using the method of Jondal et al. [14], and the monocyte and B-cell subpopulations were determined applying the peroxidase method described by Kaschka-Dierich et al. [15]. The proportion of null-lymphocytes was calculated as the difference between 100% and the sum of the percentages of the other subpopulations. Based on the methods used we cannot exclude that the lymphocyte subpopulation characterized as null-cells contains a certain amount of immature T-cells which are known to form rather unstable E-rosettes [12].

For clinical reasons, it was not always possible to keep the correct intervals between blood samplings. Thus additional statistical operations were necessary.

Statistical Calculations

Although we had planned to utilize a multivariate one-factor randomized block design, after collection of the data there were a number of observations missing, for reasons not due to the treatment. Minimizing the portion of missing values in some balanced manner, seven blocks (= patients) and six treatment levels (= days) were extracted and the missing values estimated according to the method of Yates [27]. For each of the five cell populations an analysis of variance (ANOVA) was calculated separately, thereby diminishing the degrees of freedom for the residual according to the number of estimated values [16]. To guarantee an overall probability of error of not more than 5%, each test was executed at the 1% level [18]. In cases of significant differences in treatment levels, a simultaneous test procedure (STP) including all pairs of treatment levels according to Gabriel [11] was performed. This had the effect of maintaining the test level. Because the values for T- and B-lymphocytes as well as monocytes were relative proportions, an arc-sin transformation was executed for these data before they were analyzed in the ANOVA [16].

Results

Nineteen MS patients out of 22 responded normally to corticotrophin (Table 1), which accords well with previous results [2, 26]. Only one patient showed no reaction to ACTH (no. 21 in Table 1). Another patient (no. 22 in Table 1) had an exceedingly high plasma cortisol level before stimulation and responded normally to ACTH. It is surmised that stress factors of unknown origin exerted a stimulating effect on

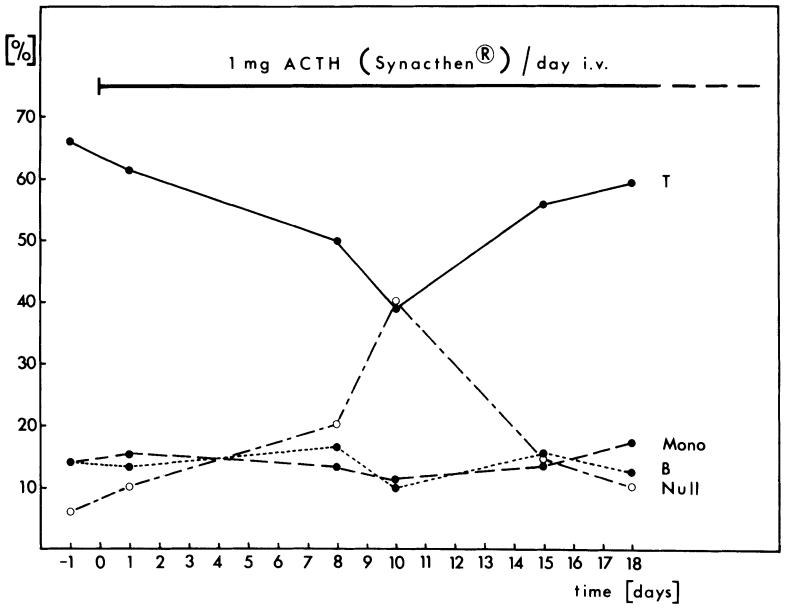

Fig. 1. Mean values of T- and B-lymphocytes, monocytes, and null-cells under treatment

Table 2. Outcome of the analysis of variance (ANOVA)

Cell population	F [a]
Leukocytes	1.90
Lymphocytes	1.94
% T-cells	27.85 [b]
% B-cells	2.30
% monocytes	0.58

[a] To compare with the 99% critical value of the F distribution with 5 and 20 degrees of freedom ($F_{5,20;\,.99}=4.10$)

[b] Significant

Table 3. Treatment level means of the different cell populations

Cell population	– 1	1	8	10	15	18
Leukocytes/mm³	6714	6329	8086	6643	7700	7142
Lymphocytes/mm³	2043	2114	1871	2286	2714	2157
% T-cells	66.2	61.2	50.1	38.8	56.1	59.4
% B-cells	14.2	13.3	16.6	10.1	15.8	12.8
% monocytes	13.7	15.2	13.3	11.1	13.4	17.4

the adrenocortical function in this case. These two patients with extreme functional states of their adrenal glands were excluded from the study.

The outcome of the statistical analysis of our data is presented in Tables 2 to 4 and in Fig. 1.

In the course of ACTH therapy, usually in the second week of treatment, we observed a transitory decrease in the percentage of E-rosette forming lymphocytes, with a concomitant increase in the percentage of null cells. By the end of the third week of treatment, there was no difference in comparison with the initial values.

Table 4. Significant differences of treatment level means for the T-lymphocytes

	−1	1	8	10	15	18
−1			*	*		
1				*		
8	*					
10	*	*			*	*
15				*		
18				*		

Discussion

As yet very few follow-up studies of lymphocyte subpopulations of patients undergoing ACTH or corticosteroid therapy have been performed [8, 13, 25]; to our knowledge none have been performed on MS patients. It could be demonstrated that ACTH not only affects the circadian changes [8], but also the long-term levels of some lymphocyte subpopulations [26]. Depending on the degree of glucocorticosteroid-resistance of the species examined, several mechanisms have been suggested to be responsible for glucocorticosteroid action on lymphoid cells [7]. In our study the transient drop in the T-lymphocyte proportion might be due to a corticosteroid-induced inhibition of cellular metabolism. If this is correct, the transient increase in a subpopulation characterized as null-cells (which might contain a certain amount of immature T-cells) might be explained by the assumption that their metabolism is less differentiated and therefore less sensitive to corticosteroids. The following increase of the T-cell subpopulation to a normal percentage, which was shown to be accompanied by a decrease of the null-cell population, might reflect some kind of adaptation, either merely numerical or metabolic. Based on studies of the short-term effects of glucocorticosteroids on lymphocyte subpopulations, Fauci et al. [9, 10] concluded that the principal mechanism of these effects is a redistribution of circulating lymphocytes to the bone marrow. Similar studies have been published by Cooper et al. [8] and Yu et al. [28], also based on short-term observations of lymphocyte subpopulations. In fact, different mechanisms of corticosteroid action might be responsible for short-term and long-term effects on lymphoid cells, redistribution and cell sequestration preferentially being involved in short-term effects and inhibition of cellular metabolism in long-term effects.

The quantitative effect of ACTH and corticosteroids on lymphocyte subpopulations is accompanied by changes in lymphocyte functions [3, 8, 9], which may be due to selective alterations in the circulation patterns of certain lymphocyte subpopulations [3].

Our study does not allow any conclusions as to whether the pathogenic mechanism of MS is affected by ACTH or whether the effect on certain lymphocyte subpopulations is a prerequisite for the therapeutic effect of glucocorticosteroids in this disease.

Summary

An analysis of white blood cell populations in patients undergoing ACTH therapy is presented which was performed by following up 22 subjects who had been admitted to the hospital with acute bouts of MS. From our observations we conclude that in the dosage schedule described, corticotrophin does neither affect the total leukocyte and lymphocyte counts nor the percentages of B lymphocytes and monocytes. We found a transitory decrease in the percentage of E-rosette forming lymphocytes (T-cells) which was accompanied by a transitory increase in the percentage of null-cells. Several mechanisms of these changes are discussed in relation to their possible significance for therapeutic success in MS.

Acknowledgments. The skilful technical assistance of Mrs. Sieglinde Dalloul is gratefully acknowledged.

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Cytological Findings in Cerebrospinal Fluid as Criteria of Process Activity in Multiple Sclerosis

J. SAYK, R.-M. OLISCHER, and R. LEHMITZ [1]

The immunocompetent cell activity in cerebrospinal fluid in MS depends on immune biological process activity. Modifications of humoral and cell permeability can arise from the ependymal and endothelial barrier surfaces. The characteristic cells of process activity in MS are the small lymphocytes, the T and B cells, which have an increased number of ribosomes and basophilia of the cytoplasm, tendency to transformation, and an increase in the nucleoli number (Fig. 1 a).

Immunocompetent plasma cells (Fig. 1 b) with their characteristically changed nucleus versus plasma-relation may be mono- or polynuclear. Increased nuclear and plasma activity of the binuclear cells may be characterized by numerous small vacuoles, indicating synthesis of gamma-globulin. Plasma cells predominate in the initial stages of multiple sclerosis (MS), in later stages of the disease they are rare or completely missing. A correlation between the clinical process and plasma cell activity is not evident.

The blastlike cells (Fig. 2), which are also found in the CSF of MS patients (1), are the most interesting cells, remarkable for the size of their nuclei, several nucleoli, and pronounced pyroninophilia of the cytoplasm. They are found in 15% of MS cases, first described by Voss (2) as a manifestation of the earliest stages and

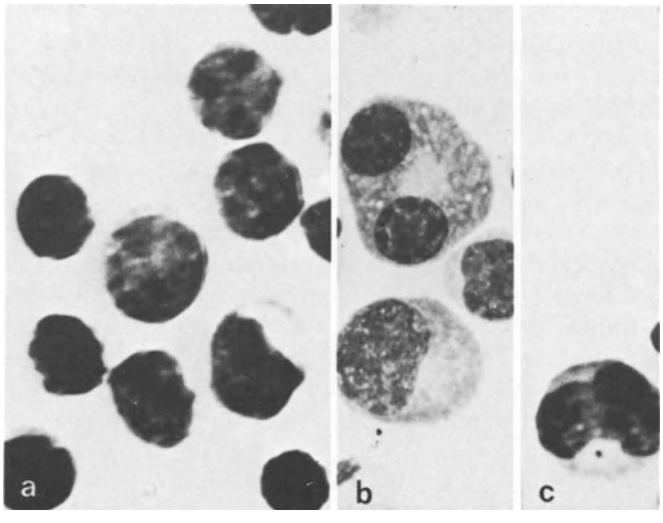

Fig. 1. Cells in the CSF in multiple sclerosis. **a** Large and small (B and T) lymphocytes. **b** Mono- and binuclear plasma cells. **c** Monocyte with perinuclear vacuoles

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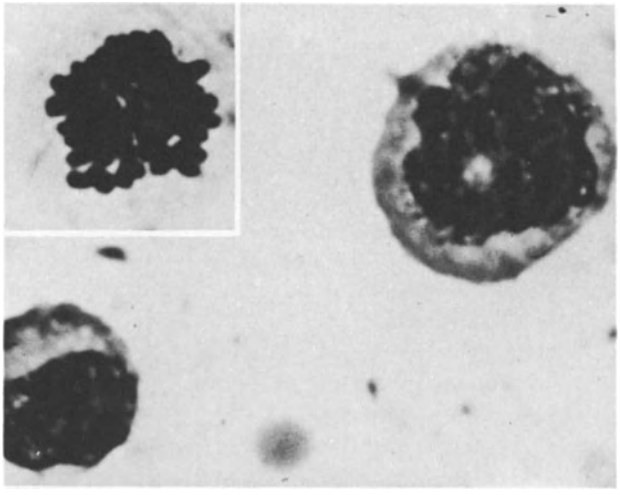

Fig. 2. CSF-cell activity in multiple sclerosis. Blastlike cells in mitosis

exacerbations of MS. They may be due to increased proliferation of lymphatic cells due to a slow virus reaction.

Monocytes thus far have received little attention, although they are increased in 42% of early stages of MS. They show signs of cell activation, cytoplasmic inclusions, increased granulation, and perinuclear vacuoles (Fig. 1 c). Their number decreases in the course of the disease. Considering their function in the reaction with antigens, it is apparent that the immune activity of monocytes must be most pronounced in the early stages of the disease.

The identification of cytological criteria of process activity in CSF depends on the quality of the technical methods and the exact evaluation of results. With the sedimentation chamber method the morphological features of cells are very well preserved, however, there is a loss of 30% of cells, especially of small lymphocytes, leading to an alteration of relative differential cell values. Turbulences at the base of the chamber produce slight cell alterations which make the identification of surface activity and interpretation of cytochemical findings more difficult.

Figure 3 shows the cytological findings in 20 cases with relapses and remissions, degree of disability I to V (ambulatory to bed-ridden), over a period of 6 years. They are selected cases with the appearance of blastlike cells in the course of their disease. Six of these patients died due to complications. In all cases lumbar puncture was done 1–4 times annually. There is a correlation between cellular findings and the degree of disability. All patients had received glucocorticoids, some azathioprine and cyclophosphamide.

1. During the clinical remissions there was no cytological correlate in the initial stages of MS. There was a fairly uniform value of 35–40% monocytes during the 1st year of the disease. A slight increase correlates with relapses and onset of paralysis. In advanced stages with slight progression, and hardly any indication of bouts, the values decreased. This may indicate a mediator function of monocytes. The perinuclear and endoplasmic activation, increase of granular, perinuclear vacuoles ap-

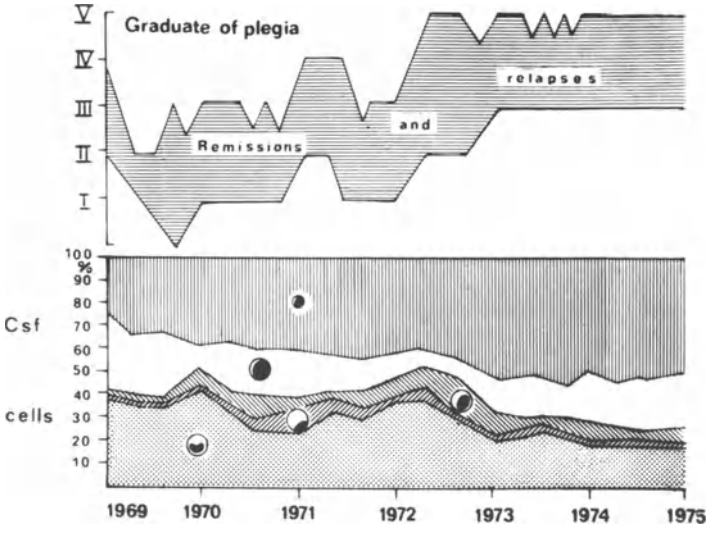

Fig. 3. Degrees of clinical paralysis and CSF-cell findings in 20 patients with multiple sclerosis. Long-term observation

pear to support this assumption and the possibility of cytokine production, which may lead to membrane damage and demyelination.

2. The blastlike cells show a similar curve, with values almost paralleling those of the monocytes, however, the number of cells is less, with values of 5–15%. The increase of blastlike cells in the early stage of MS with severe bouts and rapid progression is a phenomenon deserving greater attention.

3. In the 20 cases immunocompetent plasma cells show the highest values of about 10% before the onset of severe paralytic bouts. We found no correlation between plasma cell and IgG values. It appears that "process activity" precedes the clinical manifestations of bouts.

4. The small T-lymphocytes, of special interest in MS, are hard to differentiate from B cells in CSF. For this purpose we have developed a special rosette test. In the course of MS there is an increase of small lymphocytes, with no appreciable alterations with respect to the large lymphocytes.

In our attempts to improve cytological techniques, after years of development we have now constructed the suction chamber (Fig. 4). The principle of construction is very simple, it consists of a ceramic shell with a glass slide attached. The basic principle is the spontaneous sedimentation of cells in 30–35 min within the range of so-called sedimentation constants. After 35 min a "sorption-stop" with which the inner wall of the chamber is coated previous to use, dissolves. This exposes the porous surface of the chamber (porosity of the ceramic material "porolyte" = 0.5–1 μm). The cell-free CSF is now filtered through the chamber walls in 30 min. The bottom of the chamber, the slide surface, on which the cells have sedimented, remains undisturbed. Loss of cells and distortion by turbulences are eliminated by this procedure.

A particular advantage of this method is that it has now been possible to construct an apparatus for sedimentation-cell electrophoresis. This also is an astonish-

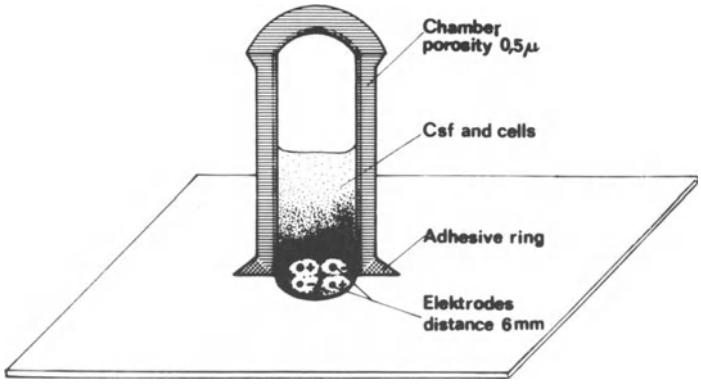

Fig. 4. Suction chamber: sedimentation cell, electrophoresis, arrangement of electrodes at the base of the chamber

ingly simple construction. The electrodes are inserted at the bottom of the suction chamber. The chief problem was arrangement of the electrode channels in the slide and the electrode carrier. The current applied varies from 0.1–0.5 mA and depends on the sedimentation diameter and electrode interspace. This was held constant at 6 mm in a square arrangement.

Since the apparatus was completed only 2 months ago, results are still limited. In two of nine MS cases examined, four to seven small T-lymphocytes were grouped in a rosettelike fashion around 1–2 monocytes close to the cathode. In a case of tubercular meningitis, rows of lymphocytes showed linear arrangement around 1–2 lymphoid cells between anode and cathode, close to the cathode (Fig. 5 a). In two

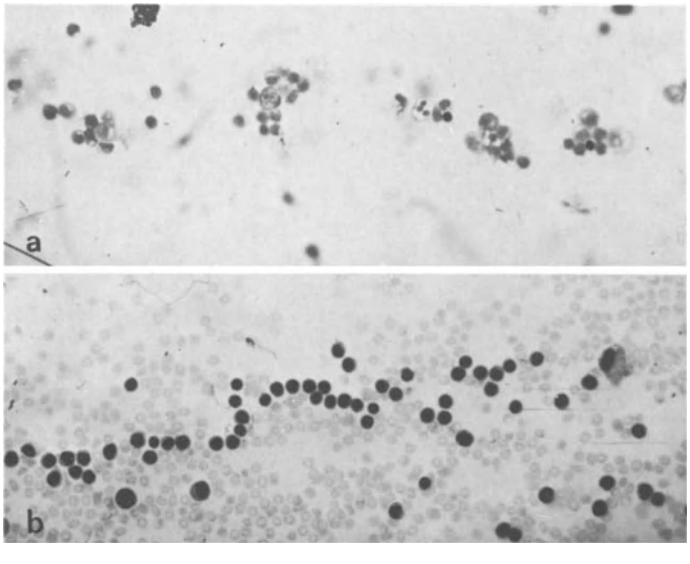

Fig. 5. Sedimentation cell electrophoresis. **a** Findings in tubercular meningitis. **b** Findings in subarachnoid hemorrhage

cases of subarachnoid hemorrhage findings were inconspicuous; the monocytes and macrophages were scattered in an indifferent fashion within the electrostatic field. In a case of subarachnoid hemorrhage without evidence of an aneurysm, in which we suspected an allergic-hyperergic hemorrhage, the findings were remarkable (Fig. 5 b): the small lymphocytes were arranged in rows, while the large lymphocytes, monocytes, and macrophages presented no indication of electrostatically induced surface activity. It thus seems possible, that in cells spontaneously sedimented in an appropriate electrostatic field, surface activity of varying nature can be elicited. This may be a useful indicator of cell activity in MS, perhaps of cell-bound mediator functions in the complicated chain of events leading to demyelination.

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Discussion

D. Dommasch: I wish to corroberate Dr. Sayk's statement about blastlike cells in CSF in MS in a study done together with Dr. Schulz and Dr. Grüninger (Würzburg) using ^{3}H-thymidine autoradiography. We found an increased proliferation of mononuclear CSF cells during exacerbations with labelling indices around 2%. These labelling indices dropped when the exacerbation was over.

Multiple Sclerosis: Presence of Lymphatic Capillaries and Lymphoid Tissue in the Brain and Spinal Cord

J. W. PRINEAS [1]

The persistence of perivascular inflammatory cell cuffs which are rich in plasma cells in some old MS plaques suggests that the putative MS antigen may be continuously expressed in such lesions [1, 2, 4]. This report describes recent findings [5] concerning the manner in which these cells are organized in plaque perivascular spaces.

Materials and Methods

Tissue fixed for electron microscopy by in situ brain perfusion was obtained from two MS patients, a patient with motor neuron disease, and a patient with adrenoleukodystrophy. Immersion-fixed CNS biopsy tissue obtained from a third MS patient was also studied.

Results

In each of the five cases, light and electron microscopy revealed that perivascular spaces in histologically normal CNS tissue frequently contained lymphocytes and macrophages. Perivascular plasma cells were also common in unaffected tissue in two of the MS cases. It was commonly observed that these lymphocytes and macrophages were not free in the perivascular space but were confined within thin-walled channels. However, plasma cells, when present, were always situated outside these channels.

The cytoplasmic processes of the flattened cells that formed the channel walls were extremely thin, often measuring less than 0.1 μm in thickness. Ultrastructurally the channels were indistinguishable from lymphatic capillaries in other tissues, both with respect to their contents (lymphocytes and macrophages) and to their fine structure, which included the presence of numerous hemidesmosomes on their abluminal surface [6].

Perivascular spaces in the MS plaques that were studied revealed similar thin-walled channels containing lymphocytes and macrophages (Fig. 1). However, the channels in this location tended to be larger, more numerous, and more irregular in shape than those observed in normal white matter, and they were separated from

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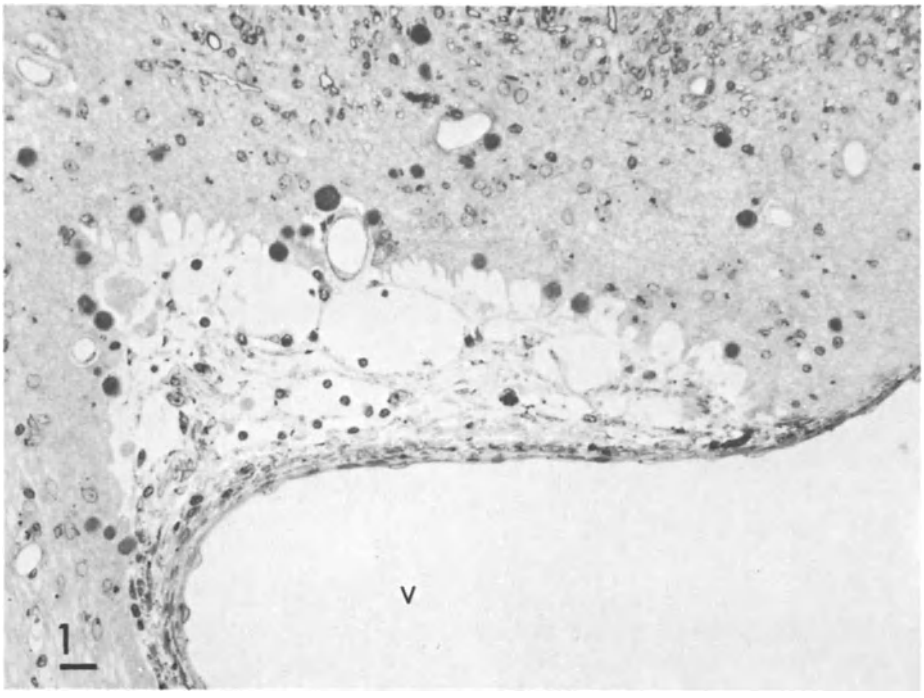

Fig. 1. Perivascular space around a large vein (V) in an old MS plaque contains a number of thin-walled channels. One μm thick epoxy section stained with toluidine blue, scale bar 20 μm

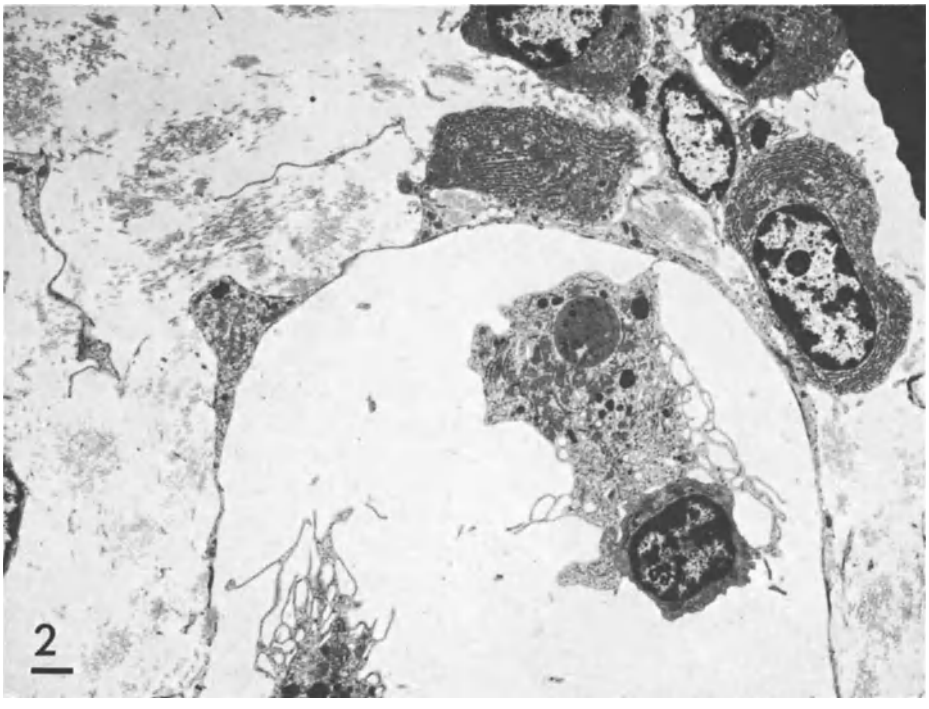

Fig. 2. An electron micrograph of a perivascular channel in an old MS plaque shows a lymphocyte and parts of two macrophages within the channel. Outside the channel, plasma cells can be seen clustered around a reticular cell. Scale bar 1 μm

each other by collagenous trabeculae which contained isolated plasma cells or groups of plasma cells clustered around cells of the same type as those that formed the walls of the channels (Fig. 2).

The tissue organization just described is similar to the antibody-secreting medullary region of a normal lymph node [3, 6]. This observation supports the view that the perivascular compartment in MS plaques may be an important site for antigen processing and expression, and that this may occur continuously in some old plaques [1, 4, 7].

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A Histological and Histochemical Study of the Macroscopically Normal White Matter in Multiple Sclerosis

I. V. ALLEN [1]

Summary

In a combined histological, biochemical, and histochemical study of the macroscopically normal white matter in multiple sclerosis (MS), 72% of samples were histologically abnormal. The significance of this fact in the interpretation of previous biochemical studies and in the design of future studies is discussed. Acid phosphatase was studied histochemically in the microscopically normal white matter in MS: there was an increase in acid phosphatase-containing cells as compared with normal and neurological control material. The significance of this finding is discussed and it is suggested that irrespective of the primary or secondary nature of this phenomenon, it may render the white matter susceptible to the pathogen in this disease.

Introduction

Pathological descriptions of MS frequently refer to histological abnormalities in the white matter outside macroscopic plaques. However, there have been few systematic studies of the macroscopically normal white matter, despite the frequent suggestions that in MS there is an inherent biochemical defect in the so-called normal white matter [4]. Such a defect, while not necessarily the immediate cause of the disease, might render the myelin susceptible to the pathogen. Unfortunately, the biochemical studies which have been carried out to test this hypothesis have produced conflicting results. Norton [8], having reviewed the biochemical evidence, suggests that while there is undoubted biochemical abnormality in many samples of macroscopically normal white matter in MS, the variation in results implies that there is no inherent lipid defect, but rather that the abnormalities are the result of microscopic lesions. Others, however, take the view that there is a primary biochemical abnormality of myelin, and relate this to genetic, dietary, and environmental factors [9]. It has also been reported that lysosomal hydrolases are increased in some samples of macroscopically normal white matter in MS [3, 5]. However, Hirsch et al. [6] found, on a small number of samples, no consistent increase in lysosomal hydrolase activity in normal-appearing white matter. Unfortunately, many of the biochemical studies on macroscopically normal white matter in MS have, largely for technical reasons, inadequate histological control. The present study was undertaken as a combined histological-biochemical investigation in an attempt to obtain some idea of the inci-

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dence of histological abnormality in apparently normal samples of MS white matter taken for biochemistry. Our second aim was to relate biochemical abnormalities to the histological findings using, in addition, histochemical techniques.

Materials and Methods

Case Material and Sampling

Samples were obtained from 14 MS cases (Table 1) and 11 controls (Table 2). The MS cases were, with one exception, of the chronic variety. In only a few cases was the death-necropsy interval greater than 6 h. Control cases were selected because of

Table 1. MS cases

Case	Sex	Age	Duration of Illness (years)	Death/necropsy interval (h)	Histological study	Histochemical study
D 15	F	29	10	4½	–	+
D 17	F	60	25	6	–	+
D 24	M	38	6	13	+	–
D 25	M	23	1	4	+	–
D 27	F	63	27	4	+	+
D 35	M	45	22	3½	+	+
D 36	F	71	42	4½	+	–
D 37	F	60	16	3¾	+	–
D 39	F	48	15	5¼	+	–
D 40	F	64	18	4½	+	–
D 41	M	59	22	4½	+	–
D 48	M	84	50	8	+	–
D 49	F	57	38	5½	+	+
D 54	F	56	31	2	+	+

Table 2. Control cases

Case	Sex	Age	Diagnosis	Death/ necropsy interval (h)	Histo-logical study	Histo-chemical study
C 7	M	49	Demyelination following CO poisoning	3	–	+
C 16	M	66	Myocardial Infarction	9	–	+
C 18	F	61	Myocardial Infarction	5	+	–
C 24	M	64	Myocardial Infarction	5½	+	–
C 27	F	65	Cor Pulmonale	7	+	–
C 32	F	84	Hemorrhagic Diathesis	6	+	–
C 35	M	62	Myocardial Infarction	5	+	–
C 36	M	48	Myocardial Infarction	5	+	–
C 37	F	28	Bronchopneumonia	3½	+	–
C 38	M	67	Myocardial Infarction	4	+	–
C 43	F	58	Myocardial Infarction	3	–	+

the short death-necropsy interval and the absence of neurological illness (with the exception of the neurological control). Necropsies were performed in a mortuary adjacent to the laboratory and immediately after removal the brain was brought to the laboratory for careful dissection. Coronal slices of the fresh brain were examined and samples of macroscopically normal white matter were taken in the MS cases as far as possible from visible plaques (this was not easy and white matter which was apparently unrelated to a plaque in the coronal plane could be close to a plaque in the sagittal plane). Normal white matter from control cases was similarly sampled.

Biochemical Study

Samples for biochemical analysis were wrapped tightly in parafilm and stored at – 20 °C in airtight tubes for up to 15 months. In some samples, blocks for histological control were taken immediately; however, in the majority, blocks were taken when the samples were prepared for homogenization. Since it was considered of vital importance to obtain representative blocks for histological control, the specimens for biochemical analysis were carefully dissected before material was taken from the center of the sample for histological examination. The following enzymes were analyzed on tissue homogenates prepared in 0.1% Triton as described previously [7]: n-acetyl-β-D-glucosaminidase (β-Glm), β-galactosidase (β-gal), β-glucuronidase (β-Glon), cathepsin D (cath D), and aryl sulphatase (ASS).

Histochemical Study

Nine samples of microscopically normal white matter from six cases of MS, seven samples from two normal controls, and two samples from one neurological control (Tables 1 and 2) were studied. The neurological control was a case of carbon monoxide poisoning with extensive myelin damage resulting in death after 3 months. The longest interval between death and cooling of the tissues was 9 h. The fresh brain was sectioned as for the biochemical studies and the blocks for histochemistry were placed on small pieces of moistened cork and snap frozen in isopentane precooled with liquid nitrogen. Cryostat sections cut at 8 μ, were air dried and placed in the incubation solution for 45 min for the demonstration of acid phosphatase (APP) by the naphthol AS-B1 phosphate method [2] using pararosanilin hydrochloride (Hopkins and Williams, basic red 9 C.1. 42500). The incubated sections were then stained by a variety of techniques previously modified in this laboratory [1] which permit the simultaneous demonstration of APP, myelin, lipids, and various cells, particularly astrocytes. The simultaneous demonstration of APP and myelin, etc. is feasible because APP is visualized using the relatively insoluble dye pararosanilin which does not wash out during the subsequent procedures.

Results

Technical Results

In a few blocks, freezing artifact was seen but most were well preserved and in all the histological features were distinct, although in those where artifact was widespread, assessment of the density of various cell types was more difficult. The combined demonstration of APP, myelin, lipids, and astrocytes produced reasonable histological preparations in which interpretation was relatively easy. The main technical difficulty was encountered in the Spielmeyer preparations in which the stain often failed to penetrate the section, leaving large pale artifacts. The combined APP/Spielmeyer preparation did not produce this artifact so frequently.

Histology Results

Of the 54 specimens of macroscopically normal white matter available from the 12 MS cases used for the lysosomal enzyme and fragility studies, 72% were histologically abnormal (Table 3). The eight control cases used for these studies had an incidence of 36% histological abnormality, but the nature and severity of the abnormality in the MS cases differed markedly from that of the controls. By far the most common abnormality in the MS cases was gliosis, which in this study was assessed subjectively. The gliosis, even with H and E staining only, could often be attributed to proliferation of astrocytes, and although no attempt was made to quantitate the abnormalities, subjective assessment was relatively easy. Unsuspected demyelination was found in 13.0% of the samples from the MS cases. The incidence of perivascular small round cell infiltration was only 9%, and the degree of infiltration in the five samples showing this change was slight and usually associated with perivascular macrophage infiltration and deposition of lipofuscin. The other histological abnormalities in the MS cases listed in Table 3 were found in only a few samples. Although 19 of the control samples were histologically abnormal, the degree of abnor-

Table 3. Histology of macroscopically normal white matter

		MS (54)	*Control (52)*
Histologically Normal		15 (27.8%)	33 (63.5%)
Incidence of Histological Abnormalities:			
Gliosis		32 (59.3%)	4 (7.7%)
Demyelination		7 (13.0%)	0
Small round cell infiltration		5 (9.3%)	0
Macrophages		7 (13.0%)	0
Perivascular deposits:	Lipofuscin	16 (29.6%)	4 (7.7%)
	Iron	0	3 (5.8%)
	Calcium	1 (1.9%)	0
Thickened blood vessels		3 (5.6%)	6 (11.5%)
Corpora amylacea		4 (7.4%)	6 (11.5%)

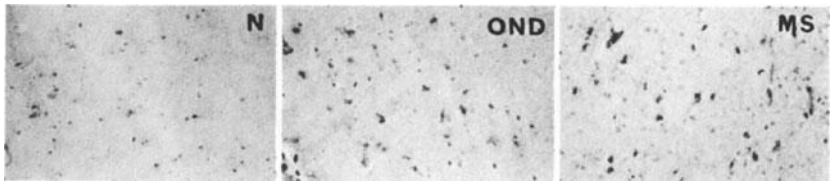

Fig. 1. Microscopically normal white matter. Normal control (*N*); Neurological control (*OND*); *MS* App. ×100

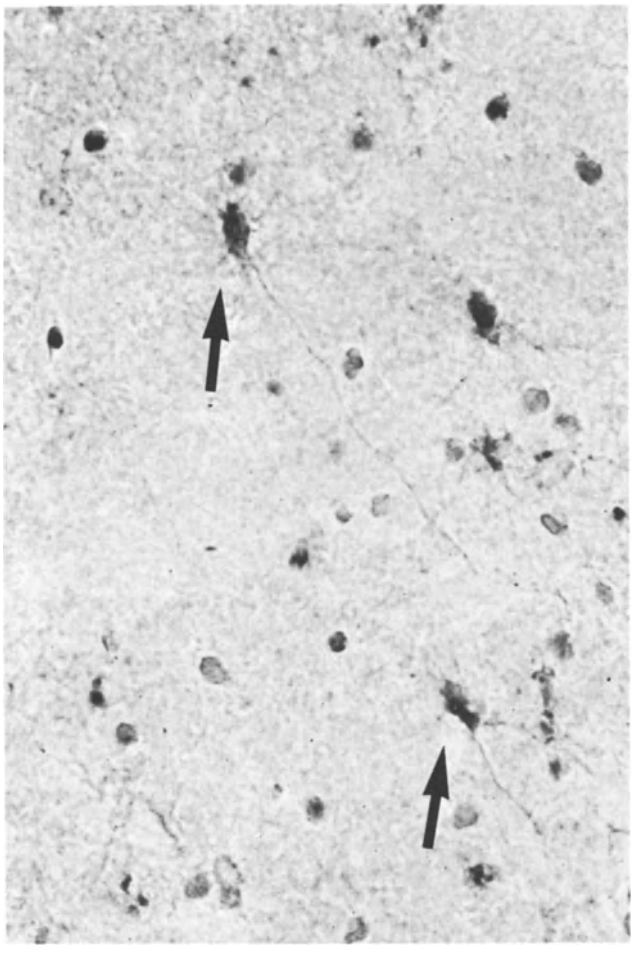

Fig. 2. MS microscopically normal white matter. *Arrows* indicate astrocytes. APP/PTAH. ×250

mality was slight and in most cases consisted of minor vascular changes or corpora amylacea deposition. Most of the control samples would have been passed as normal in routine histological examination.

Biochemical Results

Biochemical assay was carried out on 38 samples; of these, 16 were histologically normal, eight showed mild to moderate gliosis, and four were demyelinated. β-Glm was the only lysosomal enzyme of those studied which was significantly increased in the 16 specimens of histologically normal white matter [7].

APP Histochemistry

This study was done in an attempt to demonstrate the cellular source of lysosomal enzymes in the microscopically normal white matter in MS. Nine blocks of macroscopically normal white matter from six MS cases were used. Of the nine MS specimens, one showed slight gliosis, seven contained a few small round cells in perivascular spaces, and three contained a few small deposits of lipofuscin. The overall degree of histological abnormality was slight, and most specimens in routine histology would have been passed as normal. The nine blocks avilable from the control cases were all histologically normal, and a striking finding in the neurological control was the normality of the white matter except immediately surrounding the demyelinated areas. Despite the fact that the MS and control specimens were both essentially normal histologically, APP-positive cells were much more numerous in the MS cases (Fig. 1). In the MS cases, a combination of APP staining with Spielmeyer/Scharlach R confirmed the increase in APP-positive cells despite the normal myelin stains. Combined APP and phosphotungstic acid hematoxylin staining (PTAH) showed that most of the APP-positive cells were astrocytes (Fig. 2). Although oligodendrocytes were not specifically stained, the techniques used did not demonstrate APP in interfascicular oligodendrocytes.

Discussion

Several significant findings emerge from this study. It is apparent that reasonable histology can be obtained from samples of unfixed brain deep frozen for biochemistry. Although freezing artifact was seen in many samples and sometimes made cellular recognition difficult, it did not prevent overall histological assessment. The majority of the histological specimens (72%) from the macroscopically normal white matter were histologically abnormal. The most frequent abnormality was diffuse gliosis. Although references to astrocytic proliferation in the macroscopically normal white matter in MS are frequent in the literature, there apparently has been no attempt to quantitate this finding and it is usually assumed that it is a secondary phenomenon. Microscopic demyelination was also found, but was relatively uncommon

(13%). Perivascular lipofuscin deposition was found in some 30% of samples. The incidence of perivascular small round cell infiltration was low (9%) and such infiltrates contained few cells and were often associated with macrophage infiltration and lipofuscin deposition. Probably, much of the controversy over biochemical results on the macroscopically normal white matter has arisen because of inadequate histological control. Many biochemical techniques, particularly for lipid analysis, require large amounts of tissue and, therefore in these, histological control may be difficult or impossible until microtechniques have been evolved. At the present time, since such techniques are not available, the validity of biochemical results on macroscopically normal white matter where large samples are required must be questioned because of the high incidence of histological abnormality found in the present study. Detailed analysis of our published biochemical results [7] shows the importance of histological control. Of the enzymes studied, only β-Glm was significantly elevated in the microscopically normal white matter. However, if the results are calculated using all samples of the macroscopically normal white matter, then there is a significant elevation, not only of β-Glm, but also of β-Gal and β-Glon. These latter results are presumably accounted for by histological abnormalities. In the histochemical study in microscopically normal white matter in MS it would obviously have been preferable to have assessed β-Glm activity, since in the biochemical study this was the only enzyme which was significantly increased. Unfortunately, it proved technically impossible to combine this reaction with the other staining techniques. It was therefore decided to visualize APP using the relatively insoluble dye pararosanilin. Sections in which APP was demonstrated could then be stained for myelin, lipid, glial cells, etc. The finding of an increase in APP-containing cells in MS microscopically normal white matter as compared with controls may be significant. Moreover, the demonstration that the majority of these cells are astrocytes raises the problem of the significance of an elevation of lysosomal enzymes in this cell. Clearly this increase in APP-positive cells may merely reflect a level of gliosis which is not detectable by routine histological methods. Further study of this point is necessary and we are collecting material, particularly from cases of MS which are predominantly spinal and in which one would therefore expect relative sparing of the cerebral white matter from secondary gliosis. Irrespective of the primary or secondary nature of this phenomenon, it clearly has important implications. The increase in lysosomal enzymes in astrocytes in the microscopically normal white matter in MS may render this tissue more susceptible to the pathogen whatever that may be.

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The Pathology of the Active Lesion

C. W. M. ADAMS [1]

In previous communications we have reported on certain active lesions where the pathological process seemed to be relatively recent [1, 2]. The clinical history in these five cases was 1–3 weeks in duration and we assumed that the underlying lesion was probably 3–5 weeks old. It is unusual to obtain good correlation between the clinical history and post-mortem appearances [2] but, in these particular cases, the symptoms had been mainly bulbar (viz. – dysphagia), and corresponding lesions were found in the pons and upper medulla.

The prominent characteristics of the lesions in these five active pontine-medullary cases were as follows:

1. There was marked myelin breakdown within small plaques. Abundant Marchi-positive cholesterol esters were found within microglia or other phagocytic cells. In one case, frozen material was not available, but paraffin sections showed substantial foam-cell infiltration

2. There was glial hyperplasia. This was seen as an increase in the glial population either diffusely throughout the lesion or zonally at its rim [6]. The cells concerned in this hyperplasia were in part microglia (or other phagocytic cells) together with oligodendroglia [6]. Oligodendroglia, however, largely disappear from the center of established lesions. Astrocytic activation and hyperplasia also contribute to the increased glial population of the active lesion.

3. There was marked perivenular accumulation of mononuclear cells, including monocytes, within these small plaques. The precise further identification of these mononuclear or lymphocytoid cells was not easy with haematoxylin-eosin.

4. There was perivenular infiltration, predominantly with mononuclear cells outside distinct plaques (Fig. 1). These infiltrates were shown by serial section not to be direct extensions from the plaque, yet normally they were situated within 2 cm of an established plaque. These perivenular infiltrates outside plaques were frequently accompanied by intense oedema (Fig. 2), myelin pallor, and myelin disorganization. However, they did not show evidence of myelin breakdown (cholesterol esters) when stained with Sudan dyes and the Marchi reagent.

5. There was meningitis with a predominantly lymphocytic population, but with a variable macrophage component [3]. This meningitis was prominent around vessels and in the depths of sulci.

6. Lumsden [7] reported that acute lesions usually showed a shelving edge, whereas old inactive lesions were sharp and punched out in contour. Indistinct lesions and shelving edges were common in our active cases, in contrast to other lesions which appeared inactive.

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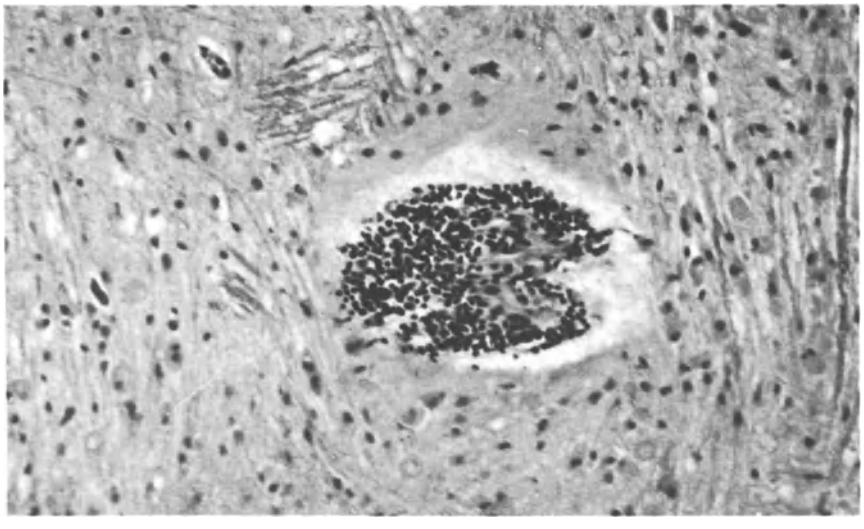

Fig. 1. Perivenular cuffing with mononuclear cells in "normal" myelin outside plaque. H and E. × 120

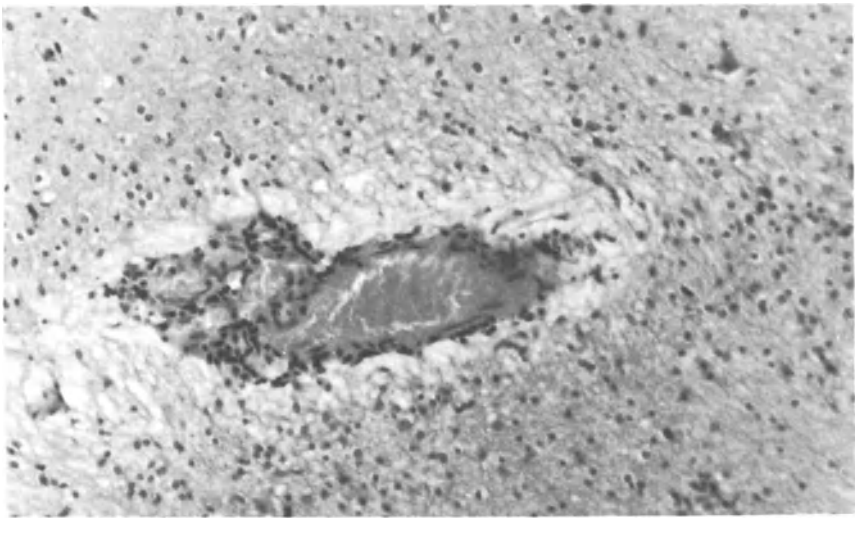

Fig. 2. Intense oedema and some mononuclear cuffing around a vein in "normal" myelin outside plaque. H and E. × 120

The foregoing observations delineated certain characteristics of active lesions. It then seemed relevant to compare the reliability and incidence of those features in supposedly active lesions in our ongoing series of 82 cases and in the 143 cases described by Guseo and Jellinger [5]. We took the presence of myelin breakdown and glial hyperplasia as primary evidence of lesion activity. Table 1 shows the relative reliability of the various other features discussed above in distinguishing acute from

Table 1. Features of active plaques

	Probability of active vs. chronic	Incidence in active
Myelin breakdown (1, 2)	100%	100%
Glial reaction (1)	100%	100%
Cuffs ouside plaque (1)	92%	63%
Meningitis (2)	79%	53%
Shelving edge (1)	78%	89%
Cuffs inside plaque (1, 2)	75%	71%

(1) Adams (1975, 1977) 82 cases
(2) Guseo and Jellinger (1975) 143 cases

chronic lesions. It can be seen that the perivenular infiltrate (cuff) outside the plaque is the most reliable of the features, but is only seen in just over half of active lesions. By contrast, a shelving edge is less reliable but, nevertheless is seen in nearly all active lesions.

These other features of lesion activity may be particularly valuable when no frozen sections are available to determine the presence of the sudanophilic cholesterol esters that are formed during myelin breakdown. In these circumstances, lipid accumulation can only be inferred by the presence of foam cells in paraffin sections, but this latter is an insensitive method for the histological detection of lipid. Likewise, difficulties may be encountered in deciding whether glial hyperplasia is present. A modest increase in glial population may be difficult to determine without using counting techniques. The recognition of astrocytic activation (gemistocytes, nuclear changes, etc.) may, however, provide a useful clue.

It could be argued that some active lesions may be present before sudanophilic cholesterol esters are formed. We saw no definite evidence of such lesions. Nevertheless, a number of mononuclear infiltrates in surrounding white matter showed areas of myelin pallor. It is uncertain whether such pallor represents focal oedema or early myelin breakdown in the stage before cholesterol esters are formed. It could also be argued that myelin breakdown may still be prominent after the lesion has ceased to be active. This can be inferred from the known persistence of myelin breakdown products in the central nervous system for periods of at least a year after induction of Wallerian degeneration [4, 8].

Conclusion

The presence of myelin breakdown and neuroglial hyperplasia are usually taken as evidence of active demyelination in MS. It is hoped that certain additional features discussed here may be used as a further guide in distinguishing between active and burnt-out lesions. These features are: perivenular cuffs inside and outside plaques, perivenular oedema, mononuclear meningitis, and a shelving edge to the lesion.

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Discussion

C. S. Raine: Dr. Adams, the oligodendrocyte was conspicuous in its absence of mention from your last table and from your description. What are your feelings now about the oligodendrocyte in the actively growing MS lesion?

C. W. M Adams: Well, I didn't really want to answer that. I did say that it is a disputable point. I frankly don't know. They are still on what's light-microscopic ground. Some of these cells seem to be oligodendroglia. There are also histochemical grounds for thinking that they are. But I would be interested to hear what other people have to say about it. I'd rather like to "duck" the issue.

H. M. Wiesniewski: With respect to Dr. Prineas's presentation, I think that we described a similiar type of pathology with Madrid in the last issue of *The Journal of Neuropathology and Applied Neurobiology* in chronic relapsing guinea pigs. We concentrated on the anterior root, because it is the area were you can nicely tease in their fibers. You, by cutting serially, got the same. We teased in their fibers and got the same type of pathology. If you study the dynamics, it is a nodal, paranodal type of pathology, which is very interesting in terms of distribution remnants of the myelin debris sitting there for a prolonged period of time. And the morphology which you presented here is identical. We saw the same in the CNS of the same guinea pigs. So it's not human only.

H. Debuch: I have two short questions for Dr. Allen please. Is it right that you looked at the brains from 12 MS patients and you found, as far as I understood, demyelination in 7% or at least disturbed myelin sheaths? Now these 7% – do they belong to different patients or do they come from some samples from only one or two patients? My second question would be, did you also show or look for cholesterol esters in your samples of the so called non-white matter?

C. W. M. Adams: First of all, in answer to your first point, the unsuspected demyelination which was present in a relatively small percentage of patients of the samples we looked at was not at all from one case. I think this came from four cases. Secondly, did we look for cholesterol esters? The answer is no. I might say that we were very limited in the techniques which we could do on these samples because we tried to keep the histological sample as small as possible so that we could keep enough for biochemistry. So it was a bit limited.

Dr. McKhann: Dr Adams, I'd like to ask you a question. I think your point about so-called normal white matter being abnormal is very good. I'd like to ask you about the interpretation. Regarding most patients with MS, you didn't tell us how these patients died, or what they died of. It is conceivable that they might have had repeated anoxic episodes. So do you think the more diffused gliosis that you are seeing is an integral part of the disease or secondary to related conditions?

C. W. M. Adams: I know the cause of death in these patients, and it was, in the MS cases, as one would expect, in the vast majority, terminal bronchopneumonia.

You might compare that with the controls, where you could have expected that an anoxia could have been an important factor in several of them. Because quite a number of the controls had myocardial infarction, one or two had cardiac arrests, and so on. However, there is not doubt about it. Biochemically one could argue that this might produce some of the lysosomal enzyme effects. And we have shown in a parallel study that lysosomal fragility in MS in the histologically normal white matter is abnormal as compared with controls. Personally, I don't think that any of these terminal events in these patients explain the astrocytic prolification and I don't think that they explain the biochemical findings because the histology and the biochemistry can be fused. They can go together because of the astrocytic response. So I don't think drugs, anoxia, temperature, and so on were significant factors because those held true, if you like, for both controls and MS patients.

E. Schuller: I will simply record, Dr. Adams, that we find a typical meningitis pattern in 15% of our MS patients.

E. J. Field: Dr. Allen, I want to ask you directly. May I take it, that from what you said, you lean to the view that MS is a generalized disease of the brain with focal accentuations around the blood vessels, of certain blood vessels?

I. V. Allen: I don't think anything we have done has cleared up the possibility or the problem here as to whether this is a primary or secondary phenomenon. You might have noticed that most of the 12 cases were of long duration. There was only one case where the disease had been present for 2 years. So you could therefore argue that with this chronicity, that might all be a secondary effect. And I think I would therefore be unprepared to commit myself as to whether this is primary or secondary. I just think it's a fact that we have got to take into account not only in biochemical studies but in immunological studies on brain tissue. I think we have got to think of the disease at the time we are seeing it at post mortem as generalized. And the plaques, if you like, are manifestations of the disease. The cases which would obviously help to resolve this are not, I think, acute cases. Because in acute cases the pathology tends to be very diffuse, severe, and widespread. This might make interpretation difficult. But the cases which we are trying to collect are cases which are predominantly spinal and in which therefore one would not expect the cerebral white matter to suffer so much from secondary effects of gliosis. And in this group I think this would be an interesting phenomenon to study.

T. Fog: This is a question to Dr. Prineas and Dr. Adams. Many years ago when I studied the neuropathology of MS I had the chance of having a very peracute case. I found in this case in the spinal cord very acute changes which were represented only by a great edema along the veins and relatively sparse infiltration. And I think that perhaps the beginning of the whole affair may be the edema at first and then the cells afterwards. I found a discrepancy between the number of cells in these very acute cases and the great edema changes, which were not artificial changes.

I compared it to the periphlebitis retinae where in acute cases you can see the edema around the vessels directly in the ophthalmascope and then later the scar building around the rings.

J. W. Prineas: Thank you. That's a very interesting point. One of the slides did show edema round a venule and that particular one had a relatively slight cellular reaction around it. I am particularly interested that you have observed edema even before cellular infiltration. It might be a very relevant point.

H. M. Wiesniewski: With respect to diffused gliosis, one has to remember that the old plaques show increased blood-brain barrier permeability, and there is continued leakage of globulins and albumins. As a result, you have, I think, diffused gliosis, because if you are studying the MS material with anti-gamma globulins and go very far off the beaten track, you find a lot of globulins there. So, in my feeling, we are dealing with a continuous chronic stage of brain edema in this situation. I can show it beautifully in chronic guinea pigs. With respect to the acute edema, I think, it's the same case. The permeability and the infiltration always go together. However, the edema fluid, as was shown by Clarkson and many others, travels along the perivascular space for quite a distance so that cells can sit in one place and you have a fantastic perivascular edema for a long distance. So that one does not preclude the other. That's the beginning of disease edema, which is much more conspicuous.

G. Pálffy: In a very acute case which occurred after the use of Lister vaccine I have found a lot of eosinophilic infiltration around the vessels. My question to Dr. Adams is whether he found any difference between the composition of the cells in perivascular infiltration in other acute and hyperacute cases.

C. W. M. Adams: I can briefly answer that, and I didn't see any difference and certainly I haven't seen any eosinophils at all.

A. Wajgt: (Shows two slides) In connection with Dr. Arnason's presentation, I would like to make a few remarks concerning surface T- and B-lymphocyte markers in MS. A study was performed with 31 MS patients. There was no difference in T-lymphocyte count in MS and controls. There was also no significant difference in surface Ig bearing cells. Between MS and controls, as shown by membrane fluorescence, the difference in complement receptor-bearing cells known to be B cells was significant at a P below 0.001 (in a third-column EAC test). But from the biological point of view, the difference between 18.1 and 24.4% of positive cells it is rather meaningless. There was a statistically significant difference in EA receptors on the lymphocytes' surface. The most pronounced difference was of 7S, and this means IgG of the receptor-bearing cells. A rosette test was performed using serial twofold 7S antibody dilution and anti-sheep red blood cells to sensitize the erythrocytes. The conclusion is that there is a very significant drop in 7S IgG receptor-bearing cells, between MS patients and normal controls and this means, taking into account Morreta's new report, that it may be just a substrate which is depleted in MS. This means there is a significant diminution in 7S as the receptor or T cells in the acute phase of the disease.

Glia Cell Alterations in Multiple Sclerosis

A. ARGYRAKIS [1]

Summary

Early sterile autopsy material from cerebrum specimens of 12 MS patients was investigated using an electron microscope. The most interesting finding concerned certain inclusion bodies in cells (possible the so-called third type of neuroglial cells) from the margin of early lesions. These inclusions consisted of clusters of trilamellated tubular profiles with a thickness of about 17–20 nm. Such inclusions appeared in some oligodendrocytes within and outside the lesions (i.e., in macroscopically normal white matter), and in some macrophages. The nature of the inclusions and the origin and role of these cells in the pathogenesis of multiple sclerosis (MS) requires further elucidation. Possibly they contain a neurogenic or viral antigen in MS, or else they might be the morphological expression of metabolic abnormalities.

Introduction

Results from light-microscopic investigations of early lesions in MS do not seem to fit into a coherent picture. Electron-microscopic findings so far are rare and mainly concern chronic lesions, lesions of uncertainage, and macroscopically normal white and gray matter. So investigation of early lesions seems to be indicated, especially as their morphology promises important insights into the pathological mechanism in MS.

The following deals with some findings of ultrastructural investigations of glial cells at the margin of early lesions.

Materials and Methods

Early sterile autopsy specimens from different parts of the brain were obtained between 1 and 3 post mortem from 12 patients with several clinical varieties of MS.

Sections were photographed, and then small blocks containing early lesions, chronic lesions, lesions of uncertain age, and macroscopically normal looking gray and white matter remote from plaques were cut out, fixed in cacodylate-buffered 3% glutaraldehyde, and further processed for electron microscopy.

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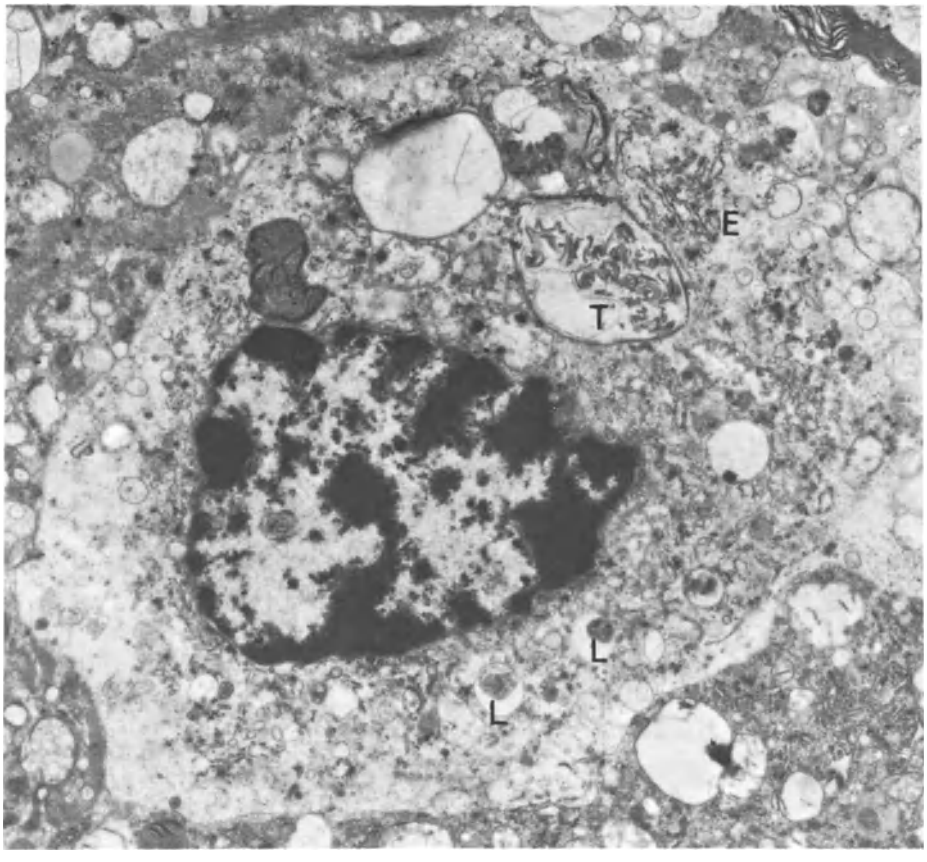

Fig. 1. Glial cell from margin of an early MS lesion with membrane-bounded tubular inclusions (*T*). *E* = granular endoplasmic reticulum, *L* = lysosomes. ×24,000

To obtain specimens from the demyelinated zone, the zone of abrupt reduction of myelin density, and the hypercellular normally myelinated margin, these blocks were further subdivided into samples from the center and the margin of the lesions and remote parts, and the resulting sub-blocks were embedded in epoxy raisin for ultrastructural investigations (using standard techniques).

Electron-Microscopic Findings

The findings discussed here are based on observation of the specimens obtained from the margin of early lesions. Besides the usual lipid-laden phagocytes, the most interesting cells in this region were inclusion-bearing cells which appeared to be glial cells. These cells differ from typical oligodendrocytes in that they have a rather light cytoplasm containing many lysosomes and longer cisternae of the endoplasmic reticulum (Fig. 1). In the cytoplasm, some microtubuli and some parts of granular

Fig. 2. Cluster of tubular profiles from another inclusion-bearing glial cell; note trilamellated structure of the tubuli (*arrows*) and partially double surrounding membrane (*M*). × 182,700

endoplasmic reticulum were visible. The cisternae were partly bounded by double membranes and contained aggregates of electron-dense longitudinally oriented or semicircular profiles (Fig. 1). At higher magnification, these profiles appeared as trilamellated microtubuli with a thickness of about 17–20 nm (Fig. 2). Some of them appeared to lie free in the cytoplasm. There was some granular amorphous material embedded between these tubuli.

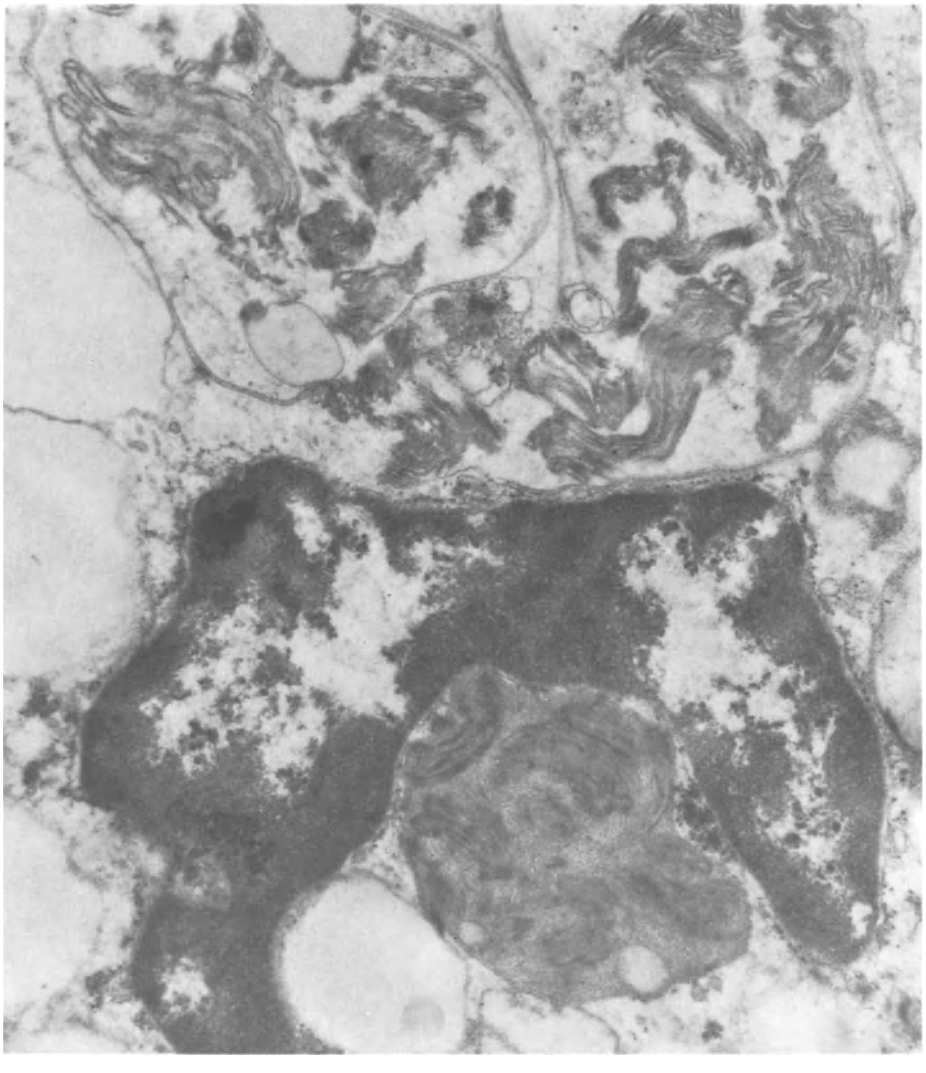

Fig. 3. Lipid-laden cell from margin of early MS lesion with clusters of similar tubular profiles. ×23,200

Numerous astrocytes were swollen, others appeared to be normal. Structures like the aggregates of tubuli were sometimes also observed in oligodendrocytes in the margin of lesions and remote from the lesions as well as in typical lipid-laden phagocytes (Fig. 3).

Discussion

The exact nature and origin of the above-described cells is hitherto unknown; Prineas [6] believes that they correspond to the third type of neuroglial cells de-

scribed by Vaughn [8] and Vaughn and Peters [9], which might be classic microglial cells and are similar in their fine structure to the inclusion-bearing cells in MS. Some evidence speaks for the participation of these cells in myelin destruction in Wallerian degeneration [5].

The nature and origin of the described tubular inclusions are also unknown. Structures like these have not been observed in the usual lysosomal disorders. Prineas [6] suspects that the possible neurogenic or viral antigen in MS might be included in this tubular material. On the other hand, these inclusions are might be a morphological expression of a changed enzymatic activity in these cells or might consist of secondary storage material in the cell organelles, both due to a metabolic disorder.

Some biochemical and histochemical evidence for this latter hypothesis has been provided by Einstein et al. [4], Rinne et al. [7], and Adams [1].

Further investigations are necessary at this point. At any rate, the possibility that histological abnormality may be common in macroscopically normal MS white matter should be taken into account in all biochemical research on white matter [2].

As long as the nature of these inclusions and the role of the inclusion-bearing cells in the pathogenesis of MS remains unclear, the hypothesis of some authors [3] that the whole of the white matter is involved in MS remains an open and highly interesting question.

Acknowledgments. This research was supported by the Deutsche Forschungsgemeinschaft, Schwerpunktprogramm "Multiple Sklerose und verwandte Krankheiten".

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Ultrastructural Changes in Peripheral Nerves in Multiple Sclerosis and Subacute Sclerosing Panencephalitis

A. ARGYRAKIS [1]

Summary

Early sterile autopsy material of femoral and sural nerves from two multiple sclerosis (MS) patients and biopsy specimens of sural nerve from one subacute sclerosing panencephalitis (SSPE) patient were investigated using an electron microscope. In all cases, many Schwann cells showed numerous coated vesicle-like particles, dilatation of endoplasmatic reticulum, glycogen deposits, and storage of morphologically variable inclusion bodies. Numerous myelin sheaths displayed different stages of demyelination which seemed to start at the nodal regions. These findings indicate that the peripheral nerves are involved in MS and SSPE.

Introduction

Morphological, especially ultrastructural studies of peripheral nerves in multiple sclerosis have not played a prominent role so far, because earlier studies under the light microscope did not show significant changes, and there seemed to be no clinical evidence for deficiencies in the peripheral nervous system (PNS). Among the exceptions to this are Pollock et al. [4], who produced morphological evidence for peripheral myelin reduction in MS patients.

The following is an ultrastructural examination of peripheral nerves of MS and SSPE patients.

Material and Methods

Early sterile autopsy specimens of fermoral and sural nerves were obtained between 2 and 3 h postmortem from two patients with chronic progressive MS. Biopsy specimens of sural nerve were obtained from an 18-year-old patient with serologically confirmed SSPE, whose conduction velocity in the sural nerve was in the normal range. In the MS cases, no neurophysiological examinations had taken place, and in all cases there had been no corticosteroid treatment during the last months before death.

Small blocs of tissue were fixed in cacodylate-buffered 3% glutaraldehyde and further processed for electron microscopy using standard techniques.

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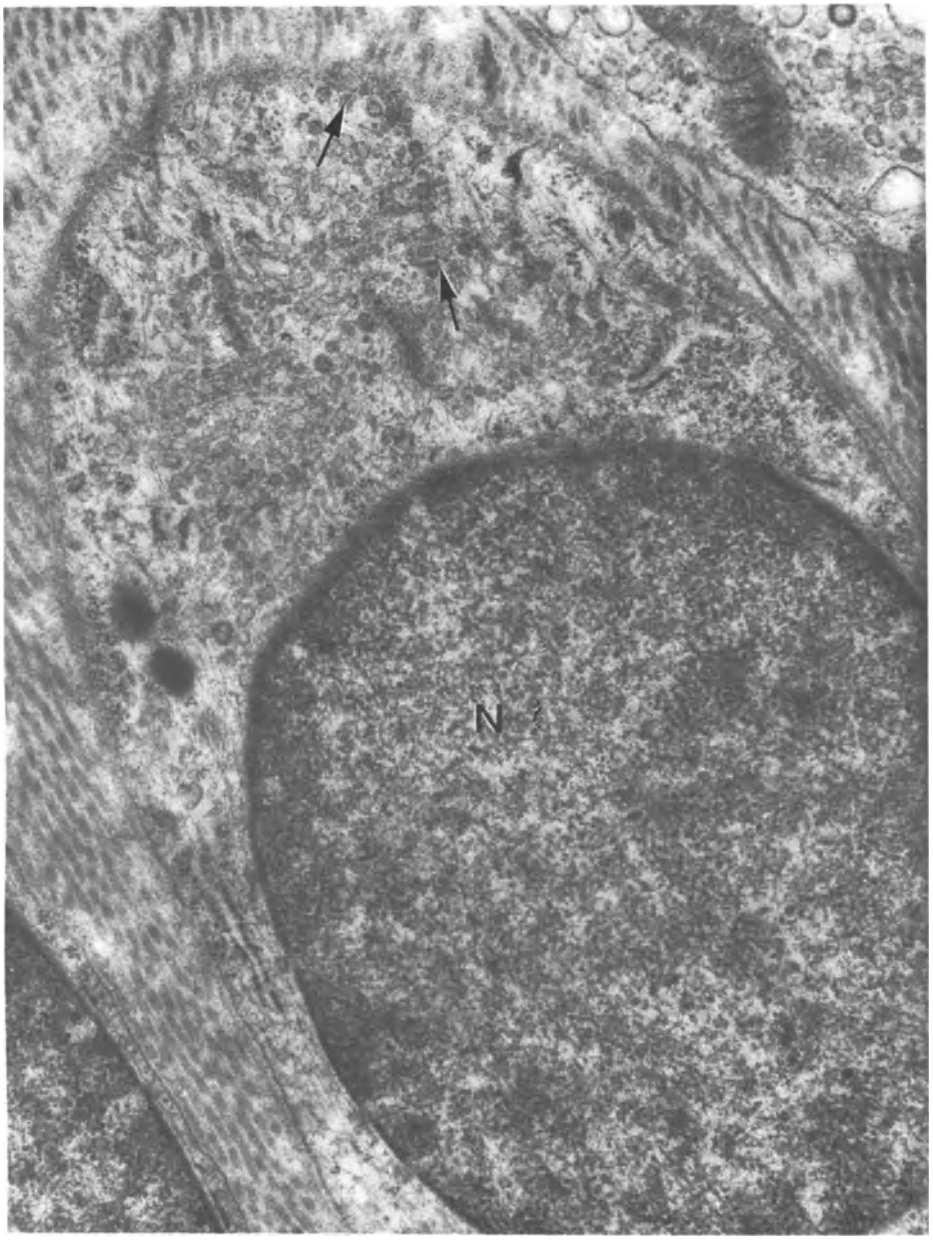

Fig. 1. Coated vesicle-like inclusions in the Schwann cell endoplasmic reticulum (*arrows*). *N* = nucleus of Schwann cell. MS, sural nerve early autopsy material. × 24,000

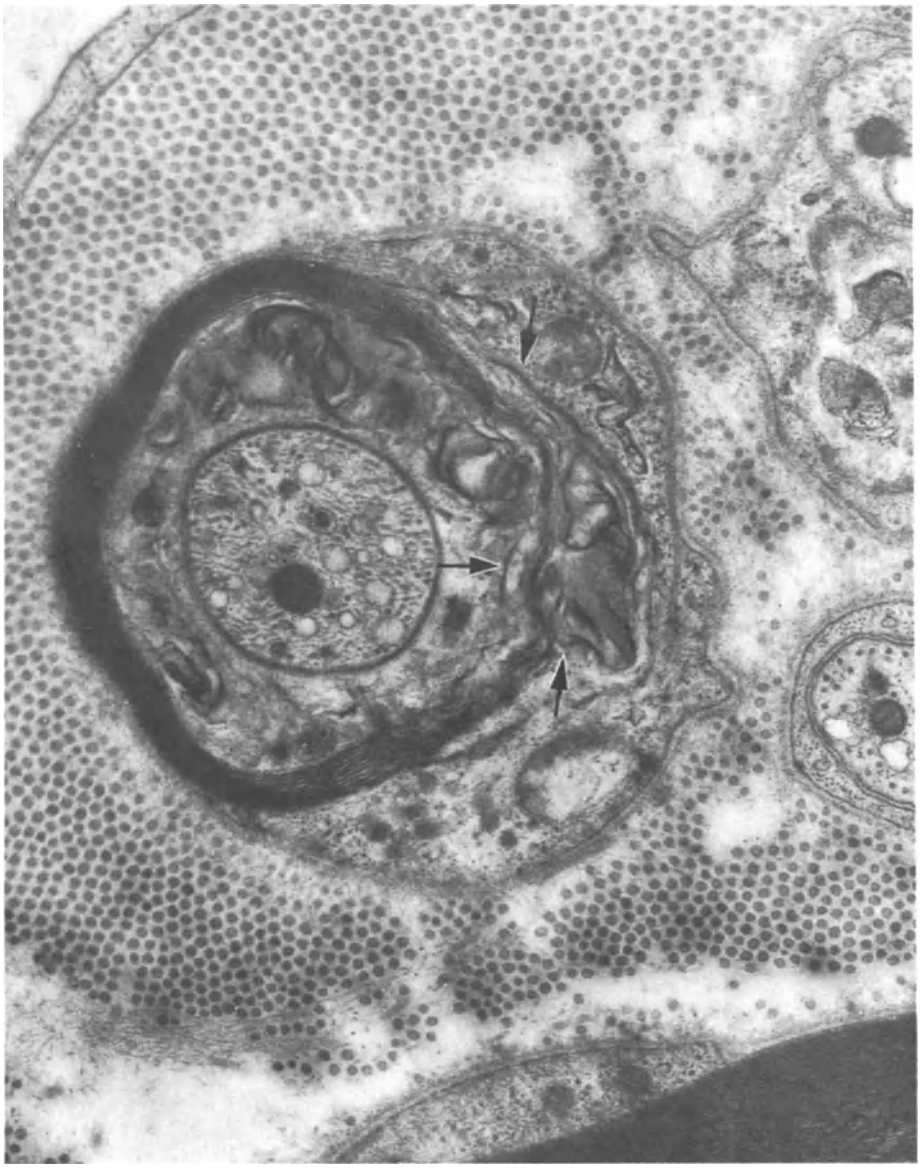

Fig. 2. Cross section of a nerve fiber at the node of Ranvier; the myelin sheath begins to degenerate (*arrows*). The axon is intact. SSPE, Sural nerve biopsy. ×24,000

Electron-Microscopic Findings

In both MS cases as well as in the SSPE case, numerous Schwann cells showed ultrastructural changes.

Most prominent among these were coated vesicle-like particles in the endoplasmatic reticulum (Fig. 1).

Moreover, the endoplasmatic reticulum showed dilatation, numerous glycogen granules, and increased storage of various morphologically differing inclusions bodies. Some of these, e.g., the so-called "tufaceous" bodies, up to now were considered as characteristic morphological inclusion bodies of metachromatic leukodystrophy (MLD), a lysosomal disorder.

Additionally, the Schwann cells contained numerous myelin ovoids and π granules. A considerable number of myelin sheaths appeared to be in different stages of demyelination starting at the nodal regions (Fig. 2). The number of myelin lamellae seemed normal.

Further prominent features were collagen pockets and platelike processes of Schwann cells without axons.

The onion-bulb formations typical of chronic neuropathies could be not observed in any of these cases, and the axons also did not show any pathological changes.

Discussion

The clinical diagnosis of the MS patients was confirmed by early sterile autopsy. One of the cases is more fully described as case no. 4 in Bauer et al. [1].

Autopsy in the SSPE case was not possible because the patient had left Germany before his death.

The role of the coated vesicle-like particles, which, as we have noted, formed the dominant pathological finding, is not clear. It may be, as Ghadially [2] states, that they have to do with the transport of proteins into the cells. The changes in Schwann cells described here – dilatation of endoplasmic reticulum and storage of glycogen granules as well as the increase of various inclusion bodies – are also found in other diseases of the peripheral nerves, i.e., they are not specific for MS or SSPE, but they seem to indicate that Schwann cells are primarily involved in these pathological processes. And if the Schwann cells are involved, so will be the myelin sheaths.

Most nerve fibers, as we stated, were morphologically intact, others displayed various degrees of demyelination. The demyelination was of the segmental type, starting at the nodal region. The conduction velocity in the peripheral nerves may, however, stay within the normal range for part of the process because the number of demyelinated fibers is still too small to affect functioning. On the other hand, Hopf [3] has noted reduced conduction velocities in the slower fibers in MS patients, and concludes that MS is a generalized disease at least in the initial stages and in acute relapses.

Numerous myelin ovoids were present in the Schwann cell cytoplasm in all cases. Myelin ovoids also occur in Schwann cells of normal peripheral nerves; the significance of this is not clear. Neither we nor Pollock et al. [4] observed significant axon changes. In contrast to Pollock et al., we did not observe a generalized reduction of myelin lamellae in these disorders. The reduction of myelin lamellae described by Pollock et al. may be due to a remyelination process.

Because of the different age of CNS lesions in one and the same MS patient, one might expect different developmental stages in the changes of the PNS. Further research on this point is desirable.

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c-AMPase and Lipids in Multiple Sclerosis and Normal Brain Myelin

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2′,3′-cyclic nucleotide 3′-phosphohydrolase (EC 3.1.4.16) activity, which is thought to be a "myelin marker" enzyme [3], has not yet been estimated in myelin samples of multiple sclerosis (MS) brains, as far as we know. Reports of white matter specimens show differences of enzyme activities in different species [4, 7]. The present study was carried out to see whether there are also differences between myelin preparations from normal and pathological brains.

Furthermore, several authors have reported contradictory results with respect to the lipid composition of myelin prepared from white matter of normal human brains and so-called "normal appearing" white matter of patients suffering from MS. While Suzuki et al. [8] and Fewster et al. [2] did not find any significant differences concerning the lipid pattern between the myelin preparations of normal and pathological brains, Clausen and Hansen [1] and Woelk and Borri [10] were able to distinguish such alterations.

Methods

Normal appearing white matter from five brains of patients with MS – 2.0–3.0 g each – and white matter from five normal control brains – 1.5–2.0 g each – were dissected from post mortem specimens (4–6 h after death in pathological and 24–48 h after death in normal brains). These samples were used for isolation of myelin according to the method of Norton and Poduslo [6]. These myelin preparations served for our enzyme and lipid studies. The method of Glastris and Pfeiffer [3] was used to determine enzyme activity; no detergents were added, and there was no sonification to stimulate the activity. Protein content was determined [5]. An aliquot of the myelin sample was dialyzed against distilled water and its dry weight was estimated. Analysis of myelin lipid patterns, fatty acids and aldehydes were performed as described by Winterfeld and Debuch [9]. Further details will be described elsewhere [11].

Results

The yield of myelin (μg dry weight per mg white matter dry weight) from the five pathological brains was significantly lower than that from the white matter of the

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Table 1. Composition of myelin (μg/mg myelin dry weight) from the white matter [a]

	Normal	MS
Protein	290.23 ± 11.166	308.38 ± 10.958
Total lipids	665.81 ± 42.929	669.38 ± 18.055
Cholesterol [b]	172.21 ± 21.129	190.94 ± 16.889
Glycolipids [c]	249.40 ± 18.908	226.14 ± 31.506
Phospholipids	244.20 ± 32.613	252.29 ± 13.512
Phosphatidylcholin (PC) fraction	59.33 ± 0.982	66.00 ± 9.388
Phosphatidylethanolamine (PE) fraction	88.67 ± 7.517	90.56 ± 2.547
incl. Ethanolamineplasmalogens	56.35 ± 6.084	55.72 ± 0.582
Phosphatidylserine (PS) fraction	41.02 ± 8.912	29.71 ± 3.765
Phosphatidylinositol (PI)	8.68 ± 2.941	7.36 ± 1.081
Sphingomyelin	39.96 ± 7.029	41.49 ± 4.494

[a] For preparation see Methods
[b] No cholesterolester present
[c] Calculated

Table 2. Fatty acids of PC, aldehydes and fatty acids of PE fractions from myelin

	% of total		μg/mg myelin dry weight	
	Normal	MS	Normal	MS
PC-fraction: Fatty acids				
C 16 : 0	33.3 ± 2.61	35.9 ± 3.05	14.08 ± 1.104	16.88 ± 0.519
C 16 : 1	2.4 ± 0.43	2.1 ± 0.65	1.02 ± 0.182	0.98 ± 0.305
C 18 : 0	16.2 ± 2.21	14.0 ± 1.32	6.84 ± 0.935	6.57 ± 0.620
C 18 : 1	45.6 ± 1.28	47.0 ± 1.92	19.27 ± 0.542	22.06 ± 0.902
PE-fraction: a: fatty acids				
C 16 : 0	6.8 ± 1.52	5.1 ± 0.59	3.08 ± 0.545	2.26 ± 0.274
C 18 : 0	9.0 ± 0.82	9.9 ± 2.99	4.13 ± 0.747	4.46 ± 1.455
C 18 : 1	48.7 ± 2.61	53.3 ± 2.46	22.27 ± 2.651	23.91 ± 2.536
C 20 : 1	10.3 ± 1.38	8.9 ± 1.68	4.72 ± 0.904	3.99 ± 0.874
C 20 : 4	7.9 ± 1.85	6.0 ± 1.27	3.64 ± 1.075	2.69 ± 0.713
Σ C 22 unsat.	17.3 ± 2.43	16.9 ± 3.37	7.84 ± 0.777	7.58 ± 1.575
b: aldehydes				
C 16 : 0	26.9 ± 4.05	26.3 ± 3.09	6.07 ± 0.913	5.86 ± 0.689
C 17 : 0	4.8 ± 3.12	3.8 ± 1.17	1.08 ± 0.715	0.85 ± 0.261
C 18 : 0	15.7 ± 2.19	15.9 ± 1.24	3.54 ± 0.494	3.55 ± 0.277
C 18 : 1	52.5 ± 6.73	48.7 ± 6.15	11.84 ± 1.518	10.86 ± 1.371

control brains. We found 358.8 ± 52.41 μg dried myelin in the latter and 230.2 ± 68.74 μg dried myelin in the former samples. As could be calculated from the data in Table 1, the ratio of total lipids per total proteins was 2.29 ± 0.148 in normal and 2.17 ± 0.059 in MS samples.

The specific activity of 2′,3′ CNPase determined with the 2′,3′ c-AMP as substrate was strongly reduced in MS specimens. In samples of myelin preparations from MS,

only 4.7 ± 1.11 μmol 2′ adenosinemonophosphate was produced per min/mg myelin protein, whereas that value in the normal controls was 7.2 ± 1.07 μmol.

According to the distribution of the major lipids (Table 1), only slight differences can be seen. However, comparison of the individual phospholipid classes showed that phosphatidylserine (PS) fractions were significantly reduced and phosphatidylcholine (PC) fractions were slightly increased in the pathological samples.

The fatty acid composition of the PC and phosphatidylethanolamine (PE) fractions and the aldehydes of the PE fraction are summarized in Table 2.

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Recent Trends in MS Therapy – Reports on Results

Long Term Immunosuppressive Treatment with Azathioprine in Multiple Sclerosis. A 10 Year Trial with 77 Patients

G. AIMARD, C. CONFAVREUX, and M. DEVIC [1]

Introduction

It is now well accepted that long term corticotherapy is ineffective in the global course of multiple sclerosis (MS) [6, 12, 14]. There are many arguments in favor of an immunological process as the source and development of the disease. Hence immunosuppressive agents are a logical tool in its treatment and they were proposed and introduced by Girard and Aimard as early as 1962 [8, 1, 9]. In 1966 Lejars [10] analyzed 30 cases with a month intensive treatment with cyclophosphamide and a mean of 2½ years follow-up. The results were promising. Since 1967, cyclophosphamide has been discarded owing to its notorious side effects, including loss of hair, and thus azathioprine has been used. A general review [4] was made of the 349 cases in Professor Devic's department collected between 1957 and June 1976 and classified as MS according to MacAlpine's clinical criteria [11]. This has provided an opportunity to test the effect of long term immunosuppressive treatment with azathioprine in MS. This has already been the topic of communications to the "Congrès de Melsbroëck" [2] and to the "Société Française de Neurologie" [3].

Materials and Methods

The dosage of the drug has been 150 mg per day on an average. In cases of drug tolerance problems it has been 100 mg per day. Hematologic supervision has been performed regularly whatever the duration and the tolerance of the treatment. Alarm counts, leading to an at least temporary interruption of treatment, were 3.5×10^5 red blood cells (RBC)/mm^3, 3.5×10^2 white blood cells (WBC)/mm^3, 35% polynuclear neutrophils, and 1×10^5/mm^3 for thrombocytes.

The immunosuppressors have been selected only for "definite" cases with the evidence of an actual malignancy shown by a high frequency of bouts (the treatment was never started at the first bout) or by a rapid worsening of the basic disability.

We have treated only 77 of the 349 MS patients with azathioprine. This low proportion arose because some of our medical team didn't prescribe azathioprine as, in the early stages, they were less convinced of its efficiency than its inconveniences. Conversely, this afforded a control group of nontreated patients, followed jointly.

Lastly, none of the treated patients have received a continuous corticotherapy. This medication has been given only in the case of a new bout according to a stan-

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dard 3 month regressive scheme: 30 mg daily of prednisolone the first month, 15 mg daily the second month, and 15 mg one day out of two during the third month.

Results

Drug tolerance problems led to cessation of the treatment in 12 cases. This includes:

1. Four cases with hematologic complications: two of anemia with leucopenia; and two of thrombopenia with hemorrhagic manifestations;
2. One sural thrombophlebitis soon after the instauration of azathioprine. In this case, the causal relation remains questionable;
3. Four gastrointestinal disturbances;
4. Three cases in which the case reports provide no clear explanation for stopping the treatment.

Incidents have usually occurred during the first weeks of treatment. None of them has been irreversible or would have, in and of themselves, precluded resumption of the treatment. Concurrent infectious diseases do not seem to have been more frequent in the treated group; only one case of shingles is reported. Lastly, no tumor has developed in the treated group, whereas a lymphoma complicated the course of a nontreated case [5]; in the opposite situation, there is no doubt that the causal relation would have been emphasized more than the hypothesis that the phenomenon was coincidental.

In 27 cases, a well-tolerated azathioprine treatment was given to patients during the progressive phase. In all 27 patients, this was a mistake, for their disability continued to worsen and three of them died. Nevertheless, no case seems to have been worsened by immunosuppressors. It should be emphasized that treatment has been given in very advanced, perhaps too advanced cases, and its lack of efficacy still needs to be demonstrated in initiating progressive forms.

In the 38 other cases with a good tolerance, the treatment was given during the remittent phase. The results are entirely different. On the average, the treatment has been started 5 years after onset of the disease and has been continued for an additional 5 years. Seventy-eight untreated patients in the control group had a remittent phase lasting at least 5 years and a mean follow-up of 10 years. These two groups – 38 treated patients and 78 controls – differ according to the following criteria:

1. The subsequent occurrence of the progressive phase was seen in only three cases in the treated group (7.8%), but in 28 controls (35%). The difference is significant ($\chi^2 = 10.23$; $P < 0.01$). The mean duration of the disease before the onset of progression was 7 years (extreme = 3–17 years) in the control group, i.e., 3 years less than the mean time of follow-up of the 35 treated patients who have escaped a secondary progressive course.

2. The worsening of the disability score, assessed according to the MacAlpine classification, is noted in nine cases in the treated group (23.5%) and 41 in the untreated group (52.5%). Here too, the difference is significant ($\chi^2 = 9.44$; $P < 0.01$). If, discarding the cases with a secondary progressive course, only cases with aggravated bouts are considered, we find six cases among the 35 treated ones (17%), and 13 among the 50 untreated ones (26%).

Table 1. Chronological occurrence of bouts in the nontreated group (78 patients)

Untreated patients	Number of patients	Duration between subsequent bouts	
		mean (years)	maximum (years)
$B^1 B^2$ [a]	76	3.8	20
$B^2 B^3$	62	2.6	10
$B^3 B^4$	33	2.4	7
$B^4 B^5$	14	1.9	4
$B^5 B^6$	5	1.4	2

[a] Abbreviation for "duration between the first and the second bout" at the remittent phase

Table 2. 35 remittent cases (3 cases with a secondary progressive course have been discarded) with a long term immunosuppressive treatment with azathioprine

	Period	
	Before treatment	With treatment
Mean number of bouts	3.6	0.8
Mean duration between bouts (years)	1.4	6.9
Mean duration of the period (years)	5.0	5.7

Table 3. Subgroup of 18 treated cases with "stopped" bouts

	Period	
	Before treatment	With treatment
Mean number of bouts	3	0
Mean duration between bouts (years)	1.7	–
Mean duration of the period (years)	5.2	4.2

Table 4. Subgroup of 17 treated cases with delayed bouts

	Period	
	Before treatment	With treatment
Mean number of bouts	4.3	1.7
Mean duration between bouts (years)	1.1	4.3
Mean duration of the period (years)	4.8	7.3

3. Lastly, the occurrence of new bouts has been analyzed for patients still in the remittent phase at the time of the study. Whereas in the control group the duration between two subsequent bouts became shorter with a continuation of the disease (Table 1), in the other group, new bouts seemed to be delayed by the treatment (Table 2). This feature is even more noteworthy, since in the 5 years before treatment, a mean of 3.6 bouts have been reported for the subsequently treated patients

and since the mean period between bouts is only 1.4 years, which reflects a worse than average course of the disease in the cases [4]. Among these 35 patients, there are:

- Eighteen with "stopped" bouts (Table 3): the mean duration of the period before treatment was 5.2 years, with a mean number of three bouts and a mean period of 1.7 years between bouts. The mean duration of treatment has been 4.2 years.
- The remaining 17 patients have had delayed bouts (Table 4). The mean duration before treatment has been 4.8 years with a mean number of 4.3 bouts and a mean period of 1.1 years between bouts. The mean duration of the treatment period has been 7.3 years, with a mean number of 1.7 bouts and a mean period of 4.3 years between bouts. But for these patients, the frequency of bouts before treatment was particularly high, and, in three cases, the new bout has occurred following a temporary interruption in treatment.

Conclusion

Our results show that long term immunosuppressive treatment in MS is usually well tolerated but ineffective in progressive forms of the disease. Conversely, it seems effective at the remittent phase compared with a control group. It delays the onset of the secondary progressive phase, slows down the instauration of disability, and delays the occurrence of new bouts.

These results agree with those of Sigwald et al. [15], Oger et al. [13], and Frick et al. [7]. They need further confirmation, but already, despite its notorious inconvenience, the use of long term immunosuppressive treatment is warranted in remittent forms of MS as soon as certain diagnosis has been made.

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Long-Term Treatment of Multiple Sclerosis with Azathioprine

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Scientific criteria for the efficacy of a remedy for MS are difficult to obtain. Whether a drug is effective only for the duration of its use or whether it also has a lasting effect on the subsequent course of the disease requires examination.

The relapsing and chronic progressive courses of MS must be considered separately. In the chronic progressive cases, primary and post-relapse chronic progressive courses are not differentiated.

The relapsing course of multiple sclerosis (MS) enables statements to be made on the annual relapse rate, which is a reliable standard for assessment of the course of the disease [8]. The period of observation during the treatment should be so long that at least one relapse may occur if the individual relapse rate of the case in question, determined from the disease course before beginning the treatment, is used as a base.

The course of the disease is taken into account for the assessment of therapeutic success in MS. The findings which have been obtained, in investigations at intervals of a few months over a period of years form the base. For this purpose, the degree of severity of the functional deficiencies is determined according to the Bronx scale and the neurological symptoms are scored. The course of the disease is represented by a curve. Although the course of MS is considered incalculable, this opinion is not correct, as first shown by Torben Fog [1] and confirmed by us. For the individual case there is a high constancy in the course of the disease.

The simplest way of assessing therapeutic success in MS seems to be the statistical comparison of several patient collectives after alternating treatment in the manner of a double blind study. This requires such a large number of patients that the conditions have only been fulfilled once so far, in an American study on ACTH [9]. If the relapse rate and disease course in a group of patients before, during, and after treatment are taken as standards for assessment, then the patients become their own controls, a procedure, which, according to the above statements, not only seems to be permissible but also the only practicable one [4, 5].

In the assessment of the therapeutic effect on the course of the disease, the time of onset of action must be taken into account for each drug. It may be assumed that azathioprine exerts its full effect on the disease process about half a year after therapy has been begun. For this reason we assessed the therapeutic effect of azathioprine based on clinical data which had been established 6 months after beginning treatment. Relapses of the disease which had led to the introduction of the treatment subsided within half a year. The interpretation of spontaneous remission of the relapse as a therapeutic effect must be avoided. It would also be wrong to take the in-

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tensity of the symptoms in relapse as a point of assessment of the further course of the disease, because an improvement might be simulated.

Over the last 10 years we have carried out a long-term treatment of 88 patients with azathioprine. The first results of treatment have already been published [2–7]. The daily dosage is 100–150 mg; the dose was determined individually, with frequent blood examinations. Important side effects did not occur.

Sixty-four patients with relapsing courses were treated for at least 1 year; the average was 28 months, and 9 patients were treated a second time for a similar period. Altogether, 73 relapsing courses could be assessed in this way. The relapse rate before treatment was 0.42 and fell to 0.16 during treatment. The differences before and during therapy are significant; statistically, the relapse rates of the patients were significantly reduced by long-term azathioprine therapy. Therapeutic success was assessed according to the course of the disease as shown in the curve. In doing so we have taken as a basis the clinical data which were observed 6 months after the beginning of treatment, for the reasons already given. During treatment, eight of 73 disease courses observed improved, 15 deteriorated, and 50 remained unaltered. The results gives a favorable impression: it correlates with the significantly lowered relapse rate. We know that disease course and relapse rate develop similarly in MS. The impression of an extenuated disease course is therefore strengthened by the fact that of the 27 relapses which occurred, 21 regressed completely, leaving no deterioration in the clinical condition.

Twenty-four patients with chronic progressive course were treated for an average of 34 months. Deterioration occurred in 14 cases. The poor prognosis of the chronic progressive form of MS is thus confirmed: ten cases remained unchanged; the expected progression did not occur. A favorable therapeutic effect can be seen here. Improvement was not seen in any of the cases.

After the treatment was discontinued, a relapse rate of 0.33 was found after an observation time of at least 1 year in 47 patients with relapsing courses in an average 33 months. The relapse rate was therefore lower than before therapy (0.42), but distinctly higher than during treatment (0.16). These differences are significant. The relapse rate after therapy increases in a statistically significant way.

Of the 47 relapsing disease courses, 38 had improved during treatment or had remained unchanged. These 38 courses showed a deterioration in 19 cases after treatment was stopped. The interval between the end of therapy and the beginning of deterioration was 20 months on the average. During therapy, about 19% of these cases had deteriorated; after the end of the treatment around 50% had deteriorated. This difference in the frequency of deterioration in the disease course is also significant. It is therefore important that the duration of treatment and follow-up time correspond to an average of 27 to 33 months.

As already stated, the disease course of MS can be represented by a curve obtained from a regression analysis using the scores of the neurological symptoms. This curve, however, not only shows an improvement or a deterioration of the disease, but it also permits an assessment of whether the course of the disease has changed significantly in time. If the therapy, for instance, causes a decreasing or a nonincreasing curve, this would suggest success. With regard to our patients, there is not sufficient data from the pretreatment period available to allow an appropriate comparison of the course of the disease before treatment and during treatment. We have

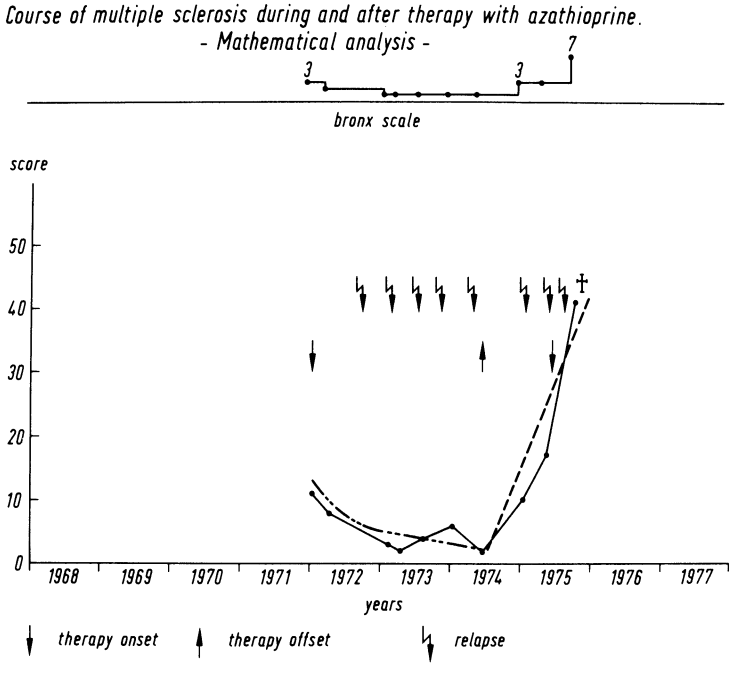

Fig. 1. During therapy there was improvement, although relapses occurred. After the offset of therapy there was deterioration and death

therefore investigated whether differences in the disease courses during and after therapy are significant. In 19 cases with deterioration after ceasing azathioprine treatment we were able to make a statement. The differences in the disease course for each case during and after therapy turned out to be significant. This also can be considered as an index for the efficacy of azathioprine. Figures 1 and 2 give examples: the regression curves (in dashed lines) correspond to the intervals: treatment and no treatment, respectively.

We summarize our experience as follows: Immunosuppressive therapy with azathioprine can only be successful as a long-term treatment lasting for at least 2 years.

Azathioprine therapy should be begun as early as possible. Our general experience that prospects for success are most favorable with early treatment has also been confirmed for MS. This indication is given as soon as the diagnosis is confirmed, even in the first manifestations; if the initial disease relapse does not regress completely, there is a disseminated symptomatology and marked CSF changes. "Benign types" and advanced stages of the disease are exceptions.

During azathioprine therapy of relapsing courses of MS, there is a significant reduction in the relapse rate and an extenuated disease course. The chronic progressive course of MS does not show the expected progression in about a third of the cases.

After discontinuing azathioprine therapy there is a significant deterioration in the course of the disease in about half of the cases of relapsing courses which had improved or remained unchanged during treatment, with an average interval of

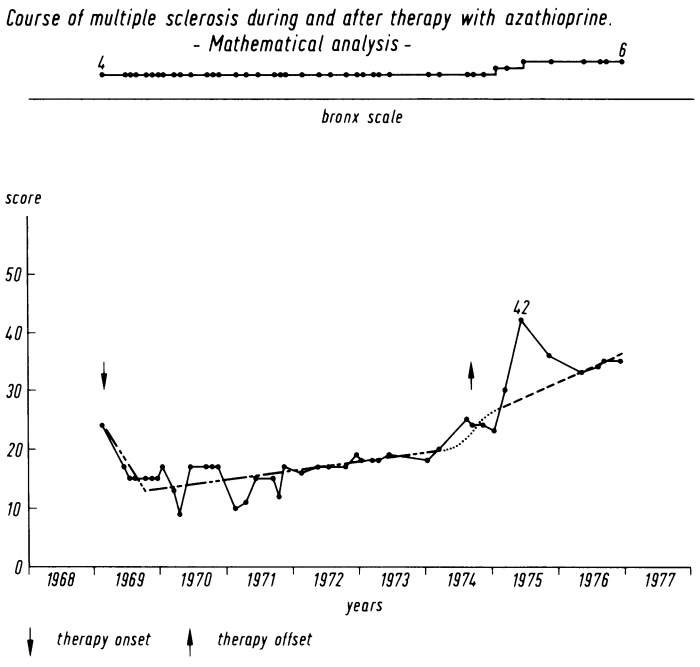

Fig. 2. During 5 years of treatment, there was only slight progression. After the offset of therapy there was significant deterioration in the course of MS

about 20 months. The relapse rate after discontinuing azathioprine therapy is also significantly higher than during therapy. MS takes a more unfavorable course after the end of azathioprine therapy.

Immunosuppressive therapy in its present form is problematic and unsatisfactory; it should be understood as a precursor of a specific intervention in the immunopathogenesis of MS.

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Long-Term Azathioprine Therapy in Multiple Sclerosis

D. DOMMASCH, M. LURATI, E. ALBERT, and H. G. MERTENS [1]

Azathioprine has been used in most of the therapeutic trials of long-term immunosuppression in multiple sclerosis (MS). Beneficial effects of the drug have been reported by several groups of investigators (cf. articles in this chapter by Aimard, Frick, Hommes, and their co-workers). In our previous studies of this treatment [1, 2, 5, 10, 11], a positive therapeutic effect was seen in relapsing/remitting courses of MS. Possible alleviating effects on chronic progression of the disease could not be demonstrated with statistical significance. This is an evaluation of azathioprine therapy in 52 MS patients who were treated for a minimum of 3 years or longer.

Patients

We tried to evaluate the course of MS in those patients whose treatment had been started between 1968 and 1972 and lasted for at least 3 years. Most of the patients attended routine follow-up examinations in our department. Some patients had to be traced through their family physicians or other hospitals, others were repeatedly invited for follow-up examinations.

Treatment

Azathioprine was administered orally in a dosage of 100–200 mg daily (approximately 2 mg/kg body weight). Leukocytes in peripheral blood were counted once a week in the early phase of therapy, later on every 2 weeks, and after 2 months at 30-day intervals. With a reduction of total leukocyte counts below 3000/μl, azathioprine treatment was reduced by ⅓ or ½. With less than 2000 leukocytes/μl, therapy was interrupted and a careful check of all blood findings was made. In some cases, especially in those with predominant lymphopenia, the bone marrow was examined. Significantly reduced thrombocyte counts were only observed in connection with granulocytopenia.

Evaluation

The course of the disease was evaluated individually for each patient. We used all the information that was available, including case histories on pretreatment periods and examination protocols from other neurologists. Each bout of the disease was reg-

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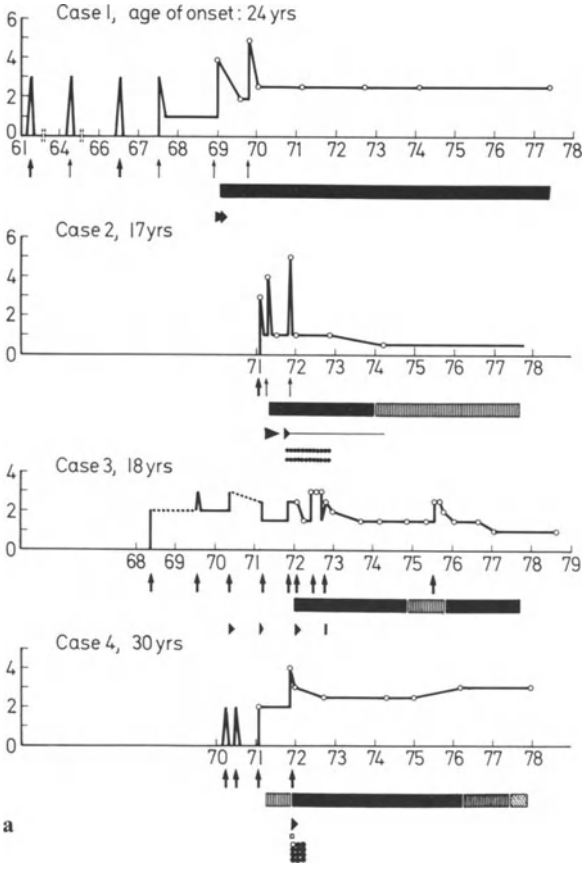

Fig. 1 a, b. Clinical courses of relapsing/remitting and relapsing/chronic progressive MS with long term immunosuppressive therapy with azathioprine. The vertical scale is Kurtzke's disability status scale; each dot on the curve represents the findings in a neurological examination. The beginning of each year is marked by the numbers below the horizontal line. *Thick arrows* indicate an exacerbation of the disease with new symptoms; *thin arrows* indicate a relapse with a recurrence of symptoms previously observed. *Black horizontal bars* show the period of treatment with azathioprine at a daily dosage of 150 mg; *striped and dotted bars* indicate a reduced dosage of 100 or 50 mg per day. *Triangles* and *horizontal lines* below these bars indicate corticosteroid treatment. *White squares* represent intrathecal injections of triamcinolone (40 mg); *white circles* represent intrathecal injections of 25 mg of methotrexate. *White squares* indicate i.v. injections of triamcinolone. *Black circles* indicate i.v. injections of methotrexate (25 mg). **a** Four representative cases of subgroup 1.1; **b** Four representative cases of subgroup 2.2

istered (large arrows in Fig. 1, 2) and also every relapse with only an enhancement of old symptoms (small arrows in Fig. 1, 2). Kurtzke's disability status scale [6, 7] was used in an assessment of chronic progression of the disease and also of remission or progression in the intervals between relapses. According to the individual course of the disease the patients were divided into three groups. Subgroups were formed of patients with comparable periods of treatment and/or post-treatment observation periods (Table 1).

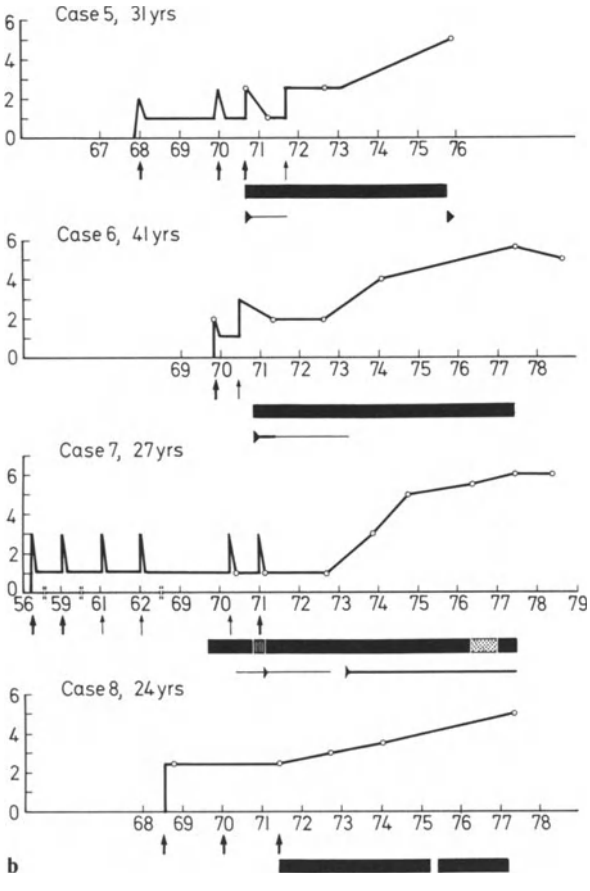

Table 1. Course of MS in 52 patients with periods of 3 and more years of azathioprine treatment

1.	Relapsing/remitting	
1.1.	Therapy continued after 3 years	5
1.2.	Therapy discontinued	
1.2.1.	Relapses	11
1.2.2.	Chronic progression	2
1.2.3.	Unaltered after end of therapy	8
2.	Relapsing/chronic progressive	
2.1.	Progressive before onset of therapy	11
2.2.	Progressive after onset of therapy	8
3.	Primary chronic progressive	7

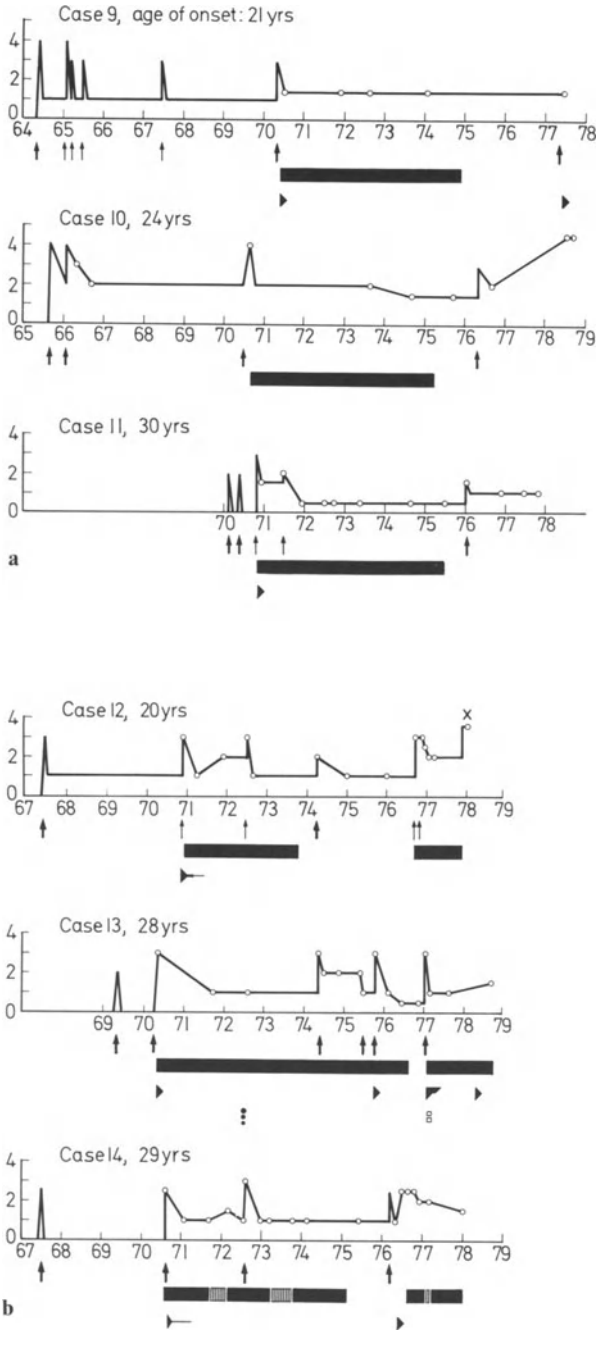

Fig. 2 a, b. Relapses after discontinuation of long term azathioprine therapy. Symbols as in Figure 1. Six cases of subgroup 1.2.1. **a** various intervals between the discontinuation of treatment and new exacerbations; **b** resumption of treatment after new exacerbations. × = accentuation of neurological symptoms and functional impairment during an acute viral infection, case 12

Results

Relapsing/Remitting MS

Eight patients with a relapsing/remitting course of the disease had been treated over periods of 3½ to 8 years before they were examined for this study. All of these patients had fewer relapses during the treatment period than in a comparable pre-treatment period. Relapses occured most often in the first year of immunosuppression and were very seldom observed in later years. Four characteristic courses are demonstrated in Fig. 1 a.

After discontinuation or a longer interruption of therapy, relapses were observed in 11 patients, some with severe new symptoms. Most of these relapses were seen 1–2 years after the interruption of azathioprine treatment in patients who had had significantly fewer relapses while being treated (Fig. 2 a). Chronic progression after interruption of azathioprine treatment was seen in two patients. Eight patients remained unchanged when their immunosuppressive therapy was discontinued; they have now been followed up for 3 to 4 years.

Relapses after reduction or interruption of therapy induced us to resume this treatment in six cases (three of these are shown in Fig. 2 b).

Relapsing/Chronic Progressive MS

Eight patients whose disease was predominantly relapsing/remitting at the onset of azathioprine treatment later developed a secondary chronic progression. Four of these patients are being described in Figure 1 b. Secondary chronic progression was apparently not influenced by immunosuppression at all. The disease showed further chronic progression in 11 patients after the onset of azathioprine therapy. (Table 1, subgroups 2.1. and 2.2.)

Primary Chronic Progressive MS

There was no significant change in the course of the disease in seven patients who had shown a primary chronic progression before the onset of treatment, although some of the graphs of their disability status scale indicated "plateaus" during the treatment period.

Discussion

Effects of immunosuppressive treatment on the course of MS can only be demonstrated in long-term studies. Short periods of treatment, unsuitable evaluation criteria, a small number of patients, and a low dosage of the immunosuppressive drug may be some of the factors responsible for negative results of therapeutic trials. Long term treatment as well as long pre- and post-treatment observation periods

could not be performed in a double blind study. Patients and their physicians had to be warned of the side effects of immunosuppression, and regular blood tests were performed. This report and our previous reports on azathioprine in MS [1, 2, 5, 10, 11] are therefore "open" studies. Specific difficulties in the evaluation of any treatment of this disease due to the high variability of its clinical course have been demonstrated. Periods of the disease before therapy were taken as control periods against comparable periods during and after treatment. Subgroups of patients were formed according to the various courses of their disease. Using this method, therapeutic effects may be evaluated in a relatively small number of patients. Individual courses of the disease are more uniform and easier to assess than an assumed "interindividual" course.

In the evaluation of the individual cases, we used Kurtzke's disability status scale [6, 7] and separately registered exacerbations of the disease with new symptoms and those with a recurrence of symptoms previously observed. In the evaluation of the relapsing/remitting course of the disease, Kurtzke's scale was used mainly so as not to overlook a beginning chronic progression (Figs. 1, 2).

More elaborate systems of assessment of the course of MS are scarcely useful in long term studies covering 10 or more years of the disease (including pre- and post-treatment periods). Kurtzke's disability status scale [6, 7] is most widely known and easily understood. It is seldom difficult to define a new exacerbation of the disease, but one must carefully differentiate between exacerbations with new symptoms and relapses with only an enhancement of previously observed symptoms [1, 2, 10, 11]. A mere accentuation of old symptoms because of stress or other exogenous influences must be distinguished from a relapse and be omitted in calculating the frequency of relapses.

In *chronic progressive* MS the rate of deterioration per year may be calculated and compared with respect to pretreatment and treatment periods. In our previous studies [10, 11] we found a somewhat lower rate of deterioration during azathioprine treatment but did not consider this as a sufficient proof of a positive therapeutic effect of immunosuppression in chronic progressive MS. The evaluation of the seven cases with chronic progression in this report also failed to demonstrate any significant change in the primary progressive course of the disease.

Secondary chronic progression in relapsing/progressive MS had been observed in 11 patients before the onset of therapy (subgroup 2.1.). It was not influenced by the treatment. In eight patients (subgroup 2.2.) chronic progression developed in the treatment period, in two patients (subgroup 1.2.2.) it developed, after the discontinuation of the treatment. These findings do not indicate a positive effect of azathioprine in treatment against chronic progression or against its development from strictly relapsing courses.

The evaluation of long term treatment of *predominantly relapsing* MS confirmed our previous postive findings [1, 2, 5, 10, 11] and again corroborates the results published by Frick and co-workers [3]. When the treatment period and a corresponding pretreatment period were compared with regard to the frequency of relapses in the same patient, a lowering of the relapse rate was significant. This can best be demonstrated in patients with a long duration of treatment.

A strong argument against azathioprine treatment of relapsing/remitting MS is that its effectiveness does not last beyond the treatment period. On the other hand,

new exacerbations after the discontinuation of therapy (subgroup 1.2.1.) tend to demonstrate the efficacy of the treatment. We resumed azathioprine treatment in six patients who had experienced severe relapses after the discontinuation of therapy (out of 11 patients in subgroup 1.2.1.). Eight patients have so far not shown further relapses or deterioration after the discontinuation of the treatment (subgroup 1.2.3.). Frick [3] has described the adverse effects of the discontinuation of azathioprine therapy in relapsing as well as in chronic progressive MS. *Side effects* of long term immunosuppression in the 52 MS patients of this report included a somewhat higher occurence of presumably viral infections. There were no serious complications of the treatment in these patients. Twelve patients who had been treated for approximately 2½ years and were therefore not included in this evaluation had stopped the treatment because of either a malignant course of the disease or because they had become well. Side effects were not the main reason. Some treatments were stopped because of leukopenia, which was always reversible. Comparably long periods of azathioprine treatment (with a much more marked effectiveness in the cure of the diseases) have been monitored in our hospital in patients suffering from myasthenia gravis or polymyositis, also without serious side effects [8, 9].

Azathioprine therapy of MS remains unsatisfactory in a large number of patients; chronic progression is apparently not influenced by this immunosuppressive drug. Positive effects of immunosuppression are demonstrated in predominantly relapsing/remitting MS cases. Further therapeutical trials with immunosuppression (e.g., "intensive" immunosuppression) [4] are needed.

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Azathioprine in Multiple Sclerosis. A Controlled Prospective Study

U. PATZOLD [1], P. HALLER [1], D. TROSTDORF [1], P. POCKLINGTON [2], and H. DEICHER [3]

The "immunosuppressive" treatment of multiple sclerosis (MS) by means of cytostatic medication has been proposed for the past few years [1–3]. A general use of cytostatics in the treatment of diseases which, like MS, display chronic inflammation, can only be justified if it can be proved in carefully controlled studies that this therapy arrests the steadily worsening sufferings of the disease or its fatal course. Such proof has as yet, owing to major methodological difficulties with regard to MS therapy studies, not been given conclusively.

In the spring of 1976 we initiated a computer-assisted longterm prospective therapy study to investigate whether a so-called immunosuppressive therapy with a low-dosage cytostatic has a beneficial influence on the further course of MS when compared with that of a control population.

Methodology

The study was conceived as an open-controlled follow-up study with which it was intended to compare the effectiveness of azathioprine with that of a "placebo".

The study included all patients originating from the district of Hannover who were undergoing treatment for definite MS at the Neurological Department of the Medical University of Hannover. All patients in the study had declared themselves to be in agreement with the course of treatment after having been informed of its possible side effects. The patients were studied in the order of their arrival at the department after conclusion of in-patient treatment.

The decision as to which form of therapy should be given was taken at random – not taking into account specific disease characteristics. The patients date of birth was used as the randomizing factor.

The cytostatically treated patients received a daily 2 mg/kg body weight azathioprine dosage. The "control" population was ordered to comply with a low fat diet of polyunsaturated fatty acids (sunflower oil, sunflower margarine). During severe relapses, all patients received the same treatment with prednisone (100 mg for 5 days, 75 mg for 3 days, 50 mg for 3 days, 25 mg for 3 days, 10 mg for 3 days, followed by a slow phasing-out process).

To evaluate the success of the treatment, the course of the disease throughout the treatment period was examined. We assumed that if the therapy was to be con-

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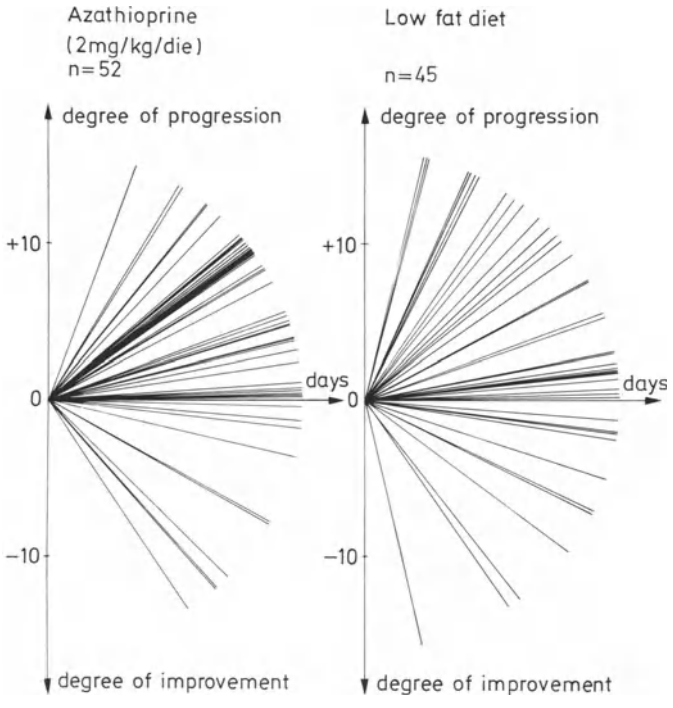

Fig. 1. Worsening or improvement of neurological signs during treatment with azathioprine compared with a control group

sidered effective, then the disease should not worsen, or the worsening should be less rapid than it would be if the patients were consuming a diet of fatty acids.

We proceeded as follows: At the start of therapy and then every 4 weeks to 2 months, sometimes more often, the current neurological state was examined, always by the same physician, and recorded on an Optical Mark Reader (OMR) [4] form set covering five individual forms. Here only signs observed by the examiner himself are recorded; subjective complaints and anamnestic data are not considered. The OMR forms then are subjected to strict automatic plausibility controls and findings are interpreted to generate a medical report [6].

The severity of the disease at each examination is then calculated automatically using an objective weighting scale, which is specially tailored to the findings on the OMR form [5], thereby allowing an estimate of the severity of the clinical signs. From the calculated values, the individual course of the disease for each patient can be described and calculated by using a regression analysis (Fig. 1). For the period reported here, the course of the disease can be approximated using the linear regression $y = a + b\,x$. The regression coefficient serves as a guide as to what degree the course of the disease worsens or improves. Using a U test (Wilcoxon), the regression coefficients of the two groups were compared with one another.

Furthermore, the rates of relapse per year under therapy were calculated. A relapse is defined as a worsening lasting for more than 24 h, or the reoccurrence of

Table 1. Data from patients not treated with azathioprine

Patient no.	Age at onset	Duration before therapy (years)	Course before therapy	Severity of disease starting therapy (value of rating scale)	Duration of therapy (days)	Worsening or improvement (*b*)	Relapses during therapy
41	19	0.1	interm./progr.	3	830	0.05244	6
21	33	0.3	intermittent	4	651	0.00785	3
25	35	1	intermittent	5	759	0.00460	–
5	21	1	intermittent	6	219	−0.00323	1
20	34	1	intermittent	7	659	0.00449	–
26	25	2	intermittent	8	696	0.02187	4
59	47	2.6	interm./progr.	10	529	0.00195	1
27	43	1	interm./progr.	10	500	0.15732	2
99	36	0.5	intermittent	11	239	0.01418	–
69	19	0.1	intermittent	12	481	0.00771	2
56	24	0.1	intermittent	12	605	0.03494	2
7	26	4	intermittent	13	265	−0.00641	2
112	32	0.5	intermittent	13	277	0.15237	1
86	38	1	intermittent	15	336	−0.02049	–
44	40	0.2	intermittent	17	641	0.00123	2
48	31	3	intermittent	18	641	0.00054	4
57	46	0.5	intermittent	20	564	0.00345	2
60	36	1	progressive	21	574	0.03308	–
34	22	1	intermittent	22	621	0.01486	1
120	42	0.3	intermittent	23	156	0.00609	2
35	47	9	intermittent	23	247	0.02158	–
61	34	1.5	interm./progr.	24	489	0.07914	–
53	34	12	interm./progr.	26	606	0.00471	1
23	27	5	interm./progr.	27	576	0.07998	1
50	16	0.1	interm./progr.	27	609	0.06055	4
102	33	1	interm./progr.	28	281	−0.00538	2
24	46	0.8	interm./progr.	39	662	0.08710	3
28	37	0.1	intermittent	39	671	0.02861	2

Table 1. Continued

Patient no.	Age at onset	Duration before therapy (years)	Course before therapy	Severity of disease starting therapy (value of rating scale)	Duration of therapy (days)	Worsening or improvement (*b*)	Relapses during therapy
122	45	0.5	intermittent	39	154	−0.17324	–
1	32	1	intermittent	39	452	0.03830	2
90	26	14	interm./progr.	40	95	0.16885	–
32	53	2	interm./progr.	40	237	0.00526	1
83	31	9	intermittent	42	348	−0.01340	2
30	22	6	intermittent	42	649	−0.01963	5
109	32	12	intermittent	45	274	−0.00510	1
87	25	1.2	intermittent	49	346	0.04292	3
78	17	2	intermittent	53	397	0.05486	1
118	30	2	interm./progr.	55	330	−0.03024	2
104	25	12	interm./progr.	62	138	0.08770	1
54	33	23	interm./progr.	65	258	−0.05847	1
92	28	33	progressive	70	198	0.08330	1
85	41	15	interm./progr.	71	436	0.06136	–
46	23	29	interm./progr.	93	192	−0.05185	1
110	42	5	interm./progr.	106	277	0.05990	–
9	29	7	progressive	118	232	0.07914	–

Table 2. Data from patients treated with azathioprine

Patient no.	Age at onset	Duration before therapy (years)	Course before therapy	Severity of disease starting therapy (value of rating scale)	Duration of therapy (days)	Worsening or improvement (*b*)	Relapses during therapy
16	21	0.1	intermittent	4	739	−0.00331	2
76	26	0.5	intermittent	6	465	0.00823	1
119	16	3	intermittent	6	203	−0.00112	–
74	38	1	intermittent	7	301	0.05212	3
40	17	4	intermittent	8	642	0.00100	1
37	27	1	intermittent	8	615	−0.00458	–
63	39	5	intermittent	10	389	0.02834	2
33	35	6	intermittent	10	125	0.03225	–
103	25	0.2	intermittent	12	198	0.01037	–
84	42	2	intermittent	13	182	0.03508	–
58	40	2	intermittent	13	599	0.00276	1
80	31	2	intermittent	15	394	0.02129	1
38	23	0.5	intermittent	28	302	0.04345	5
11	28	1	intermittent	18	727	0.01337	2
107	31	1	intermittent	23	272	−0.04587	1
71	26	0.5	interm./progr.	23	487	0.00200	2
47	43	1.2	interm./progr.	24	625	0.01265	3
91	26	14	interm./progr.	25	323	0.10229	2
77	25	0.8	intermittent	26	470	0.00618	3
115	40	5	progressive	27	250	0.02912	–
70	49	3	progressive	29	492	0.00999	–
31	29	6	progressive	46	260	0.06438	3
81	30	2	intermittent	31	421	0.02884	3
12	43	8	progressive	36	747	0.03343	3
3	18	18	interm./progr.	82	251	−0.04010	1
72	48	0.4	intermittent	31	224	0.03154	1
108	39	1	interm./progr.	44	321	0.06680	–
89	18	9	interm./progr.	45	352	0.03408	1

Table 2. Continued

Patient no.	Age at onset	Duration before therapy (years)	Course before therapy	Severity of disease starting therapy (value of rating scale)	Duration of therapy (days)	Worsening or improvement (*b*)	Relapses during therapy
82	20	1	intermittent	46	498	−0.02294	3
42	20	12	interm./progr.	48	680	0.02443	–
52	27	4	interm./progr.	48	595	0.04955	2
49	43	2	progressive	66	280	0.00162	1
100	15	7	interm./progr.	50	231	0.05023	2
10	19	6	interm./progr.,	51	606	0.02548	2
65	23	1	interm./progr.	51	515	0.01515	–
64	39	4	progressive	53	553	0.02429	3
117	26	3	interm./progr.	55	291	0.00976	1
114	16	20	intermittent	55	198	−0.06060	–
4	32	6	interm./progr.	83	150	0.02937	2
62	38	10	interm./progr.	64	284	0.02971	2
95	26	1	interm./progr.	59	314	−0.04541	–
121	25	4	interm./progr.	60	151	−0.00942	1
19	48	8	progressive	62	855	0.02993	1
73	27	8	interm./progr.	75	259	0.02434	1
45	22	10	interm./progr.	91	261	0.03094	2
93	21	3	interm./progr.	71	300	−0.02297	–
79	41	12	interm./progr.	83	280	0.00051	–
116	23	1	interm./progr.	61	547	0.02961	1
113	20	17	interm./progr.	85	216	0.03362	1
75	43	5	interm./progr.	85	447	0.00079	1
18	41	14	progressive	139	611	0.01432	–
13	17	20	interm./progr.	159	686	0.01262	2

symptoms that 4 weeks previously were not present or had, during this time, not become worse [7].

Preliminary Results

Our conclusions are based on results from 97 patients with varying degrees of illness. In both populations, relatively fresh cases, severe long-standing cases, and cases with intermittent and chronically progressive course were present. There were not important distinctions between the two groups with respect to age at the onset of disease manifestation, period of illness prior to therapy, or the duration of treatment (Table 3).

Table 3

	n	Mean age at onset	Mean duration of disease	Mean duration of therapy	Mean relapse rate per year	Mean degree of progression (*b*)
Azathioprine	52	29.6 years	5.4 years	408 days	1.08	0.01635
Controls	45	32.8 years	5.0 years	433 days	1.2	0.02610

The clinical data recorded for the patients treated, the number of relapses, and the degree of worsening or improvement expressed by the regression coefficient *b* can be seen in Tables 1 and 2. The average rate of relapse per year was almost identical for both therapy groups. The worsening appears to be somewhat less accelerated for the group undergoing immunosuppressive therapy (Fig. 1). The average regression coefficient was smaller for the patients treated with azathioprine than for the control group, although no statistically significant differences between the two groups can as yet be ascertained.

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Prognostic Factors in Intensive Immunosuppressive Treatment of Chronic Progressive MS

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Introduction

Since 1971 a short course of intensive immunosuppression has been induced in 86 chronic progressive multiple sclerosis (MS) patients [4].

This is a report on prognoses that were found when the complete data from 39 of these patients were reviewed in October 1976, 1–5 years after treatment.

Patients and Methods

The patients are of the same group as described in the previous article in this volume (see also [4]).

Immunosuppression was induced by 400 mg of cyclophosphamide and 100 mg of prednisone per day, till a total of 8 grams of cyclophosphamide was reached, usually within 3 weeks. Kurtzke's scales were used to score the clinical situation of the patients [5]. The Standard Neurologic Examination (SNE), Functional Systems (FS) and the Disability Status Scale (DSS) were scored immediately before (I), directly after (II), and 3 months after the treatment (III), as well as in October 1976.

At I, II, and III blood and cerebrospinal fluid (CSF) total IgG and albumin were determined. The progression rate was calculated as described previously [4].

Histocompatibility testing was performed with 180 different sera available from the Seventh International Histocompatibility Workshop and Conference in Oxford 1977 [3].

Results

To evaluate the results of the treatment the DSS scale was used. In comparison with the pretreatment DSS score (I), in October 1976, 13 patients showed a decrease of one or more points, indicating improvement (DSS+ group).

In October 1976, 14 patients scored at the same level as before treatment (DSSo group), and 12 patients scored one or more points higher on the scale, indicating deterioration since treatment (DSS– group).

These three groups were compared with regard to data collected 1–5 years before at I, II, and III, to detect significant differences (Table 1). Most important seemed to be the comparison of data from the DSS+ and DSS– group.

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Table 1. Table of parameters between three groups of MS patients whose DSS score showed improvement (DSS +), no change (DSS 0), or progression (DSS–) 1–5 years after a short course of intensive immunosuppression

	DSS +	DSS 0	DSS –	Significance + versus 0	Significance + versus –
Number of patients	13	14	12		
Mean of % decrease SNE between I and October 1976	49	35	–6	$0.02 < p < 0.05$	$p < 0.01$
Duration of stationary period (months)	27.3	17.3	10.8	$0.02 < p < 0.05$	$p < 0.01$
Age at treatment	34.1	39.3	40.9	n.s.	$0.05 < p < 0.10$
Duration disease before treatment in years	5.7	8.6	10.2	n.s.	$0.05 < p < 0.10$
Duration of control in months	27.3	29.8	26.8	n.s.	n.s.
SNE score I	78.2	82.3	87.7	n.s.	$0.02 < p < 0.05$
SNE score II	61.8	72.1	76.8	n.s.	$0.02 < p < 0.05$
SNE score III	59.3	75.0	77.2	$0.02 < p < 0.05$	$p < 0.01$
SNE score October 1976	60.0	71.4	92.8	n.s.	$p < 0.01$
IgG spinal fluid at I in mg/l	94.7	97.6	86.4	n.s.	n.s.
IgG serum at I in mg/l	11,377	11,185	12,396	n.s.	n.s.
Total protein in spinal fluid mg/l I	483	522	482	n.s.	n.s.
II	422	511	448	n.s.	n.s.
III	413	487	447	n.s.	n.s.
DRw2-positive homozygote	8/13	1/13	1/11	$p = 0.01$	$p = 0.025$
Progression rate	17.0	9.2	8.1	n.s.	n.s.

The mean SNE scores of each DSS group at I, II, and III and in October 1976 were calculated. In Figure 1 the mean course of the disease in each DSS group is graphically represented. From Table 1 and Figure 1 it can be seen that the percentage decrease in SNE score in October 1976 was much higher in the DSS+ than in the DSS– group.

The duration of the stationary period following treatment was much longer in the DSS+ than in the DSS– group. The age at treatment was lower and the dura-

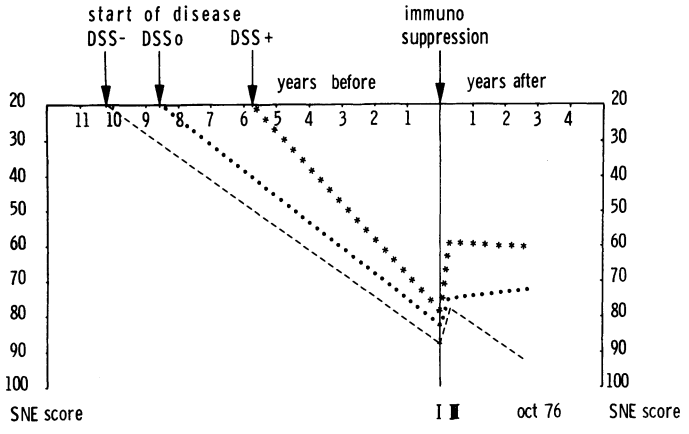

Fig. 1. Course of the disease as calculated by mean SNE scores for three DSS groups. Score II (immediately after the treatment) is left out

tion of the disease in years before treatment shorter in the DSS+ than in the DSS– group.

The DSS+ group is mainly DR_{w2} homozygous, the DSSo group mainly DR_{w2} heterozygous, and the DSS– group mainly non-DR_{w2} in histocompatibility testing (Table 2).

The progression rate of the DSS+ group is higher than in the DSS– group, although this difference does not reach statistical significance (Table 2).

If total IgG content of serum and CSF at II and III is expressed as a percentage of the starting value at I, the percentages of CSF and serum IgG do not show significant differences in the three DSS groups at II (Fig. 2). However, the percentages of

Table 2. Progression rates and histocompatibility typing in three DSS groups

DSS +			DSS 0			Dss –		
Patient number	P.R.	DRw	Patient number	P.R.	DRw	Patient number	P.R.	Drw
9	7	2	8	7.5	2.4	10	10	3
13	7.3	2	11	4.7	–	12	4	1.6
15	3.8	3	14	3.5	–	24	4.9	4.5
17	16.3	2	21	2.2	1.3	29	8.7	6.7
18	7	2	22	4.3	2.3	31	13.2	†
28	12	2	23	14.5	2.8	32	21.5	3
35	3.7	2	27	49	2.3	36	2.8	3.4
38	2.5	2.3	30	3.8	2.8	37	3.0	1.4
44	28	–	33	1.9	n.d.	40	2.0	2.4
46	15.5	2	34	10.6	7	42	10.4	2.4.7
48	22.5	1.2	49	10	2	45	1.3	2
56	37	2	50	5	1.6	54	15.3	2.7
57	58	3.4	51	3.5	4			
			53	8	3			

– = no DRw detectable

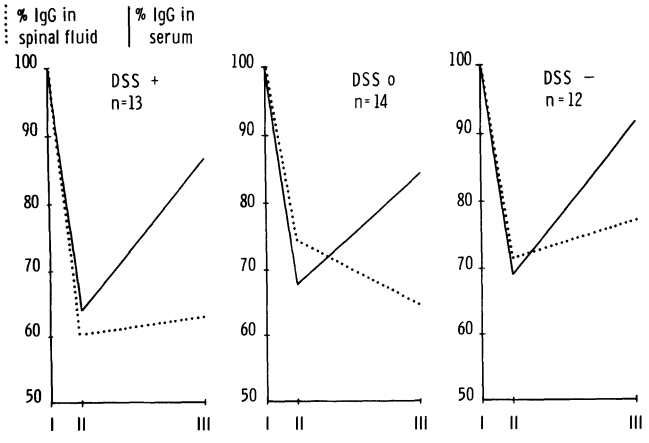

Fig. 2. Total IgG in serum and CSF expressed as percentage of I (immediately before treatment) in three DSS groups

serum and CSF at III are strongly different in the DSS+ group ($P < 0.01$), as well as in the DSSo group ($0.05 > P > 0.02$).

In the DSS– group at III the percentage IgG in serum and CSF is not statistically different (Fig. 2).

The CSF percentage of IgG in the DSS+ group at III is lower than in the DSS– group ($0.10 > P > 0.05$).

This indicates that in the DSS+ group the CSF-IgG percentage after treatment remains low in comparison with serum IgG percentage and in comparison with CSF-IgG percentage in the DSS– group. In the group that improved after immunosuppression, the CSF total IgG percentage remains low at least 3 months after the treatment.

Summary and Conclusion

Some prognostic aspects were found with regard to intensive immunosuppressive treatment in chronic progressive MS. Patients showing clear improvement 1–5 years after treatment have the following characteristics.

1. The disease starts at a relatively early age (mean 28 years).
2. The disease duration before treatment is relatively short (6 years).
3. The progression rate is high before treatment.
4. The clinical condition before treatment is good (low SNE score).
5. The improvement during treatment is strong and lasts for a long period after treatment.
6. The decrease of CSF-IgG percentage induced by the treatment remains for 3 months after the treatment.
7. The histocompatibility typing is DR_{w2}-positive homozygous.

Together with the results of the other report in this volume, strong indications are found that progression rate, long term effect of immunosuppression, changes in

spinal fluid total and "local" IgG content, and DR_w histocompatibility typing are interrelated.

In this study some evidence is found that in chronic progressive DR_{w2}-homozygous MS patients with a high progression rate, immunosuppressive treatment has a stronger and longer lasting effect on the IgG producing system in the CSF of these patients, produces strong clinical improvement, and inhibits progression of the disease.

We suggest that the genetically determined activity of the disease process is the same system that determines sensitivity to immunosuppressive treatment, and the same system that regulates "local" IgG production. As the DR_{w2} locus on chromosome 6 is close to the immune-response locus, some disturbance of the immune-response may be the primary cause of MS.

Since in the same region as DR_{w2}, complement C_2 and C_4 deficiencies are encoded, as well as congenital adrenal hyperplasia characterized by 21-hydroxylase deficiency [2], this leads to the question of whether a genetic enzyme deficiency in MS is the cause of immune-response disturbances and sensitivity to immunosuppression. This question is even more pertinent as Bertrams et al. [1] have demonstrated C_2 deficiency in MS in relation to DW_2-positive (I.R., DR_{w2}-positive) HLA typing.

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Intensive Immunosuppression with Cyclophosphamide in Remittent Forms of Multiple Sclerosis. A Follow-Up of 134 Patients for 2–10 Years

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Since 1968, immunosuppression with cyclophosphamide has been applied in 201 MS cases experiencing frequent relapses and hospitalized in the Belgian Center for Multiple Sclerosis (MS).

The details concerning our clinical experience have been published previously [7, 8]. This work is a reappraisal of the long term follow-up in order to assess more precise indications.

Materials and Methods

Cyclophosphamide was administered IV in order to effect intensive immunosuppression in a short time (1–2 weeks). Since this substance exerts its action by killing lymphocytes, the efficacy of immunosuppression was estimated by looking at the drop in lymphocyte count.

The total dose was adapted to maintain a lymphopenia of under 1000/mm^3 for 2–3 weeks. When leukocyte count was under 2000/mm^3, infections were carefully avoided by keeping the patient away from sources of infection.

Side effects were easily controlled. Nausea and vomiting were most frequently encountered; when present, hair loss was reversible in every case, cystitis was unfrequent, and blood infusion was indicated in six cases (3%).

A continuous follow-up was possible in 134 cases for 2–10 years and for over 4 years in 113 of them (84%).

Definition of success was based upon the annual relapse rate (ARR) calculated over a period of 2 years before and after treatment, and upon the stabilization period of the disease after immunotherapy.

Results

Changes of Annual Relapse Rate

Patients were distributed in three groups, according to the duration of the disease before treatment. A fourth group consisted of 13 severely handicapped patients with a long evolution of MS before immunosuppression. Even at this stage of evolution, they still suffered relapses and remissions.

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Table 1. Annual relapse rate before and after treatment as calculated over a 2-year period (119 patients)

Duration of disease before treatment (years)	Number of patients	Annual relapse rate	
		Before treatment	After treatment
1	10	2.2	0.3
2 – 5	43	1	0.23
6 – 10	53	0.75	0.26
Fixed handicap	13	0.46	0.46

Table 2. Reduction of annual relapse rate (expressed in %)

Duration of disease (years)	Decrease (%)
1	87
2 – 5	77
6 – 10	66
Fixed handicap	0

Table 3. Mean stabilization period (121 patients)

Duration of disease (years)	Mean stabilization period (years)
1	2.1
2 – 5	2.25
6 – 10	2.8
10	3

Table 4. Minimal stabilization period (106 patients)

	Number of patients	%
2 years	74	70%
3 years	42	40%
4 years	14	13%

ARRs calculated over a period of 2 years before and after treatment in 119 patients are summarized in Table 1. The reduction in these ARRs is expressed in percentages in Table 2.

There is conclusive evidence that a single intensive immunosuppression produced with cyclophosphamide in a short time, without any combined corticotherapy or maintained immunotherapy, can decrease ARRs by 66 to 87% over a period of 2 years.

By contrast, in the group of patients with a long evolution of MS before treatment and a heavy handicap, immunosuppression definitely failed to influence the course of the disease.

Stabilization of the Disease

The mean stabilization period, i.e., the time interval between immunosuppression and the next subsequent relapse, was determined in 121 patients. The results according to duration of the disease before treatment are summarized in Table 3. The patients experiencing a minimal stabilization period of 2, 3 and 4 years respectively, are listed in Table 4.

Our observations seem to indicate that, after a single intensive immunosuppression, most MS patients with a remittent form remain free of new symptoms for 2 years (70%) and some of them (40%) for 3 years. But it also seems evident that this interference with the course of the disease is limited in time.

Discussion

Because of the spontaneous variability of the disease, appreciation of the efficacy of therapeutic trials in MS remains a crucial problem. For this reason, many authors are reluctant to use patients as their own controls, but where immunotherapy is concerned, side effects make double blind studies impossible.

In calculating ARRs, we tried to minimize the risk of interference with spontaneous variability in the clinical course by restricting our considerations of pre- and posttreatment relapses to the 2 years before and after entry into the trial. Moreover,

Table 5. Spontaneous annual relapse rate as calculated over a 2-year period (91 patients)

Duration of disease (years)	Annual relapse rate	Decrease (%) ARR vs 2 preceding years
1 – 2	1.44	–
3 – 4	0.95	35%
5 – 6	0.78	18%
7 – 8	0.68	13%
9 – 10	0.60	12%

the spontaneous reduction of ARR according to the evolution of the disease was determined in a group of 91 patients not undergoing immunotherapy and regularly examined at the Belgian National Center for MS (Table 5). It is obvious that there is a tendency for ARRs to diminish with time when MS is progressing. This tendency is more marked during the first 4 years. However, it never exceeds a reduction by 35% when successive periods of 2 years are considered. The mean reduction of the ARR in our treated patients is 75% during the same period.

Moreover, to overcome any effect due to the variability of individual disease courses, our evaluations were based on an important group of patients with a prolonged follow-up. If we consider the criteria of Lhermitte et al. [11] "a drop by 50% of the ARR in 50 patients with a follow-up of 2 years," we observe that the number of patients in our series is more than twice as much and that the ARR is reduced by 75% on the average (Table 6).

Another argument for the efficacy of immunosuppression in MS is that in a group of 34 MS patients who showed no clinically detectable response to treatment with basic proteins, the ARR calculated according to the same criteria remains almost stable before and after treatment [8].

Table 6. Criteria for appreciating a therapeutic trial effect on the annual relapse rate

	Number of patients	Follow-up period	Decrease ARR (%)
Theoretic criteria (Lhermitte 1973)	50	2 years	50%
Belgian center patients series	119	2 years	76%

Conclusions

There are some discrepancies concerning the efficacy of cyclophosphamide therapy in MS [1, 2, 4, 6, 9, 12].

After a clinical experience of 10 years, we are deeply convinced that intensive immunosuppression with cyclophosphamide can influence the natural history of MS in about 70% of patients.

The reduction of ARR is more pronounced in patients with a short evolution of the disease before treatment (1–3 years). This efficacy gradually diminishes in patients with an evolution of up to 10 years and there is virtually no interference with the disease in patients with an evolution over 10 years or severely handicapped. Moreover it has been stressed by Drachman et al. [4] that cyclophosphamide therapy is ineffective in acute cases with a dramatic and rapid downhill course.

In our experience, cyclophosphamide immunosuppression is indicated in patients with frequent relapses and with a short evolution of the disease. By contrast, patients with an acute exacerbation did not improve. In these cases, corticotherapy remains the most effective treatment.

Exacerbation of the disease was never observed during immunosuppression, and most of the patients (60%) experienced improvement in neurological signs and functional disability.

Unfortunately, the efficacy of this therapy cannot be predicted and 30% of MS patients failed to respond to cyclophosphamide immunosuppression. In our experience, there is no correlation between the drop in lymphocyte count and clinical efficacy.

According to Lamers et al. [10], there is a marked decrease in CSF intrathecal IgG production after immunosuppression. Moreover, reappearance of high IgG levels should be associated with a reactivation of the disease.

The intrathecal synthesis of IgG was determined in 14 of our patients, before and after immunotherapy, using the isotachophoresis technique [3] and the regression line formula [5].

Using both techniques, intrathecal IgG production was decreased in half of the patients and increased in the others. It thus seems that, just as there is no significant correlation between local synthesis of IgG and the evolution of the disease, so there is no correlation between the reduction of IgG synthesis and the clinical efficacy of immunosuppression.

The crucial problem is how to extend the 2–3 year remission period observed in 70% of the MS patients after cyclophosphamide therapy. Preliminary clinical experiences conducted at the Belgian Center for MS indicate that a subsequent maintained oral immunosuppression might prolong the quiescent period of the disease.

Summary

Based on our experience with 201 patients, IV cyclophosphamide immunosuppression is clinically permissible and does not exacerbate MS. Side effects are trivial and easy to control.

A marked reduction of the annual relapse rate (by 75%) was observed in 70% of patients, and an improvement of neurological signs occurred in 60%.

Cyclophosphamide immunotherapy seems to influence the natural history of MS and to produce a stabilization of the disease for 2–3 years. In our experience, this immunosuppression exerts its action immediately after the beginning of the treatment.

Relapses occurring after treatment were less severe and easier to control with corticotherapy.

Favorable effects have been observed more frequently in patients with a short evolution and frequent relapses. Immunotherapy had no effect in acute cases or in patients with severe chronic neurological deficits.

However, the interference of immunosuppression in MS is of limited duration. There is hope that combined IV and oral immunosuppression will be able to extend the remission period in MS patients.

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A Pilot Study of Amantadine Treatment of Multiple Sclerosis

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There is evidence that multiple sclerosis (MS) is caused by a virus [4, 5]. If true, then treatment with antiviral agents such as amantadine might affect the course of MS [2].

The choice of experimental design for early studies on antiviral therapy of MS is difficult. One wants the most efficient design but does not want to miss a beneficial therapy even for some but not all patients. The National Advisory Com-

Table 1. Wilcoxon matched-pairs signed-ranks test on the difference score (d) between amantadine and placebo treated patients

Pair	Treatment[a]	Illness severity score at		Entry score[b] minus score at 40 weeks	Difference score (d) amantadine minus placebo	Signed ranks	Rank[c] with less frequent sign
		entry	40 weeks				
1	Am	51	59	−8	−2	−3	
	Pl	53	59	−6			
2	Am	42	35	7	7	5.5	5.5
	Pl	31	31	0			
4	Am	55	55	0	2	3	3
	Pl	58	60	−2			
5	Am	48	56	−8	−1	−1	
	Pl	34	41	−7			
6	Am	50	49	1	2	3	3
	Pl	47	48	−1			
10	Am	51	48	2	−7	−5.5	
	Pl	49	40	9			
12	Am	69	73	−4	−9	−7	
	Pl	70	65	5			
							T = 11.5[c]

[a] Am means amantadine received; Pl means placebo received

[b] A negative number indicates worsening of neurologic function

[c] In order to reject the null hypothesis that no difference exists between the patient with amantadine and the patient with placebo we would require T to be equal to or less than 2 ($P = 0.05$, two tailed test; $P = 0.025$, one-tailed test). Because T = 11.5, we accept the null hypothesis that no differences exist

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mission on Multiple Sclerosis has suggested three levels – preliminary, pilot, and full [6]. We have adapted the matched-pairs design used by Bloomfield et al. [1] for our pilot study.

For the measurement of drug effect, an "illness severity score" was derived from the physicians' (GWE and LWM) assessments of the neurologic condition of the patients [3]. "Weights" were assigned to activity (worsening in the preceding 6 months), course, Kurtzke's Functional Systems ratings, and Disability Status Scale scores. The sum of the weights was the "illness severity score." The illness severity score was used as the primary indicator of change. In deference to other investigators [7], the physician's subjective estimates of worsening, no change, or improvement, a standard neurologic examination, and Kurtzke's Functional Systems ratings and Disability Status Scale score were also used but will be reported elsewhere.

The study was conducted in a double-blind manner. One patient from each pair received 100 mg amantadine twice daily for 40 weeks; the other received a placebo. Analysis was performed using Wilcoxon's matched-pairs signed-ranks test on the difference score between pairs of patients [8].

Table 1 contains an elevated T value, indicating no difference between those receiving placebo and amantadine. This result was obtained using only seven pairs of patients!

We conclude that double-blind placebo-controlled therapeutic trials can be carried out with greater economy of numbers of patients if the matched-pairs design is used. Amantadine in a dosage of 200 mg daily for 40 weeks does not appear efficacious in the treatment of MS.

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Discussion

T. Fog: I should like to begin with a discussion of whether the substances have any effect upon the immune system at all. As far as I can see, only the two groups which have used cyclophosphamide have shown that it has any influence upon the immune system. May I have a discussion about that problem first.

D. Dommasch: May I answer this? Leukopenia of course is an indication for immunosuppression. But, in MS it is difficult to find specific tests which really measure the amount of immunosuppression. All the data in the literature actually differ. Whether you take lymphocyte subpopulations or stimulation tests, or others, there is no general agreement as yet. But I don't think you can doubt the fact that azathioprine in the dosages that were administered in all of these studies is immunosuppressive, because you can judge this from other diseases. Our own experience in neurology stems from myasthenia gravis, where we have obtained a certain beneficial effect, actually in all of our patients without any doubt. And there are enough other certified autoimmune diseases where you have a therapeutic effect. So I don't think you have to prove the immunosuppressive effect as such in each MS patient.

T. Fog: May I add one question too. Does this substance pass the blood-brain barrier? Is it necessary that it does pass the blood-brain barrier?

D. Dommasch: Even if it did, this would not be sufficient evidence for action in the brain because it is the metabolites that actually work in an immunosuppressive way, and they do not pass the blood-brain barrier. At least we don't know. Prof. Frick knows, because he determined it.

E. Frick: Azathioprine passes the blood brain barrier about 20–25% of the time. It can be found in the cerebrospinal fluid by immunosuppressive action. I think the best method to show immunosuppression is the changes in bone marrow. As you know, in the bone marrow there is a suppression of leukocytes and lymphocytes, and this is the best. The titer of IgG in the serum is not decreased by azathioprine but the turnover of IgG is lowered, considerably lowered. That is well known in azathioprine theapy as we do it.

O. R. Hommes: Well, I just wanted to remind you of the slides in which I showed that IgG levels were down to around 60% in the blood and at the same level in the spinal fluid. In the spinal fluid, IgG levels went up again but at a slower rate than in the serum. I didn't want to go into this, but it seems to indicate that the effect of immunosuppression on the IgG synthesizing system in the brain is higher, is more intense, than on the IgG synthesizing system in the blood. We have, at least now, found two ways of indicating this. I showed you one.

H. J. Bauer: I have a general question to all of the speakers on the azathioprine and the other treatments. Have they used steroids or haven't they? Now, when Aimard and the group in Lyon started, I know that they worked with a combination of steroids, and Dr. Hommes had very high doses of steroids as we could see on one of

his slides. Could we have some information on that? Then with respect to monitoring the azathioprine therapy, that is, counting the white blood cells, we have had one case where the white blood cells remained constant in toto but the number of lymphocytes, the relative percentage of lymphocytes, went down to 2%. Then I took this patient off and put her on a little later again and she went down to 5% again, so that there was a particular vulnerability of this one component. Another question to Dr. Ellison. How do you measure process activity?

D. Dommasch: Well, I showed the corticosteroid treatments on my graph – each individual application. We had corticosteroids in acute relapses. The next question was the white blood counts. We observed the same phenomenon, in quite a few cases not as low as this lymphopenia in the differential count, but in our MS patients as well as in our myasthenia patients. Here is quite a large number of patients with lymphopenia with normal or slightly subnormal white cell counts, but this is not constant. Even when you continue the same therapy for a few months, it may reverse. But as Prof. Frick pointed out, the bone marrow should be looked at then. This is probably most important.

H. J. Bauer: Did the lymphopenia make you stop the treatment?

Answer: Yes, we do stop.

D. Dommasch: May I add another question to Dr. Ellison, because anyway he will answer Dr. Bauer's question. I want to add, one always envies such controlled sticking to the rules studies. But do you really know whether amantadine works, is beneficial, or not? Because don't you have to follow up the matched pairs for a longer period? Do they really match, if you take strictly relapsing and remitting courses? I think it is almost impossible to find a matched pair in the early stages of the diseases and can you really say at this time that amantadine is insufficient?

O. R. Hommes: I just wanted to give some information about the lymphocytes and granulocytes in intensive immunosuppression. Both go down rather quickly in the massive, intensive type of immunosuppression. Within 10, 12, or 13 days we have in several patients an inhibition of the bone marrow. But the lymphocytes start to go down even when we stop the cyclophosphamide at that moment. We go on with the prednisone, then the granulocytes go up again and the lymphocytes stay low when we start with the normal count of granulocytes. We start the cyclophosphamide treatment again. Then the granulocytes go down again and the lymphocytes start to go down slower and slower. There seems to be, as Prof. Bauer suggests, a vulnerability between the lymphocytes as a cell line and granulocytes.

G. W. Ellison: Dr. Bauer asked, how do you measure process activity? In the study on amantadine the major criteria was progression as measured by the slope of the clinical course. My feelings about the use of immunosuppressive drugs are that IgG synthesis in the spinal fluid, the presence or absence of oligoclones as Dr. Hommes has measured, the serum IgM level, and probably the mixed leukocyte reaction would be useful criteria. On the horizon I would guess that antibody-dependent cytotoxity would be a good measure of immunosuppression.

Regarding the question of long term follow-up, we have reduced the patients in our clinic to a computer-generated curve. We are also forced to look at what happens before, during, and after their treatment. So, as long as you can keep the pair matched, you can follow them. And that of course is one of the major problems. Because, as you have all demonstrated, long term follow-up is very difficult to achieve.

And by long term, we mean 5 years. Are they really matched? When we ask this question, we try to analyze it statistically, and by looking at the degree of variation between our assessments of patients within the pairs, we have a rough indication of how well matched they really are. And they are quite well matched, surprisingly. Now, whether the mathematical proof has anything to do with real life, I'am not certain of yet. But as far as we can tell, this really works and they really do come out pretty closely matched. I have other problems as to whether amantadine is really insufficient treatment. The trend, as you probably gathered, was that the amantadine patients did better than those who received placebo, even over 40 weeks. However, to show a mathematically significant change with our amantadine would have required 16 years of treatment. So all I can say is that 200 mg per day for 40 weeks does not appear to modify the course. I also didn't go into the side effects of amantadine. It has anticholinergic effects, and two of our dropouts were because they went into urinary retention from the drug. So it is not a completely inocuous drug. We use it pharmacologically to treat Parkinson's disease. It has effects on neurotransmitters. I think that the drug has an effect upon patients with MS, for one of the patients had a marked improvement. She threw away her cane and began to walk again. At the end of the study we took away her amantadine and she was very upset, so we gave her dextroamphetamine and this had an almost identical effect upon her. She began to feel great and started walking again. But she always reminds me, "that's not the same. The amantadine is better, doctor!"

T. Fog: Is there anybody who will speak about myelin basic protein in spinal fluid during this treatment?

(No response).

G. W. Ellison: There is a little difference. I was referring to antibody dependence other than cytotoxicity by the patients' lymphocytes, so we might take antibody to thyroglobulin or something on that order and see how MS patients' lymphocytes respond. This would, I think, be a more sensitive indicator than using the antibody titer in the MS serum.

E. Frick: Yes, you know there exists a direct cytotoxicity and an antibody-dependent toxicity. Both are cytotoxic reactions and the direct cytotoxic reaction against MBP or against myelin cultures has been shown but only three or four times. I have carried out studies to show a direct cytotoxicity with this test system, but I had no success. Perhaps (and this is my opinion) there may be a lot of suppressor cells which diminished the reaction. I can't exclude a direct cytotoxicity, but I suppose that the reaction is charged by suppressor cells or by other suppressor factors in the serum. And you must differentiate between the direct cytotoxicity, which is difficult to show in MS, and the antibody-dependent cytotoxicity which can be easily studied by everybody.

L. P. Weiner: It is both wonderful and surprising that your experience in treating MS patients differs from a 10-year history with renal transplant collagen vascular disease, where the same doses of these drugs are used, and the incidence of complications is enormous, many of which are very serious. And just a brief review of the literature in terms of either disseminated cases with azathioprine or in renal transplants – Vascular disease or CNS infections include many cases of herpes simplex, varicella zoster, cytomegalic virus, which often result in the rejection of transplants, progressive multifocal leucodystrophy (PML), disseminated mycobacteria,

disseminated *E. coli* fungus infections such as Candida, Nocardia, and Aspergillus, and Protozoan infections such as Toxoplasma and Pneumocystus carinii.

And one cannot make light of the reticulum cell sarcoma which has been evaluated over a large number of cases in the past 10 years in renal transplants and patients with azathioprine. The incidence is 2.2 per 1000 cases of lymphoma, a risk rate 30 or 40 times that expected. Reticulum cell sarcoma is 330 times more common, and the frequency of lymphoma of the brain is 52% versus 1% expected. So the fact that your controls got a reticulum cell sarcoma (referring to Frick) does not make me very comfortable. I think that certainly it may well be that a renal transplant patient reacts differently; it may well be that you are suppressing different things with the same drug in different patients. But I think that the incidence in the rate of complications is surprising.

D. Dommasch: I didn't mention the side effects in my report because of the limited period of time. We saw one cytomegalovirus infection and in one not MS but myasthenia patient who had spent the summer vacation in the Mediterranean we had a severe bacterial infection, which bought her back to Germany, where she actually died from this infection.

We did not see a reticulum cell sarcoma or lymphoma of the brain. One of our patients has been diagnosed elsewhere, but this was after a very short time following the beginning of the treatment. We have one carcinoma of the breast in our group of MS patients, but not in the group I presented here. This was found 7 months after azathioprine treatment had started. I wish to add a remark about the occurrence of malignant neoplasms in MS patients anyway. It has commonly been thought that tumors occur rarely in MS patients as compared with normal controls, but in the last 2 years we have seen two malignant tumors in untreated MS patients who never had even had corticosteroids.

O. R. Hommes: I think that Dr. Wüthrich published a case of lymphosarcoma of the brain after cyclophosphamide treatment. I don't know if he is here and can comment on this. The other thing is that Pughley and Campbell published in 1977 a review of malignancies that were found after treatment with cyclophosphamide. I think the findings in their 66 cases are very important. I'm just quoting from memory, and I'm not exactly sure. I think that they found 66 cases in the literature and 44 of them had tumors that came on in those patients who were treated with cyclophosphamide because of malignancies. So they got a second malignancy after cyclophosphamide. The rest were treated for nonmalignant conditions; these people got a malignancy after very high doses, or a total of about 80 g of cyclophosphamide. So if we give these patients 8 g, we remain far below that level. Pughley and Campbell say that they think as soon as you reach 80 or a 100 g total of cyclophosphamide you come into the range of a malignancy.

R. E. Gonsette: Concerning our treatment with cyclophosphamide, it was over a very short period of time and the side effects were rather trivial. A blood transfusion was necessary in five patients out of 200, and we had cystitis in perhaps five or six patients too. So, as I said, the side effects were very trivial and easy to control. Concerning the problem of malignant tumors in MS patients: in our files at the center we have about 2000–3000 MS patients and we have reviewed this problem for another study. In our experience the occurrence of malignant tumors in MS is exactly the same as in what we call a normal population.

R. Wüthrich: As we said, we had a patient observed after 2 years of treatment with azathioprine. The treatment was started in Würzburg and he died then of lymphosarcoma of the central nervous system. But this is a unique case and its the only one we've found in the literature with treatment for MS. So I don't think this is something frequent.

T. Fog: Dr. Frick, will you comment on your experience with side effects?

E. Frick: We had no serious side effects. As we said, we had three tumors, one carcinoma of the ovarium, a rectum carcinoma, and a stomach carcinoma. But the carcinoma appeared one-half year after the beginning of the treatment, and we believe there is no connection. The rate of carcinomas in MS patients in Germany is unknown. But I don't think that the rate is the same as demonstrated in the Israeli population by Alter and co-workers.

T. Fog: What about the control of bladder infections and decubitus?

E. Frick: No, we had no infections. When the patients can't walk and they are bedridden, azathioprine therapy is not indicated, as you know. The "burned-out" cases should not have immunosuppressive therapy. Also, when the bladder is infected, one should not apply this therapy.

D. Dommasch: Just to make it complete, you should also always exclude tuberculosis or other chronic infectious diseases.

G. Aimard: In our communication we have spoken of side effects. In our experience there were 12 cases with side effects. None were irreversible. We mentioned one case of lymphoma, but it was in the untreated group.

T. Fog: As far as I can see the different clinics are continuing their treatment in some cases at any rate. What bothers me is that in the progressive cases, as far as I could find out here by listening to what was said, the efficiency of treatment has been almost nil. So I think that if a substance has any effect upon this disease, it must be in these more malign cases too.

O. R. Hommes: I think we should at least make a few comments on this point – because what is a progressive patient? What we call a progressive patient is a patient who has a progressive disease without bouts. But he can have progression during a period of 10 years, and he also can have progression during a 1-year period. Now what we show is that if the patient is young and has a progressive course with rapid deterioration, then the prognosis for immunosuppression is good. Because the progression rate in a group of DSS+ patients was rather high. So we should treat patients that are young and deteriorating very quickly. Also, I think that patients who type as HLA Dw2 have a good prognosis.

T. Fog: What I mean by progressive cases is not the slow progressing spinal cases mostly: rather I mean the malign cases which may or may not have bouts but which are progressing. That is my definition of progressive cases. You could call them malign cases.

O. R. Hommes: I think that the most malign cases should be treated as soon as possible.

D. Dommasch: Dr. Gonsette doesn't say so himself, but I think one could infer from his report that he stopped or at least alleviated some malign courses, isn't that correct? With maximum immunosuppression as he described it, at least in the paper published, we had the impression that some malign courses were stopped or allevi-

ated and did not progress as much as they had before after the first cyclophosphamide therapy.

O. R. Hommes: I would like Dr. Gonsette to comment on repeated intensive immunosuppression in his patients, because some of these patients deteriorate after a certain time and he starts to immunosuppress them again.

R. E. Gonsette: This is a very delicate problem. We were very reluctant to induce a second immunosuppression. But some of our patients asked for a second course of immunosuppression 3 or 4 years after the first treatment. They were very quiescent for 3 years and then they had two or three relapses and they asked for a new immunosuppression. Up until now we have had about 25 patients with two or even three immunosuppressions, extending over a period of about 8–12 years. It is very difficult in such a small number of patients to get an idea of the action of this immunosuppression. We feel that the second immunosuppression is effective, but less effective than the first one. In our report we calculated the efficacy of the second immunosuppression and this was lower. But I think that we cannot come to any definite conclusion.

T. Fog: I also consider it important that some speakers have found that after they stopped the treatment, rapid progression followed. That is, if you begin with treatment, you have to continue, because you run a risk for the patient. Could anyone comment on this? It seems very difficult to decide when to stop treatment.

E. Frick: I don't believe there is a higher risk for the patient. We give azathioprine for 2 or 3 years; this period of treatment appears necessary to achieve an effect. There are other cases where we see there is no effect and we stop the treatment. In most cases, as I told you, in the relapsing form in about 80%, there is an effect, and the relapse rate is diminished. If the course has not worsened during a period of 1 to 2 years, and the last relapse was 2 years ago, we stop the therapy and wait 1 to 1½ years before beginning again. Then we resume the therapy even if the disease has not worsened. And we can continue with a new period of therapy of 2 or 3 years. I think that this therapy should be carried out in comparison with the azathioprine therapy of myasthenia gravis, which is continued over many years.

G. Aimard: In our experience it is necessary to maintain continuous, long term immunosuppressive therapy. We do not stop therapy.

Dr. Tourtellotte: Could a double-blind study be considered?

D. Dommasch: I rather doubt it. Over this period of time, using an immunosuppressive drug, you would have to include so many safeguards that it probably would not be practical. And the study would not continue to be blind. And if I may cite Dr. Ellison, who found cholestatic effects of amantidine, when you have to control the blood cells and the differential blood count so often, the first problem is to have a place for treatment and to always control the differential blood cell counts over many years. And there are other problems in setting up a double blind study with the immunosuppressive agents.

O. R. Hommes: I would like to comment on this because it comes up every time. At least in the intensive immunosuppressive patients, it is impossible because they all lose their hair. There is no question – this cannot be a double blind. Regarding constant low dosage of immunosuppressive therapy, I doubt the possibility of a double blind study even in these patients, because even they show changes, and they are well aware of the effect of using this very strange and very potent drug.

Dr. Kelly: Just one observation I thought perhaps needed to be made when we are talking about treatment in this condition: It seems to me that sometimes we tend to forget that we are talking about a disease that has a natural history of 20–30 years, and therefore any treatment that is going to be used is a life sentence, if it is going to be proved to work.

A Synthetic Copolymer of Amino Acids in a Clinical Trial for MS Therapy

R. ARNON [1]

This report describes a limited clinical trial in which a synthetic basic copolymer of amino acids has been used for multiple sclerosis (MS) therapy. This copolymer, denoted Cop 1, is composed of alanine, lysine, glutamic acid, and tyrosine, and is immunologically cross-reactive on the cellular level, with myelin basic protein (MBP), the encephalitogenic protein which induces acute experimental allergic encephalomyelitis (EAE) [9]. The rationale behind this study was the assumption that there is a resemblance between MS and EAE, and hence that a material capable of suppressing EAE might exert a beneficial effect in the case of MS as well.

Previous studies from our laboratory have demonstrated that Cop 1 is effective in suppressing EAE in guinea pigs and rabbits [5–8] as well as in primates such as rhesus monkeys [7] and baboons [2]. Furthermore, in recent experiments done in collaboration with Dr. Wisniewski (unpublished data), Cop 1 proved capable of preventing and suppressing chronic relapsing EAE in guinea pigs, a disease which perhaps represents a closer experimental model for demyelinating diseases [10]. Toxicity experiments have indicated that Cop 1 does not cause any adverse effects in mice, rats, or beagle dogs. It was therefore considered suitable for feasibility studies in clinical trials for MS therapy.

In a preliminary trial with acute disseminated encephalitis (ADE), a disease closely similar to EAE in clinical course, pathological manifestations, and immunological features [3, 4], all three patients treated with Cop 1 showed complete recovery within 3 weeks of initiation of treatment, whereas of two untreated patients, only one recovered completely (albeit after a longer time) and the second retained some of the neurological symptoms for a long period after recovery from the acute phase [1].

Four patients in a severe stage of MS were subjected to treatment with Cop 1 for a period of several months [1]. Cop 1, at a dose of 2–3 mg per injection was administered IM in an aqueous solution in physiological saline. Each patient received a course of initial treatment while hospitalized, which was given every 2–3 days for 3 weeks, followed by weekly injections, at home, for a period of 2–5 additional months.

The results are described in the following: The condition of patients before treatment: Case 1 (B. T.), a 40-year-old man suffered from MS for 8 years, was bedridden, and had a spastic paraparesis, moderate optic neuropathy, and severe cerebellar disturbances. In the last 2 years he showed chronic progressive deterioration with 8–9 relapses per year. Case 2 (J. S.), a 23-year-old man, has suffered for 3 years from a fulminant nonremittent form of MS. He was paralyzed in both legs and had

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severe cerebellar, ocular, and bulbar dysfunctions. He was unable to eat or talk, was fed by stomach tube, and suffered from pressure sores. Case 3 (A. M.), a 30-year-old woman, had MS for 2 years and had severe weakness in her legs and a very serious incoordination of her limbs. She had disturbances of vision and dysarthria of the cerebellar type. Case 4 (S. C.), a 40-year-old woman who had MS for over 10 years, was in a static phase, with sporadic relapses and remissions.

The results of the treatment are summarized in Table 1.

Table 1. Results of the treatment

Case no.	Duration of treatment (months)	Globulins in CSF (%)	Lymphocyte transformation with BP	Clinical changes observed
1	3	17.1 → 7.3 → 14.5	1.3 – 2.7	Initial remission for 3 months, then relapse
2	6	23.0 → 23.8	1.9 → 2.9 → 1.7	Static (following rapid deterioration before treatment)
3	4	16.9 – 13.2	0.8 → 2.6	Improvement in speech and vision
4	6	17.4 – 17.0	1.5 – 1.0	Static

The clinical condition of cases 2–4 remained static during treatment and, although no improvement was observed, there was no further deterioration in their motor function. Case 3, and to a lesser extent case 2, showed mild to moderate improvement in speech and visual functions. Case 1 showed initial remission for 3 months, but thereafter suffered a relapse while still under Cop 1 treatment.

No side effects were noted in any of the patients. There were no changes in blood pressure, heart rate, or EEG. No allergic or toxic reactions were observed. Blood count and urine and blood tests gave normal results, and liver and kidney functions were normal.

There was no change in the levels of serum immunoglobulin which were normal. CSF IgG decrease in case 1 was from 15.9% to a normal value of 7.0; smaller a decrease was observed in Case 3, while in the two other patients no change was observed. The immune response to MBP, which was negative before treatment, became positive in 3 cases, but no adverse effects were noted.

For several reasons, it is difficult to draw conclusions from this study regarding the efficacy of treatment: (a) the number of patients is small; (b) they were all in a very advanced stage of the disease, in which the damage to the nervous system might have been irreversible; and (c) there is no information about whether the dose is suitable. The most important observation is that Cop 1 did not cause any side effect or any toxic or allergic reactions. These findings render this treatment suitable for further and more extended clinical trials. Such trials with patients with less severe stages of MS, and using varying doses of Cop 1 have been initiated recently, but no results are available as yet.

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Studies on Myelin Basic Protein Administration in Multiple Sclerosis Patients

1. Rationale and Preliminary Report of Immunologic Observations

J. SALK [1], F. C. WESTALL [1], J. S. ROMINE [2], and W. C. WIEDERHOLT [2]

For many years acute experimental allergic encephalomyelitis (EAE) has been studied as a presumptive model for multiple sclerosis (MS) [1, 2, 13]; more recently, a chronic form of EAE has been described [14, 15]. However, a comparison of MS with either form of EAE has revealed both similarities and differences [9, 10, 13, 15, 20]. Since EAE in animals can be suppressed by the administration of myelin basic protein (MBP) and its derivatives [3, 6, 7, 11, 12, 16, 18], or by synthetic polypeptides [19], an investigation was undertaken to determine the relationship between EAE and MS in this respect by studying the effect of MBP administration in patients with MS.

MBP Studies in Animals

A method for the suppression of EAE with MBP in animals was designed that would be acceptable for use in humans. Hence, MBP in aqueous solution was studied to determine the conditions for effectively suppressing EAE. It has been found that the effective suppression of acute EAE in guinea pigs and rabbits depends upon dose, timing, frequency, and duration of MBP administration following sensitization; also, the quantity of MBP required is related to the severity of the experimentally induced disease, which in turn depends upon the amount of encephalitogen and upon the composition of the adjuvant in the sensitizing inoculum [17].

The MBP used in these studies is of porcine origin and was prepared by The Lilly Research Laboratories (Indianapolis, Indiana) employing a modification of the procedure of Diebler et al. [5]; toxicological tests in guinea pigs and monkeys revealed no abnormality after 90 days of daily subcutaneous administrations of 1 mg or 10 mg/kg. The EAE-suppressive effect in guinea pigs sensitized with human brain tissue suspension is shown in Figure 1. The results of MBP clearance studies in guinea pigs are shown in Figure 2. The level of MBP in the serum of guinea pigs one hour after different doses is shown in Figure 3.

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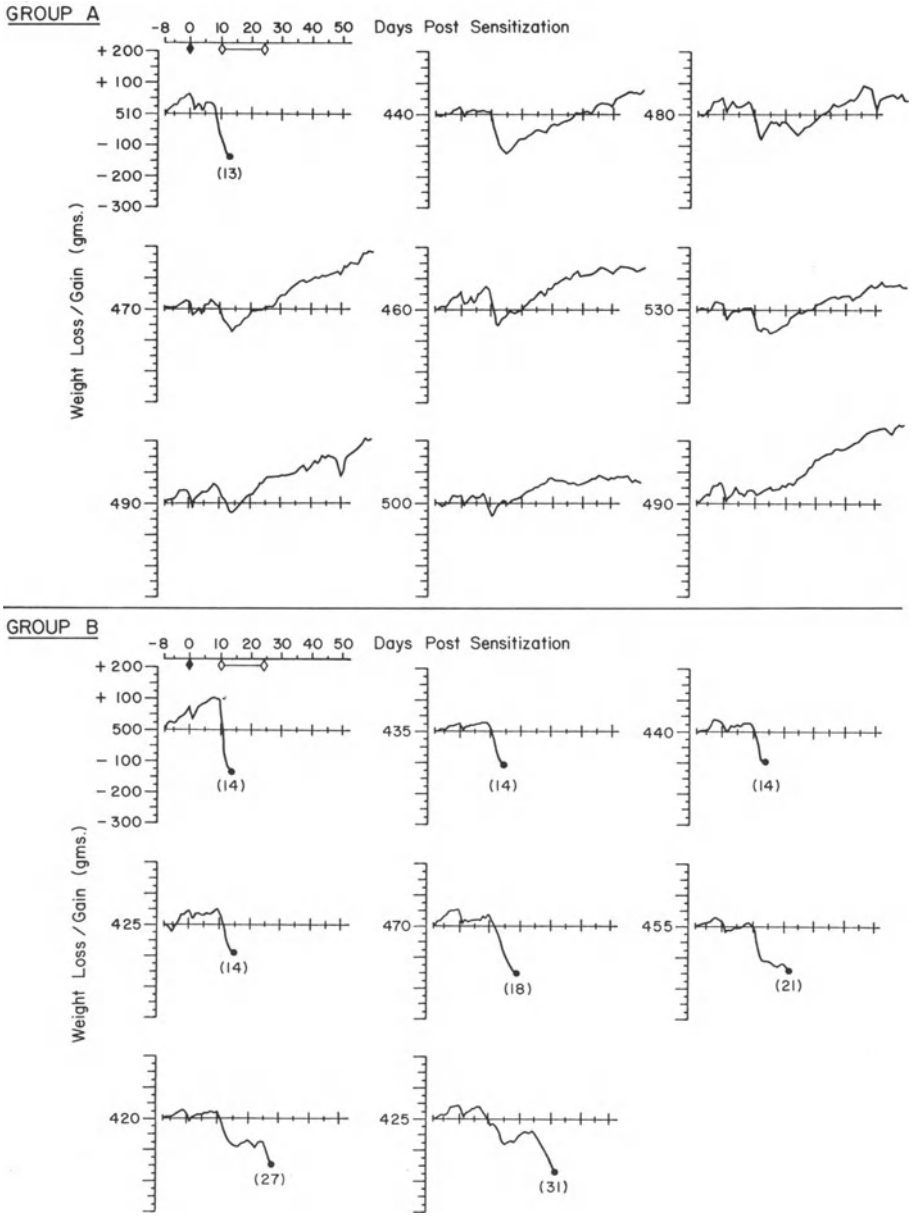

Fig. 1. EAE-suppressive effect of porcine MBP in guinea pigs sensitized with whole brain tissue suspension in complete Freund's adjuvant (CFA). Sensitization (using 3.3 mg of human brain tissue in CFA) was by footpad inoculation on day 0; subcutaneous injections of 500 μg/0.5 ml of porcine MBP in saline was given in Group A and 0.5 ml of saline as control in Group B, from the tenth to the 24th days post sensitization. Animals were weighed daily 8 days before and 59 days after sensitization. The starting weight for each animal is noted and the weight curve for each animal is presented separately. Numbers in parentheses indicate day of death or of sacrifice of moribund animals (from exp. 43–76)

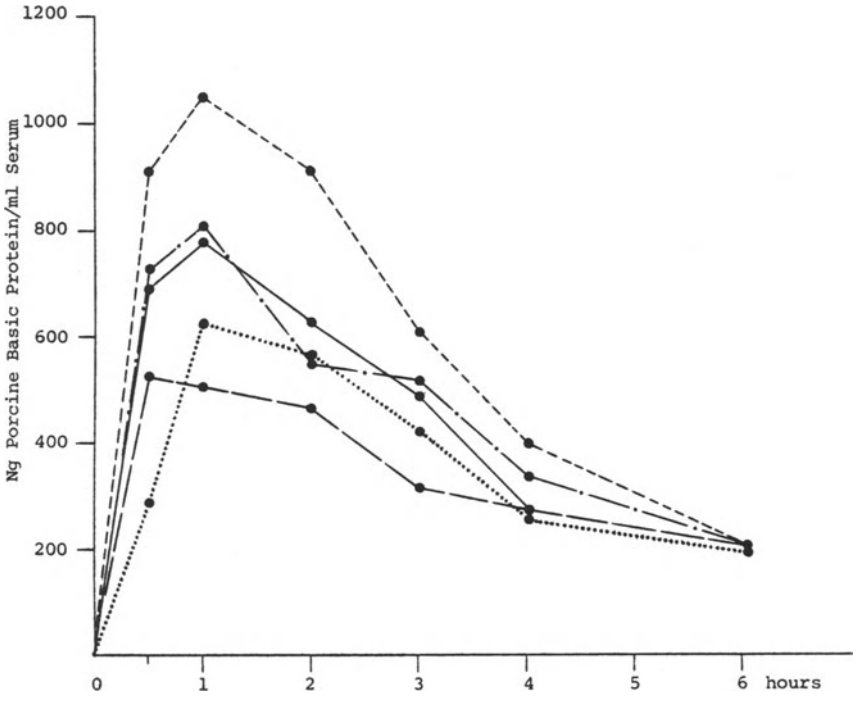

Fig. 2. Clearance rates of porcine MBP in five untreated guinea pigs each given 2000 μg/kg IP (from exp. 49–75B)

MBP Studies in Humans

The primary objective of this preliminary phase clinical study is to observe for adverse effects of MBP administration in patients with MS prior to consideration of a pilot placebo-controlled therapeutic trial. As part of this study, short- and long-term observations are being made for any immunologic as well as clinical effects.

The initial regimen of MBP administered to the first three patients was similar to the 2 week course used in the animal experiment referred to in Figure 1. Based on clinical observations, which are detailed in an accompanying report (see Romine et al., this volume), the plan was modified and MBP is now being administered over an indefinite period of time. The quantity of MBP administered has ranged from approximately 0.5–3.0 mg/kg/day in single or divided doses. Clearance rates of MBP as well as MBP levels in the blood 1 h after injection have been measured with results similar to those observed in guinea pigs (Figs. 2, 3). The periods of MBP administration and the dosage schedules in each patient are shown schematically in Figure 4 and are detailed in the accompanying report (see Romine et al., this volume).

The pattern of MBP administration in this study differs from that in studies by Campbell et al. [4], and by Gonsette et al. [8], in which 5 mg of human MBP was

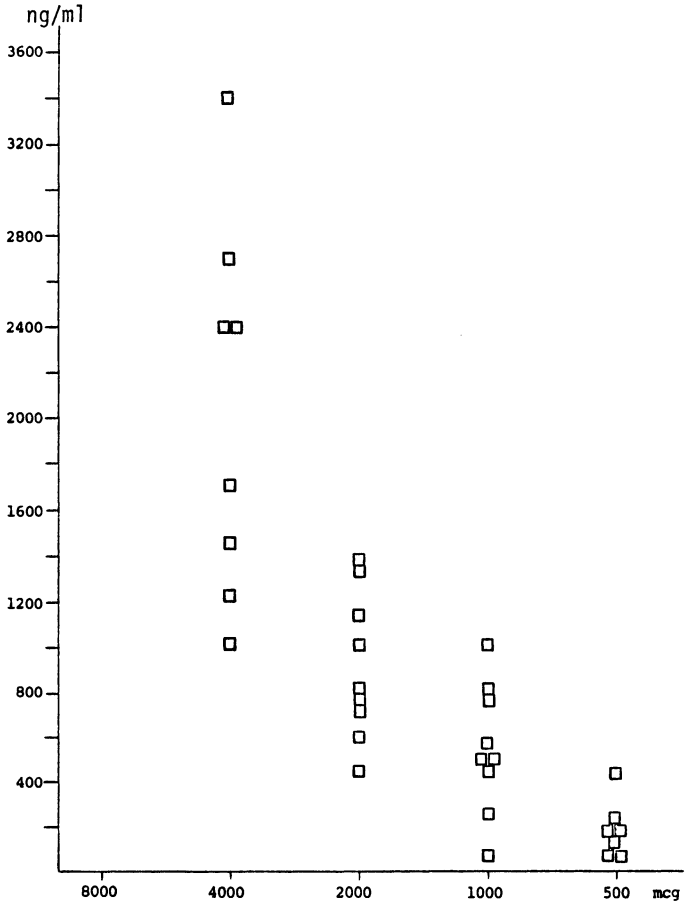

Fig. 3. MBP serum levels in guinea pigs 1 h after subcutaneous injection of indicated dose of porcine MBP. Animals in this experiment had been sensitized with 3.3 mg human brain tissue suspension in CFA 20 days previously and were given the indicated dose of MBP twice daily from the fourth to the 20th days post-sensitiziation. These observations were made following the dose administered on the 20th day (from exp. 5–76)

given weekly, a schedule of injections which is similar to that employed for desensitization of atopic patients, without evidence of either harmful or conclusive beneficial effect.

Immunologic Observations

The occurrence of local delayed-type hypersensitivity reactions, commencing near the end of the second week of MBP administration, were observed in some patients. In Patient no. 3, a single such occurrence was noted following the eleventh consecutive daily dose of MBP. Similar reactions at the injection site were not observed in Patients no. 1 and 2. However, Patient no. 5 and all of the subsequent four patients experienced a variable degree of local reaction. These occurred with diminishing in-

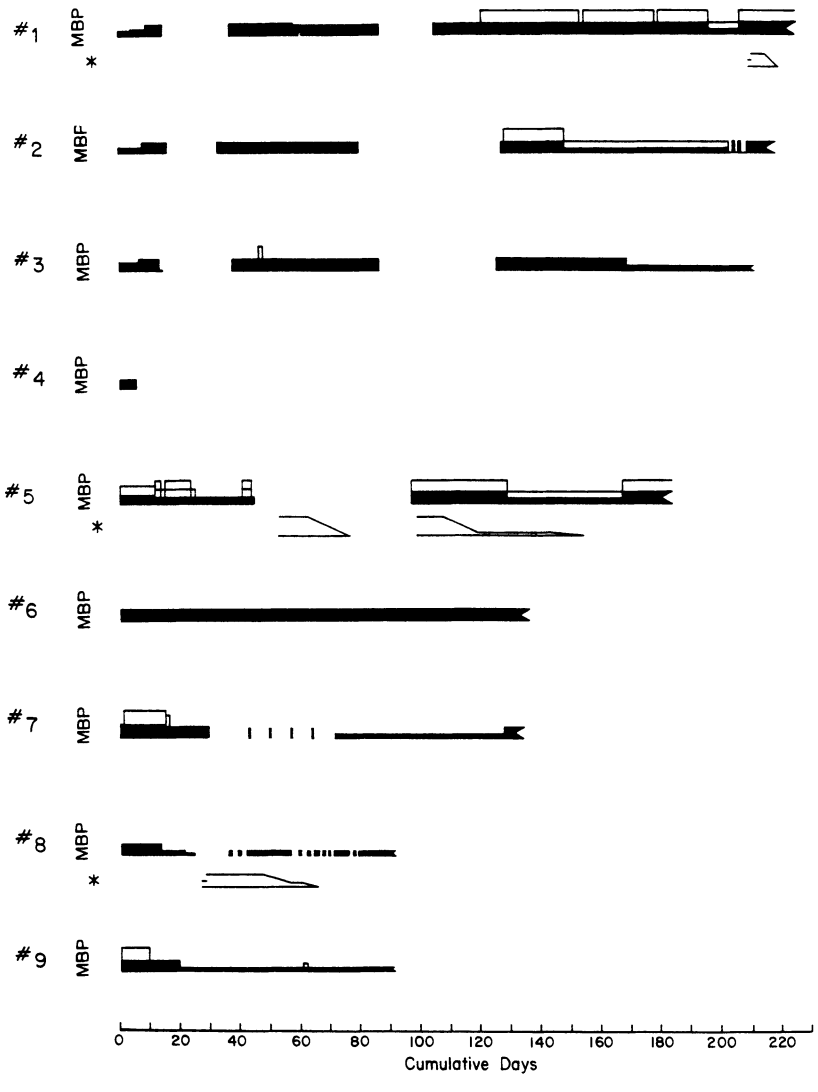

Fig. 4. MBP = Course of myelin basic protein administered. The height of the black areas indicates the relative quantity of MBP given either as the only dose per day, or as the first dose if more than one dose per day was administered. Relative quantity of any further dose(s) in a given day is indicated by the height of the enclosed white areas. *= Relative quantity and course of prednisone administered

tensity, usually over a period of several days and then only periodically and mildly, with a greater tendency to occur at the site of a previous reaction. In Patient no. 8 such reactions were suppressed during a period when prednisone was being administered.

The absence of reactions in the first two patients, and the single isolated reaction in the third, may have been due to the use of smaller starting doses of MBP. The possibility that such reactions might be minimized or prevented by administering smaller doses of MBP in the first and second weeks requires further study.

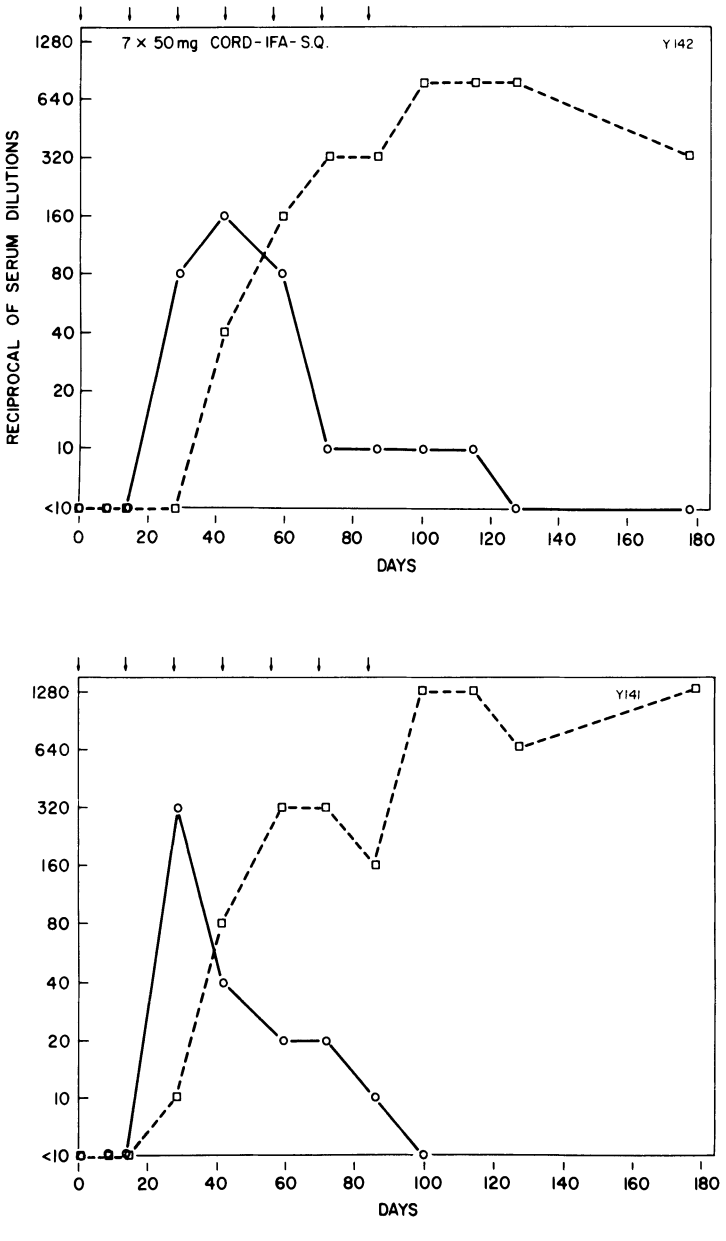

Fig. 5. Anti-MBP IgG (- - -) and IgM (——) response in serum of rabbits inoculated subcutaneously with 50 mg of guinea pig cord suspension in incomplete Freund's adjuvant. *Arrows* indicate times of repeated injections. Antibody titers were determined in passive hemagglutination test using MBP-sensitized chicken red blood cells. To distinguish IgG and IgM responses, antibody measurements were made in untreated serum and in mercaptoethanol-treated serum

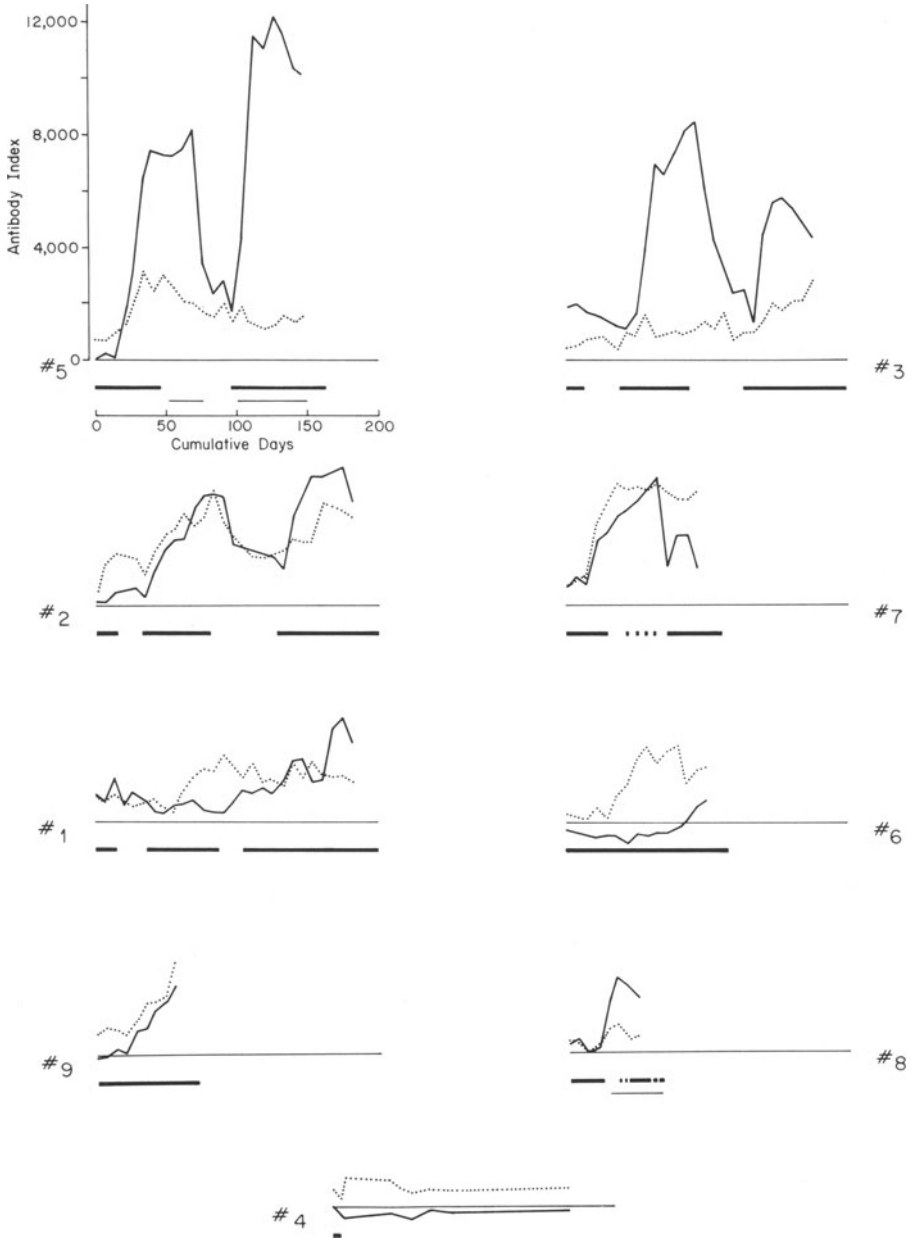

Fig. 6. Anti-MBP IgG (- - -) and IgM (——) response in serum of MS patients to inoculation of porcine MBP in aqueous solution. Time course of administration of MBP and of prednisone are indicated by *heavy* and by *light lines*, respectively below the baseline. Antibody determinations were made in a sandwich-type radioimmunoassay. *Vertical scale* reflects the relative amount of antibody contained in 0.1 ml of 1/10 dilution of patient's serum, expressed in terms of counts per minute (CPM) of ^{125}I-labeled goat anti-human IgG or IgM bound; values for CPM are corrected by subtraction for background. Serums from all patients were tested simultaneously for comparability; IgG and IgM tests were made on different days

Compared to the pattern of humoral antibody response to MBP in animals, atypical patterns of IgM and IgG anti-MBP responses have been observed in these patients. The anti-MBP response in rabbits to injection of whole spinal cord suspension in incomplete Freund's adjuvant is illustrated in Figure 5. An early and transient IgM response is typically followed by a more sustained IgG response. However, this pattern of response to MBP clearly differs from that seen in the patients, as shown in Figure 6. Whether or not MS patients have a similar atypical response to other antigens will be determined by further investigation with antigens that can also be studied in non-MS subjects.

Low levels of antigen-antibody complexes have been observed in some patients; when the administration of MBP is discontinued, they become undetectable. The details of the results of these and other immunologic studies will be presented in a later report.

Summary

A preliminary clinical study has been undertaken to determine the feasibility of studying the effect of MBP administration in patients with MS. As compared to studies reported earlier by others, larger and more frequent doses have been employed and, thus far, have been well tolerated. Immunologic studies have revealed atypical patterns of anti-MBP IgM and IgG response, thus adding to the accumulating evidence of defective immunoregulation in patients with MS.

Acknowledgments. The earlier work referred to in this report was supported by grants from the National Foundation, from the National Multiple Sclerosis Society, and by numerous generous donors. We wish to acknowledge the invaluable collaboration of The Lilly Research Laboratories in the preparation and toxicological testing of the porcine MBP used in these studies. The current work is being supported by grants from the National Multiple Sclerosis Society to The Salk Institute for Biological Studies (RG 1225-B-9) and to the University of California, San Diego (RG 1226-B-2), and from the National Institutes of Health to the General Clinical Research Center at University Hospital (P. H. S.-DHEW, NIH Grant RR 827), San Diego, California. The facilities and services of the Veterans Administration Hospital, San Diego, continue to be of inestimable support. We desire to acknowledge also the devoted and indispensable help of Donald Wegemer, Francis Yurochko, Elsie Ward, and Monica Winnick in the laboratory studies referred to in this report, and of Lorraine Friedman and Barbara Robinson in keeping records and in the preparation of reports, and of Kathy Ketring in the clinical phase of these studies.

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Studies on Myelin Basic Protein Administration in Multiple Sclerosis Patients

2. Preliminary Report of Clinical Observations

J. S. ROMINE[1], J. SALK[2], W. C. WIEDERHOLT[1], F. C. WESTALL[2], and Ch. K. JABLECKI[1]

The rationale for the study of myelin basic protein (MBP) administered to patients with multiple sclerosis (MS) and preliminary immunologic observations have been presented in the accompanying report (see Salk et al., this volume). The principal objectives of this preliminary clinical investigation are: (1) to determine whether MBP administration is accompanied by any adverse clinical effects; and (2) to determine whether a controlled pilot therapeutic test should be undertaken.

Procedures

Nine patients who satisfy the criteria proposed by Schumacher et al. [4] for a clinically definite diagnosis of MS were included. Schematic diagrams illustrating the antecedent course of illness of each patient, classified according to the categories of Fog and Raun [1], and their condition upon entry into the study are summarized in Table 1. All but patient no. 6 were ambulatory and patients no. 1, 4, 5, 6, and 9 had experienced recent worsening of neurologic function.

Before entry into the study and at approximately weekly intervals thereafter, a standard neurologic examination of each patient was performed by the same examiner (JSR). These observations were converted to the numerical rating system described by Kurtzke [2]. An estimate of overall condition (EOC) as to whether the patient's general neurological function had improved, worsened, or remained essentially the same, as compared to the previous examination, was also recorded.

Objective changes too fine to be reflected in the Kurtzke ratings are included in the formulation of the EOC, as was done in the ACTH therapeutic trial reported by Rose et al. [3]. The EOC also includes such individualized daily measurements as the maximum number of finger or foot movements per time interval, and the subjective impressions of the patient and/or the examiner.

Hematological, blood chemical, and urine analyses were made weekly.

Porcine MBP was administered subcutaneously. The starting dose varied from 37.5 mg once a day to 85 mg twice a day; the continuing dosage varied from 25 mg once daily to 75 mg twice daily. The total amount of MBP administered to each patient, up to August 31, 1978, and the then current dosage schedules are shown in Table 2. MBP administration was discontinued after five injections in patient no. 4 for psychiatric reasons.

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Table 1. Patients under study

Patient	Sex	Age (yrs.)	Duration (yrs.)	DSS[a] at Study Entry
1	M	34	3	3
2	M	56	16	6
3	M	38	13	3
4	M	35	11	3
5	F	21	5	6
6	M	33	7	7
7	M	41	9	3
8	F	41	4	3
9	F	26	1	3

[a] Disability status score = Point of entry into study

Table 2. Elapsed time since entry of each patient into the study and the quantity of MBP administered through August 31, 1978

Patient	Elapsed time		MBP Cumulative dosage mg	Present daily dosage mg
	From	Days		
No. 1	19 Jan	225	20,717	75×2
No. 2	26 Jan	218	12,409	75×1
No. 3	2 Feb	211	8,939	25×1
No. 4	9 Feb	204	310	–
No. 5	2 Mar	183	14,380	75×2
No. 6	19 Apr	135	10,125	75×1
No. 7	26 Apr	128	5,365	75×1
No. 8	2 June	91	2,263	25×1
No. 9	2 June	91	3,925	25×1

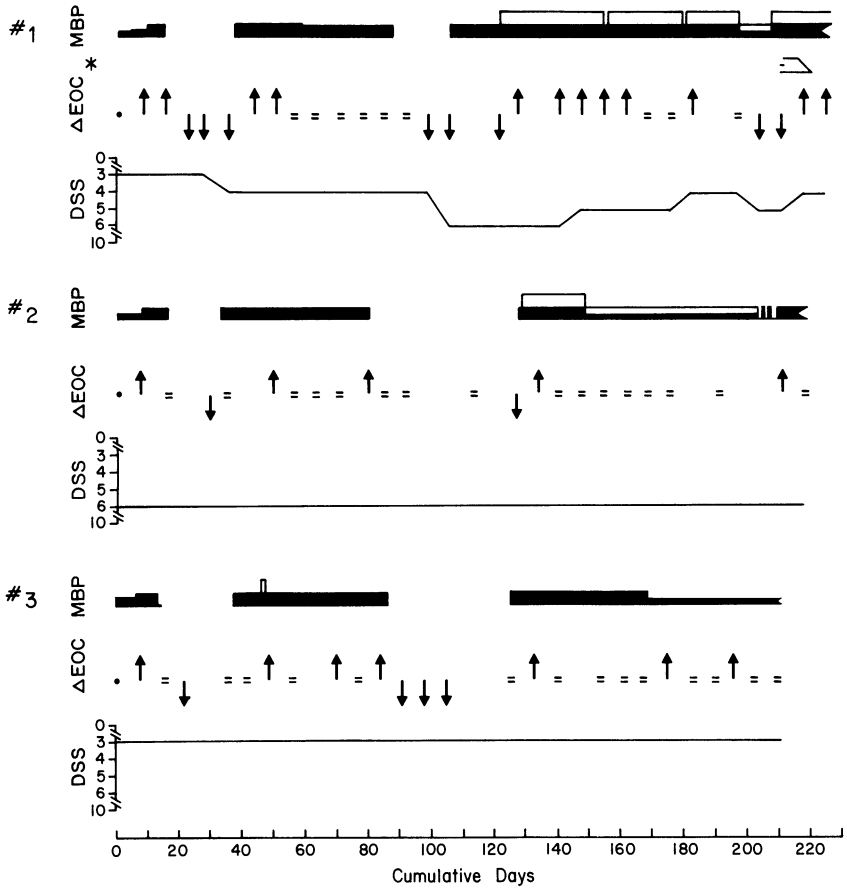

Fig. 1. *MBP* = Course of MBP administered. The height of the black areas indicates the relative quantity of MBP given either as the only dose per day, or as the first dose if more than one dose per day was administered. Relative quantity of any further dose(s) in a given day is indicated by the height of the enclosed white areas. * = Relative quantity and course of prednisone administered. *ΔEOC* = Change from previous Estimate of Overall Condition. An "up" arrow signifies improvement; an = sign signifies no change; and a "down" arrow signifies worsening. *DSS* = Disability Status Score

Clinical Observations

Changes in clinical condition are summarized in Figures 1, 2, and 3. These include the EOC as well as the Disability Status Score (DSS) ratings.

Patients no. 1, 2, and 3 received daily injections of MBP for 2 weeks starting with 37.5–50 mg/day and increasing to approximately 75 mg/day. As gauged by the EOC, all showed improvement at the end of the first week. Approximately 1 week after discontinuation of injections, a decline in neurologic function was observed in the three patients; in patient no. 1 this was also reflected in the DSS. Overall clinical improvement followed resumption of daily injections. This second course of injections was terminated when anti-MBP IgG antibody and low levels of antigen-anti-

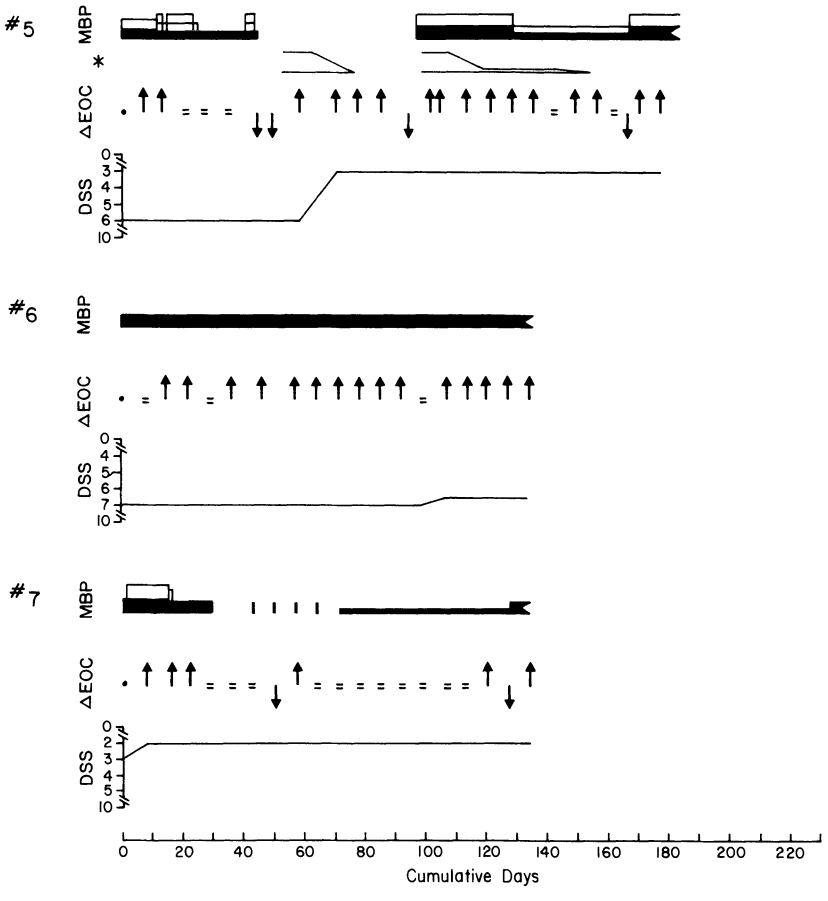

Fig. 2. See legend to Figure 1

body complexes were detected. In all three patients, the EOC declined at variable intervals thereafter, as did the DSS in patient no. 1. A third course of injections was followed by improvement in the EOC in all three as well as in the DSS in patient no. 1. Subsequently, the daily dosage of MBP was reduced; patient no. 2 remained stable; the EOC in patient no. 3 continued to improve; and in patient no. 1 worsening occurred both in EOC and in the DSS score. A trend of gradual improvement followed resumption of the previous higher dosage level of MBP in patient no. 1 and soon after initiation of a short course of prednisone, more rapid clinical improvement occurred. At the time of this report this patient was stable on MBP alone after discontinuation of prednisone.

Patient no. 5 has severe and widespread involvement of the neuraxis. Initially she received 50 mg of MBP either once daily or every 8–12 h; some improvement was observed. MBP was discontinued when the presence of anti-MBP antibody and of antigen-antibody complexes were detected. Subsequently, her clinical condition deteriorated. Marked improvement followed the initiation of a course of prednisone; soon after prednisone was discontinued, her condition worsened again. Prednisone and MBP were resumed simultaneously and this was followed by rapid

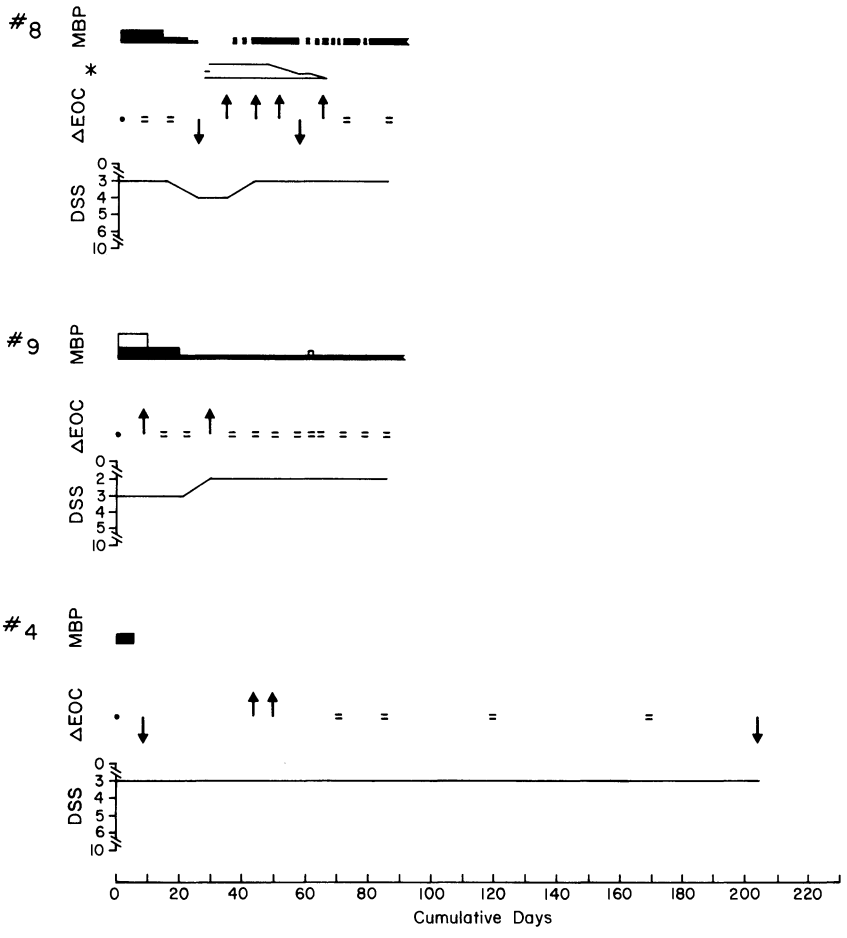

Fig. 3. See legend to Figure 2

clinical improvement which has continued up to the time of this report after prednisone was terminated and while daily injections of MBP were continued.

Patient no. 6 has received 75 mg of MBP daily without interruption for almost 5 months, during which time he has experienced gradual and steady improvement.

Patient no. 7 initially received 85 mg of MBP twice daily for 2 weeks followed by 75 mg once daily for an additional 2 weeks. The frequency of injections was reduced to once weekly to observe the effect on antibody response to MBP. Subsequently, daily injections were resumed. During this period, the DSS remained stable with some fluctuations in EOC.

Patient no. 8 initially received 75 mg of MBP daily. The dosage was reduced because of delayed skin reactions at the injection site. These continued to occur and, when a low grade fever appeared, administration of MBP was interrupted. The fever was associated with worsening in neurologic function and a course of prednisone was initiated. The fever cleared promptly, and neurologic function began to improve. While still receiving prednisone, MBP injections were resumed. The prednisone was discontinued and 25 mg of MBP was continued daily. Mild local skin re-

actions have recurred infrequently, but fever has not reappeared and the DSS has remained stable.

Patient no. 9 first received 75 mg of MBP every 12 h, and showed some improvement within the first week. Local skin reactions appeared in the second week, but diminished when the dose was reduced to 25 mg daily, and then disappeared with continued daily injections.

Patient no. 4, who received only five injections, has shown some fluctuations in neurological status but has remained essentially stable.

A detailed follow-up report of continuing clinical and laboratory observations in these patients will be made at a later date.

Summary

In this group of MS patients, daily subcutaneous administration of porcine MBP has thus far been well tolerated over periods varying from 3½ to 8 months. A determination as to whether or not MBP has any beneficial effects must await the outcome of a placebo-controlled clinical trial. In view of the absence of significant adverse side effects or of evidence of worsening attributable to the administration of MBP, such a study appears warranted.

Acknowledgments. This work is supported by grants from the National Multiple Sclerosis Society to the University of California, San Diego (RG 1226-B-2) and to The Salk Institute for Biological Studies (RG 1225-B-9), and from the National Institutes of Health to the General Clinical Research Center at University Hospital, San Diego, California (P. H. S.-DHEW, NIH Grant RR 827). Clinical facilities and laboratory support have also been provided by the Veterans Administration Hospital, San Diego, California. The Lilly Research Laboratories, Indianapolis, Indiana, have generously provided the porcine MBP used in these studies. We desire to acknowledge the able and devoted assistance of Kathy Ketring, R. N., in the management and coordination of many aspects of this project and of Sandy Britton and Barbara Lorenz for their accurate and tireless recordkeeping and manuscript preparation. We also wish to acknowledge the important role of Dr. Lenora V. Brown, clinical neurologist, as official clinical monitor.

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Discussion

W. A. Sibley: I have very little to add over what I said yesterday about Dr. Cuzner's paper indicating that there was an increased level of proteolytic enzymes in active MS, and our preliminary data indicating that plasminogen activator may be elevated. Also, in active cases of MS we have talked this afternoon about various forms of treatment of MS. It would seem reasonable, although we have not yet tried it, to use inhibitors of some of these proteolytic enzymes in the treatment of MS, especially if they are nontoxic. One such agent that comes to mind is epsilon aminocaproic acid, which is an inhibitor of both trypsinogen and plasminogen activator. And since there are reports in the literature of at least in vitro demyelination with both of these enzymes, and if one is confronted by a patient with fulminant MS or by a demyelinating disease such as acute hemorrhagic leukoencephalitis, it would seem reasonable to try this additional avenue of symptomatic treatment, even though one would have to agree that it would have nothing to do with the basic etiology of the disease.

A. Kohlschütter: I would like to ask Dr. Arnon whether her animals developed antibodies that reacted with MBP.

R. Arnon: No, they developed antibodies that reacted with the polymer, but not with the basic protein.

H. J. Bauer: Just a short remark on some very preliminary work on the copolymer preparation of Drs. Arnon and Sela. We have examined 16 patients with 20 courses of a very short term treatment, 4 weeks at the most, and some of these 3 weeks. We had two cases with bouts who moved up in the disability scale by one to two. But bouts do that anyway sometimes, so it does not prove anything as yet. Three of the cases had subjective improvement, two showed to improvement, and four proceeded to get worse, so that we had to discontinue the treatment and give them a course of steroids. Of the chronic cases, we had only one that moved up in the scale. Two had subjective improvement, four unchanged, and two showed further deterioration. That is the way it looks; it is very preliminary and I wouldn't like to comment any more on this. It's an open study, but I think what we need is some laboratory parameters and the two that have been suggested at this meeting are Frick's test and MBP.

Dr. Kelly: I wondered whether it wouldn't be better not to muddy the trial of MBP if you gave the patients prednisone all the time so as to prevent your skin reactions instead of some patients having it and some not.

J. Salk: In the first three patients, skin reactions did not occur. In those patients we were a bit more firm and started with smaller doses. We suspect that that may be one way to avoid the skin reactions and then build up the dosage subsequently after they have developed tolerance to the delayed hypersensitivity response. And it is clear that we can get beyond it. One of the first things we wish to do in the next few

patients is to find a way to overcome the delayed hypersensitivity type reactions that occur beginning around the eleventh day. In one patient it occurred for just one day, in others for a few days, and in some it lingered and then disappeared. You are quite right, and we have thought about the possibility of using prednisone as a way of overcoming that. But I think that we may be able to succeed with smaller doses for induction but, if necessary, your suggestion is one that we've thought about as well.

R. Arnon: I would just like to add something about the dose. We actually don't know what dose to use – we based the dose that we are using on results in guinea pigs and in our trial we gave only 2–3 mg per day or per injection. Probably the dose had to be higher, but we were afraid to give more in the beginning in the first trial. But now that we know there are no harmful signs, we have increased the dose to 5 and then to 10 mg, and eventually maybe we'll have to give 20 mg based on the results of Dr. Salk, since the fact that very high doses are used, may mean that by that better results can be obtained using the polymer.

Essential Fatty Acids and Immunity

J. MERTIN [1]

Introduction

Certain polyunsaturated fatty acids cannot be synthesized by mammals and are therefore important factors in the mammalian diet. As components of the phospholipids of the plasma membrane, and as precursors of prostaglandins they are essential for the growth and function of all tissues. The major essential fatty acid (EFA) is linoleic acid, which serves as the parent substance for the ω-6 family of EFA. Other members of this family – in the sequence of their biosynthesis – are γ-linolenic, dihomo-γ-linolenic, and arachidonic acid.

In multiple sclerosis (MS), the concentrations of EFA were found to be decreased in the central nervous system (CNS) and in the cells of the blood, including the lymphocytes [11]. These observations have prompted clinical trials of EFA treatment of MS patients. In two double-blind trials [1, 14] supplementation of diet with EFA was found to have a beneficial effect on the clinical course in patients with relapsing-remitting disease. There is some evidence for the involvement of cell-mediated immune mechanisms in the pathogenesis of MS [4]. On the other hand, EFA have been shown to affect cell-mediated immunity [7, 8, 9]. Thus, it is attractive to speculate that the therapeutic effects of EFA might be due to the effects of these substances on the patient's immune system. In this paper I will review findings which indicate a role of EFA in the regulation of cell-mediated immunity, and recent observations linking these effects with the derivatives of EFA, namely, prostaglandins.

Essential Fatty Acids and Cell-Mediated Immunity

Table 1 summarizes the effects of linoleic acid treatment on the immune function in mice. Depending on the duration of the treatment, immune activation, immunosuppression, or destructive changes in the lymphoid organs were observed. In splenectomized animals, EFA-induced immunosuppression was abrogated [13]. Further experimental evidence for the effects of EFA on cell-mediated immunity comes from studies on experimental allergic encephalomyelitis (EAE). In guinea pigs, significant reduction in EAE incidence, extent of histopathological changes, and in the clinical severity of the disease was seen when oral treatment with linoleic acid was commenced at day 7 after antigen inoculation and continued for 2 weeks [6]. Also,

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Table 1. Effect of linoleic acid subcutaneously (99% pure, 10 μl/day) on the immune function in mice [7, 8]

Duration of treatment	Effect
< 10 days	*Immune activation:* Increased ^{125}IudR uptake by thymus, bone marrow, lymph nodes, and spleen; splenomegaly and increased lymph node weight; reticuloendothelial system activation
10 – 16 days	*Immunosuppression:* Prolongation of skin allograft survival[a]; depression of thymus weight; decreased cytotoxic response of spleen cells; decrease in the number of Thy-1-positive cells in the spleen
> 16 days	*Cytolysis:* Destructive changes in the lymphoid organs, especially in the spleen

[a] Linoleic acid-induced prolongation of skin allograft survival is no longer seen in splenectomised mice [13]. Immunopotentiation was observed in mice fed with a linoleic acid-deficient diet [10]

Table 2. Approximate amount of daily EFA supplementation to the diet in various treatment regimens in patients and in experimental animals

Trial	Supplement	Linoleic acid (mg/kg body wt.)	γ-Linolenic acid (mg/kg body wt.)
Millar et al. [14] MS patients	Sunflower seed oil	250	–
McHugh et al. [5] kidney transplant patients	Naudicelle[a] oil	38	4
Bates et al. [1] MS patients	Naudicelle oil	42	5
	bread spread	280	–
Meade el al. [6] EAE guinea pigs	99% pure linoleic acid	2,000	–
Mertin et al. [12] EAE rats	Naudicelle oil	500	54

[a] In combination with steroids and azathioprine

sensitization of lymph node cells to myelin basic protein (MBP) was significantly decreased in animals treated according to this regimen.

However, the daily EFA doses used in the treatment of EAE in guinea pigs were rather high when compared with those found to be effective in the clinical trials mentioned above (Table 2). Therefore, in a recent series of experiments we have also investigated the effectiveness of lower EFA doses. These experiments were carried out in age-, sex-, and weight-matched groups of Lewis rats. EFA was given orally in the form of a plant oil (Naudicelle; Bio Oils, Nantwich, UK) which contains 73% of linoleic acid and about 8% of γ-linolenic acid. The control animals were treated with liquid paraffin. The treatment period was usually from day 7 to day 21 after antigen inoculation. Disease activity was determined by a clinical scoring sys-

Table 3. Experimental allergic encephalomyelitis (EAE) in rats: clinical scoring system [a]

Score	Symptoms
0	No clinical disease
1	Atonia of the abdominal muscles, 'limp tail' sign
2	As in 1, plus mild weakness of the hind (or front) legs and/or ataxia
3	Distinct weakness of hind (or front) legs, or hemiparesis
4	As in 3, plus neurogenic overflow bladder
5	Complete paralysis of hind legs
6	As in 5, plus neurogenic overflow bladder
7	As in 5 or 6, plus marked weakness or paralysis of the front legs, the animal lying on its side and being almost unable to move
8	Death due to EAE

[a] In all experiments EAE-induced loss of body weight is also determined

Table 4. Treatment with indomethacin (Experiment A) or splenectomy (Experiment B) abolishes suppression by essential fatty acids (EFA) of experimental allergic encephalomyelitis

	(A) Normal Lewis rats group			(B) Splenectomised (Sx) Lewis rats group			
	a	b	c	sham Sx	sham Sx	Sx	Sx
Number of rats							
X (female/male)	8 (0/8)	8 (0/8)	8 (0/8)	13 (6/7)	13 (6/7)	13 (6/7)	13 (6/7)
Treatment							
Carrier solution	–	+	–	–	–	–	–
indomethacin	+	–	+	–	–	–	–
liquid paraffin	+	–	–	+	–	+	–
EFA	–	+	+	–	+	–	+
Clinical disease activity							
Number of rats per group showing signs of EAE	8	4	7	13	5	13	13
Day after sensitisation on which first clinical signs were observed within the group	10	18	10	12	13	11	11
Clinical score per group on the day disease activity was greatest	25	6	12	35	12	47	56

tem (Table 3) and by EAE-induced changes in body weight. Histological evaluation of the pathological changes in EFA-treated animals and their controls was carried out objectively in a "blind" fashion. In all experiments, EFA treatment significantly suppressed EAE in Lewis rats. (Table 4, Fig. 1). Protection by EFA treatment was also seen when EAE was passively transferred.

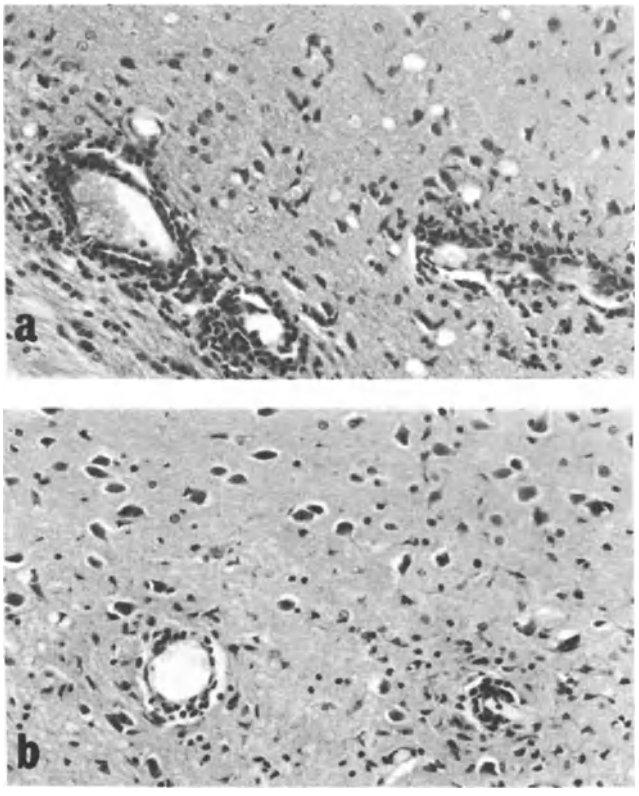

Fig. 1. Suppression of EAE in Lewis rats by treatment with essential fatty acids (EFA). Perivascular infiltrations in the brain stem (×45) of a control rat **a** and an EFA-treated animal **b**. In animals treated with EFA, fewer infiltrations were observed and these were usually smaller than those in the paraffin-treated controls

Abrogation of EFA-Induced Immunosuppression by Inhibition of the Biosynthesis of Prostaglandins

The mode of action of EFA in immunoregulation is still unknown. Increased EFA concentrations cause changes in the fluidity of cell membranes [15] and the activity of membrane-bound enzymes [2]. Both mechanisms might affect the reactivity of immune competent cells. Another possibility is that EFA effects on cellular immunity are mediated by prostaglandins.

According to findings by Webb and Osheroff [16] and others, the spleen appears to be major site for the production of immunologically active prostaglandins. In our experiments, suppression of EAE by EFA was abrogated in splenectomized rats (Table 4). Furthermore, combination of EFA treatment with indomethacin – a drug known to inhibit the biosynthesis of prostaglandins from EFA – also abrogated the EFA-induced suppression of EAE in Lewis rats [12].

Discussion

The results of the experiments which employed EAE as a model for cell-mediated autoimmunity imply that the immunosuppressive effect of EFA is mediated by prostaglandins produced by the spleen. It has recently been reported [17] that mitogen-stimulated lymphocytes can release prostaglandin E which, in turn, acts as the signal for a T-lymphocyte subpopulation to release suppressor factor. By treatment with EFA the increased availability of prostaglandin precursors may facilitate this mechanism. Kirby et al. [3] have shown that in MS inhibition of the in vitro lymphocyte response by prostaglandin E requires higher prostaglandin concentrations when compared with the lymphocytes of normal control persons. Other workers have observed that administration of indomethacin to MS patients worsens their clinical symptoms (Rudge and Davison, personal communication). These observations, together with our findings and the increasing awareness of the regulatory function of prostaglandins in various physiological systems neccessitate further research into the role of EFA and their derivatives in the pathogenesis and the treatment of MS.

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Discussion

E. J. Field: There is no doubt that unsaturated fatty acids (UFA) do indeed act as immunosuppressants when they are first administered. Although the control experiments in some early animal graft work were poorly controlled [2], UFA do seem to exert an immunosuppressant effect in vivo in acute experiments both regarding transplants and EAE, as they do in vitro.

However, long term administration in humans is different. For the first 6 months of administration of γ-linolenate (a metabolic product of linoleic acid), there would seem to be some immunosuppression borne out by the use of UFA by McHugh et al. [5]. They found, however, that after about 6 months, the use of UFA made no significant difference in the rejection rate of human kidneys. The same failure of suppression after some months is shown in Table 1. It can be seen that the acute suppression of lymphocyte antigen is not maintained after 6–8 months on Naudicelle but that the surface of the lymphocyte apparently returns to normal in its reactivity to antigens. Long term immunosuppression with UFA does not therefore occur in the human. The beneficial effect of γ-linolenate in early ambulant cases of multiple sclerosis (MS) with recurrent episodes, which Dr. Mertin tells us has occurred in the Newcastle double-blind trial by Bates et al. [1], is most encouraging in its support of Millar et al.'s [6] original claim for linoleic acid.

Table 1. Some results from UFA administration

Patient	Age	% EF	% Thyr.	% PPD	% BSA	% LA + Thyr.	% LA + PPD	% Red. LA + Thyr.	% Red. LA + PPD	
MS										
I. N. (F)	33	24.07	26.13	22.33	−0.20	1.62	1.52	93.80	93.19	
D. L. (M)	18	14.60	18.23	17.53	0.0	−0.1	0.0	100.00	100.00	
OND										
S. K. (F)	46	–	21.07	24.46	0.0	8.56	10.31	59.37	57.85	C.V.A.
B. D. (M)	41	20.48	18.12	16.13	0.71	8.64	8.45	52.32	48.25	Spino-cerebellar atrophy
Normal										
E. F. (M)	62	–	19.13	18.08	0.0	8.37	7.06	56.25	60.94	
E. G. (F)	10	–	22.12	19.43	0.0	10.53	10.63	52.40	45.29	
Naudicelle										
T. M. (M)	23	17.51	22.75	23.01	0.01	11.90	10.58	47.69	54.02	8/12 year
G. P. (F)	23	17.19	21.08	17.53	0.78	8.65	8.09	58.97	53.85	2 years

The real value of γ-linolenate will, I think, turn out to be in the prophylactic treatment of children borne with the defective handling of UFA postulated by Thompson [7, 8] and which so leads to abnormal cell surfaces on which the macrophage electrophoretic mobility – linoleic acid depression and erythrocyte UFA tests are based. Exhibition of γ-linolenate before myelination has been completed (and especially at a very early age) should convert the oligodendrocyte surface to normal and lead to the laying down of normal myelin nonsusceptible to MS. Full details and some theoretical considerations have been set out elsewhere [3].

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The Effect of Diet on Serum Linoleic Acid in MS

D. SEIDEL [1] and R. HEIPERTZ [2]

Following results presented by Baker and Thompson [2, 3] some time ago, we investigated the fatty acid composition, in particular linoleic acid of serum, in patients with multiple sclerosis (MS) and controls and we found no significant difference. We have since extended this research to lymphocyte preparations and again no significant difference was found.

Various dietary regimens have been proposed that are claimed to benefit MS patients, and some of these diets are supposed to alter the fatty acid composition of serum. In all these diets emphasis is placed on polyunsaturated fatty acids in particular linoleic acid.

In our investigation total linoleic acid was determined by Gas liquid chromatography (GLC), with diethylene glycol succinate 10% (DEGS) as stationary phase using C 17:0 fatty acid as internal standard, the results were expressed in μmol/ml serum. Repeat determinations on 60 serum specimen gave a coefficient of variation (CV) of 7.8% for the same specimen (methodological variation). Diurnal variation in two healthy subjects over 24 h independent of food intake and blood glucose levels yielded a CV of 18.7% (intraindividual variation). The 15 healthy controls gave a mean fasting linoleic acid level ($\bar{x}$) of 3.34 μmol/ml ± 0.56 (SD), the range was 2.35–4.10 with a CV of 17.3% (interindividual variation).

In comparison with results quoted from Baker et al. [3] (with normal value 3.68 and MS 3.06 ($\bar{x}$), difference 16.8%) and Paty et al. [14] (normal 3.01, MS 2.81, difference 6.6% (data expressed in μmol/ml serum)) our results indicate that normal variation among normal controls would be much more likely to influence serum linoleic acid than the diagnosis MS, where alterations appear small and insignificant. The comparison of MS patients without diet to controls had been completed in 1977 without significant results by our team [9], we now compared patients on specific diets to our controls.

Thirteen patients on the so-called Evers diet [7] (composed mainly of cereals and high in sunflower oil) between for 2–14 years gave a mean linoleic acid content of 3.06 ± 0.64, the correlation of duration of diet to serum linoleic acid concentration was $r = -0.45$, indicating that adherance to the diet would tend to decrease serum linoleic acid content.

Twenty-six patients on Naudicelle capsules (six capsules per day, equivalent to 0.4 g gamma-linolenate and 2.7 g linoleate) for at least 6 weeks gave a mean of 3.18 ± 0.71 with no significant difference to controls. Five patients were reexamined after 6 months, again with no significant difference.

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On the basis of these results, which are still preliminary and have to be checked with a large number of patients on various diets, it appears probable that the beneficial action of the diet on the clinical course of MS is not mediated by an alteration of serum polyunsaturated fatty acid composition. In this connection the "Thompson anomaly" [i.e., the inborn mishandling of unsaturated fatty acids (UFA)] [17, 18] appears to be an interesting hypothesis, however, it should not be overlooked that some of the epxerimental evidence is controversial and other results still need corroboration.

Thus, in contrast to the previous assumption that MS serum and blood cells contain less linoleic acid than controls, numerous studies have been performed supporting our results presented in this symposium and published previously [9], viz. that there is *no* significant difference.

The question of an altered lipid pattern and fatty acid composition of normal-appearing cerebral white matter in MS as a quasi predisposing factor for contracting the disease has resulted in considerable controversy [1]. Some of the controversial results can probably be explained by the difficulty of excluding microscopic demyelinated plaques in unfixed white matter. Formalin fixation on the other hand causes alteration of the lipid and fatty acid profile, but not in the sense of abnormally constituted cell surface membranes. Possibly current research by our team will offer more detailed information.

It has been mentioned during this symposium that inborn mishandling of UFA is supposedly compensated for by diet or Naudicelle capsules. It has been suggested that children with high risk from MS families could be prophylactically treated to eliminate the predisposition, and UFA substitution is claimed to influence the manifest disease [13]. Such a repair of oligodendroglial membrane by UFA seems unlikely on the basis of animal experiments with labeled fatty adics which demonstrated that UFA can only cross the blood-brain barrier (BBB) in the immature animal for a very short postnatal period, and then only in negligible quantity [6, 10]. That massive oral UFA substitution can alter the fatty acid pattern of brain lipids seems even less likely [12], neither is there any diminution of UFA in brain lipids with a special UFA-restricted diet [11].

Numerous clinical studies have been unable to confirm previous reports [13] of a positive effect of UFA supplementation on the progression of neurological findings, the relapse rate or the severity of relapses [4, 15]. Experimental doses of UFA employed in animal experiments to suppress the severity of EAE in guinea pigs [12] and the susceptibility to EAE in rats [5] are far higher than any therapeutic doses used in humans. The assumption that gamma-linolenic acid represents the active principle in Naudicelle capsules with a higher specific activity than its precursor linoleic acid is only derived from in vitro experiments [16]. The passage of gamma-linolenic acid through the BBB at the time of peak myelination is certainly no better than of linoleic acid [8].

In conclusion it should be pointed out that available scientific evidence produces a far more complex picture of etiology, diagnosis, and therapy of MS than the simple hypothesis of fatty acid metabolism anomaly warrents. It is too early to pass judgment on this hypothesis and considerable research will be necessary to define the rule of UFA, fatty acid metabolism, and availability of prostaglandins (derived from UFA) as a basic pathogenetic principle or simply a peripathological phenomenon.

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Metabolism of Myelin-Typical Long-Chain Fatty Acids

D. SEIDEL [1] and R. HEIPERTZ [2]

Introduction

Myelin lipids have a high content of galactocerebrosides and sulfatide esters (together approx. 30%). These contain a high proportion of long-chained fatty acids (approx. 90% of total fatty acids), the predominant ones being C_{24} acids [5].

Experimental Methods

After intracerebral application of labelled (15,16-^{3}H)-C_{24}-acid (lignoceric acid) in adult rat brain, the incorporation into complex lipids and the step-by-step oxydative degradation of this typical myelin fatty acid was investigated [9].

Radio GLC was utilized for detection of degradation fatty acids (FA); the radioactive marker ended in two pools of C_{16} and C_{18} FA, which were then incorporated into glycerophosphatides (Figs. 1, 2).

The main mechanism for degradation is beta oxidation, but alpha oxidation is also possible. As an intermediary product of alpha oxidation, long-chained alpha-hydroxy FA are formed, which are practically completely utilized for incorporation into the stable cerebron fraction of brain cerebrosides. After elimination of one C atom, the odd-numbered FA which are abundant in brain lipids are formed.

A complex galactocerebroside was synthesized with the labelled lignoceric acid (15,16-^{3}H-lignoceroyl-psychosine = kerasin) and its degradation was studied in vivo in rabbit brain [8]. Only a small proportion of lignoceric acid is released, which is then degraded over the same pathways (alpha and beta oxidation) ending in the C_{16} and C_{18} pools (Table 1).

Discussion

Myelin stability is largely dependent on a high proportion of sphingolipids with long-chained FA. Research by other authors [2] has demonstrated that the biosynthesis of long-chained FA in the glial cells starts from an endogenous pool of short-chained FA (C_{16} and C_{18}) and depends on the mitochondrial or microsomal chain-elongation mechanism, while *de novo* synthesis is considerably less important. Our

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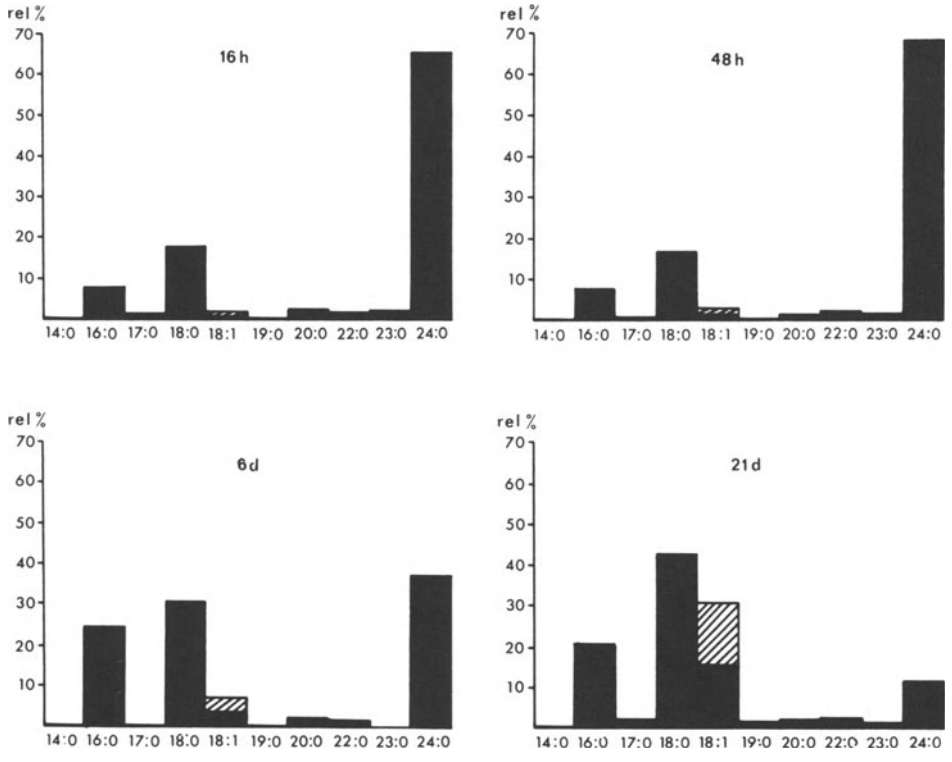

Fig. 1. Relative distribution of labelled FA in the combined sphingolipid and glycerophospholipid fractions in adult rat brains after the administration of (15,16-^{3}H)-lignoceric acid. Animals were killed after various times

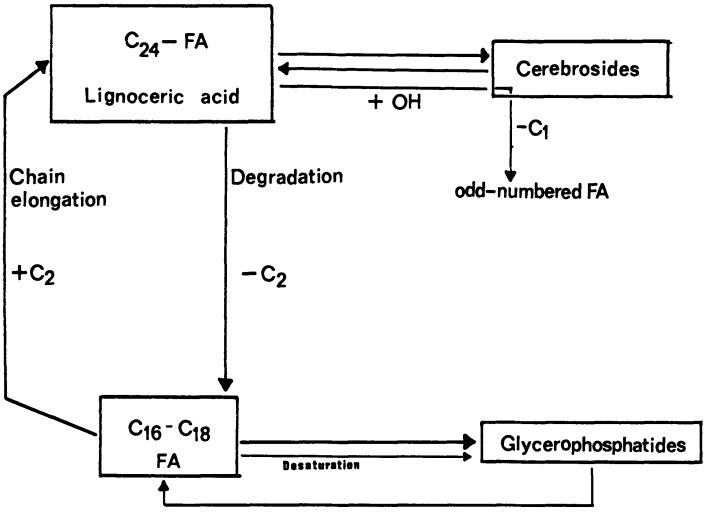

Fig. 2. The fate of lignoceric acid (C_{24} FA)

Table 1. Distribution of labelled FA in various lipids of adult rabbit brain after the application of labelled [cerebroside (15,16-^{3}H)-lignoceroyl-psychosine] [a]

Lipid classes	After 8 days			After 21 days		
	CER	PC	NF	CER	PC	NF
Fatty acids C_n						
14 : 0	0	0	0	0	tr.	1.1
15 : 0	0	0	0	0	0	tr.
16 : 0	0	1.2	2.8	0.3	5.8	15.2
16 : 1	0	0	0	0	0	3.2
17 : 0	0	0	0	tr.	0.4	tr.
18 : 0	0.2	10.8	3.9	0.8	16.2	11.6
18 : 1	0	tr.	2.8	1.4	25.3	33.1
20 : 0	0	0	0.3	0.2	2.8	2.7
20 : 1	0	0	0	0	0	2.4
22 : 0	0.9	2.4	0.3	0.3	3.5	3.2
23 : 0	0.2	0	0	0.6	1.4	tr.
24 : 0	98.7	85.6	90.0	91.5	44.5	27.4
24 : OH	0	0	0	5.1	0	0

[a] CER: cerebrosides; PC: lecithin; NF: neutral lipids; tr.: trace amounts

results on the degradation of C_{24} FA show that this process terminates in pools of C_{16} and C_{18} FA [1, 9]. Further degradation was not detected (Fig. 1, Table 1). Apparently, the glial cell functions on an economic "recycling" principle with regard to long-chained FA synthesis.

It has been postulated that a disturbance of the chain-elongation mechanism with acyltransferase specific for C_{22} to C_{26} FA might affect the biosynthesis of membrane-stabilizing sphingolipids and result in myelin instability and ultimately demyelination. Such a pathogenic principle is considered to be the cause of myelin deficiency in animal mutants, e.g., quaking mice [4], with experimental myelin destruction and with demyelination, e.g., MS [3] and unspecific myelin loss or myelin degeneration, e.g., Huntington's chorea and adult metachromatic leukodystrophy (MLD) [6, 7]. In all these cases there is a more or less pronounced loss of very long-chained FA of sphingolipids in the affected areas of white matter, with a corresponding increase of short-chained FA.

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Observations in 166 Patients with Multiple Sclerosis After Thymectomy

D. BRAGE [1]

The observation that symptoms of multiple sclerosis (MS) improved after thymectomy in a patient with myasthenia gravis and MS prompted the author to have thymectomies performed on 166 patients with MS. These patients were characterized regarding the following items:

1. Sex. 56% of the patients were females, 44% were males.
2. Age. The age distribution was as indicated in Table 1.
3. Duration of the disease was as indicated in Table 2.
4. Clinical forms of the disease were as indicated in Table 3.
5. Disability status. The disability status of the patients was as indicated in Table 4.
6. Geographical zone. Two-thirds of the patients lived in the pampas, one-fourth in the littoral zone, 4% at the seaside, and 5% in the mountains.
7. Climate. 90% of the patients lived in a humid climate, 10% in dry zones.
8. Living conditions. 55% of the patients lived in cities, 45% in rural areas.
9. Heredity. In 4.8% of the patients more than one member of the family had MS.
10. Allergic Background. 13% of the patients had a history of allergies.
11. Blood group. The blood groups of the patients were as indicated in Table 5.
12. T lymphocyte level in the peripheral blood of the patients reached an upper normal limit in 4%, was normal in 73.5%, and was decreased in 22.3%.

Table 1. Age distribution of the patients

10 – 20	20 – 30	30 – 40	40 – 50	50 – 60	60 – 70 years
1.2	16.87	32.53	37.35	10.85	1.2

Table 2. Duration of the disease

1 – 6 months	6 months to 1 year	1 – 3 years	3 – 5 years	5 – 10 years
–	3.01	10.85	15.66	31.93

10 – 15 years	15 – 20 years	20 – 25 years	25 – 30 years
24.1	8.43	6.02	–

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Table 3. Clinical forms of the disease

1	2	3	4	5	6	7	8
63.25	1.81	1.81	–	–	12.65	4.22	–

9	10	11	12	13	14
1.21	9.64	2.41	1.2	1.2	0.6

1 = Optic-pyramidal-cerebellar; 2 = Optic-pyramidal-cerebellar-sensory; 3 = Optic-pyramidal; 4 = Optic; 5 = Sensory; 6 = Optic-pyramidal-vegetative-cerebellar; 7 = Pyramidal; 8 = Cerebellar-optic; 9 = Optic-pyramidal-cerebellar-mental; 10 = Cerebellar-pyramidal; 11 = Cerebellar-pyramidal-bulbar; 12 = Pyramidal-optic-cerebellar-bulbar; 13 = Pyramidal (vegetative).

Table 4. Disability status scale

3	4	5	6	7	8	9	10
–	5.42	19.28	19.88	30.12	10.84	13.26	1.2

Table 5. Blood groups of the patients

A+	A–	0+	0–	B+	B–	AB+	AB–
45.78	6.03	36.14	3.61	4.22	2.41	1.81	–

Table 6. Percentage of active remaining thymic tissue

0%	10%	20%	30%	40%	50%	60%	70%	80%	90%
5.42	33.14	19.88	19.88	7.23	5.42	5.42	2.41	0.6	0.6

Table 7. Time elapsed since thymectomy

1 year	2 years	3 years	4 years	5 years
25.9	7.23	22.89	35.54	8.44

13. IgG in the cerebrospinal fluid (CSF) was increased in 59% of the patients and normal in 41%.
14. The percentage of remaining active thymic tissue in the patients after thymectomy was as indicated in Table 6.
15. Upon examination, the time elapsed since thymectomy of the patients was as indicated in Table 7.

16. Further course. The further course of the disease was favorable in 45.8% of the patients, uncertain in 21%, and unknown in 33.1%.
17. Further symptomatology was favorable in 33.7% of the patients, uncertain in 24%, and unknown in 42%.
18. Complications. There were no complications observed in any of the patients.

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Discussion

E. J. Field: Few of you possibly understood what Prof. Brage was driving at. What he's been doing is total thymectomy for MS, and I want to show you what happens. In myasthenia gravis there is sensitization to a lot of antigens. If you stand in the operating theater, and when the surgeon cuts the last thymic vein you take blood samples and then you take samples at ½ hourly and hourly intervals, you find that immediately after the thymus is removed, within a very few hours there is a failure of the lymphocytes to react to antigens to which they previously have very successfully reacted.

I then went back to the laboratory and took adult guinea pigs – and I'm not talking about neonatal rats or neonatal mice – the thymectomized patient was an adult man of 30. Here is the sham thymectomy, here is the thymectomy, and again the animal, which is highly sensitized to PPD, refuses point blank to respond to stimulation. The lymphocytes refuse point blank to respond to PPD stimulation. You can restore that stimulation by adding normal serum. Now it seems to me that if MS is due to recurrent attacks of experimental allergic encephalomyelitis of one sort or another, then total thymectomy ought to eliminate these attacks completely. With a patient who does this all the time, if you take his thymus out, and of course this is a serious operation, you should have no attacks whatsoever. The only thing is, if you leave the smallest scrap of thymus behind, 2 g out of 34 are enough to maintain the thymic function and keep your T cells going.

B. G. W. Arnason: The group in Khazan in the Soviet Union have been doing thymectomy for MS for the past 7 or 8 years. They have not reported any dramatic changes to date in terms of patient recovery. But they told me 2 or 3 years ago that some of the patients did have flareups after thymectomy. This would suggest that thymectomy is not an absolute answer for MS.

E. J. Field: Could I reply to one remark you made there. First of all, we have developed a test to see how much the surgeon has taken out, and within 24 h you can give a prognosis as to the surgical result of thymectomy. It is surprising how much thymic material is left behind.

B. G. W. Arnason: That astonishes me, since in myasthenia patients it may take 10 years for them to show a beneficial effect from thymectomy.

Epidemiology of MS

Correlation of the Course of MS and Histocompatibility Antigens

N. E. RAUN, T. FOG, A. HELTBERG, and P. PLATZ [1]

Studies of the HLA system in multiple sclerosis (MS) during the last few years have shown that the *Dw2* locus is present in 50–70% of all MS cases (totally 932 patients from nine different studies) [3], compared to 25% in the normal population. Thus presence of the *Dw2* gene gives a relative risk of approximately 4 for developing MS. While an association between *Dw2* antigen and MS has been demonstrated among black American individuals, in whom *Dw2* is otherwise rare, this has not been found in other ethnic groups such as Israeli, Arab, Japanese, or Italian MS patients, but associations to other HLA-D antigens have been found. We thus conclude that HLA studies show that genetic factors definitely predispose to MS. However, twin studies show that the predisposing gene *Dw2* itself or any gene closely linked to *Dw2* is of only minor importance for the acquisition of MS, and family studies show that these genes are neither necessary nor sufficient providing a correct diagnosis of MS has been given in all cases studied.

In a previous study on *Dw2* we found a correlation between the course of MS and the presence of this gene. Those patients having the *Dw2* determinant, show a more progressive course of disease. This was confirmed in our Transfer Factor study [1]. In this study, 32 cases were followed in monthly neurological examinations for more than 1 year, and a coefficient of progression was calculated in each case. However, due to the limited number of patients, only a moderate statistically significant correlation was found. No general agreement seems to exist on this point, therefore we wanted to confirm our findings, and have made a new evaluation of our material.

Materials and Methods

Of 112 definite MS cases from the MS Rehabilitation Center in Haslev and some cases from the Neurological Department, Kommunehospitalet, Copenhagen, 106 patients were HLA-D typed at the Tissue Typing Laboratory, University Hospital, Copenhagen. This material is probably biased, as most MS patients at the Rehabilitation Center in Haslev have moderate handicaps and are subject to physiotherapy; the most benign and the most severe cases are underrepresented.

Each patient was assigned a disability status by one of us (TF) according to the following criteria:

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Disability degrees

0. *Practically* without signs and symptoms.
1. *Slight troubles* (in reading, paresthesias, slight sphincter disturbances, etc.) Capable of working.
2. *Moderate troubles,* shortened gait capacity, with or without a stick, localized signs, for example, spastic paraparesis, even of a severe degree, but no other signs and symptoms beyond the mid thoracal level, or slight hemiparesis, capable of non-manual work.
3. *Severe* gait disturbances, severe hemiparesis, but able to walk with one or two sticks or crutches; moderate speech disturbances, severe sphincter disturbances, incommoding coordination disturbances, for example, in writing; but able to eat, severe paresis of both lower and slight paresis of upper extremities.
4. *Severe handicap,* personal dependence to a certain but not a total degree on a wheelchair in combination with other CNS signs, high degree of incontinence, unable to feed self, severe paresis of both lower and one upper extremity.
5. *Personal dependence* in dressing and undressing, in eating, but able to move about in a wheelchair.
6. *Totally invalidated.*

The rate of progression is determined by dividing the disability status degree by the duration of the disease. The duration is defined as the number of years from presumed onset until the year of presumed stationary stage or the year of the last neurological examination in still progressing cases. These latter dominate. The course of the great majority of these patients is known through yearly admissions to the Rehabilitation Center in Haslev.

The distribution of patients of the different disability grades may be seen in Table 1.

Table 1. Estimated rate of progression for MS patients with different disability status [a]

No. of patients			
Disability status scale	Below median	Above median	
V – VI	3	16	19
IV	8	17	25
III	20	19	39
I – II	24	5	29
Total	55	57	112

[a] Extended median test $\chi^2 = 24.6$, df = 3, $P < 0.001$

Results

It follows from Table 1 that patients with a high degree of disability have had a more progressive course than those with a low degree of disability.

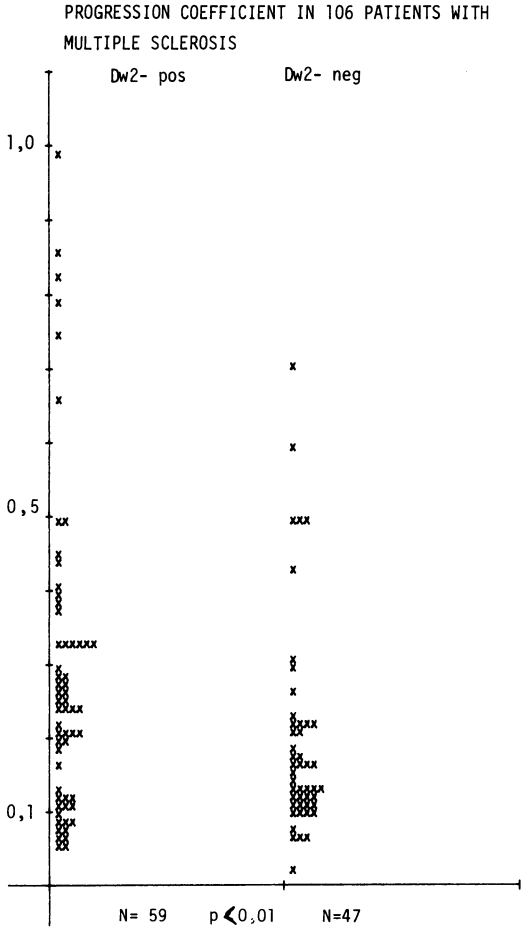

Fig. 1. Distribution of estimated rate of progression among *Dw2*-positive and *Dw2*-negative MS patients. *Dw2*-positive patients have a significantly higher rate of progression as tested by a Mann-Whitney rank-sum test adjusted for ties

Table 2. Estimated rate of progression for MS patients with LD-type *Dw2* and non-*Dw2* [a]

No. of patients			
Rate of progression	*Dw2*	non-*Dw2*	
Above median	38	16	54
Below median	21	31	52
Total	59	47	106

[a] Median test $\chi^2 = 9.52$, df = 1, $P < 0.005$

Fifty-five percent of the HLA-D typed patients were *Dw2*-positive. The rate of progression among *Dw2*-positive and *Dw2*-negative patients is shown in Figure 1. Based on a rank-sum test, it is concluded that *Dw2*-positive patients show a significantly higher rate of progression. The results of a median test are presented in Table 2.

We also tested whether there was any association between degree of disability and presence of *Dw2*, but this was not significant (χ^2 test, $P > 0.20$).

Discussion

Even if this material is not representative of the MS population in general owing to the dominance of moderately handicapped patients, individuals have not been selected for admission to the Rehabilitation Center based on rate of progression, but rather on their degree of disability. With reservations regarding possible bias, it has been shown that the majority of the moderate to severely handicapped patients have had a higher coefficient of progression than the more benign cases. Although there are variations from patient to patient, this confirms the impression that patients with an initial high rate of progression have a relatively bad prognosis.

It should be borne in mind that the course of the majority of patients is linear or shows a flat exponential course. Although the disability status is a very rough estimation of neurological status, Kurtzke [2] showed that there is a good correlation between neurological score and disability status.

Our reservations regarding a possible bias due to the selection of patients do not apply to the effect of *Dw2* gene. It has been demonstrated that *Dw2*-positive patients have a faster progression course than *Dw2*-negative patients. This may be of practical importance in the prognosis of single cases, but should not, of course, be overestimated.

It should also be emphasized that although presence of the genetic factor HLA-*Dw2* is of only minor importance for the development of MS, it may in some unknown way contribute to a lower resistance to a possible environmental factor. A study of possible differences in clinical manifestations between patients with and without the *Dw2* is warranted, but has not yet been performed.

Summary

To 112 MS patients, mostly with moderate to severe handicaps, we have assigned a disability status. Hence a rate of progression has been estimated in each case and it is shown that patients with a high degree of disability have had a more progressive course than those with a low degree of disability. One-hundred and six patients were HLA tested for *Dw2*. Fifty-five percent were *Dw2*-positive and these patients had a statistically significant higher rate of progression than those who were *Dw2*-negative.

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HLA Patterns in Multiple Sclerosis

J. J. F. OGER and B. G. W. ARNASON [1]

HLA-A and -B Locus Antigens in Caucasians of North European Extraction

The increase in frequency of tissue types A3 and B7, accompanied by a decreased frequency of B12 in persons afflicted with MS and of North European Caucasian extraction appears established [2, 5, 17, 22]. The augmentation found is modest, however, (relative risk 1.2 to 1.7) and the considerable variation between the results of individual studies should be noted. These discrepancies between laboratories may be partly explainable based on differing specificities of antisera used to detect HLA types and the difficulties inherent in obtaining consistent diagnostic criteria, particularly in the case of large series involving cooperation among several centers. The linkage disequilibrium between A3 and B7 which is characteristic of the North European Caucasian population as a whole has been found to be exaggerated in MS victims [6, 17]. This may suggest that a gene in linkage disequilibrium with *A3-B7* on chromosome 6 may have a major role in the development of the disease.

In addition to the alterations cited above, which have been found in the vast majority of studies, certain other HLA antigens have been noted to be overrepresented in individual studies, e.g., BW5 [5], B-18 [22], A-9 [9], and BW35 [15]. Overrepresentation of B8, in contrast, although only occasionally attaining statistical significance [2, 23], has nonetheless been found to be present in almost all studies published to date together with an *A1-B8* linkage disequilibrium greater than expected [21, 24].

HLA-D Locus Antigens in Caucasians of North European Extraction

The finding of Jersild et al. [19], confirmed by Bertrams et al. [7] and by Oger et al. [23] among others, of an increased representation among MS patients of the *D*-locus antigen *Dw2* (once again with an increased linkage disequilibrium between *B7* and *Dw2*) is of particular interest, inasmuch as *Dw2* has been found to be more increased than B7 in all published studies. Indeed, Opelz et al. [28] were able to detect an increase of *Dw2* in a group of 330 MS patients in which they failed to observe a significant increase of B7. At the level of the *D* locus, the relative risk of contracting

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MS for those carrying *Dw2* is increased 4.5 times overall [16]. It is of interest to note that *Dw3*, which is in linkage disequilibrium with *B8*, was also found to be increased by Grosse-Wilde et al. [16]. An antigen found on B cells which is closely related but possibly distinct from the *D* locus (Seventh International Histocompatibility Workshop, Oxford (1977)) was shown to be increased by Winchester et al. [34] and independently by Terasaki et al. [33]. In the series of Terasaki et al., the *DRw2* antigen was present in 83% of the MS patients tested versus 32% of the controls.

The various disturbances in antigen frequency of the major histocompatibility complex (MHC) briefly outlined above may signal the existence of a susceptibility gene for MS located on chromosome 6 and situated between the centromere and the *D* locus.

Ethnic, Family, and Immunological Studies

Three kinds of study merit discussion with regard to the relationship between this putative "intrinsic propensity gene for MS" and environmental factors. (1) Studies of Caucasians not of North European extraction and non-Caucasian populations; (2) family studies; and (3) studies of correlations between HLA type and immunologic abnormalities within the MS patient group.

These will be addressed sequentially.

Non-North European Caucasian Populations

Brautbar et al. [8] showed that Jewish MS patients in Israel show no increase in A3-B7-*Dw2*, yet BW40 appears increased. In studies of DR-locus antigens, *Dw4* has been found increased in Jordanian MS patients, suggesting that susceptibility to MS in Mediterrean populations carries an association with a gene distinct from that tied to MS in North Europeans.

Non-Caucasian Populations

Studies of black Americans show that in this group MS patients show an augmentation of *Dw2* as well as some increase in A3 and B7 [13], while these same antigens are relatively rare in the black American population taken as a whole.

In Japan, the incidence of MS is much lower than in Europe or in North America. B7 and *Dw2* are rarely found in Japanese MS patients or in Japanese controls, and BW22 is found increased in the MS patients. In a recent study [31], it has been found that the linkage disequilibrium between *B7* and *DRw2* does not hold for Japanese; rather B7 is in linkage disequilibrium with DRw1 in this population. An association between DRw1 and MS has been reported at the Oxford Workshop.

There have been conflicting interpretations of these data. Some have held the findings to indicate that the populations being studied have not yet reached genetic

equilibrium. This is doubtless true for black Americans, but seems less probable for European Caucasians. Others have argued that mutation permitted the appearance in a remote ancester of a susceptibility gene for MS. Crossing-over from generation to generation has lessened the linkage disequilibrium between HLA genes and the susceptibility gene. It has been postulated that the original mutation in North Europe affected a person carrying *A3*-B7-*Dw2*-*DRw2*, but in other races the mutant carried other HLA types.

Family Studies

These have given differing results. Olsson [27] in a study of 12 Swedish families with more than one case of MS, found haplotypes shared by affected members in most families. In the United States, Alter [1] found a shared haplotype in several families, but Drachman et al. [12] did not. In an interesting study from the NIH [14], some of the pitfalls which can invalidate family studies in MS were addressed. Attention was drawn to the necessity of correcting for age of unaffected siblings. When strict diagnosis criteria were applied, 50% of the families originally labelled as MS had to be set aside. In the remaining families, no clear association between MS and HLA type was found. Taken together, the family studies published to date fail to indicate any consistent segregation of HLA types between affected and unaffected members, and do not support the thesis that any single gene in the HLA complex rigorously determines susceptibility to MS.

Immune Function and HLA Patterns

Correlations have been sought by many groups; most studies have been negative. Measles antibody levels in relation to HLA type have been explored by several groups, with conflicting results. A tentative relationship between HLA-3 and hemagglutination-inhibiting antibody (HIAb) levels to measles was reported by Arnason et al. [2]. Jersild et al [18] found higher measles antibodies in bearers of *A3-B7* and/or *B18*. Cazzulo and Smeraldi [9] found higher measles titers in bearers of *A3* and *A9*. Paty et al. [29] found complement-fixing antibody levels to be higher in bearers of *A3-B7* and/or *B18*, but HIAb titers were normal. Fewster [15] found females carrying *A3* to have higher HIAb titers than any other subgroup. In contrast, some workers have failed to find any significant associations between HLA type and measles antibody titer [5, 26]. The situation remains confused at the time of writing. The leukocyte migration test has been employed to study cell-mediated immunity (CMI) to measles antigen [11], but no relationship to HLA antigens was found. Paty et al. [30] studied CMI response to herpes simplex virus using a lymphocyte transformation technique. Eleven of 12 patients showing low response to HSV were *Dw2*; of these, five failed to respond altogether. The data require confirmation but are potentially important in showing an HLA-associated response to a virus with high pathogenicity for the CNS.

Preliminary evidence for absence of the increased IgG synthesis in the CNS characteristic of MS in some HLA-B8 bearing MS patients was presented by Oger et al.

[24]. A correlation between the IgG/Total CSF protein-IgG index and HLA type was sought. Some relationship was found between HLA type and this indirect measure of intrathecal secretion of IgG. It was speculated that two different genes might be implicated in the pathophysiology of MS. Further analysis of these parameters indicates that intrathecal IgG secretion is particularly prominent in certain patients bearing *B7* when these are chosen using correspondence analysis [3]. High secretors include B7-blank, B7-B5, B7-BW18, B7-B12, and B7-BW15. (IgG/TP-IgG = 16.7 ± 2.66.) Some patients chosen by the same method are B8 and low secretors include B8-blank, B8-BW15, B8-BW18, and B7-B13. (IgG/TP-IgG = 7.48 ± 0.83.) The difference between groups is significant ($P < 0.01$).

Part of the data just presented conform with the results of Stendhal [32], who on ten patients found higher values for intrathecal secretion of IgG in *Dw2* patients than in any other group. No mention was made by Stendhal of contrasting patterns of HLA type distribution between high and low secretors.

A corrollary of these observations is that a substantial proportion of patients bearing B8 show no increase in CSF IgG even though they have the fully developed clinical picture of MS with multifocal white matter involvement, and frank exacerbations and remissions. The consequences in terms of diagnostic criteria to be used for selection of patients for HLA studies are evident.

The clinical response to azathioprine in relation to HLA type has been studied by Oger et al. [25]. There is some evidence that the group (21 patients) who remained totally stable during long term immunosuppressive therapy (minimum 3 years) show an exaggerated frequency of those B-locus antigens associated with MS (B7: 52%, B8: 43%, B12: 10%), whereas the group (37) patients who have shown a tendency to progress show less of these same abnormalities (B7: 38%, B8: 27% B12: 22%).

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Multiple Sclerosis Among Immigrants to Britain and in the Islands of Sicily and Malta

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Milton Alter has talked to us this morning on the geographical distribution of multiple sclerosis (MS) and the need for modification of accepted concepts. MS is almost certainly uncommon in Asia, for instance in India, China, and Japan, among the Africans of Africa, and in the West Indies. On the other hand, it is known to be common in Northern Europe, in the northern part of the United States, and in Canada.

Between 1972 and 1975 a study was undertaken to ascertain the hospitalized prevalence of MS among immigrants to the United Kingdom; both from areas of the world where the disease is common, such as Europe, Ireland, and the old Commonwealth countries of Australia, Canada, and New Zealand; and from areas of the world where MS is believed to be uncommon, that is, among the large number of Indian and Pakistani immigrants, immigrants from Africa, and immigrants from the West Indies. In this study all patients hospitalized in Greater London and the West Midlands between the years 1960 and 1972 were included. The majority of immigrants came from countries which are now called the "new commonwealth countries" – these are, in the main, countries which were formerly colonies of the British Empire. Many of them have settled in Greater London and the West Midlands, that is, the area in and around Birmingham and Wolverhampton [1, 2].

Table 1 shows that MS was as common, or nearly as common, among immigrants from Europe and the old commonwealth countries as in the United Kingdom born. Among immigrants from Ireland, the hospitalized prevalence of MS was two-thirds of that in the United Kingdom born. This lower risk in the Irish may be because it is really less common among Irish immigrants, or it may be that those Irish immigrants to England who develop the symptoms of MS return to Ireland.

Table 1. MS in Greater London residents (first admissions, 1960 – 1972). By birthplace

	Actual	Expected
Europe	152	158.1
Ireland	168	225.3
Old Commonwealth	17	24.8
America (N & S.)	12	16.5
Cyprus	23	35.0
USSR	4	7.1

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Table 2 shows that immigrants from Italy, 24 MS patients in London, had almost the same risk of developing MS as the United Kingdom born; if they had the United Kingdom born rates, 27 would have been expected. A high proportion of the Italian immigrants came from southern Italy and Sicily. Immigrants from Cyprus (Table 1) had two-thirds of the risk of the English, 23 MS patients when 35 were expected. Immigrants from Spain had a similar reduced risk, eight patients when 16.4 were expected.

Table 3 shows that among immigrants from the new Commonwealth countries of America (the West Indies), Africa, Asia, and Europe (Malta) the risk of being

Table 2. MS in Greater London residents (first admissions 1960 – 1972), Europe

	M	F	T	Expected
Germany	3	31	34	29.2
Italy	12	12	24	27.2
Poland	18	7	25	25.4
Austria	1	8	9	11.0
France	4	7	11	9.8
Belg/Lux.	0	2	2	3.9
Netherlands	1	2	3	4.3
Hungary	3	4	7	6.1
Spain	0	8	8	16.4
Other	8	21	29	24.8
Total	50	102	152	158.1

hospitalized with MS is very small. The small number of immigrants with MS who were born in these countries were nearly all of Caucasian stock. For instance, there was only one Indian MS patient and no Pakistanis among the large number of Indian and Pakistani immigrants from Asia resident in Greater London, and there were no Africans with MS. The very low risk of MS among Asian, African, and, to a lesser extent, the West Indian immigrants is confirmed from studies of deaths certified as having MS. Among the immigrants born in the islands of Malta, there were no hospitalized MS patients, although ten (9.7) would have been expected if they had had the United Kingdom born risk.

The southern Mediterranean area would appear to be one of particular interest, as it is at the junction between the high MS prevalence found in Europe and the low MS prevalence suspected to occur among the peoples of Africa. We have therefore undertaken studies of the prevalence of MS in the island of Sicily and in the islands of Malta on behalf of the Specialized Working Group of Epidemiology of the Commission of the EEC.

In Sicily previous MS prevalence studies have been undertaken by the medical faculties at the universities of Palermo, Messina and Catania, and it was thought that the prevalence of MS in the provinces studied was low, in the region of 4 to 8 per 100,000. The low MS prevalence found until now may have been due, at least in part, to the difficulties involved in studying large populations to find the MS preva-

lence. We decided to undertake an intensive study in a small city in central Sicily, Enna, which has a population of 29,000 people. There is a good hospital in the city, and the senior neurologist of Enna province, Professor Pino Grimaldi, as well as Dr. Reginald Kelly, the chairman of the Multiple Sclerosis Medical Advisory Committee of the United Kingdom and Dr. Lucien Karhausen of the EEC, collaborated with me in the study. In Enna, we have so far found 15 patients with probable MS, a prevalence of 53 per 100,000. This prevalence is of the same order of magnitude as that reported in studies in Northern Europe and in the United Kingdom and is the highest prevalence yet reported in any study in Sicily or in Italy. Because Enna is on

Table 3. MS in Greater London residents (first admissions 1960 – 1972, low MS areas)

	Actual	Expected
New Commonwealth		
America	16	130.0
Africa	4	25.8
Asia (excl. Cyprus)	13	85.7
Europe (Malta)	0	8.4
Burma	1	3.9
China	0	2.8
South Africa	3	11.5
London and West Midlands		
Malta	0	9.7

high ground in central Sicily, two further studies are being undertaken in two small coastal towns, Agrigento and Monreale. The high prevalence of MS in Enna and among the Italian immigrants resident in England suggests that the true prevalence of MS in Italy and Sicily is much higher than was previously reported.

A similar study is being undertaken in the islands of Malta in collaboration with Dr. Luis Vassallo, the senior neurologist on the islands, and Dr. Marta Elian. The Department of Medicine, under the professorship of Professor Frederick Fenech, is collaborating with the study, and we have the cooperation of the physicians, ophthalmologists, and general practitioners on the islands. There is a strong British tradition in medicine in Malta, and in the past there has been close collaboration between the Maltese Medical Association and the British Medical Association. The main hospital, St. Luke's, is a state-supported hospital, and patients with neurological diseases in which there is any doubt as to the diagnosis are referred to London teaching hospitals for a further opinion at state expense. Patients suspected of having MS are usually sent to the National Hospital, Queen Square.

On the islands of Malta only 14 patients to date have been found with probable MS and no patients with only retrobulbar neuritis. One of these 14 cases was diagnosed as Devic's syndrome. Including the Devic's syndrome patient, this is, at the moment, a prevalence of 4.2 MS patients per 100,000 population, a very low prevalence indeed. Nine of the 14 probable MS patients have had the diagnosis con-

firmed in London, seven of them at the National Hospital, Queen Square. There are three further patients with possible MS, two of whom have also been investigated at the National Hospitals for Nervous Diseases in London.

We have, therefore, a contrasting situation between the high prevalence of MS in Enna city in Sicily and a low prevalence of MS in the neighbouring islands of Malta. Both Sicily and Malta have been invaded in turn by people of Pheonician, Greek, Roman, Carthaginian, and Arab stock, as these islands are on the crossroads of the Mediterranean. The people of Malta speak an Arabic tongue, and there may well be a difference in the genetic strain of the Maltese people in comparison with those of Sicily, with a more semitic element in Malta. It will certainly be of great interest to study the HLA blood group composition of the people of Malta and of Sicily.

Further studies of the genetic makeup of the population of Enna and other cities in Sicily in comparison with that of the people of Malta and a comparison of the differences in the environment of the two peoples should throw great light on our understanding of the etiology of MS.

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The HLA-D System in Multiple Sclerosis

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Introduction

A considerable number of studies on the immunogenetic association between multiple sclerosis (MS) and the HLA blood group system have now been reported [2, 3, 6]. Most of these studies have dealt with the phenotypic distribution of HLA-A and -B antigens among MS patients compared with those in healthy controls, showing that HLA-A3 and B7 are significantly increased among MS patients. Due to the fact that HLA-D typing was introduced [13] and internationally accepted [19] relatively late and this method is based on a rather complicated and time consuming cell culture technique, the number of HLA-D typed MS patients is comparatively small. Interestingly enough, the reported HLA-D typing data in MS patients gives clear evidence that the HLA-Dw2 allele is even more strongly associated with MS than HLA-A3 and B7, respectively [9, 10]. This report summarizes our data from the three independent studies on HLA-A, B, and D typing in more than 300 MS patients.

Materials and Methods

MS Patients

This study comprises three independent series of tests in 111, 100, and 165 MS patients, respectively (total 376), most of them living in the northwestern part of Germany. All MS patients were tested during the chronic state of the disease. The diagnosis was based on both clinical and biochemical criteria. The first two series were done in cooperation with Dr. W. Schuppien, Camillus-Klinik, Asbach/Linz, and the third series with Prof. Dr. H. Bauer and Prof. Dr. G. Ritter, Neurologische Klinik und Poliklinik der Universität Göttingen. In order to avoid HLA mistypings on lymphocytes caused by therapeutic regimens, no immunosuppressive drugs were given for at least 4 weeks before HLA typing.

HLA-A, B Typing

A set of more than 90 highly selected antisera was used. The first two series were typed in the microlymphocytotoxicity assay described by Kissmeyer-Nielsen and

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Kjerbye [11], and the third one according to the method of Terasaki and McClelland [17].

HLA-D Typing

Peripheral blood lymphocytes were biologically frozen with 10% dimethylsulfoxide and stored frozen under liquid nitrogen until assay. Typing for HLA-D antigens was performed in a microculture system using 1×10^5 responding cells and 2500 R-irradiated HLA-D homozygous typing cells (HTC). Details of this method are given in a previous publication [8]. The mixed lymphocyte culture (MLC) stimulation results obtained as counts per minute (cpm) were recalculated in so-called stabilized relative responses (SRR) according to Thomsen et al. [18] with some modifications [14]. Based on HLA-D population data in healthy controls [7], a SRR value below 30% was chosen as a typing response, i.e., presence of the respective HLA-D antigen. For the antigens HLA-Dw1, w2, w3, w4, w5, and w11, and RE established HTC's from the Sixth and Seventh International Histocompatibility Workshop in Aarhus and Oxford were used. The HLA-D determinant DE has recently been identified [7].

Statistics

For 2×2 comparisons the X^2 test was used. The correlation coefficient was calculated as $\sqrt{X^2/N}$. Calculation of Delta values were done according to Ceppelini et al. [5] and relative risk values and their statistical significance were calculated using the method of Svejgaard et al. [16].

Results

In Table 1 the HLA-A, B, and D antigen frequencies recorded in the three independent MS studies are listed and compared to HLA-A, B, and D antigen frequencies found in a healthy control population. For the HLA-D antigens we could test in the first series for four alleles, HLA-Dw1, w2, w3, and w11, whereas in the second and third series six and seven HLA-D alleles, respectively, were investigated. We plan to extend the third series of MS patients to a number of more than 200 patients. Until now we have typed 165 patients for HLA-A and B and 98 patients for HLA-D.

If one summarizes these HLA data from three studies, as done in Table 2, there are three statistically significant deviations in the HLA-B and the HLA-D antigen frequencies compared to healthy controls: HLA-B7 with a frequency of 27.0% in the control population was found in 38.2% of the 376 MS patients, and HLA-Dw2 is carried by about the half (46.0%) of the MS patients compared to 20.7% in healthy controls. The HLA-A3 antigen was found with exactly the same frequency (30.2%) in MS and the control population. There was a decrease of HLA-A2 and B12 in the MS patients group, with 41.6% and 16.4%, respectively, compared to 51.5% and 22.6% respectively in the controls. These differences, however, were only statistically significant for the decrease of HLA-A2 (X^2 for HLA-A2, 14.0; $P = 0.005$, X^2 for

Table 1. Distribution of HLA-A, B, D antigen frequencies in three independent studies of MS patients (total 376 patients)

	Controls ($N = 5046$) [a]	MS/1st Study ($N = 111$)	MS 2nd/Study ($N = 100$)	MS/3rd Study ($N = 165$)
HLA-A				
1	28.1	37.8	27.0	32.1
2	51.5	36.0	47.0	41.8
3	30.2	27.0	31.0	32.7
9	20.5	18.9	29.0	20.0
10	11.2	9.0	7.0	10.3
11	9.8	10.8	11.0	9.1
28	7.1	2.7	3.0	9.1
29	4.7	3.6	8.0	6.7
w 19	9.0	9.0	10.0	7.3
w 32	6.1	10.8	9.0	10.3
HLA-B				
5	13.4	17.1	15.0	9.7
7	27.0	45.0	35.0	34.5
8	19.2	24.3	17.0	25.4
12	22.6	13.5	18.0	17.6
13	6.2	5.4	3.0	7.3
14	5.0	2.7	6.0	6.7
15	14.4	14.4	13.0	13.7
17	8.2	5.4	7.0	4.8
18	8.5	9.9	7.0	7.3
27	8.1	11.7	10.0	6.1
37	2.0	3.6	12.0	1.2
40	12.5	1.8	10.0	10.3
w 16	3.4	2.7	14.0	6.7
w 21	4.3	5.4	2.0	1.8
w 22	3.4	0.9	5.0	1.2
w 35	17.3	13.5	16.0	10.3
HLA-D	($N > 200$)			(N = 98)
w 1	9.8	8.1	11.0	13.7
w 2	20.7	54.0	41.0	42.9
w 3	10.7	21.6	13.0	15.8
w 4	6.0	n. t. [b]	9.0	3.1
w 5	9.3	n. t.	n. t.	5.2
w 7	8.3	n. t.	n. t.	2.1
w 11	4.6	7.2	n. t.	n. t.
RE	9.8	n. t.	6.0	9.5
DE	2.6	n. t.	8.0	4.2

[a] HLA-A, B antigen frequencies are taken from Albert et al. [1]
[b] n. t. = not tested

Table 2. Comparison of HLA-A, B, and D antigen frequencies in the combined MS patients group with those of healthy controls and calculation of relative risk (RR) values and their statistical significance (χ^2, P)

	Controls	MS patients	RR	χ^2	P
HLA-A					
1	28.1	32.3			
2	51.5	41.6	0.7	14.0	0.005
3	30.2	30.2			
9	20.5	22.6			
10	11.2	8.8			
11	9.8	10.3			
28	7.1	4.9			
29	4.7	6.1			
w 19	9.0	8.8			
w 32	6.1	10.0			
HLA-B					
5	13.4	13.9			
7	27.0	38.2	1.7	22.3	<0.001
8	19.2	22.2			
12	22.6	16.4	0.7	7.5	n. s.
13	6.2	5.4			
14	5.0	5.7			
15	14.4	15.0			
17	8.2	5.7			
18	8.5	8.1			
27	8.1	9.3			
37	2.0	5.6			
40	12.5	7.4			
w 16	3.4	7.8			
w 21	4.3	3.1			
w 22	3.4	2.4			
w 35	17.3	13.3			
HLA-D					
w 1	9.8	10.9			
w 2	20.7	46.0	3.2	51.5	<0.001
w 3	10.7	16.8			
w 4	6.0	6.0			
w 5	9.3	5.2			
w 7	8.3	2.1			
w 11	4.6	7.2			
RE	9.8	7.7			
DE	2.6	6.1			

HLA-B12, 7.5 $P = 0.17$). The calculation of so-called relative risk factors (RR) revealed values of 1.7 and 3.2, respectively, for the increased antigens HLA-B7 and HLA-Dw2 and for both the decreased frequencies of HLA-A2 and HLA-B12 revealed a RR value of 0.7.

Since in our first series of MS patients we observed no linkage disequilibrium between HLA-Bw35 and Dw1 [9] which is present in all Caucasoid populations tested so far [12, 19], the second and third series of MS patients were also subjected

Table 3. Comparison of linkage disequilibrium parameters between HLA-Bw35 and Dw1 in MS patients and in healthy controls

MS-Patients/1st Study ($N=111$):				
	HLA-Dw1 +	HLA-Dw1 −		
HLA-Bw35 +	3	12	$\chi^2=$ 3.3	$P=$ n. s.
HLA-Bw35 −	6	90	Delta = 0.0089	$r=0.17$
MS-Patients/2nd Study ($N=100$):				
	HLA-Dw1 +	HLA-Dw1 −		
HLA-Bw35 +	3	8	$\chi^2=$ 1.3	$P=$ n. s.
HLA-Bw35 −	11	78	Delta = 0.0083	$r=0.11$
MS-Patients/3rd Study ($N=98$):				
	HLA-Dw1 +	HLA-Dw1 −		
HLA-Bw35 +	4	7	$\chi^2=$ 3.6	$P=$ n. s.
HLA-Bw35 −	12	75	Delta = 0.0129	$r=0.19$
Healthy Controls ($N=389$):				
	HLA-Dw1 +	HLA-Dw1 −		
HLA-Bw35 +	26	41	$\chi^2=76.0$	$P=0.001$
HLA-Bw35 −	12	310	Delta = 0.0284	$r=0.44$

to a 2 × 2 comparison. As can be seen from Table 3, all three series revealed no statistically significant gametic association between HLA-Bw35 and Dw1, which is in clear contrast to the findings in healthy controls (see bottom of Table 3).

Usually the gametic association or linkage disequilibrium between alleles of two closely linked loci is given as a so called Delta value. This value, however, is influenced by the absolute frequencies of the traits under study; therefore the correlation coefficient as a second measure for linkage disequilibrium is introduced. The comparison of both the Delta values and the correlation coefficients for HLA-Bw35 and Dw1 in the MS and the healthy population revealed that in three independent studies there was no linkage disequilibrium between these traits among MS patients. In contrast, the 2 × 2 comparisons for HLA-B7 and Dw2, and HLA-B8 and Dw3, known to be in linkage disequilibrium, showed no differences between the MS and the healthy population.

Discussion

The described association of the HLA-B7 antigen and the even stronger association of the HLA-Dw2 antigen with the disease phenotype of MS in three independent studies with a total of more than 300 patients is in concordance with data already reported [15]. For the most prominent increased frequency of HLA-Dw2 in MS, the most attractive explanation is the hypothesis that there exist so-called MS-suceptibility gene(s) within the HLA complex coding for gene products which are in linkage disequilibrium to HLA-Dw2, and secondly, that these gene products together with other factors are the prerequisite for the development of MS. Nothing, how-

ever, is known about the exact nature and function of these disease-susceptibility genes, since at present we are unable to recognize their gene products directly like the HLA antigens at the cell surface.

A new finding is the fact that among MS patients no linkage disequilibrium between HLA-Bw35 and Dw1 was found. It is not clear, whether this observation holds only for MS patients from West Germany, since all the other reports on HLA-A, B, and D typing in MS have been more or less concentrated on the phenotypic comparison of each HLA antigen frequency. According to Bodmer [4] linkage disequilibrium between alleles of the different HLA loci is most likely the result of selective forces favoring particular HLA-A, B, and D allele combinations or haplotypes, e.g., HLA-A3, Bw35, Dw1; HLA-A3, B7, Dw2; or HLA-A1, B8, Dw3 in the Caucasoid population. This in turn would mean that HLA haplotypes with positive linkage disequilibrium provide a better biological fitness than others, possibly in the way that their gene products are functionally interrelated. If this is true, the observed loss of linkage disequilibrium between HLA-Bw35 and Dw1 in MS might be a reflection of an impaired immunological defense mechanism against environmental agents such as a virus due to a less effective cooperation between the HLA-Bw35 antigen and HLA-D antigens others than Dw1 at the cell surface or vice versa.

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HLA-Dw2-Associated C2 Hypocomplementemia in Multiple Sclerosis: A New Genetic Marker?

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Summary

C2 serum levels were determined by hemolytic titration in the sera of 200 multiple sclerosis (MS) patients, one MS family, two blood donor families, and ten unrelated blood donors, all typed for HLA-A, -B, -C, -D, and Bf alleles. The results suggested the existence of HLA-Dw2-associated heterozygous C2 deficiencies in about 50% of HLA-Dw2-positive and in about 24% of random MS patients. The data were discussed with respect to a polygenic regulation of C2 serum levels and to a possible pathogenetic significance in MS.

Introduction

Immune complexes and decreased serum and spinal fluid complement levels found in MS patients [22, 23, 33] have been the main arguments for the hypothesis of an autoimmune pathogenesis of MS for many years [2]. A new aspect of hypocomplementemia in MS became evident when in families with homozygous C2 deficiencies and severe systemic lupus erythromatosus (SLE)-like syndromes, the linkage of C2 deficiency gene(s) with the HLA-Dw2 allele could be demonstrated [1, 12]. Since in MS a very striking association with HLA-Dw2 exists [18], and C2 levels were found to be decreased in MS sera and spinal fluid [22, 23] we looked for HLA-Dw2-associated heterozygous C2 defects in 200 MS patients. The results suggest the existence of such complement defect genes in at least 50% of the HLA-Dw2-positive and in about 24% of all MS patients.

Materials and Methods

Patients and Controls

C2 measurements were performed in the sera of 200 HLA-A, -B, -C, and -D-typed MS patients, ten HLA-A25-, -B18-, and -Dw2-positive clinically healthy blood donors, one MS, and two blood donor families. At the time of bleeding all patients had

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a chronic state of the disease. No immunosuppressive drugs were given. Serum samples for complement determinations were stored at – 70 °C. Control frequencies of HLA-A, -B, -C, and -D alleles were taken from previous publications [5, 18].

Complement Measurements

C2 serum levels were determined functionally by means of hemolytic titration according to Boros and Rapp [8] and Borsos et al. [9]. Sheep erythrocytes (E) were sensitized with anti-sheep erythrocyte antibodies (A) and EAC$\overline{4}$ and EAC$\overline{14}$ intermediates prepared. After incubation of these intermediates with patients' and control sera diluted with sucrose veronal-buffered saline (VBS-sucrose), for 2.5–3.0 min. (t-max) at 30 °C, guinea pig serum, diluted 1 : 33 with 0.04 M EDTA veronal buffer, was added. The generated EAC$\overline{142}$ cells were lysed, the degree of lysis was measured spectrophotometrically at 412 nm, and the number of effective molecules of C2 was calculated. Total hemolytic complement activity (CH50) was measured according to the method of Mayer [24] in a microliter system modification [32].

C3c serum levels were measured by radial immunodiffusion according to Mancini on commercially available plates (Behring Werke, Marburg).

HLA Typing

HLA-A, -B and -C typing was performed by the microlymphocytotoxicity technique according to Kissmeyer-Nielsen and Thorsby [19] with 108 highly selected predominantly monospecific antisera. Frequencies of HLA-D alleles (Dw1, Dw2, Dw3, and Dw7) were determined by the mixed lymphocyte culture technique using homozygous typing cells [16, 17] established at the Seventh International Histocompatibility Workshop (Oxford 1977).

Bf Typing

Bf phenotype frequencies resulting from thin layer agarose gel electrophoresis [30] were subjected to immunofixation using specific antisera to C3 activator protein (Behring Werke, Marburg).

Results

The ABC and D typing results of the 200 MS patients showed a significant association between MS and HLA-B7 and Dw2: B7 was found in 39.5% of the patients and only 26% of the controls ($P < 10^{-5}$), and Dw2 occurred in 49.5% of the MS population and in only 20.7% of the healthy controls ($P < 10^{-10}$) (Table 1). Accordingly, these data confirm the results of previously published studies [4, 6, 18].

When we determined the C2 serum levels of these 200 MS patients, we found a range from 0.8–7.2 × 10^{11} effective molecules (eff mol)/ml. As shown in Figure 1a, the quantitative distribution of the C2 serum levels showed a unimodal curve with its maximum at 3 × 10^{11} eff mol/ml serum. Considering, however, only the C2 values of the 97 MS patients being positive for HLA-Dw2, a bimodal distribution could be shown. The first peak was found at 2.5 × 10^{11} eff mol/ml and the second at

Table 1. Phenotype frequencies (%) of the MS-associated HLA determinants in 200 MS patients and controls

Antigen	Frequency (%) in		Relative Risk (RR)	*P* value
	MS patients	Controls		
HLA-A3	55/200 (27.5%)	288/1000 (28.8%) [b]	0.9	n. s. [a]
B7	79/200 (39.5%)	260/1000 (26.0%) [b]	1.8	$<10^{-5}$
Dw2	97/200 (49.5%)	84/405 (20.7%) [c]	3.5	$<10^{-10}$

[a] n. s. = not significant
[b] Bertrams et al. [5]
[c] Grosse-Wilde et al. [18]

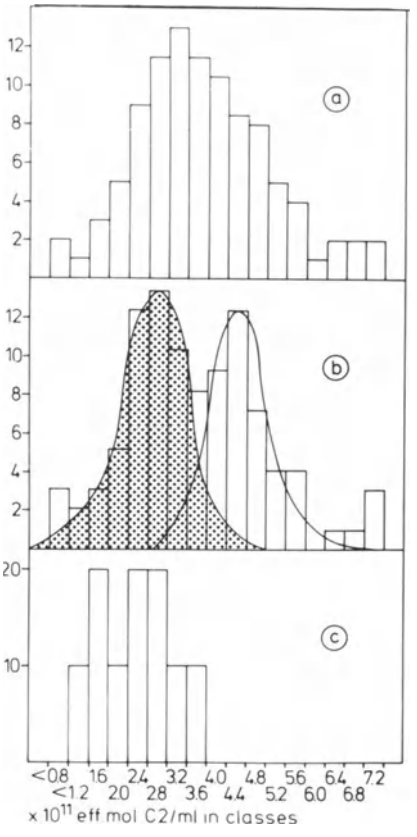

Fig. 1a–c. C2 hemolytic activity in the sera (%) of 200 random MS patients (**a**), of 97 HLA-Dw2-positive MS patients (**b**), and of 10 HLA-A25-, -B18-, -Dw2-positive healthy blood donors (**c**)

4.4 × 10^{11} eff mol/ml (Figure 1b). According to these findings, about 50% of the HLA-Dw2-positive MS patients had C2 serum values suggesting a heterozygous C2 deficiency, associated with HLA-Dw2.

To verify this suggestion we measured the C2 serum levels of ten healthy blood donors who were positive for HLA-A25, -B18, and -Dw2, since these alleles are associated with C2 deficiency in nearly all patients with a homozygous C2 defect. As shown in Figure 1c, the expected low C2 acitivites were indeed found in all ten sera. Thus an HLA-Dw2-linked heterozygous C2 defect appears to exist in clinically healthy individuals as well. To determine whether C2 hypocomplementemia resulted from activation of the complement system or reflects a genetic complement defect, we looked for the relation between C2 and C3c and CH50 activity in the sera of HLA-Dw2-positive MS patients with low C2 serum levels (< 3.2 × 10^{11} eff mol/ml). The lack of a correlation between C2 and C3c, (Fig. 2b) as well as CH50 (Fig. 2a) in these sera may be further indirect proof of an HLA-Dw2-associated heterozygous C2 defect. In the case of functional consumption of C2, a correlated decrease of other complement components like C3c and also of CH50 might have been expected.

Since segregation analysis is the most relevant method to show whether the reason for the observed C2 hypocomplementemia is functional consumption or genetic deficiency, we performed several family studies among MS patients and clinically healthy blood donors. The pedigrees of one MS family and two blood donor families given below support the genetic basis of C2 hypocomplementemia.

In MS family BR (Fig. 3), the patient (II-2), and her brother (II-3), sister (II-4), and son (III-1) had low C2 serum levels, while normal C2 values were found in the sera of the proband's mother (I-2) and her husband (II-1). In this family a C2° gene may be linked to the haplotype HLA-Aw24-Bw35-Dw2-BfS (II-2, II-3, III-1).

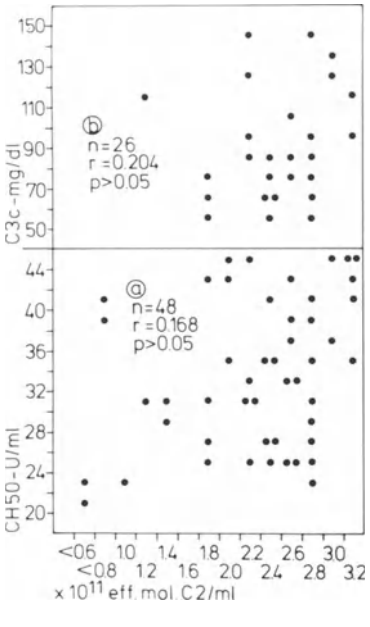

Fig. 2a, b. Test for correlation between C2 hemolytic activity and the total hemolytic activity (CH50) (**a**) as well as the C3c protein levels (**b**) in the sera of 48 (**a**) and 26 (**b**) HLA-Dw2-positive MS patients with C2 < 3.2 × 10^{11} eff mol/ml.

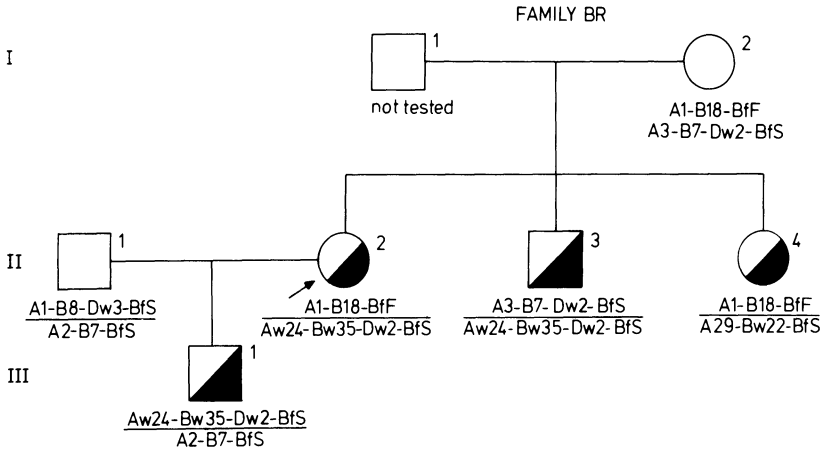

Fig. 3. Pedigree of family BR. The propositus (↗) suffered from MS. *Shaded areas* represent decreased C2 serum levels ($< 3.2 \times 10^{11}$ eff mol/ml). HLA and Bf typing results are given as haplotypes below each individual

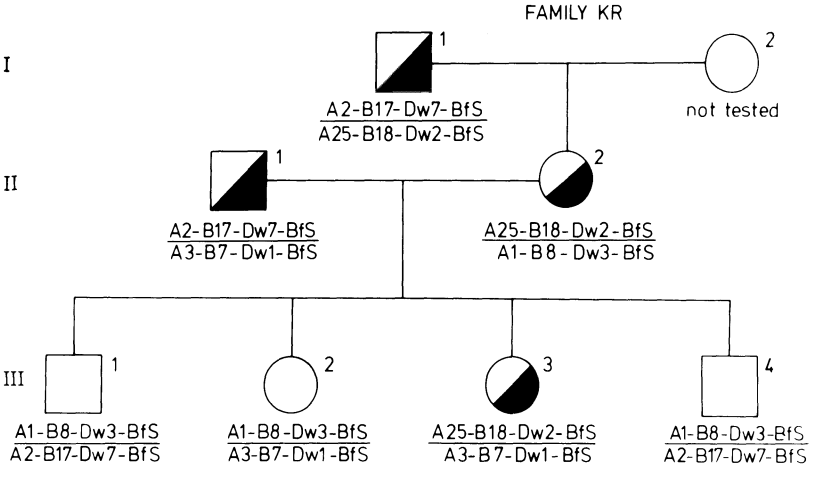

Fig. 4. Pedigree of family KR with healthy blood donors. *Shaded areas* represent decreased C2 serum levels ($< 3.2 \times 10^{11}$ eff mol/ml). HLA and Bf typing results are given as haplotypes below each individual

The low C2 serum level of individual II-4 may have resulted from functional complement consumption. In the healthy blood donor family KR (Fig. 4), the C2° gene may be linked to the haplotype HLA-A25-B18-Dw2-BfS in the blood donor (II-2), her father (I-1), and her daughter (III-3). As in the MS family, one individual (II-1) has a low C2 serum level, although lacking the haplotype HLA-A25-B18-Dw2-BfS. However, this finding is no argument against the existence of an HLA-Dw2-associated C2° gene in this family, since the possibility of functional C2 consumption cannot be ruled out.

In a further blood donor family HO (Fig. 5) an HLA-Dw2-associated C2 deficiency may exist. Low C2 activity existed in the sera of the blood donor (II-2), his parents

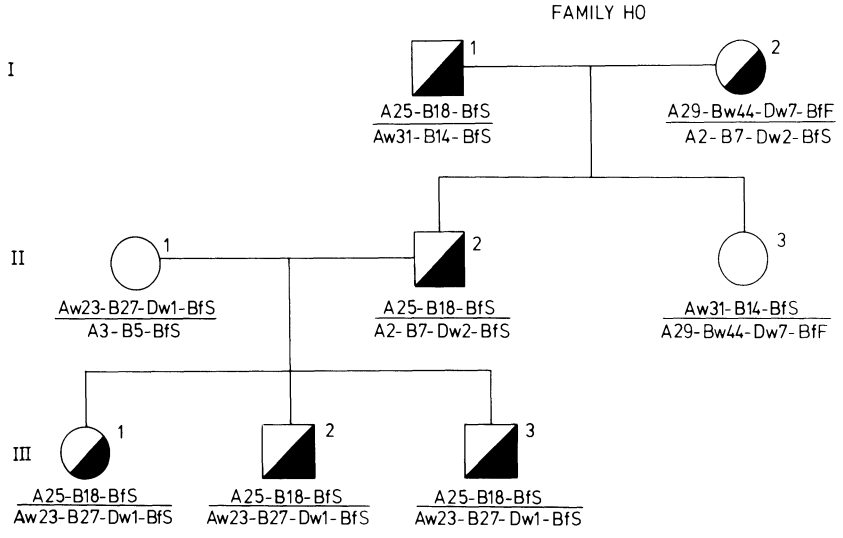

Fig. 5. Pedigree of family HO with healthy blood donors. *Shaded areas* represent decreased C2 serum levels ($< 3.2 \times 10^{11}$ eff mol/ml). HLA and Bf typing results are given as haplotypes below each individual

(I-1, I-2), and all three HLA-identical children (III-1, III-2, III-3). Interestingly enough, the blood donor's father (I-1) is HLA-Dw2 negative but positive for HLA-A25-B18-BfS, while his mother (I-2) is positive for HLA-Dw2 but negative for HLA-A25-B18-BfS. Since both parents had low C2 serum levels, both may carry a C2° gene. In the father it may be linked to the haplotype HLA-A25-B18-BfS and in the mother to HLA-A2-B7-Dw2-BfS. The low C2 levels in the sera of the three HLA-identical children (III-1, III-2, III-3) can be explained by the haplotype HLA-A25-B18-BfS. The blood donor (II-2), carrying the haplotypes HLA-A25-B18-BfS and A2-B7-Dw2-BfS accordingly should be homozygous deficient for C2. Since, however, this individual had C2 activities of about the half of the normal amount, the question arises whether additional genes may be the prerequisite for the manifestation of a homozygous C2 defect.

Discussion

The findings (a) of linkage between C2° and HLA-Dw2 in individuals homozygous for C2° [1]; (b) of C2 serum levels showing about half the normal C2 amount in individuals heterozygous for C2° [1]; and (c) of an association between MS and HLA-Dw2 as well as C2 hypocomplementemia [7] have been the basis for the working hypothesis of the present study: "C2 hypocomplementemia in MS is the manifestation of a heterozygous C2 deficiency state associated with HLA-Dw2." This hypothesis was supported by the bimodal distribution curve of C2 serum levels of 97 HLA-Dw2-positive MS patients (Fig. 1b), a lacking correlation between C2 values and C3c as well as CH50 activities in sera of HLA-Dw2-positive MS patients showing

decreased C2 serum levels (Fig. 2b, 2a), and by a common segregation of low C2 values and the HLA-Dw2 allele and/or the HLA-A25-B18 haplotype in one MS and two healthy blood donor families (Fig. 3, 4, 5). A close linkage also seems to exist between C2° and the BfS allele, which segregates together with the suspected C2°-linked HLA haplotype in all three families. The recently published findings of Nerl et al. [25] of an association between HLA-Dw2 and low C2 as well as C4 serum levels in clinically healthy individuals agree with the results of the present study in MS patients. Thus in healthy persons and MS patients, an association between HLA-Dw2 and the C2° gene(s) may exist. Based on the strong association between the HLA-Dw2 allele and MS, the frequency of the C2° gene(s) in MS should be higher than in healthy individuals. Supposing the existence of a C2° gene in about 50% of HLA-Dw2-positive MS patients, the frequency of the C2° gene among MS patients can be estimated as the product of the frequency of HLA-Dw2 among MS and the frequency of C2° among Dw2-positive MS patients: $0.485 \times 0.5 = 0.24$. This phenotypic frequency of C2° allows a tentative calculation of the frequency of C2° homozygote MS patients, which should be about 0.014 (1.4%). Since for this calculation only the HLA-Dw2-positive MS patients were considered, the calculated frequency of homozygotes of 0.014 may be underestimated. Nevertheless, no individual was found among the tested 200 MS patients completely lacking C2 serum activity.

This discrepancy between observed and expected cases of C2°/C2° was also found by Nerl et al. [25] among healthy controls, suggesting a more complicated regulation of the C2 serum levels.

It may be that not one but two or even more loci may act together in the regulation of C2 synthesis. Such a complementation of histocompatibility locus-linked immune response (*Ir*) as well as immune suppressor (*Is*) genes could be demonstrated in inbred strains of mice and rats [13] and seems to be a generalized immunogenetic phenomenon.

Interestingly enough, O'Neill et al. [26] have recently shown that the polymorphic variants of C4, which is linked to HLA like C2 [31] are coded for by two closely linked genes within the HLA complex. Thus the existence of more than one locus for the synthesis of C2 with complementing regulator and suppressor genes indeed is an attractive suggestion.

Another important question is the pathogenetic significance of the heterozygous C2 deficiency in MS. Although the relatively high frequency of HLA-Dw2-associated low C2 serum levels in healthy controls, observed by us [7] and others [25] does not indicate a pathogenetic significance, a heterozygous C2 defect may nevertheless influence the course of the disease in individuals possessing the disease-susceptibility gene for MS.

Since it is known that C2 among other immunological factors plays an important part in virus neutralization [11], C2 hypocomplementemia may contribute to the disturbance of immunological reactions against viruses resulting in virus persistence observed in MS: cellular immunoreactivity against paramyxoviruses in particular, is significantly decreased [10, 14, 15, 20, 27, 28, 34, 35]. Paramyxoviruses could also be isolated from cultured brain cells [21] and jejunum [29] taken from MS patients.

The heterozygous C2 deficiency may thus be a further genetic marker for MS, which accordingly is associated with the Caucasian "super haplotype" HLA-A3-B7-Dw2-BfS-C2° [3].

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A Sib-Pair Double-Case Study of the Genetics of Multiple Sclerosis. An Interim Report on 34 Pairs of Affected Siblings [1]

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Summary

Under the hypothesis of a genetically determined multiple sclerosis (MS) susceptibility associated with markers of the HLA system, 34 pairs of siblings both of whom are affected by MS were analyzed for joint segregation between the disease and HLA-A,B haplotypes. The essential methodological features of this sib-pair double-case approach are discussed in detail. The results in six out of 34 sib-pairs are not compatible with joint segregation of MS with a HLA-A,B haplotype. At present it seems likely that the HLA-linked "MS-susceptibility gene(s)" should have a dominant action with low penetrance and it or they should be fairly frequent in the population. Therefore, cases that are an exception to joint segregation should be expected.

Introduction

Only a few years ago Myrianthopoulos [12], in his comprehensive review of almost 100 years' scientific work concerning the "Genetic Aspects of Multiple Sclerosis", stated that "the relative role of genetic and environmental influences in the etiology of MS has not been assessed with certainty", and that there was "a lack of sufficient evidence for clear-cut genetic determination of MS". In 1972, however, the first evidence was presented for an association of MS with certain immunogenetic markers of the HLA system [13, 3, 10]. In the meantime, this association has been established beyond any doubt, especially in Caucasians having the haplotype HLA-A3, B7, Dw2 [7]. These findings strongly suggest that some genetically determined MS-susceptibility factors exist which may be involved in the etiology of the disease. Such factors should be closely linked to the HLA loci on chromosome 6. To confirm this hypothesis, family HLA studies are required. However, the studies which have been published so far have failed to yield conclusive proof [2, 4, 5, 9, 14, 20].

All clinical twinship and sibship studies which are based upon a comparison of affected and "healthy" family members have at least two limitations. First, almost 20% of all MS cases remain clinically silent during the "affected" individual's whole lifespan, as has been revealed by occasional findings at autopsy and also by an extensive study of post mortem material [8, 11]. These silent cases, as well as very be-

1 Dedicated to Professor Gustav Bodechtel on the occasion of his 80th birthday

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nign cases, distort the results of any ill-or-healthy comparison and have rendered twin studies inconclusive. Secondly, the danger exists that a "healthy" control may at some time in the future develop the disease. To determine whether or not this will be the case, follow-up studies must be continued for decades. To avoid these methodological difficulties, we decided to concentrate our efforts on a HLA sib-pair double-case approach.

The essential feature of this type of study is to examine families in which at least two siblings are affected by the disease. The assumptions which are involved in this approach are

1. that a disease susceptibility gene or gene complex exists,
2. that this gene or gene complex is closely linked to well-known genetic markers,
3. that due to the polymorphism of the genetic marker systems and due to a lack of linkage disequilibrium, marker haplotypes may differ between families,
4. that the inheritance pattern of the susceptibility gene or gene complex follows the dominant model with weak penetrance,
5. that some environmental factor must cooperate for the clinical manifestation of the disease.

The essential point of this approach is to demonstrate that the two siblings, both of whom are affected by the disease, share at least one common haplotype. Parents, unaffected siblings, or other relatives need not be considered, except to enable genotype determination or to allow a better clinical diagnosis to be made. Should there be only one single sib-pair where no evidence can be found for joint segregation of the disease with at least one haplotype, this one exception alone is crucial to the assumptions made above, unless it can be explained by one of the mechanisms described in the discussion. Thus, the sib-pair approach is a very powerful tool for the detection and chromosomal assignment of disease susceptibility genes. For an estimation of penetrance levels, however, this approach will provide no information.

In MS, a pitfall of both the sib-pair approach and of any other type of family study is the occurrence of familial spinocerebellar ataxias and other syndromes very similar to MS and known to be classical inherited disorders. Even if spinocerebellar heredoataxia, etc. can be ruled out by careful clinical reexamination of the patients, applying rigid classification criteria, the question remains as to whether familial double cases of MS are representative of the majority of sporadic cases.

Patients and Methods

A survey of 14,000 patient members of the West German MS society [18] and a questionnaire which was sent to 4000 neurologists in West Germany [19] revealed some 60 sib-sib double cases. From this crude material there remained 34 sib-pairs (i.e., 68 patients) who were willing to participate in our study and who had been classified as suffering from MS by university clinicians or by neurologists at specialized MS centers. All these patients have manifested the disease for more than 10 years.

Blood samples for HLA typing were taken by the patients' family doctors and sent to the laboratory by express mail. Healthy relatives were typed if they were

available and willing to cooperate. HLA typing was performed for ten specificities of the A locus and 16 specificities of the B locus using the NIH microlymphocytotoxicity assay of Terasaki and McClelland [17].

Chi-square values were calculated with Yates' correction, and P values were corrected for multiple comparisons.

Results

In 17 out of a total of 34 sib-pair double cases, we were able to deduce the genotypes. In all these families, both affected sibs had one haplotype in common. Five other sib-pairs were HLA identical. In 12 additional sib-pairs we were unable to deduce the genotypes to date; in six of them we could conclude from the phenotypic marker distribution that both sibs most probably share one haplotype; in the other six sib-pairs, however, no evidence for joint segregation of HLA markers with the disease could be found. Thus, it is necessary to explain the six "exceptional families", as will be attempted in the discussion.

Among the 22 sib-pairs who either share one haplotype on the genotypic level or who are HLA identical, we observed the HLA-A3, B7 haplotype in five pairs, i.e., in 23% (versus 6.38% in the control population as published by Albert et al [1]; chi-square with Yates' correction = 7.18; $P = 0.007$, P corrected for multiple comparisons = 0.18) which confirms the results described in the literature. The next most frequent haplotypes were HLA-A2, B15 and HLA-A2, B7. Each of them occurred in each of two pairs and other haplotypes occurred only once.

Discussion

The probability that two randomly selected sibs, whether affected by a disease or not, share at least one haplotype, is 0.75. Assuming that a genetically determined susceptibility for MS segregates with HLA markers and acts in a dominant way, the probability that two affected sibs will share at least one haplotype should be expected to be 1.0. The ratio we observed is $28 : 34 = 0.82$, which does not differ significantly from 0.75. A similar result was obtained in a multiple case family study by Hens and Carton [9], in which two out of eight affected sib-pairs did not have one haplotype in common. Bertrams and Kuwert [5] found one "exceptional sib-pair" in a total of 11 and Olsson et al. [14] found none "exceptional sib-pair" in a total of four families.

Deviations from the 1.0 value may be explained by:

1. recombination between the HLA loci and the "MS-susceptibility gene(s)",
2. occurrence of extramarital haplotypes,
3. homozygosity for "MS-susceptibility" in one parent,
4. "MS-susceptibility" in both parents, and
5. difficulties in the clinical diagnosis of MS.

Presently we are reassessing all of our patients using strict standardized criteria according to the Göttingen classification system [15, 16]. Clinical evaluations are

made without prior knowledge of laboratory values. At the same time, we are analyzing the patients and their relatives for HLA-D (formerly called "MLC") specificities and for other markers on chromosome 6, such as complement factors (Grosse-Wilde and Zander, manuscript in preparation).

These studies are intended to address items 1, 2, and 5 of the explanations offered above. The weight of items 3 and 4 depends mainly on frequencies and penetrance of the HLA-linked "MS-susceptibility gene(s)". We know from the Hardy-Weinberg analysis of phenotypic data from 1000 MS patients [6] that there is no evidence for an increased frequency of HLA-B7 homozygosity in MS. Thus a recessive mode of inheritance of the HLA-linked "MS-susceptibility gene(s)" is unlikely. In a dominant model, however, there should be a parent-child transmission of the disease which is relatively infrequent, suggesting a low degree of penetrance. In this case, the "MS-susceptibility gene(s)" should be very frequent in the population. Therefore it must be expected that in a considerable number of families both parents carry the "MS-susceptibility gene(s)" (item 4), and that it or they occur in a homozygous form in an appreciable number of individuals (item 3). Thus, a number of seemingly "exceptional families", i.e., where there is no evidence for joint segregation between HLA haplotypes and MS, must be expected.

Our results clearly do not exclude the possibility of one or more additional genetic factors which may determine or codetermine MS susceptibility and which are not associated with well-known markers of chromosome 6. Alternatively, one would have to consider the possibility that the hypothetical exogenous factors may have been so powerful as to overwhelm an individual who lacks a genetically determined susceptibility. Under this aspect, these "exceptional families" may be of special interest, for they could provide a clue to enigmatic exogenous factors which must be suspected to operate in the etiology of MS.

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Discussion

B. G. W. Arnason: Dr. Kuwert, do you have any data on homozygotes or blanks for Dw2 in terms of complement level?

H. Grosse-Wilde: Our first Dw2 homozygote, back in 1972, was actually found to be homozygous deficient for C2, but he is healthy. However, aplastic anemia was found in a sibling. But this person is obviously healthy.

M. Alter: One point to Dr. Arnason: I would like to suggest that if the mechanism of action of the markers with relation to the causative agent of MS is due to viral recognition of these marker genes, it might be very useful to apply some of the elegant techniques described yesterday to specific viral titers in MS patients in relation to the HLA group, and see what those results might be.

And a brief comment to Dr. Grosse-Wilde. If the association that you describe, the "hot locus" concept, is to be effective in selection, it would be unlikely that it is due to the manifestation of MS, since the selective forces must, of course, operate prior to reproduction. And if the average age at onset of MS is, for example, age 30, we would have to look for something prior to the MS, perhaps susceptibility to viral infections again, as being the selective force accounting for your lack of disequilibrium for the Bw35, Dw1 association.

E. Schuller: Using a B-cell marker DRw system, we found a correlation suggesting that MS is a polygenic disease. This is a preliminary result from a small population, to date only 31 patients. Classically, susceptibility is linked to S3, B7, DRw2, and we found a high frequency of this genotype in our own patients. We especially analyzed the progression coefficient, which is the ratio between disability grade evaluated with the Kurtzke scale and the duration of the disease in years. It is a very classical ratio proposed by Jersild and Torben Fog. We found a rapid course in a special type of patients with the DRw3 marker in their B cells. This is significantly divergent from a slow course of the disease, with a ratio below 0.5. Mild forms of MS with a slow course are frequently associated with A2, B12, DRw7, but are, however, only significant for A2 at this time. This work is still in progress; it suggests a different type of course linked to a DRw factor. In our population of 101 MS patients we determined C3 and C4 in serum and CSF using electro-immuno-diffusion (EID), and found no difference from normal individuals. In other words, we never found hypocomplementemia concerning C3 and C4, or B factor. B factor is also determined using EID in serum and CSF. We found no diminution of these complement components.

H. Link: In relation to Dr. Arnason's presentation I would like to point out that elevated IgG is the dynamic and not the static finding, varying between normal and elevated values in the single MS patient. And as I can see it, it should be difficult to correlate elevated IgG levels to HLA antigens. In contrast, the finding of oligoclonal bands in CSF is a static phenomenon, not varying in single MS patients. And in ad-

dition, there are clinically definite MS cases without demonstrable Ig abnormalities in CSF, and it is therefore possible to separate MS patients based on the presence or absence of oligoclonal immunoglobulins. We have carried this out and have related these two groups to Dw2 and find that patients without oligoclonal CSF IgG had normal frequencies of Dw2.

We can make the same separation in optic neuritis regarding the presence or absence of oligoclonal IgG, and these results are similar. That means that in MS as well optic neuritis, without oligoclonal CSF IgG, there are normal frequencies of Dw2. These data are based on a small number of cases, and the investigations have to be extended. I will add that those MS patients who have no oligoclonal CSF IgG and normal Dw2 show an extremely benign course. Finally, we asked if the ability to synthesize IgG within the CNS, i.e., this immune response within the CNS, is coupled to any HLA antigen. Therefore we have investigated, in a very preliminary study, aseptic meningitis with oligoclonal IgG, and these results are negative.

B. G. W. Arnason: It seems to me that there is really no substantial disagreement between us in terms of what I've said. I would wonder whether MS populations in different countries are identical. We may be running risks in assuming that what happens in France and what happens in Sweden is identical and in extrapolating data from one part of the world to the other.

Certainly in Japan – Dr. Kuroiwa is here and may wish to comment on this – the disease has certain features which are somewhat different from those in Europe or in the United States. Perhaps I might call on Dr. Kuroiwa to make a brief comment.

Y. Kuroiwa: According to its clinical features, MS in Japan is evidently different in the acuteness or strength of the response. But I will be speaking later this morning and I don't want to go into much detail here. I would like to make a brief comment about HL distribution in MS and other neurological diseases, particularly myasthenia gravis, where this is different. In Europe, Causasians with myasthenia gravis carry B8. But Japanese MS is associated with B12, and this is confirmed in all of the Japanese cases. And regarding Behcet's disease, these patients often have an inflammatory disease of the CNS which is associated with B5. But this is not different from the location of Behcet's disease, which I think is similar. And also, our disseminated encephalo-myelitis (DE) cases have no Dw2 to B7 haplotype. We have more haplotypes of Dw1 to B7. So there is something evidently different. But to go back to the history of HLA studies and MS, this research started with the impression that there was a worldwide tendency in the distribution frequency of the A3 gene associated with the frequency of MS – now we are all talking about the different frequencies of HLA and MS in different countries. Now for an opposite concept: another starting point might be the association with certain HLA haplotypes. But each country, each race, must have a different association. I think this changed concept is extremely important.

B. G. W. Arnason: Thank you very much. I think that's very appropriate.

O. R. Hommes: I would like to comment on what Dr. Schuller said on the progression rate.

I think I showed you in my first presentation that there is a clear correlation between DRw2 and progression rate in MS patients in the Netherlands. The second comment I wish to make is on what Dr. Link said about the IgG values. I calculated

the total IgG values, but I didn't show the figures, because the differences were not significant between the three groups of DRw2-homozygous, DRw2-heterozygous, and DRw2 MS patient populations. But what we did, and what I showed you in the last slide of my first presentation, is that if we calculate local IgG synthesis, there might really be a correlation, and I think as clinicians we should look for these correlations. Because if we do find a close correlation between DRw2 and the progression rate of MS, this means that the locus to the immune response is so close that we should be able to find the site that produces the abnormal immune response – and the only thing we have is the local synthesis of IgG in the CNS.

E. Kuwert: Would you tell us once more how many patients you have?

O. R. Hommes: There were 34 MS patients separated into three groups of 12, 12, and 10. I said that the population is not large, but it gave at least a hint that we can find a clear difference. We know that the total IgG in the spinal fluid does not indicate the real activity because a lot of the IgG comes from the serum. Now what we should do as clinicians is look at the local IgG synthesis and correlate that to the histocompatibility pattern.

H. Kuwert: So your data corroborates finally the data of Dr. Arnason in both respects. Dr. Schuller, you mentioned that you did not find any hypocomplementemia in your 100 MS patients. Which method did you use? Just the usual quantitative protein determination by immuno-plates, or did you use functional activity determination in moles as we have shown here? And secondly, did you correlate your data with the HLA-B, -C, and -D data? Because, as we show, if you have a preponderance of patients in your group who do not have the Dw2 antigens, then such a hypocomplementemia perhaps would be darkened. So that you could not expect, in the whole group, to have a hypocomplementemia.

H. Schuller: We used electroimmunodiffusion, a quantitative method, a personal method in serum and CSF of the same patients unconcentrated CSF and diluted serum. And we found practically no case with abnormal levels of C3 or C4 in serum or in CSF in 101 patients of my population presented here. And recently we described a new method of electroimmunodiffusion showing the presence of B factor in some CSF. Its not a normal component in CSF. An increase in B factor is frequent in MS sera.

H. Kuwert: Yes perhaps later on you should also introduce functional determination, functional enzyme activity, because you are just measuring protein content by all methods you mentioned.

T. Fog: A comment to Dr. Schuller about this progression rate. If you determine the progression rate you have to define it as the duration from the presumed onset until the presumed stationary state, or if the patient is not stationary of course you may use the date on which you are measuring this rate. Because if you just take the duration from the onset until you make the determination, you will get too many patients with a slow rate. That's the difficulty, because we don't know how many patients are stationary. But if you know the patient over the years you are able to find out which of these patients are in the stationary stage. It makes a difference.

C. Jersild: Yes, it is very difficult to divide unrelated individuals into C2 heterozygotes or C2 normals, and as Dr. Kuwert showed on his slides, there is a considerable overlap in the presumably C2 heterozygotes compared to the C2 normals. So I would like to say that the only way is to do family studies. Has that been done and

what did it show? Secondly, it is known that the C2 deficiency gene is linked very frequently with the BFS, the C3 proactivator polymorphism. What did that show?

H. Kuwert: Those aspects have been looked at in two families and these factors correlated with each other.

C. Jersild: But the claim that you have C2 deficiency in the 52 cases – is that still open?

H. Kuwert: Well, in your way of thinking, yes.

H. Grosse-Wilde: I think you have summarized the C2 deficiency data and you know how complicated it is to conduct family studies at least as a test of C2. I think you should not ask such a question, because its impossible since one has such a big variation in the heterozygotes. We have tests in our normal persons selected for Dw2 and have tested their functional C2 and functional C4. We have found in both cases, in the Dw2-positive persons, a bimodal distribution, so there is at least an influence for the preselection for Dw2 to the C4 level measured by protein and the C2 measured by functional tests. I think you at least have to do polymorphism studies for C2 and C4 to show that these persons with a low level are so-called "hemizygotes" expressing only one allele since they are deficient. But perhaps you also know that there are very few studies on the correct inheritance of C2 and C4 alleles and there is some very new data that C4 is identical to the Chido or the Rogers blood group. So I think one should really wait at least for 6 months to see what the real explanations for the correlations are for the C4 and HLA systems.

C. Jersild: The variation you see among unrelated individuals is much less pronounced than within the family. There is no overlap. We can clearly classify people as heterozygotes or normals within a family.

Dr. Shibasaki: As one of Prof. Kuroiwa's associates, I would like to make a brief comment on the clinical status of MS in Japan. Having seen many patients in several western countries, I feel that we are dealing with essentially the same disease in Japan although there might be some clinical and pathological modifications, as Prof. Kuroiwa pointed out. I think this would help to avoid a possible misunderstanding that we are dealing with a completely different disease entity in Japan.

O. R. Hommes: When we find a correlation between immune activity in the spinal fluid and DRw2 typing, we think about a change in immune reaction. There could be, close to the immune response area on the chromosome 6, an area where enzyme deficiency is coded for. Now what I would like to ask is, does anyone know about an immune activity disturbance linked with enzyme deficiency, and what enzyme should we look for in MS?

B. G. W. Arnason: Would anybody like to answer this question?

H. Grosse-Wilde: I mentioned C21 hydroxylase deficiency which was caused in homozygous so-called congenital adrenal hyperplasia, and I have met the discoverer of this disease, Dr. Bongiovanni, who has said that a deficiency for this enzyme causes the disease and as we know about the data from Dr. Dupont and our own, that this deficiency is closely related to the HLA-D locus and on the top of C2 or C4, if this is correct on my slide. And I think we have reconsidered the whole region as perhaps a chain of operator genes. And it may be that the structural genes are on different chromosomes. The polymorphism of HLA might also be explained, as such, by the action of operator genes. And we can also argue that what is defined as enzyme deficiencies is not really an enzyme deficiency. The structural gene for

C21 hydroxylase is there, but the operator genes say no. The same could be true for C2 and C4. The deficiency is allelic to the polymorphism and I think there's one extrapolation from the mouse data on the *Ir* genes, the immune response genes, where we should really think that all the *Ir* genes, at least as defined in mice, are antibody regulating genes against very stupid antigens. And I think that these are like copolymers of glutamine, tyrosine, and so forth, and I think these are agents which are far more complicated and we are not going to find any defined *Ir* genes responsible for MS.

H. Kuwert: I would like to raise another point of interest. Dr. Dean mentioned that the HLA-B- or DRw-related antigens, so to speak, are present in about 84% of MS patients. I would like to know if these are the old data published by "Wernet, Kunkel et al." some years ago. Because the same question has been worked out by other groups and the figures were far lower. So the question I want to ask Dr. Dean is which paper does he relate to? Secondly, perhaps a question to Drs. Jersild, Grosse-Wilde, and Schuller. What were your data on HLA-B differences between MS controls?

G. Dean: The recent editorial in the Lancet referred to the fact that the proportion of B-lymphocyte antigens in the general population is 14%. One hears so many figures and one is never quite sure what population has been studied. But this is the claim. Certainly the recent figures for northeast Scotland, which confirm that the B7 group is very high indeed, seem to be very authentic and might well explain the difference between the Viking population from Scandinavia who settled in the Orkneys, the Shetlands, and the northeastern area of Scotland in comparison with the Celtic population in western Scotland, the Outer Hebridies, and, of course, in Ireland. I can only quote you the figures that I have received and whether there are more recent figures, I could not say.

H. Kuwert: To me it seems important to have the actual data because this marker at the beginning was finally hailed as a criterion, a diagnostic criterion for MS. If there were a relationship of let's say 80–100%, as the first paper pointed out, then it would also have great value for diagnostic questions. And so we should hear now when the other studies were performed, the more recent ones using defined antisera. I think that in the first study published, the high figure was due to some impurity or contamination by cross-reacting antibody in the antisera. So may I ask once more.

C. Jersild: It was Dr. Platz who did that study in Copenhagen, and it included about 35 patients as I recall. He found the same frequency as for Dw2. The DRw2 fitted very well. I think he had two who went one plus minus and one minus plus.

H. Grosse-Wilde: The HLA DR typing was done by Dr. Bertrams and Dr. Wernet and, in our hands, the DR typing has beaten the Dw2 a little bit. But the statistical significance was not different in the frequency of DRw2. DR means D related, this is a B-cell antigen. This is about 25% of the control population of about 200. We found about 55–60% in MS patients, so that is lower than the previous figure of 84%.

E. Schuller: We found a clear linkage disequilibrium between Dw2 and DRw2.

H. Kuwert: So also in the range of 50% in MS and 20–25% in controls?

E. Schuller: Yes.

H. Kuwert: So this brings the whole situation into a more realistic perspective.

Y. Kuroiwa: Our recent study with Prof. Sasataki in Tokyo on the DRw group shows a significant correlation association if we include DRw3, 5, 6 and 8 and if we combine these, there are I think 64% in MS and just 26% in the control group.

The Geographic Distribution of Multiple Sclerosis: New Concepts

M. ALTER [1]

Investigations over more than four decades have established that multiple sclerosis (MS) has an unequal geographic distribution [36]. Such studies, while of some intrinsic interest, are valuable chiefly because of the light they may shed upon possible etiologies, risk factors, and the pathogenesis of the disease. Before examining the fruit of almost a half century of effort in this field, it is necessary to remind ourselves of the technical problems which had to be overcome in order that data on MS distribution would be meaningful. One such problem is diagnosis. As in any scientific study, we must be sure that the entity we are looking at is relatively "pure". For MS, the difficulties in designating the affected have been (and continue to be) the lack of an objective, pathognomonic test to support the clinical impression. The problem has been resolved (though certainly not solved) by using clinical criteria to define the individual case. These criteria include occurrence of remissions and exacerbations (time scattering) as well as evidence on examination or documented evidence of multiple lesions of central white matter (place scattering) [41]. Various investigators have modified these criteria somewhat to include an age-at-onset limitation [52], and a few recommend including elevation of gamma globulin [56] in the cerebrospinal fluid or oligoclonal banding [58]. However, it is reassuring, at least for the present, that experienced neurologists have a very high rate of agreement as to the "purity" of the entity for patients who fulfill the most stringent of these clinical diagnostic criteria. Of course, discovery of an objective test for MS may radically affect current concepts of MS distribution.

Techniques of case ascertainment have evolved over the last several decades, but for the most part, data on MS distribution based on mortality data [35] have been supported by the more sophisticated morbidity data, which use living patients with MS to measure prevalence and incidence rates [24]. The morbidity data (Fig. 1) suggest a gradient of MS frequency strongly correlated with geographic latitude. The higher the latitude both north and south of the equator, the higher the apparent frequency of MS. Almost everyone agrees that this distribution is not a function of the availability of medical care facilities, though such facilities obviously influence the number of cases identified in a given area [59]. Therefore, one should not look to the absolute number of cases identified, but rather to whether the frequency in a given area is low, intermediate, or high. The cut-off limits for each of these areas is arbitrary, but most investigators agree that an area with less than ten living cases per 100,000 population at any one time constitutes a low prevalence area; one with 10–35 cases, an intermediate area; and one with more than 35 cases per 100,000 population, a high MS prevalence area. Some areas, such as the Shetland and Orkney islands [49] and Denmark [37], allegedly have up to a hundred or more

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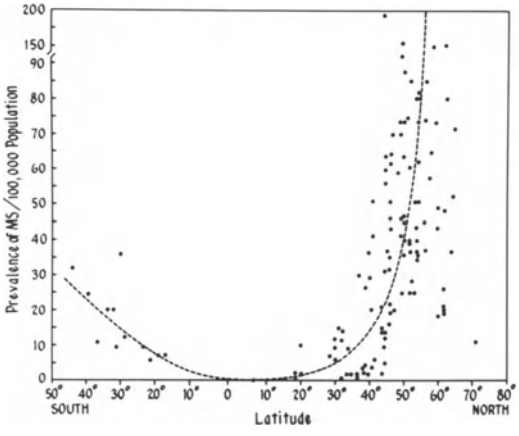

Fig. 1. MS prevalence as a function of latitude. Note appreciable scatter

living cases per 100,000 population. These areas usually have intensive surveillance programs and physicians interested in MS. In such areas, patients who might be missed elsewhere are more likely to be identified.

While there is good agreement that MS frequency is related to geographic latitude, there is disagreement as to whether the increment is smooth or discontinuous. There is some variation in absolute rates of MS in a given area and considerable scatter in rates for a given latitude (Fig. 1). The MS rates for southern Europe [14, 40, 50], for example, are consistently lower than the rates for communities in the United States at the same latitude [31, 48]. Japan is another example of a region with an apparent discrepancy between observed MS frequency and the frequency which would have been expected from consideration of latitude alone [33]. All of Japan has a low prevalence of MS even though areas in the USA at the same latitude as northern Japan have prevalence rates of about 60 per 100,000 population [48] (Fig. 2). However, no one believes that latitude per se has anything to do with causing MS. All agree that some other variable, influenced in a general way by latitude, plays a role.

Fig. 2. MS prevalence in Japan

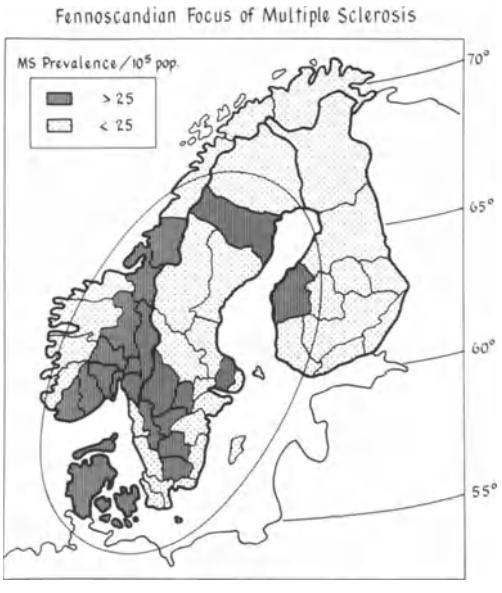

Fig. 3. An apparent focus of MS in western Finland, southeastern Sweden, southwestern Norway, and Denmark

When the "fine structure" of MS distribution is carefully examined, local variations in frequency become even more prominent [27, 34, 44]. Adjacent regions vary significantly in MS frequency (Fig. 3). While some of this variation may be due to chance, there is a body of opinion holding that the distribution of the causative agent of MS accounts for the rest of this variability. There are insufficient data as yet to resolve this question.

What is the frequency of MS at the extremes of latitude? This question has also been studied insufficiently. It appears that MS declines in prevalence toward the extreme north and is low in northern Norway [54] and in the northern parts of Canada [6]. However, the populations in these regions are small, and the medical facilities are relatively poor. Therefore it is uncertain as yet whether the apparent low rate of MS in extreme northerly areas is real. Studies extended in time based on careful medical surveillance will be needed to resolve this question.

To date, there have been no formal studies of MS in South America and only a few studies in Oriental countries [45]. Much of central and northern Africa has not been studied, though there are good data on populations which emigrated from North Africa and Asia Minor, which suggest that MS rates in these populations are low [38]. If our conception of the world-wide distribution of MS is to be complete, some studies should be carried out in the Far East, northern and central Africa, South America, and regions at extreme northern latitudes. Such studies may yield important data to support or refute currently held hypotheses about the etiology of MS.

On the basis of these admittedly incomplete data on MS distribution, several lines of speculation as to the cause of MS have been promulgated [4]. One line suggests an environmental agent correlated with latitude which might cause MS. Another line of speculation attempts to relate differences in MS distribution to genetic fac-

tors. A third concept attempts to integrate environmental and genetic factors in etiology, and is the concept most widely held at the present time.

Environmental theories of MS etiology have examined such factors as sunlight [1], temperature [39, 50], cosmic radiation [12], and trace elements [60]. These might be grouped as the geo-climatic hypotheses. They imply that MS is caused by *where* people live. Another group of hypotheses is based on *how* people live. This latter group includes the etiologic theories based on diet [7], educational achievement [43], socioeconomic status [13], and sanitary standards [10]. MS risk appears to be directly related to the sociocultural variables correlated with a high standard of living. Within a given area, individuals who are more advantaged economically have a higher risk than the disadvantaged [43]. Still another set of ideas involves infectious agents, particularly viruses [29, 61]. Two candidate viruses include measles [42] and, more recently, canine distemper (CDV) [20], which is also a paramyxovirus and cross-reacts with measles. It is now well established that MS patients have a higher measles antibody titer in serum than do controls [58]. It is of interest that sibs of MS patients who do not have clinical signs of MS also tend to have higher titers of measles antibody than do controls [17], but either genetic factors or common exposure to an etiologic agent could account for the familial aggregation of the measles titer elevation.

CDV titers are allegedly increased in MS according to one group [21], but at least one attempt to confirm this result in a small series failed [30]. The higher CDV titer would fit with those reports which have described a higher exposure to small household dogs among MS patients than among controls. However, not all investigators have found increased exposure to dogs among MS patients [32], and in any event, dogs are so ubiquitous as pets that it would be difficult to define what constitutes a significant exposure. If an individual visited a friend who owned a dog or purchased food from a store guarded by a dog, would that constitute an exposure?

The observations suggesting an association between occurrence of MS in the Faroes and occupation of these islands by British troops in World War II is relevant to the question of dog exposure and MS [21]. According to one report, dogs on the Faroes were not inoculated [19] against distemper, and an epizootic of canine distemper occurred in the Faroes after the British occupation because the British troops brought dogs with them. The appearance of MS cases followed the occurrence of canine distemper. After the departure of the British troops, MS cases occurred with decreasing frequency and then virtually disappeared. This event is now under intensive investigation and will be followed closely by all students of MS.

An additional set of data offering strong support for an environmental factor is based on studies of migrant populations, especially those moving from a zone of high risk to one of lower risk of MS, or vice versa [2, 23, 47]. These studies suggest that migrants carry the risk of their original place of residence *unless* they migrate as youngsters, before adolescence. New data from Israel [9] suggest that the critical age of migration might be as early as five years. Moreover, the fact that migrants from presumed low MS frequency areas moving to Israel, an intermediate frequency area, had an increased risk of MS, suggests that the MS agent is concentrated where MS is rare, as in poliomyelitis. Thus, early-life exposure may be protective [47].

It is wise to recall that even though the data on MS in migrants have been confirmed repeatedly, there are sources of error which may not have been eliminated.

For example, individuals who migrate when very young are usually still young when ascertained in a population survey. Therefore, not all of them will have passed through the age-at-risk of developing MS, and the low MS frequency rates among young migrants may be spurious. Moreover, it is difficult to obtain an appropriate population-at-risk. The latter must include individuals who migrated from the same place, at the same time, and at the same age as the patients.

While it is widely accepted that an environmental factor is required to produce MS, it is not known what this environmental factor might be. It is still debatable whether it is related to *where* or *how* people live. The most popular idea is that a viral infection in childhood may be important, and at least one investigator has suggested that acquisition of one or another viral infection later in childhood might account for higher rates of MS [3, 5].

Investigation of genetic factors in MS has been conducted for years, but this line of research has been particularly active in recent years, since the demonstration that certain major histocompatibility determinants are increased in MS relative to controls. In populations with Northern European forebears, HLA A3, B7, Dw2 and a B-cell antigen, Dr2, are increased in MS patients [18, 46, 53, 55]. In Japanese [51] and Israeli patients [16] other determinants are increased, and A3 and B7 may actually be decreased. It has been suggested that A3, B7 individuals with MS may have a more malignant course [28]. Patients who have only optic neuritis and A3, B7 are allegedly at greater risk of disseminating the demyelinating process [11]. There is, however, no universal agreement on these points [4]. Still others suggest that histocompatibility-linked genes control humoral and cellular immune responsiveness [22] or complement levels [15, 57] in MS patients or in individuals prone to develop MS.

Efforts to establish the existence of an MS susceptibility gene (MSS) by tracing inheritance of a shared HLA haplotype in multiplex MS families has failed [8, 25, 26]. While MS does seem to segregate with a given HLA haplotype in some families, the inherent probability of such segregation is high and application of Morton's rigorous lod score method of establishing linkage between MSS and an HLA haplotype has shown that such linkage is not statistically significant [8].

Despite these equivocal and negative results, the relationship between HLA genes and MS is intriguing and is attracting much interest. It has been shown that the geographic distribution of HLA genes associated with MS parallels the distribution of MS [22]. The cited work which suggests that HLA genes control humoral and cellular immune responses to infectious agents offers a way to relate environmental and genetic factors in MS, for example, by postulating that the risk of MS is related to HLA mediated host responsiveness to childhood viral challenge [3]. Virus may conceivably establish occult infection in the central nervous system. Non-specific triggers (e.g., emotional upset or banal illness) could upset a delicate balance in the cellular immune status perhaps by lowering the number of suppressor cells, and thus may precipitate an exacerbation of demyelination [61].

It remains to be shown in prospective studies whether A3, B7, Dw2 individuals handle infections (or immunizations) early in life differently from other individuals, and whether differences in immune responsiveness are associated with increased risk of MS. A combined field study and laboratory study approach is needed to obtain evidence to support these newer concepts about the cause and pathogenesis of

MS. This amalgamation of field and laboratory work, called experimental epidemiology, succeeds the phase of descriptive epidemiology which has provided us with data on the geographic distribution of MS. If the fruitfulness of experimental epidemiology is as impressive as that of descriptive epidemiology, a solution to the cause and prevention of MS may not be far off.

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Epidemiological and Immunogenetic Studies of Multiple Sclerosis in Japan

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Introduction

Multiple sclerosis (MS) is distributed over the world in a unique geographical pattern, with Japan and other Asian countries belonging to the low risk areas. Systematic prevalence studies, besides those of Japan [9], are lacking. Some reports of the clinical manifestations suggest a modification in Asian MS [2, 3, 16]. Since the environmental factors differ immensely between Asia (Japan) and the West, it is essential to cover these areas in obtaining various epidemiological parameters.

In this paper I would like to review the recent prevalence studies in Japan, as well as immunogenetic studies performed by the Japan MS Research Committee, Ministry of Health and Welfare (Chairman, Y. Kuroiwa) and the Kyushu University consecutive series of probable MS (75 cases).

Prevalence Studies

Population surveys on the prevalence of MS were performed in selected cities of northern, central, and southern Japan. In each area a regional director performed the standardized procedures used in the USA (Kurland) and Japan [9].

The prevalence rates per 100,000 population of recent studies were below 5 (1–4 per 100,000) (Table 1). The average prevalence rate for the northern cities (Asahikawa, Aomori, Hirosaki, and Morioka; 40–44° N) was 3.25; for middle zone cities (Sendai and Kanazawa; 37–38° N), 1.74; and for southern cities (Kagoshima and

Table 1. Prevalence rates of MS in Japan

Cities	Regional director	Latitude ° N	Population	Prev. rate per 100,000
Asahikawa	Takahata	43	323,000	2.5
Aomori	Goto and Matsunaga	41	248,000	3.6
Hirosaki	Goto and Matsunaga	41	159,000	3.8
Sendai	Itahara	38	575,000	1.9
Kanazawa	Yamaguchi	36	400,000	1.5
Kagoshima	Igata	32	417,000	0.7
Naha (Okinawa)	Hika	26	310,000	1.9

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Naha in Okinawa, 32–26° N), 1.38 per 100,000. In a chi-square test ($P = 0.018$) northern cities rate significantly higher than the southern ones. However, even in the northern cities the rates are strikingly lower than those of similar latitudes in the Western countries. This suppression of the MS susceptibility among the Japanese should be stressed.

Stableness of the Prevalence

Our first prevalence studies were performed around 1960 [9]. The rates were 1.6 in Sapporo (43° N), 3.9 in Niigata (38° N, 1960), 1.6 in Fukuoka (33° N, 1959), and 2.4 in Kumamoto (33° N, 1958) [5]. When these first studies were performed, knowledge on MS among physicians in Japan was slight. After 20 years MS is now accepted as one of the important public welfare problems by the Ministry of Health and Welfare in Japan (1972), and knowledge of the disease has increased tremendously. Both medical care in Japan and the socioeconomic status of the Japanese have improved in the last 20 years. Despite such socioeconomic and medical changes, the rate of MS is essentially unchanged. If MS is caused by an infectious agent, the response to such agents must be stable, as no rapid changes have occurred.

The prevalence rate on Okinawa (Naha) appeared to be slightly higher compared with Kagoshima. If this increased prevalence on Okinawa is true, possible factors relating to the large American base located in Okinawa should be researched (Hika, 1978).

Clinical Modification: Some Evidence

Since our first report on clinical aspects of MS in Japan [10] and our reappraisal [3] 15 years later, our concept of MS in Japan has changed. We have findings similar to those in the West as to sex ratio (slight female preponderance), age of onset (peak at 33 years), and multiplicity of CNS lesions. However, in the mode of clinical manifestation, especially as to acuteness or severity, some modification in the Oriental MS was noted.

The spinal lesion in Japanese MS tends to show acute transverse myelitis (ATM). The MS cases of the Kyushu University series showed ATM in 39% (recurrent ATM 24%). This higher rate of ATM was also seen in Hawaiian Oriental MS: ATM appeared in 67% (recurrent 40%), while in Caucasian MS, ATM was found in only 12% (recurrent 4%) [15]. This transverse myelitis might be one expression of the severe spinal lesion.

In cases with severe spinal lesions, painful tonic spasm may frequently occur [2, 14]. The frequency of this paroxysmal phenomenon in our MS series was 25%. This phenomenon was also high in Taiwanese Chinese MS cases [2] and Thailand MS (Vejjajiva, 1970), while it is rare in Caucasian MS [7]. This so-called spinal horizontal spreading phenomenon might be related to the severity and nature of the Oriental MS.

Our MS series showed bilateral visual loss in 56% of the cases. Among 68 bouts of optic nerve of our series, 35% of the cases showed complete blindness, while 56% showed severe visual loss. Combination of severe involvement in both the bilateral optic nerves and spinal cord was seen in 21% in our series.

Inflammatory Reactions of CSF

CSF Cell Count

Our MS series showed pleocytosis of over 50 per mm^3 in 5.5%, and our nationwide series (1084 cases) showed such pleocytosis in 10.4% [5]. The figures of McAlpine and Tourtellotte were as low as 0.4% and 0.3%, respectively. Such an increased cell count is not rare among Oriental MS [2] cases.

Table 2. Phenotypic frequencies (%) of HLA-A, B and C antigens in Japanese MS

		MS $N=43$	Control $N=46$	χ^2	Risk
A	2	40	41	0.15	0.93
	3	2	2	0.45	1.07
	9	72	65	0.22	1.38
	10	16	15	0.02	1.08
	11	16	22	0.85	0.80
AW	30	0	2	3.91	–
	31	0	9	5.80	–
	33	9	7	0.01	1.47
B	5	42	41	0.03	1.02
BW	51	16	17	0.18	0.92
	52	12	17	1.14	0.63
	53	2	2	0.45	1.05
other related	5	12	4	0.78	2.89
B	7	12	7	0.22	1.89
	12	14	7	0.66	2.32
	13	2	4	1.25	0.52
	15	12	11	0.05	1.08
BW	16	14	15	0.22	0.90
broad	BW22	23	20	0.01	1.21
BW	22	10	9	0.06	1.11
	54	14	11	0.01	1.33
	35	16	13	0.01	1.30
B	40	23	44	5.02 [a]	0.39
	40.1	10	15	1.56	0.54
	40.2	14	17	0.54	0.77
BW	48	0	11	7.21 [b]	–
CW	1	16	35	5.00 [a]	0.365
	3	28	35	0.86	0.73
	4	5	2	0.04	2.20
	6	0	4	4.40	–

[a] $P<0.05$ [b] $P<0.01$

CSF Total Protein

Regarding CSF total protein, 3.5% of our MS series and 8.5% of the Japanese nationwide series showed an increase in total protein of over 100 mg/dl, while in McAlpine and Tourtellotte's series only 1.0% and 2.3%, respectively showed a similar increase. Increased CSF protein among Chinese MS cases was also reported [2]. Thus, increased inflammatory reactions are seen more frequently in Oriental MS than the Western cases.

HLA Studies

Since Naito first reported the association of HLA with MS [8], there have been many studies on this problem. Our previous studies on HLA and MS showed no association between A3, B7, and MS [12]. Repeated surveys have shown that there was no association between these A and B loci (Table 2). Dw2 was positive in 13% of MS without significant difference from the control (8%). According to Sasazuki, who collaborated with our series, Dw2 was seen in 11.4% of Japanese MS without significant difference from the control (7.7%). In his studies on Caucasian MS, Dw2 was found in 53%; and in MS among American blacks, 35% [13].

Therefore, our MS showed no association with HLA A3, B7, and Dw2, which might be an important difference between Caucasian MS and that found in Japan.

Serum and CSF Measles Antibodies

Measles antibodies in serum as well as in CSF have been studied by various procedures (Shishido, H. and Kuroiwa, Y.) (Fig. 1). There was no increase of serum measles in HI and radioimmunoassay titers. CSF also showed no elevation in these titers (Fig. 1).

Discussion and Conclusions

The repeated surveys of the prevalence rates of MS in Japan have shown consistently lower rates compared with Western countries. These rates have not increased for the last 20 years, despite various environmental changes. The low susceptibility of the Japanese was reported also among the American Japanese [1]. It is quite natural to consider the racial factors for such low susceptibility, in addition to the exogenous environmental factors.

Oriental MS showed modified features of demyelinating processes in severity. Such increased inflammatory changes were also seen in the CSF reactions.

Such modified features of non-Caucasian MS were reported in the Africans [11], Indians [16], and Chinese [2].

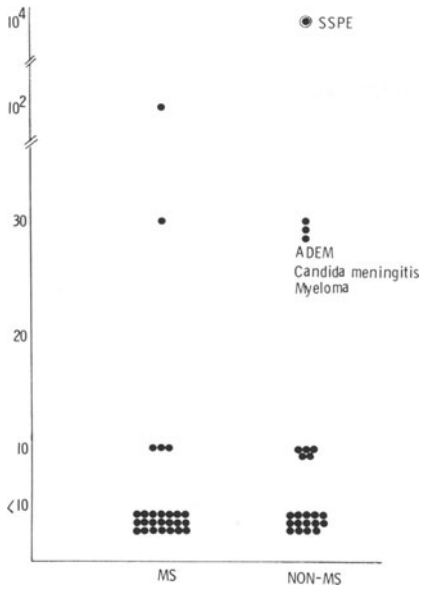

Fig. 1. Measles antibody titers in CSF by radioimmunoassay(Cooperative study with Dr. A. Shishido, Dept. of Virology, National Institute of Health of Japan, Tokyo)

As to the immunogenetic pattern of HLA, there was no association between any antigens to A, B, and D loci. Also, there was no increase of measles antibodies in serum or in CSF in Japanese MS, while in Caucasian MS, serum measles antibodies were often increased. These data show some difference in the immunological patterns of our MS.

The comparative studies between high and low risk areas might show discrimination between the essential causative factors and modifying ones.

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Cerebrospinal Fluid Findings in Japanese Patients with Multiple Sclerosis

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Recent reports [7, 8, 9, 16] have shown that multiple sclerosis (MS) in Japan, except for its low prevalence rate (estimated to be 1–4 per 100,000 population), is essentially the same as MS in Western countries. However, some [7, 8, 9] of these reports have also pointed out that a racial factor might well be involved in epidemiological and clinical statistics (symptoms and signs) in MS in Japan. Therefore, it is of interest to study the fundamental cerebrospinal fluid (CSF) findings in Japanese patients with MS. It is the purpose of this paper to report the CSF findings in Japanese MS patients.

Materials and Methods

MS patients, all Japanese, consisting of 66 consecutive cases, 25 males and 41 females, were studied at the Department of Neurology, Kyushu University Hospital, Fukuoka, Japan, from July 1964 through July 1978. The ages ranged from 16 to 50 (mean 36.1), except for three cases of childhood MS (ages 7, 8, and 14) and two senile MS cases (ages 60 and 65). A diagnosis of MS was based on the diagnostic criteria of the MS Research Committee of Japan [9], and only probable MS cases were included, except for the three childhood and two senile MS cases mentioned above. These were included as MS because of their typical clinical symptoms and signs and the exclusion of other neurological diseases. Possible MS cases were not included in this study, except in the tests using agarose electrophoresis. The definitions of exacerbation and remission at the time of spinal tap in this study are the following: exacerbation – within one month after the onset, or during progressive or active neurological symptoms; remission – more than one month (after the onset) in which the symptoms are in clear regression, or when the disease is stationary (from a clinical point of view) for more than six months, regardless of clinical disability. The CSF was obtained by spinal typ in all cases, and bloody CSF was not included in the present study. The CSF cell count was made in 164 samples taken from 66 patients with MS in exacerbation or remission, using a Fuchs-Rosenthal chamber. Total protein was measured in 172 samples obtained likewise by Kingsbery-Clark's method. Immunoglobulin G (IgG) measurement was made in 49 samples in exacerbation and 19 samples in remission, taken from 35 consecutive cases of MS by means of Mancini's method, using LC-Partigen (Behringwerke, West Germany). Control samples for normal ranges of total CSF protein, IgG, and IgG% (of total protein) were ob-

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tained from 44 surgical (non-neurological) patients at the time of spinal anesthesia. They comprised 21 males and 23 females, ranging in age from 14 to 78 (mean 39.7). Agarose electrophoresis (by arch electrophoresis, using a Pol-E-Film System, Pfizer, USA) for the detection of oligoclonal immunoglobulin band (OB) was performed concomitantly on concentrated CSFs and sera obtained from 22 consecutive patients with probable MS, ten patients with possible MS, three with acute disseminated encephalomyelitis, four with Guillain-Barré syndrome, 16 with infections of the central nervous system (CNS) [including four with subacute sclerosing panencephalitis (SSPE) and two with neurosyphilis], seven with cerebrovascular diseases, 12 with degenerative diseases of the CNS, and 34 with other miscellaneous neurological diseases (including 12 with Vogt-Koyanagi-Harada's disease and nine with polyneuropathies). CSF was concentrated nearly 100 times with a Minicon B15, Amicon, USA.

Measles antibody determinations by hemagglutination inhibition (HI) and radioimmunoassay (RIA) methods were made on 26 CSF and serum samples obtained from 23 consecutive patients with probable MS and 23 CSF and serum samples obtained from 23 patients with neurological diseases other than MS. The samples were coded with a number, and determinations were performed blind in the Department of Virology, National Institute of Health of Japan, Tokyo. The details of the RIA method are described by Sakata et al [15].

Results

The CSF cells, ranging in number from 0 to 164/mm^3, consisted of mononuclear cells (lymphocytes) in all samples except for several patients in an acute exacerbation, such as a case reported previously [3] in which polymorphonuclear cells predominated. The mean of cell count was 12.8/mm^3 in exacerbation, 6.8/mm^3 in remission, and 9.8/mm^3 overall. It was noted, however, that nine (5.5%) out of a total of 164 samples showed a cell count more than 50/mm^3. These samples were taken in exacerbation.

Normal values (mean $\pm$ 2 S) for total CSF protein, IgG, and IgG% were 8.0–40.0 mg/100 ml, 0.7–5.0 mg/100 ml, and 7.0–17.0%, respectively.

Total protein of all MS samples ranged from 10.0 to 122 mg/100 ml with a mean of 38.7 mg/100 ml. Mean (range) of total protein in exacerbation and remission was 42.7 mg/100 ml (12.0 to 122.0 mg/100 ml) and 34.6 mg/100 ml (10.0 to 92.0 mg/100 ml), respectively. Six (3.5%) of 172 samples tested showed a value higher than 100.0 mg/100 ml. Mean (range) of IgG concentration in total, in exacerbation, and in remission was 44.1%, 51.0%, and 28.6%, respectively. Mean (range) of IgG % in 28.8 mg/100 ml) and 4.8 mg/100 ml (1.0 to 18.4 mg/100 ml), respectively. The frequency of CSF IgG values higher than normal range in total, in exacerbation, and in remission was 44.1%, 51.0%, and 28.6%, respectively. Mean (range) of IgG % ion total, in exacerbation, and in remission was 15.9% (6.0% to 33.0%), 17.8% (8.0% to 33.0%) and 13.9% (6.0% to 28.0%), respectively. The frequency of CSF IgG % higher than normal range in total, in exacerbation, and in remission was 41.7%, 52.1%, and 15.8%, respectively.

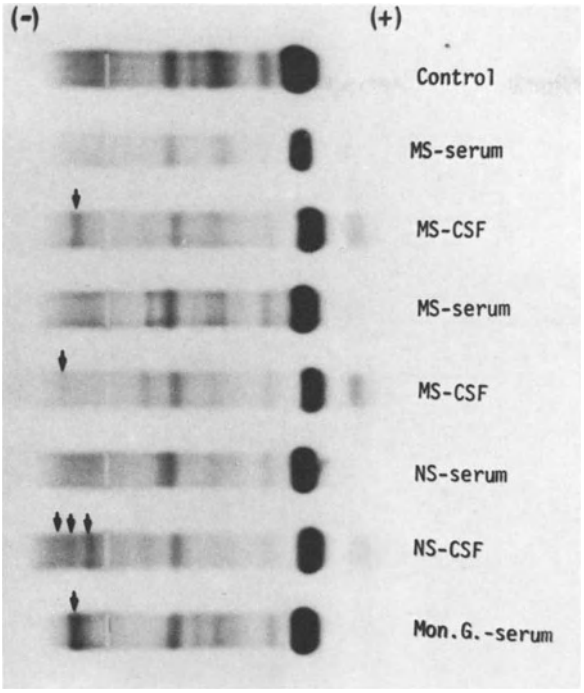

Fig. 1. Agarose electrophoresis *NS:* neurosyphilis, *Mon. G:* monoclonal gammopathy. *Arrows* indicate oligoclonal immunoglobulin bands

CSF-OB was detected in ten (45%) out of 22 patients with probable MS, and four (40%) out of ten patients with possible MS. Serum-OB was not observed in sera of any patients with probable or possible MS. All patients with SSPE and neurosyphilis showed OB in their CSF. OB was observed in serum in three out of four SSPE patients. CSF-OB was also observed in some of other chronic inflammations of the central nervous system such as chronic candida meningoencephalitis [4]. However, it was not detected in the CSF or serum of other diseases, except in the CSF of one case (a 23-year-old woman) of acute status epilepticus of unknown etiology (Fig. 1). In probable MS patients, there was no significant correlation between CSF-OB (+) or (–) and other data such as total protein, IgG, IgG %, cell number, measles antibody titers, disability scale, or the use of steroids (Fig. 2).

Measles antibody HI titers in serum were 256 in one MS patient, 128 in six other MS patients, and 32 or less in the remaining 19 patients with MS. One SSPE patient tested showed a value higher than 1024. Another patient, with subacute encephalitis of unknown cause, showed a value of 512. Measles antibody HI titers in CSF were less than 1 in all MS samples, whereas an SSPE patient showed a value as high as 32. Measles antibody titers in serum by the RIA method were 3×10^4 in three MS patients, 10^4 in three other MS patients, and less than 3×10^3 in the remaining 20 patients with MS. In the non-MS group a value as high as 10^6 was seen in an SSPE patient, and a value of 5×10^5 was recorded in a patient with subacute encephalitis of unknown cause. Other non-MS patients showed values similar to those of MS pa-

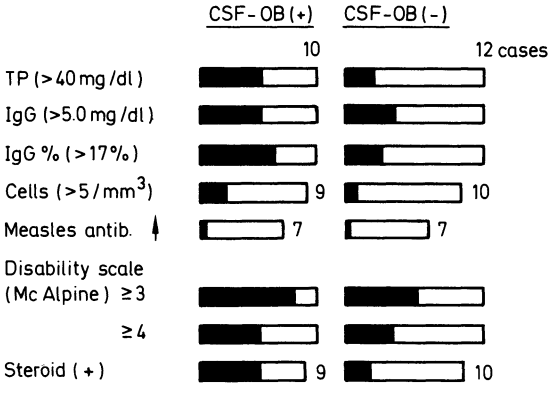

Fig. 2. CSF-Oligo band (+), (−) and other data in Multiple Sclerosis

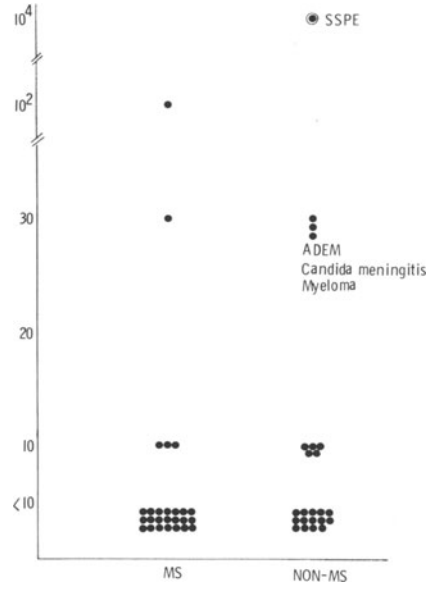

Fig. 3. Measles antibody titers in CSF by radioimmunoassay

tients. Measles antibody titers in CSF by the RIA method were 10^2 in one MS patient, 30 in another MS patient, 10 in three more MS cases, and less than 10 in the remaining 21 patients with MS. On the other hand, a patient with SSPE showed a value of 10^4, and a value of 30 was observed in one patient with acute disseminated encephalomyelitis, in one patient with candida meningoencephalitis [4], and in one patient with paraplegia due to myeloma. Other non-MS patients showed values similar to those of MS patients (Fig. 3). It was concluded that, with the exception of some specific diseases such as SSPE, there was no significant difference in measles antibody titers in serum and CSF by the HI or RIA methods between MS and non-MS patients.

Discussion

The present study shows that the CSF findings in Japanese patients with MS are largely similar to those in MS patients reported from the Western countries [2, 5, 6, 10–13, 17, 18], except for some points which seem to need comment. First, the frequency (5.5% in the present study) of a cell count higher than 50/mm^3 is higher than those in McAlpine et al.'s and Tourtellotte's et al.'s series [13, 17], which counted 0.4% and 0.3%, respectively. Secondly, the frequency (3.5% in the present study) of total protein higher than 100 mg/100 ml is slightly higher than those in the above two series, which counted 1.0% and 2.2%, respectively. Thirdly, the frequency (44.1% in all MS patients tested in the present study) of IgG values higher than the normal range and that (41.7% in all MS patients tested in the present study) of IgG% than the normal range are slightly lower than most of the results reported in the Western MS patients [6, 10, 12]. However, these differences of total CSF protein, IgG concentration, and IgG% between the present study and the results of Western MS studies seem not to be large enough to need a further comment, especially when one considers the different methods used for the quantitation of total protein and IgG, the different numbers of cases tested, and also diagnostic biases which might well be involved in collecting MS cases. However, a fourth point, the lower frequency (45% in the 22 probable MS patients in the present study) of occurrence of CSF-OB in agarose electrophoresis than that reported in Western MS patients [2, 5, 11, 12, 18] seems to need some comment. The frequencies reported in Western MS patients are as follows: Laterre et al. (agar) [11]: 76.3% of 118 probable MS patients and 86.9% of 84 definite MS patients; Link and Müller (agar) [12]: 94% of 64 MS patients; Vandvik and Skrede (agarose) [18]: 91% of 23 MS patients; Johnson et al. (agarose) [5]: eight of eight definite and five of eight presumptive MS patients; Delmotte and Gonsette (agar) [2]: 63% of 262 MS patients. Laterre et al. [11] reported that agar electrophoresis is good enough to detect CSF-OB, but that polyacrylamide electrophoresis is not a suitable method for this purpose. Vandvik and Skrede [18] reported that agarose electrophoresis is superior to cellulose acetate electrophoresis for the detection of CSF-OB. Our method (agarose electrophoresis) is very much similar to that of Johnson et al. [5], who reported a higher frequency of CSF-OB in MS patients, although the number of cases they tested is relatively small. The significances of CSF-OB in MS are considered to be following: (1) one of the diagnostic aids, (2) one of the evidences of IgG synthesis within the CNS, (3) one of the indicators of the antibody activities of IgG against certain antigens such as measles virus, and (4) one of aids in understanding the pathogenesis involved in MS by knowing the CSF-OB-positive neurological diseases other than MS.

Our present study of CSF agarose electrophoresis on various neurological diseases confirmed the reports of Laterre et al. [11]. However, the relatively low frequency of occurrence of CSF-OB in MS patients in the present study shows that the detection of CSF-OB is not as useful in MS diagnosis as has been previously reported by Western authors [2, 5, 11, 12, 18]. Bader et al. [1] also reported a relatively low frequency (40% of 380 MS patients) by agar electrophoresis.

It is of current interest in MS-CSF studies whether CSF-OB in MS has antibody activity against certain antigens, such as measles virus. In some chronic inflammatory diseases of the central nervous system such as SSPE [18], progressive rubella virus

panencephalitis [20], and candida meningoencephalitis [4], CSF-OB has been reported to have antibody activities against the etiological agents (measles virus, rubella virus, and candida, respectively) of the diseases. In MS, however, hard evidence is still lacking that CSF-OB is an antibody against a specific etiological agent [14]. The fact that only a small portion of MS patients in the present study showed high values of meales antibody titers in serum and CSF by both HI and RIA methods, and that there was no significant difference in these titers between MS and non-MS patients, indicates that the measles virus has no specific linkage with CSF-OB in MS, at least in the present series, and might not be involved in the pathogenesis of MS.

Summary

CSF findings of 66 consecutive Japanese patients with probable MS have been reported, and are considered to be largely similar to those in MS patients reported from the Western countries. However, the relatively low frequency (45%) of occurrence of CSF-OB and its unspecificity obtained in the present study indicate that a detection of CSF-OB is not as useful a diagnostic aid as has been previously reported. Raised measles antibody titers in MS serum and CSF, which have been frequently reported from Western countries, were not found in the present study by either the HI or RIA methods.

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HLA Groups in Relation to Epidemiology

G. DEAN [1]

Nearly all diseases result from the interaction of inheritance and environment – the seed and the soil. Those who inherit a certain predisposition are more likely to get certain diseases. A well-known example of this is the way rheumatic fever so frequently runs in families, although there is an environmental factor involved, a streptococcal sore throat. There have been few convincing associations between disease prevalence and the distribution of red blood cell (RBC) markers of the AB0 system, the best known are cancer of the stomach and blood group A and duodenal ulcer and blood group 0. On the other hand, there have been many reports of significant associations between a number of diseases and markers on white blood cells, the human leucocyte antigens or the HLA system [4].

It has long been suspected that multiple sclerosis (MS) results from the interaction of inherited and environmental factors. For instance, it is known to occur more frequently than would be expected by chance alone in two or more members of a familiy, even if they have been brought up apart from each other. The low prevalence of MS among Caucasians who have lived for some generations in South Africa is strong evidence that environmental factors are of great importance [1]. Immigrants to South Africa or Israel from Europe have a high risk of MS unless they immigrate below the age of 15, when their risk is very much less [2]. In contrast, immigrants from areas of the world where MS is uncommon – low prevalence areas – to high prevalence areas, such as the United Kingdom, keep their low risk of developing MS [3].

That there is an association between MS and HLA-A3, HLA-B7, and HLA-Dw2 is now beyond question, although this association does not approach the closeness of the association between ankylosing spondylitis and HLA-B27. There is an even stronger association between MS and the more recently described B-lymphocyte marker and further associations may yet be revealed [8].

HLA-A3, HLA-B7, and HLA-Dw2 and particularly the B lymphocyte alloantigen, are most common in areas of the world where MS is common, for instance, in Northern Europe and particularly in Scandinavia and Finland.

Now let us look at the distribution of MS and what we know about its possible relationship to the HLA markers. The highest prevalence of MS which has yet been described was found by Poskanzer et al. [5] in the 17,000 population of the Orkney Islands, among whom there were, in 1974, 54 MS patients, giving a prevalence of 258 per 100,000 for probable MS. Not far behind are the Shetland Islands, with a population of 18,000 and 34 MS patients in 1974, giving a prevalence for probable MS of 152 per 100,000 [5].

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Shepherd and Downie [6] in a recent large scale study in a population of 400,000 in the northeast of Scotland found a high MS prevalence of 127 MS patients per 100,000 population. This is a higher prevalence than in any other surveyed area with a comparable population. The disease was not spread homogenously, and in one district one in 400 people had MS. There is a strong Viking strain in the Orkneys and Shetlands and in the northeast of Scotland.

Sutherland [7], more than 20 years ago, pointed out that MS was much less common in the west of Scotland than in the east of Scotland. A recent intensive survey of MS has been undertaken in the Outer Hebrides, which is still largely Celtic speaking. The islands have a population of 29,000. This study is not yet complete but at present there are only 19 patients with probable MS, a prevalence rate of 66 per 100,000, and this is not likely to be significantly higher. The prevalence of MS in the Western Isles, or the Outer Hebrides, appears to be approximately one-quarter of that found in the Orkneys and less than one-half of the reported prevalence in the large population studied in the northeast of Scotland. Dr. Allan Downie, an Aberdeen neurologist, took part in both the study in the northeast of Scotland and the present study in the Outer Hebrides.

There are large areas of the world where the HLA groups have not been adequately studied. Information about the HLA and other leukocyte groupings in these areas should help us to understand to what extent genetic factors are responsible for the low MS prevalence among people of Asian and African stock, for instance in Japan and China. One area of particular interest is the southern Mediterranean. Among immigrants from Italy and Cyprus to England, the prevalence of MS is nearly as high as among the United Kingdom born but among immigrants from Malta it is very low. Later today we will report to you the preliminary results of a study on the prevalence of MS in Enna city, in central Sicily, and in the islands of Malta.

Apparently, there is a highly significant association between MS and the presence of HLA antigens A3 and B7, the mixed-lymphocyte culture determinant Dw2, and certain B-lymphocyte alloantigens. The worldwide occurrence of HLA-A3 and B7 in general populations follows a geographical pattern remarkably similar to the distribution of MS. For instance, B7 has been found in 34.4% of a control population in northeast Scotland, a prevalence greater than that found in virtually any other control series [6]. This may partly explain the high prevalence of MS in northeast Scotland but the essential additional environmental factor remains obscure.

Conclusion

Further work on the HLA antigens and other antigens, both in families where MS occurs, and in population groups, can add greatly to our understanding of the predisposing factors that are responsible for MS. However, whatever predisposing factors there are, the prospect of preventing MS depends on the strong evidence available that there is also an environmental factor involved.

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Clustering and Epidemic Occurrence of MS

J. F. KURTZKE [1]

As determined by the nearly 200 prevalence studies currently available, MS is distributed worldwide into three bands or zones: high, medium, and low [6]. Migration studies among these bands have recently come into prominence. In the United States, for example, we have evidence that changing risk areas before onset changes the likelihood of MS (Table 1). Moves from the high-frequency north to the medium-frequency south decrease the risk, and moves from south to north increase the risk [9]. These conclusions arise from case-control comparisons of US veterans with MS, matched to their unaffected peers from the military. When we seek population-based data in migrants, though, the picture becomes very complex. For example, consider the risk of MS by age 30 for two groups in Denmark, one born there, and another arriving there at age 25 (Table 2). For the former the risk is nine in 10,000. For the latter, at this same age, this ratio is only four in 10,000, or less than half, *even though they are drawn from the same high-risk population* [7].

Within the high-frequency band of Europe, there is evidence for spatial clustering of MS. Utilizing all the nationwide surveys available, highly significant variations from a homogeneous distribution were found, of such a nature that in each land but Sweden the high frequency areas were contiguous, forming single clusters or foci. There was about a sixfold difference in the MS prevalence rates within each land, so that the foci seemed to be of biological as well as statistical significance [2, 4]. Essentially the same patterning was seen when the smallest geographic units available were tested as was found with the large counties [3], and the variations were not related to the distribution of medical facilities in any land [1]. The countries demonstrating such clustering were Norway, Sweden, Denmark, Switzerland, Finland, Northern Scotland, and possibly the Netherlands. Only for the small state of Northern Ireland was a homogeneous distribution found.

In Scandinavia, the intranational distributions were such that the high-frequency areas were not only contiguous within each land, forming one focus in Norway, Denmark and Finland, and two in Sweden, but they were also contiguous across the borders, so that the disorder could be described as occurring in a single Fennoscandian focus. This focus extends from the waist to the southeastern plains of Norway, across the southern lake region of Sweden, and via Åland in the Bay of Bothnia to the southwestern part of Finland, from where it returns to the northern high focus of Sweden in the region of Umeå [5].

Not only are there clusters or foci in these lands, but for the three countries for which such data exist, the clusters are stable over time. The distributions by county

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Table 1. Migration in MS: Case/control ratios for US white male veterans of WW II and/or Korean Conflict by tier of residence at birth and at entry into service [a]

Tier of birth	Tier at entry into service			
	North	Middle	South	Total
	(case/control ratios)			
North	1.38	1.20	0.85	1.35
Middle	1.34	0.97	0.75	0.98
South	0.75	0.68	0.54	0.55
Total	1.36	0.97	0.57	1.00
	(case/control)			
North	1762/1278	119/99	39/46	1920/1423
Middle	149/111	1671/1725	78/104	1898/1940
South	21/28	49/72	501/930	571/1030
Total	1932/1417	1839/1896	618/1080	4389/4393

[a] Kurtzke et al. [9]

Table 2. Period cumulative risk of MS in Denmark. Number of new cases expected per 100,000 population of given age at entry, both sexes combined [a]

Age at entry	Period					
	5 yr.	10 yr.	15 yr.	20 yr.	25 yr.	30 yr.
0	0	1.9	3.7	19.7	51.8	89.5–
5	1.9	3.9	20.6	54.4	93.9	128.0
10	1.9	18.7	52.6	92.2	126.4	159.3
15	16.8	50.8	90.5–	124.8	157.8	180.7
20	34.1	74.0	108.5	141.6	164.6	180.6
25	40.2	74.9	108.3	131.4	147.6	156.9
30	35.0	68.7	92.0	108.3	117.7	120.5–
35	34.0	57.5	74.0	83.5–	86.3	86.3
40	23.8	40.5	50.0	52.9	52.9	52.9
45	16.9	26.6	29.5	29.5	29.5	29.5
50	10.0	13.0	13.0	13.0	13.0	13.0
55	3.1	3.1	3.1	3.1	3.1	3.1
60	0	0	0	0	0	0

[a] Kurtzke [7]

were highly correlated within Norway, Denmark and Switzerland, with coefficients of 0.8, in studies covering different generations of patients – and doctors [5]. In Figure 1 the respective MS prevalence rates for each county are denoted by their percentage of the national (mean) rate, and this percentage is plotted as the intersect of its value on the y axis for the old series and the x axis for the new. Denmark is indicated by the solid circles, Switzerland by the X's, and Norway by the open boxes. It appears also that the slope of the regression line is about the same for each country.

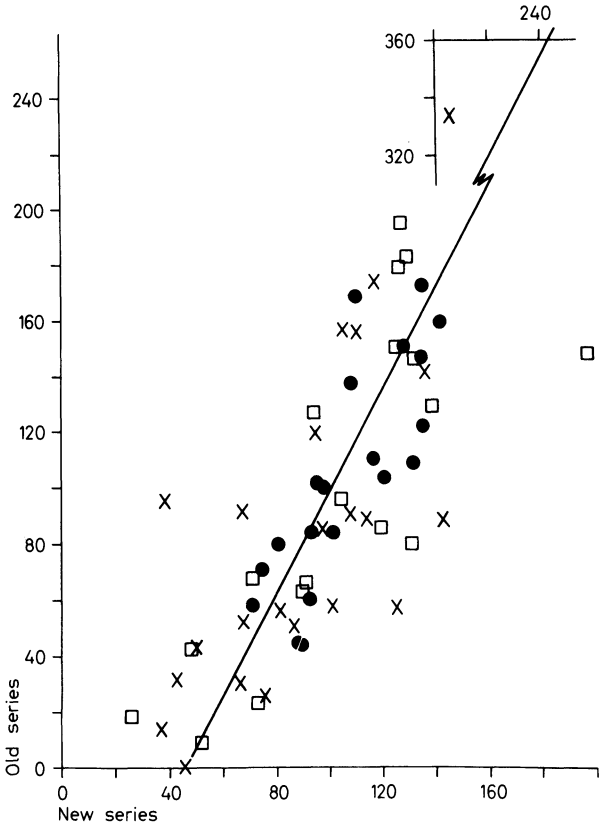

Fig. 1. Correlation of distributions of MS by county between old series and new series of prevalence surveys in three countries, each covering different generations of MS patients: Denmark (*solid circles*), Switzerland (*X*), Norway (*open boxes*). Each county rate is expressed as its percentage of the respective national rates, and the percentages for old vs. new plotted at their intersects. From Kurtzke [5]

It further appears from this slope that there has been, to some degree, diffusion of MS within these lands. Were there an equivalent degree of variation at each time, then the regression line would go through the origin at a 45° angle.

This clustering, plus the migration studies, are taken to mean that MS is a disease intrinsically dependent on *place*, the environment, and therefore MS has been defined as an acquired, exogenous (environmental) disease. I also believe that MS is predominantly the white man's burden, and that the disorder probably originated in Western Europe and has been spread elsewhere as part of the colonization of our times [6].

Aside from the evidence of diffusion just noted, there has been very little evidence that MS has changed in frequency in any given locale over time. Our information on this point, though, is quite limited, and assertions as to its stability really rest on only two studies: one for 60 years in Rochester, Minnesota [11], and one for 25 years in Northern Ireland [10].

However, we believe that we have a situation unique in the history of MS, and that it is what seems to have happened on the Faroe Islands. Dr. Hyllested and I

Table 3. MS in native resident Faroese. Total series by sex and calendar years of birth, clinical onset, and death [a]

Case no.	Year of			
	Sex	Birth	Onset	Death
1	F	1940	1959	–
2	F	1901	1945	1966
3	F	1910	1952	1963
4	F	1935	1953	–
6	M	1915	1947	1965
7	M	1938	1957	1966
8	M	1930	1959	–
9	M	1913	1943	1971
10	F	1906	1945	–
12	M	1938	1955	–
13	M	1929	1944	–
14	M	1938	1960	1968
15	F	1926	1945	–
16	F	1928	1947	–
18	M	1921	1958	–
19	M	1920	1944	–
20	M	1923	1948	–
21	F	1917	1949	–
23	F	1950	1970	–
29	F	1940	1954	1976
38	M	1895	1944	1945
41	F	1920	1946	1957
42	M	1929	1949	1970
43	F	1937	1956	–
44	M	1922	1946	1970

[a] Kurtzke and Hyllested [8]

Table 4. MS in native resident Faroese, accepted cases. Distribution by sex and age at onset [a]

Age	Total	Male	Female
10 – 14	1	–	1
15 – 19	8	3	5
20 – 24	5	4	1
25 – 29	3	2	1
30 – 34	3	2	1
35 – 39	2	1	1
40 – 44	2	–	2
45 – 59	1	1	–
Total	25	13	12
Mean	26.2	26.4	25.9

[a] Kurtzke and Hyllested [8]

Table 5. Some characteristics of the MS series, accepted cases as of various prevalence days [a]

Characteristic	Prevalence day [b]				
	1939	1950	1961	1972	1977
Number of cases	0	13	22	15	14
(number of males)	–	(7)	(12)	(6)	(6)
Prev. rate per 100,000	–	40.9	63.6	38.3	33.7
Mean Age @ P. D.	–	30.85 –	35.73	42.67	48.43
Mean Age @ Onset	–	26.85 –	25.32	23.07	23.71
Mean Duration (yrs.)	–	4.00	10.41	19.60	24.72
Time of Onset:					
Mean	–	7/46	11/50	11/52	9/52
Median	–	1946	1949	1951	1951

[a] Kurtzke and Hyllested [8] [b] July 1

Table 6. Calendar year of onset of MS among native resident Faroese [a]

Year	Patient number	Year	Patient number
1943	09	1953	04
1944	13, 19, 38	1954	29
1945	02, 10, 15	1955	12
1946	41, 44	1956	43
1947	06, 16	1957	07
1948	20	1958	18
1949	21, 42	1959	01, 08
1950	–	1960	14
1951	–	1961 – 69	–
1952	03	1970	23

[a] Kurtzke and Hyllested [8]

have been working on this problem since 1972, and the formal presentation of our work to date will take place later this month at the American Neurological Association meeting in Washington [8]. But it would not appear inappropriate to give you now some advance information as to our findings.

The Faroes are a group of small islands lying in the North Atlantic between Norway and Iceland, at 62° north latitude and 7° west longitude. Until 1948 they were a standard county or *amt* of Denmark, and they are still a semiindependent member of the Kingdom of Denmark. Their population now is some 42,000.

We have been able to identify 78 patients on the Faroes up to July 1977, in whom MS was either diagnosed or suspected. After our independent reviews of all available medical records, in 1974–77 we examined jointly all but two of the first 59 patients, or interviewed their relatives if they were dead by the time of our visits. For the other 19 cases, alternative diagnoses were so well established in the records that we did not believe it was necessary to examine them.

Table 3 is a list of 25 patients who comprise the entirety of the native-born resident Faroese who have MS. All but one met all diagnostic criteria of the Schuma-

cher Committee, and that one had a recurrent but varied myelopathy with three remissions.

As may be seen, their ages at onset are typical of any series of MS, with a mean of 26 years (Table 4). The sex ratio was equal. Clinical characteristics were also in accord with expectations.

However, when we look at these same cases over time, then major discrepancies appear. The living cases provided a prevalence rate of 41 per 100,000 population in 1950; of 64 in 1961; 38 in 1972; and 34 in 1977. There were *no* resident Faroese with the disease in 1939 (Table 5).

Duration of illness can be defined as the difference between age at onset and at prevalence day. At the several prevalence days, there were mean durations of 4 years in 1950; 10 years in 1961; 20 years in 1972; and 25 years in 1977.

If we allocate the cases by calendar years of onset (Table 6), we see a log-normal distribution with all cases but one beginning between 1943 and 1960. It is these 24 cases which then meet all criteria for a point-source epidemic. As such, this may be the result of a single cause introduced into the Faroes before 1943.

So far, we have been able to find only one unusual event that had occurred in the Faroes in this century, and that was their occupation by British forces in large numbers for five years from April 1940. Residences of all but three patients during the war were locations where troops were stationed, and even these three, who were fishermen, had extensive contact with the British in the Faroese ports.

We therefore then have good evidence (*post hoc ergo propter hoc?*) that this epidemic of MS was introduced into the Faroes by the British troops, or their baggage, during the war. If this is correct, then on the Faroes, at least, MS is a transmitted disease, most likely infectious.

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Discussion

G. F. W. Arnason: I have a question for Dr. Dean. I went to Enna once and I must say it struck me as the most atypical town in Sicily. It's like going over the surface of the moon to get there and it must be the single most isolated community in Sicily. I also somehow have in the back of my mind, without the facts, that the people in Enna are perhaps of different extraction, or they type differently in certain ways from the bulk of the Sicilians. I wonder if you have any information in that regard.

G. Dean: It is undoubtedly true that Enna is much higher than the other areas of Sicily. Chance could be a factor, but it is interesting that in the neighboring town of only 5000 people there are three definite MS patients. Because it is a small population, we feel that it is necessary to study two other towns on the coast, and we are doing so at present. These studies in Agrigento and in Monreale are not complete, but clearly the findings are going to be closer to the Enna findings than to anything which has previously been reported from Sicily or Italy for that matter.

The findings we have in Sicily, the findings in Cyprus, and the findings in Spain do suggest that the Mediterranean area may have a somewhat lower MS rate than Northern Europe. But it does appear very likely that the differences are nothing like so great as was formerly thought and that the previous very low findings which were reported from Italy largely came from the fact that big populations were studied, and it is extremely difficult to get an accurate prevalence rate in a big population. Another advantage regarding Enna and why it was chosen is that it is diplomatically halfway between the university cities, so nobody took exception to it. There is a good hospital there and a competent neurologist who speaks good English. So there were a number of logistic reasons for the choice. But clearly the study needs confirmation in two other towns and this is being done.

H. J. Bauer: I have two questions. You mention, Dr. Alter, that something has changed, so that in the native-born Israeli of European and Afro-Asian extraction the figures approach each other. What is the frequency of measles in the home areas of the Afro-Asian population before they came to Israel, and how about vaccinations? Probably they were not vaccinated against measles or any other infection previous to coming to Israel, where they were then confronted with an extremely well-functioning health system. Do you see any causal relationship?

M. Alter: What has changed for the native-born Afro-Asian is the frequency of MS. This, in the native-born of European parentage, you recall, remained high. So what has changed for the Afro-Asian offspring? Certainly the health standards are much better in Israel than in the Afro-Asian countries, so that has changed. We would like to suggest that it is not that they don't get the childhood diseases in Israel, but that they are now getting them later than they would have gotten them in their native countries. This is from the best evidence that we have.

areas includes a greater number of well-educated individuals, as judged by the proportion who went on to college and higher education, than was true of the immigrant group. So they have now shifted not only in their disease pattern, but also in their educational achievement. It is not so much where they live, but how they live,

The second thing that we have shown as having changed is the educational achievement of these individuals. MS in the group with parents from low frequency that appears to determine the risk of MS. Those are the factors we consider important.

I cannot tell you about vaccinations per se, but we have the impression, as you did, that they were not vaccinated with any frequency in their home countries, whereas they were uniformly vaccinated in Israel.

H. J. Bauer: Just a brief remark regarding John Kurtzkes presentation in relation to the Fenno-Scandinavian focus. When I was in Moscow in 1973, the physicians at the Neurological Institute told me that there was an area around Lake Ladoga which had a high frequency of MS, and that it might be an extension of the Fenno-Scandinavian focus or something equivalent. Could you comment on this, Dr. Kurtzke?

J. F. Kurtzke: Yes, it would be interesting to get the entirety of the USSR, because this is an area where we get an east-west gradient, which is against all our orthodoxy.

H. Zander: I have a question to the Netherlanders in the auditorium. I think that there is a very interesting group of immigrants from a low-risk area to a high-risk area. Regarding the Polynesians who immigrated to the Netherlands 25 or 30 years ago when this country gave up its colonies – has this population ever been investigated?

O. R. Hommes: The population has been investigated. But I'm afraid the number is fairly small and I remember that there are just one or two patients. I don't know the exact number, but it's very small.

J. F. Kurztke: This was Dassel's work. He had three patients who were in this immigrant group. But I don't know whether they were whites or Polynesians, as I never got an answer from him. The problem will not be studied any more, since a follow-up was impossible. Now those three, as I recall, all immigrated in adolescence and came down with their disease about 10–15 years after moving. We also had a group of Vietnamese that we studied in France, and three of them came down with MS, 15 years plus or minus after they emigrated at an average age of about 9 years, and the rates as calculated then were the same as they would have been for native French, but the confidence intervals are so wide that it is difficult to say that they achieved the same level as in France. However, it is clear that they did have a higher rate than they would have had had they stayed at home, even with only three cases.

G. Agnarsdottir: I just wanted to make a comment. It was alluded to briefly earlier on, that the importation of canine distemper virus might be related to the outbreak of MS in the Faroe Islands. The prevalence of MS in Iceland has been recognized to be similar to that of other Northern Euopean countries. However, canine distemper is not endemic in Iceland and dogs are not immunized against it. There have been two isolated outbreaks during recent years, the latter one in 1950, and there has been no recognized clustering of MS after this.

E. J. Field: In our work during the last 2 years, we have concentrated very largely upon whole families, and in the first degree relatives (near relatives), we were appalled to find that one in 39 people who are apparently normal gave a full MS result. I was so appalled at this that I rang up Prof. Roberts, who has the Orkney and Shetland data, and he told me that the great disparity between them is obviously a technical error somewhere. But in the Orkneys, one in 50 of near relatives of the same degree are infected with MS.

The second point is that when we were in Italy we were told that despite a common belief in England that MS is rare in Italy, it is in fact very common in South Italy and in Sicily. But they are sent up to Milan for diagnosis. It so happened that when we went back to England we had an appointment with a Sicilian family, a whole family coming over to be tested.

Thirdly, MS is very common among Arabs, contrary to what is believed. For example we have many Arab cases, and when I was in Jerusalem recently Yaffa Leibovitz pulled out a whole drawer showing me Arab cases of MS. A young Arab medical student brought his sister over together with the rest of the family and he said if I cared to come to Beirut – and I said I would when things were a little quieter – he would show me a whole lot in the hospital.

Finally, a question to the assembled galaxy of epidemiologists: Can they tell me what is the sibling order of occurrence in MS?

G. F. W. Arnason: If they are going to tell you, they will do so after the next discussion.

Epidemiological and Serological Findings in Multiple Sclerosis, Results of a 12-Year Study in Southern Lower Saxony

G. RITTER, J. WIKSTRÖM, S. POSER, W. FIRNHABER, and H. BAUER [1]

In Southern Lower Saxony, in an area of 260,000 inhabitants, all multiple sclerosis (MS) patients have been repeatedly examined according to a standardized documentation system and registered for continuous prospective study. If their condition permits, these patients can visit an MS clinic established in the Neurology Department of the University of Göttingen, or they can call on a social worker, who will visit them in their homes and initiate medical, socioeconomic, or psychological care measures as necessary. This epidemiological observation area is proving its value in two ways: by achieving a better organization and practice of long-term care, and by making a thoroughly studied population of MS patients accessible for scientific investigations.

The prevalence rate recorded for the epidemiological area was 76/100,000 on the prevalence day (December 31, 1977, possible cases included) and 63/100,000 for only definite and probable cases. The apparent increase since 1969 (51/100,000) is most likely a result of better case recording and longer life expectancy.

The incidence rate was 3/100,000 from 1961 to 1975 (Fig. 1), and the mortality rate was 2/100,000. No clustering of cases could be registered.

The mean age at onset for the group of 195 patients living in the area on the prevalence day was 31.7 years; the mean duration of the disease was 15.4 years. The female : male ratio was 1.7 : 1. The course was remitting in 50%, remitting and progressive in 39%, and progressive from onset in 11% [7]. Fifty-seven percent of the patients were only slightly disabled (disability grade 0–3 according to Kurtzke [4]).

There was no difference in prevalence between patients born in the area and patients coming from the DDR, Czechoslovakia, and from regions of Poland formerly belonging to Germany. Among foreigners working in Germany ("guest workers" mainly from Turkey, Greece, and other Mediterranian countries) no cases of MS were recorded, although statistically about four cases could have been expected.

A standardized questionnaire was used to obtain data on the social and medical situation of the 195 patients living in the area. Analysis of the 178 (= 91%) answers revealed that medical needs were met satisfactorily. The financial situation and living conditions were similar to those of the normal population. About 50% of the patients who had been employed before onset of the disease were no longer working. This percentage appeared to be rather high, since 57% of all patients were only slightly disabled (disability grade 0–3). This indicates the need for better vocational guidance and rehabilitation facilities to retrain MS patients [5].

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The hypothesis that dogs or other house pets are carriers of an MS virus [2] was tested in the epidemiological area. The results did not confirm an association between exposure and developing MS [1].

Serological studies performed on patients from the epidemiological area revealed no difference in the hemagglutination antibody titers for 6/94 parainfluenza and measles viruses. Cytotoxic antibodies against measles virus were slightly elevated in MS patients compared with healthy controls [3, 6].

HLA typing of all patients in the area and a battery of laboratory tests for the assay of cellular immune reactions have just been completed; a study on the influenza vaccination of MS patients is under way.

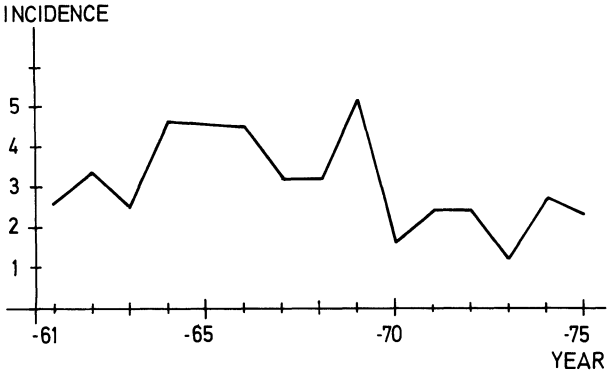

Fig. 1. The annual incidence of MS in Göttingen County per 100,000 inhabitants from 1961 to 1975

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Studies on the Course of Multiple Sclerosis

O. ANDERSEN, L. BERGMANN, and T. BROMAN [1]

Our studies are focused on the case material of patients who developed symptoms and signs of possible or probable multiple sclerosis (MS) during the years 1950–1964 and lived at this time in the city of Göteborg. After a follow-up period to 1977, i.e., 13–27 years, the diagnostic problems have often cleared up and become simplified. A more or less qualified analysis of the cerebrospinal fluid (CSF) has also been performed in the majority of the cases. The material was defined as an incidence material and contains 312 patients. This implies an overall incidence figure of 5.3 per 100,000 inhabitants per year.

All the patients have been seen by us personally, 75% from the third year of the disease. Only 7 of the 312 patients (2–3%) had such deficiences in the documentation of their symptoms that reconstruction of the course of the disease was seriously defective.

We have classified the cases according to diagnostic probability into four categories. Of these, 74% are of highest diagnostic probability (MS "beyond doubt"). Most of the rest we have classified as probable MS, but 9% represent borderline cases who usually have had only a single attack of CNS involvement. Although they have the symptoms and signs commonly seen in MS and an onset at a typical age for MS they lack further diagnostic support for or against the diagnosis, e.g., retrobulbar optic neuritis or reversible parahypesthesia.

We are at present dealing with this extensive mass of data, which implies an attempt to ascertain as many facts as possible about the various events during the course of the disease in each patient. This procedure involves a critical evaluation of all available data, which, however, is made easier as we know personally most of the patients (and often their families as well), and because we also know most of the other doctors who may have contributed to some of the earlier clinical documentations.

As we have just started with our calculations, I can only give a few impressions of some of our results. The description of the course of the disease may naturally be made from various points of view. We have as yet tried to analyze only such features as the frequency of bouts, the development of progress, the symptom spectrum at onset and at various intervals, possible seasonal variations, and a few handicap problems. As it is apparent that many of these phenomena vary with sex and age at onset, we have found it appropriate to divide our material into subgroups. Our material shows the usually reported distribution of age at onset, and I shall dispense with a demonstration of this, but I think that some circumstances concerning the sex ratio may be worth mentioning. We think that the well-known discrepancies between

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different materials may be explained largely by the worse prognosis for the male sex, and by the rather frequent deaths from causes other than MS. In mortality studies several particularly benign forms of MS may be overlooked when the patients become ill and die from other serious diseases, which was the case in 40% of all deaths in our material. In prevalence studies the shorter average life span for male patients will increase the relative number of female patients. In addition, we must be aware of the fact that several cases will not be suspected as MS until many years after the onset. In our studies the female/male sex ratio was 2.0 in the prevalence

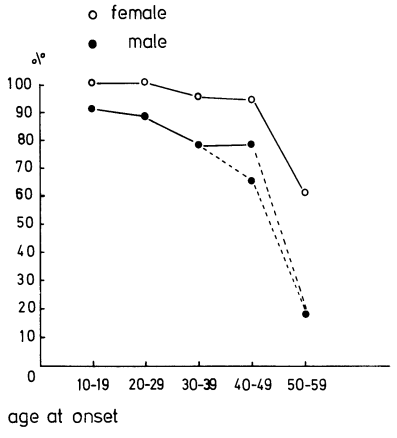

Fig. 1. Frequency of bouts at onset in various age groups. The results are calculated for the total material (312 cases). If calculations are made only for cases with highest diagnostic probability (232 cases) the results are approximately the same with exception only for *dotted line*

material from 1965, while it is 1.5–1.6 in the present incidence material and only 0.6–0.7 among the deaths during the first decades.

Most patients start with a bout at onset. The frequency of bout onset varies with sex and age as demonstrated by Figure 1. The frequency is highest among young female patients and decreases with higher age for both sexes. This means that the opposite is valid for the cases with a progressive onset. The occurence of bouts during a 10-year period subsequent to the onset was calculated in two ways. A. The relative frequency of cases displaying one or more bouts (of the material under observation that year) was ascertained for each year. After the first year it decreased to the

Table 1. Mean bout frequency during the first 10 years of the disease[a]

	Bout frequency		
Age	10 – 29	30 – 39	40 – 59
Female	4.1 [b] – 3.6 [c]	2.9 [b] – 2.7 [c]	2.3 [b] – 2.1 [c]
Male	3.0 [b] – 2.8 [c]	2.7 [b] – 2.7 [c]	2.4 [b] – 2.4 [c]

[a] All bouts are added and the mean bout frequency is calculated for all cases with bouts occurring during one decade *after* the onset bout

[b] Cases with highest diagnostic probability

[c] Cases with all categories of diagnostic probability

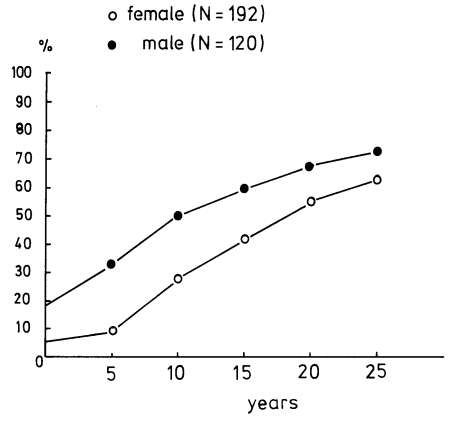

Fig. 2. Development of progress with time. The poorer prognosis for the male patients seems to correspond to an earlier development of progress. (Earlier progress is also seen in higher age groups)

20–30% level, successively falling to about 10–20% in cases with onset in young age. Lower frequency was found in higher ages. Generally, males had a lower bout frequency than females. B. The mean bout frequency for the whole 10-year period after MS onset is similarly influenced by age and sex, as shown in Table 1.

Figure 2 demonstrates the development of a progressive course in the whole material (all age groups added). It shows that about 30% developed a progressive course after about 5 years among males and in about 10 years among females. A 50% frequency is reached in about 10 years among males and in about 15–20 years among females. From a practical point of view, it may be asked how long a time must elapse after onset and the first symptoms before new symptoms appear. This is illustrated by Figure 3, based upon the total material. The diagram shows that the appearence of new symptoms (whether due to new bouts or progress) seems to follow a rather straight line on a semilogarithmic scale, with new symptoms in 50% within 2 years, about 75% within 5 years, about 85% within 10 years, and about 90% within 20 years (provided that the 9% of lowest diagnostic probability are included).

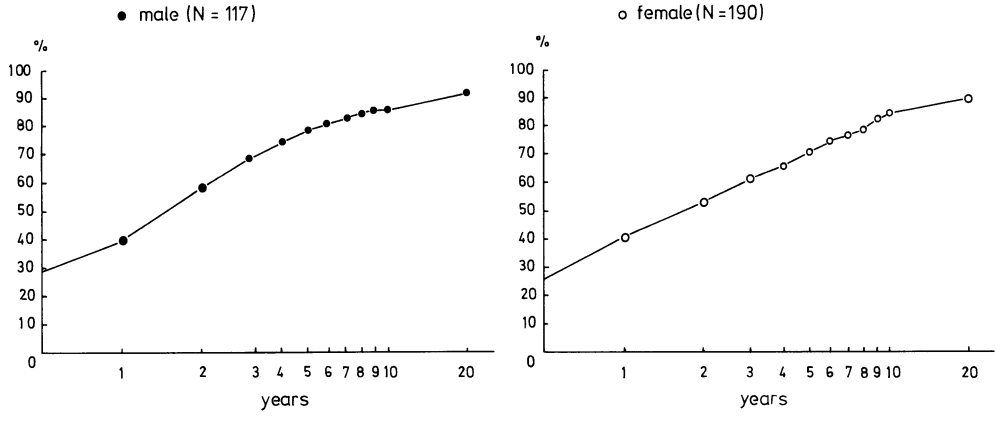

Fig. 3. Time interval between onset and appearance of new symptoms. Cumulative values (a few cases with uncertain documentation are excluded).

Table 2. Symptom spectrum in bouts at onset of the disease (analyzed in cases with well-defined bout onset)

	♀		♂	
	Mono-sympto-matic	Mono + polysymp-tomatic	Mono-sympto-matic	Mono + polysymp-tomatic
Sensory deficit	42%	52%	48%	52%
Optic lesion	32%	28%	33%	25%
Vestib. sympt.	11%	17%	5%	14%
Afferent total	85%	(97%)	86%	(91%)
Motor deficit	9%	25%	11%	34%
Cerebellar ataxia				
Sphinct. disturb.				
Mental lesion				
Oculomotor paresis	6%	11%	3%	11%
	100%		100%	

Table 3. Some characteristics concerning cases of progressive onset.

Onset with bout could not be stated in 15%.
A probable progressive onset was accepted in 13%.
Female/male sex ratio for these patients was 1/2.
The symptoms at onset (40 patients) was:

a) *Motor deficit in the lower extremities in* > 80%
(starting a para/tetra syndrome in 4/5, a hemisyndrome in 1/5 of the cases).
b) *Micturition disturbances*[a] (including impotence) in < 5%.
c) *Cerebellar ataxia*[a] in 10 – 15% of the cases.

[a] Later developing in combination with para/tetraparesis

The spectrum of symptoms at onset is of particular interest. It is illustrated for the cases with well documentated bout onset in Table 2. The survey of various symptoms shows that 40%–50% start with bouts entailing sensory symptoms, and 25%–30% start with bouts entailing optic nerve lesions. These figures are valid for the whole material, but they change somewhat with age. Bouts with optic lesions are more common in younger age groups and among females, who actually start with optic bout lesions in about 40%. An opposite trend is seen with the sensory symptoms. It is also remarkable that onset bouts with symptoms from afferent pathways amount to about 85% of all monosymptomatic bouts. The last-mentioned fact is striking in contrast with the spectrum of symptoms in cases with a progressive onset, which is illustrated by Table 3. In these cases symptoms from the motor tracts dominate in quite an outstanding way. The initial symptoms never consisted of isolated sensory loss or cranial nerve involvement. The mortality and the causes of death are shown in Table 4.

Table 4. Mortality (to the end of the follow-up in 1977)

Age groups (at onset)	All categories		Only the cases with high diagnostic MS probability	
	Female (%)	Male (%)	Female (%)	Male (%)
10 – 19	10	9	13	11
20 – 29	5	17	7	19
30 – 39	14	20	14	25
40 – 49	15	30	17	36
50 – 59	15	67	11	67

Causes of death:	Female		Male	
	MS	11	MS	9
	MS + suicide	3	MS + cardiosclerosis	1
	MS + cardiac valvular disease	1	MS + pulm. embol.	2
			MS + Asthma bronch.	1
	Cancer	3	Cancer	5
	Cardiac infarction	1	Sarcoma	1
	Subarachn haemorrhage	1	Myeloic leukemia	2
	Asthma bronch.	1	Cardiac infarction	2
			Arterial aneurysms (aorta, a. renalis)	2
			Thrombosis a. vert.	1
			Uraemia (phenacetin-nephropathia)	1

The frequency of partial or total disability during 25 years' duration of the disease shows that in the younger age groups (onset at age 10–29) about 50% have maintained their working capacity after 20–25 years.

MS in Switzerland – Canton Wallis

W. BÄRTSCHI-ROCHAIX [1]

In carrying out epidemiological studies it is tempting to study not only large populations, but also smaller and better structured communities.

A study by F. Georgi and P. Hall in 1960 proved the prevalence of marked variation in Switzerland, e.g., 105 in Basel compared with only 19 in the Wallis. These figures led me to investigate this canton thoroughly for various epidemiological criteria. Two initial questions must be answered: is the low prevalence due to an insufficient medical registration, and are there any significant regional differences of prevalence or even cluster formations?

The canton of Wallis is a geographically isolated area with 206,000 inhabitants. The horizontal and a vertical bipartition of this area is important.

Vertically, the area is divided into the plain of the Rhône with semiurban or urban settlements and industrial centers, and into the upper regions, where there are villages with agricultural structure and tourism.

Horizontally, the area is divided into the Upper Wallis (roughly 61,000 inhabitants) with an Alemannic, German-speaking culture and a larger proportion of farming settlements and some well-developed tourist centers, and into the Lower Wallis with a French-speaking culture.

During the time when multiple sclerosis (MS) was presumably developing in our patients, there was only a moderate migration within this population. We were therefore dealing with a mostly autochthonous population. Thus we divided our study into four areas: the Upper and Lower Wallis, each divided into different levels of altitude.

Selecting our material in such a way as to avoid mistakes turned out to be rather difficult. All medical practitioners were asked several times for the names of their MS patients. Another list of names was received from the regional hospitals and from the Bernese clinic in Montana (AMS department). In addition, the university hospitals concerned with diagnostic problems gave us the names of all their patients living in the Wallis. During the questionning of the patients, which I mostly did myself, and which was carried out to lesser degree by a well-informed staff, an effort was made to discover MS patients. In cases in which a diagnosis of MS did not clearly follow from the case history, we examined the patients thoroughly and compared our findings with earlier data.

After the elimination of the non-MS patients and after having fixed a date limit, 52 remained and their prenatal, hereditary and environmental influences were analyzed. The 52 patients were divided into three groups (Table 1).

Group A, the most important and interesting group, comprises 38 cases who had lived in the same place from birth until the onset of their illness. Group B comprises

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Table 1. 52 cases of verified MS

A.	38 cases	from birth to start of illness in the same village
B.	5 cases	from birth to the age of 15 years in the same village
C.	9 cases	migratory cases (18 males, 34 females)

five patients who had lived in the same place up to the age of 15. Group C is made up of nine patients who were either migrating within the Wallis or had moved into the Wallis many years before.

Our first results basically confirmed earlier investigations. The prevalence per 100,000 was 25.17, which is about half the Swiss average (about 51). The slight increase in this value compared with earlier studies was probably due to a better medical care of the population and therefore a more complete recognition of clear MS cases.

Surprisingly, the prevalence in the Upper Wallis (37.6) proved to be twice as high as in the Lower Wallis (19.4). In trying to proceed to even smaller units of space we found parishes in the Upper Wallis where cluster formation was suspected. This applied to the indigenous patients of group A as well as to the five cases of group B (Fig. 1). The prevalence reached values of 110 (group A) and 116, respectively (group B). Smaller parishes tended to have a higher prevalence. Due to the small number of cases this result is not statistically significant.

Nevertheless, in a vertical division the values are significantly different: in the Upper Wallis parishes above 1200 m show a higher prevalence than lower areas.

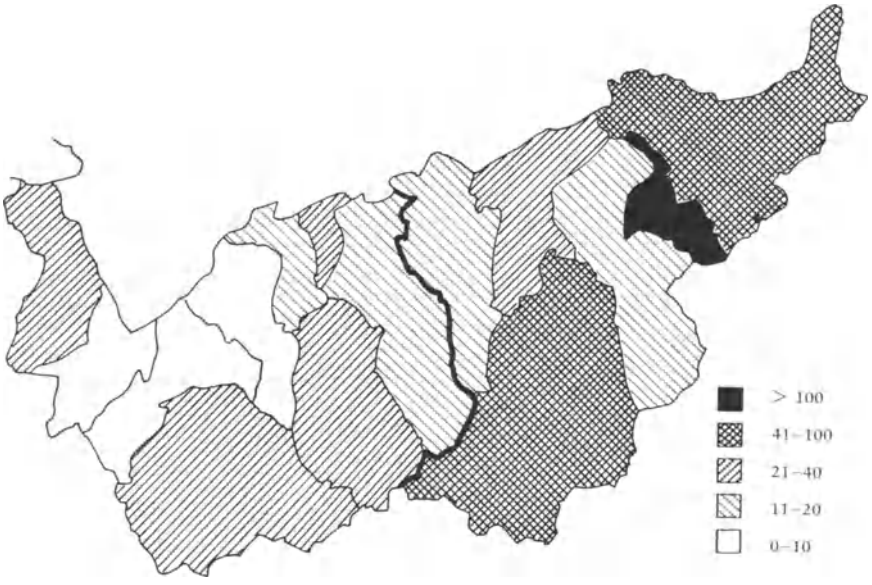

Fig. 1. Prevalence of MS in Wallis

Table 2. Infectious diseases at age 0 – 15 years

	A	B	C	ABC
Measles	19	5	4	28
No measles	17	0	0	17
Pertussis	13	3	5	21
No pertussis	24	2	4	30
Measles + pertussis	10	3	3	16
Chicken pox or rubella	6	1	2	9
No Chicken pox or rubella	32	4	7	43

Table 3. Factors of no or minor importance

Industrial environment
Relation to epilepsy, mental debility
Alcoholism
Contact with MS cases
Pregnancy and birth conditions
Number of siblings and range
Sport or no sport activities
Tourism
Chewing grass stalks
Brain or spine trauma
Psychological stress
Fruit feeding ("methanol hypothesis")
Nicotine
Caries (and fillings)

Our patients originated mainly from farm families (four times as many as the average proportion in that population). Only one quarter of the indigenous group A came from an occupation other than dairy farming or agriculture.

The number of siblings is larger in the Upper Wallis than in the Lower Wallis. The position in the succession of children is of no importance. In only four cases from the indigenous group were several members of a family affected (siblings in three cases, mother and siblings in one case), and in one case of the smaller group B the father and siblings were affected. Possible contact with MS patients was mentioned in only two cases.

Familial occurrence of epilepsy, debility, and alcoholism is of no importance. Pregnancy and birth was normal in practically all cases. Most patients had been breast fed as babies. Psychomotor development was normal in 90%. Only five cases from the indigenous group were slightly retarded. The proportion of athletic and nonathletic persons was about equal. Most of the men were serving in the army and could therefore be considered as healthy at the age of 18 to 20. One-half of the patients had had a habit of chewing grass stalks as children. This interesting information cannot be used statistically. Infection with measles was mentioned in half the patients, and only a small minority had had chicken pox or rubella (Table 2).

Up to the age of 15, the patients had access to modest to poor hygienic facilities (water supply, sewerage, heating, kitchen equipment). After reaching the age of 15

and up to the time of onset of the disease, the patients' social standard and hygienic conditions had considerably improved in most cases. Two-fifths of the indigenous population drink uncleaned milk. They eat and were eating little meat but a lot of dairy products and vegetables instead. About one-half of the patients were eating little or no fruits (an argument against the methanol hypothesis). One-third of the patients are teetotalers and one-half drink wine. Beer and hard alcoholics are not consumed to an important degree. Only one-third of the patients are considered to be smokers. Dark bread is eaten by two-fifths of the patients. Accordingly, one-half of them had healthy teeth up to the age of 15, whereas later only one patient had completely intact teeth. This is probably due to their rising standard of living (Table 3).

Summary

The prevalence of MS in the Wallis is only one-half the Swiss average. In the Upper Wallis it is twice as high as in the Lower Wallis. The patients originate mainly from modest farming communities. In the Upper Wallis cluster formations within small areas of parishes also occur outside industrial areas. The prevalence here can be up to 100 or more. The possibility of a hereditary factor is raised since we are dealing for the most part with indigenous patients. Also, intermarriage occured.

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Epidemiological Research in MS in Italy and Optic Neuritic Risk Cases of MS

C. L. CAZZULLO [1], D. CAPUTO [1], G. BERTONI [2], A. ZIBETTI [1], and C. FROSIO [2]

Epidemiological Research in Multiple Sclerosis in Italy

Italy is situated between 35° and 47° north latitude, in the Mediterranean Basin, in an area whose population should be at a low risk of contracting multiple sclerosis (MS). However, despite our restricted range of latitude, the Italian population is in a peculiar condition from an epidemiological point of view, whether due to extreme ethnic heterogeneity or hygienic-sanitary conditions or extreme dietary habits which even vary in neighboring regions.

It may also be important to note the intense flux of migration from the southern Italian agricultural economy to the northern industrial regions. However, in the northern alpine valleys, there still exist areas whose populations migrate from areas bordering on Italy, and whose members have adjusted to Italian dietary conditions and customs.

Yet Italy does not have a national epidemiological research program. Epidemiological studies have been made in 1959 (Macchi) in Parma province with a prevalence rate of 12.33/100,000, in 1966 (Montanini) in Varese province with a prevalence rate of 19/100,000, in 1964 (Mapelli) in Sassari province (prevalence, 20.11/100,000), in 1963 (Mapelli) in Nuoro (24.85/100,000), in Cagliari (Caruso, 1968) (11.61/100,000), in 1969 (Paci) in Terni province (12.95/100,000), in 1967 (Mapelli) in Ferrara (12.95/100,000), in 1969 (Paci) in Perugia (14.86/100,000), and in 1974 (Tavolato) in Padova (16.04/100,000).

These data, however, are extremely partial and can be contested in two ways: the first way pertaining to the problem of the different time periods of the studies; the second dealing with different research and diagnostic methods, which make the resulting data hard to compare and show some not completely forgivable discrepancies.

In 1975 the Italian Neurology Society tried to analyze epidemiological data on MS using uniform criteria for each province. The data presented showed the following prevalence rates: Aosta, 20.16; Torino, 18.76; Varese, 19.15; Padova, 20.31; Firenze, 12.65; Perugia, 18.27; Bari, 14.58; Napoli 7.34; Messina, 5.23/100,000 (Fig. 1) [6]. Recently the Center for Study on Multiple Sclerosis performed an epidemiological study in Varese and Novara provinces. For this research, all ms cases were collected between January 1, 1966 and December 31, 1975 consulting hospitals,

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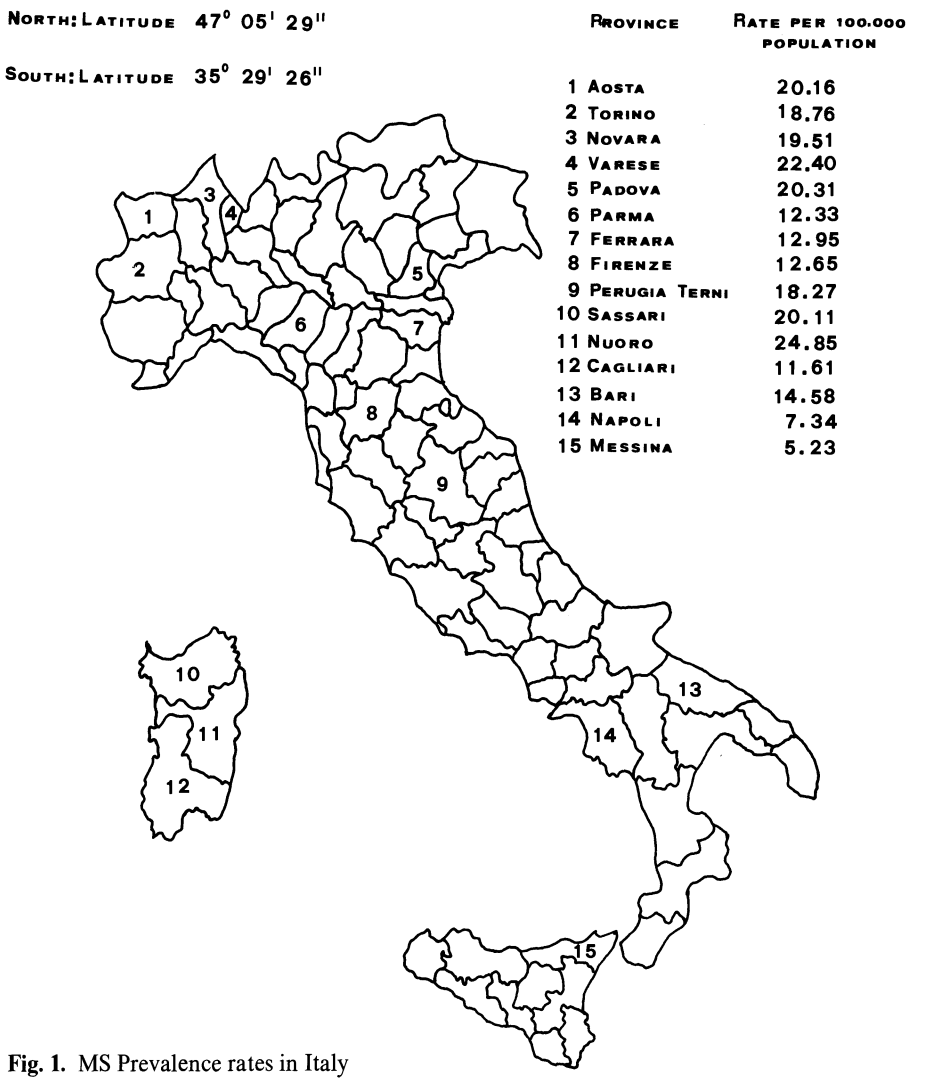

Fig. 1. MS Prevalence rates in Italy

nursing homes, National Health Services, and local sections of the Italian Association for MS.

The diagnostic criteria adopted are those proposed by Alter and Kurtzke [1]. The disease is differentiated into three categories: definite, latent probable, and possible.

Clinical symptoms have been evaluated using the Kurtzke rating scale.

Geomedical Conditions

Varese Province. Varese province is situated between 45° 34′ and 46° 7′ north latitude. The total area is 1,198.71 km^2. The province is divided into three areas, a flat area in the south, a hilly area, and a mountainous area in the north. The average

yearly temperature is 10.87 °C with a maximum in the summer period 34.4 °C and a minimum of – 6.5 °C. The average yearly humidity is 66.6% and the average yearly precipitation is 1,114.8 mm.

The total population of the province on December 31, 1975 was 790,046 inhabitants with a demographic density of 659 inhabitants per km^2. The four most important towns exceed 50,000 in population. The other centers of the province are less populated. Occupational classification of the population indicates that 32.37% are employed in industry, 7.67% in commerce, 5.2% in building, 1.76% in agriculture, 1.60% in transport, and 1,83% in public administration.

Almost all of the population benefits from drinkable water from the aqueduct system. Private water sources are used only in sporadic mountainous areas. Typhus and Salmonella endemic foci are not known.

Over a 10-year period 210 patients with MS were found. Nineteen of them died before December 31, 1975 and they are not included in our sample. We therefore examined 167 patients affected by total and latent probable MS and 24 patients affected by possible MS for a total number of 191 patients: 110 females (57.6%) and 81 males (42.4%).

Considering the population of Varese province as of December 31, 1975, 790,046, the prevalence rate in that period is 24.176 per 100,000 inhabitants.

The prevalence rate calculated only for the cases of manifested and probable MS is 22.40/100,000. Studying the distribution of the patients in Varese province, we noticed that the prevalence rate is higher in mountainous areas (33.14/100,000 inhabitants) than in hilly and flat areas (22.83 and 23.12/100,000, respectively) [22].

Novara Province. Novara province occupies 3,594 km^2. It comprises a mountainous region which includes a real alpine area, a morainic area, and a southern plain area. The main rivers and lakes are: the Ticino, the Sesia, part of the west side of Lake Maggiore, and Lake Orta. The climate presents different features from area to area. In the alpine area, winter is long and cold, summer is cool, and precipitation is modest in summer and in autumn; on the plain, temperature variations and winter fogs are more frequent; in the lake area there is a submediterranean climate with a mild winter, cool summer, and plentiful precipitation. The economy has a well-balanced structure with an efficient and highly mechanized agriculture, a flourishing industry in standard sectors and in consumer goods, commercial activities, and a well-developed tourist trade in the mountain and lake areas.

Table 1. The main epidemiological data from Varese and Novara Province

Varese area		Novara area
1,198.71	km^2	3,594
790,046	Population	507,394
167 (22.4 × 100,000)	MS	99 (19.51 × 100,000)
71 (42.4%)	Males (MS)	43 (43.4%)
96 (57.6%)	Females (MS)	56 (56.5%)
33.14 × 100,000	Mountainous district	25.08 × 100,000
22.83 × 100,000	Hilly district	15.20 × 100,000
23.12 × 100,000	Flat district	19.42 × 100,000

The population of Novara province on December 31, 1975 was 507,394: 245,080 males and 262,306 females. The MS prevalence rate is 19.51% (17.54% for males and 21.35% for females). The average incidence rate in the 10 years studied was 0.8%, with a minimum of 0.6% and a maximum of 1%. We divided Novara province into three areas: area A (a mountainous area north of 45° 58′ latitude), area B (hilly between 45° 58′ and 45° 40′), and area C (flat plains south of 45° 40′).

In area A the prevalence rate is clearly the highest: 25.08% while area B has the lowest (15.20%). The prevalence rate in the flat area represents the average for the province.

We did not notice any differences between the prevalence rate of the city of Novara (the only center with a population exceeding 100,000), which was 19.60% and the rest of the province, which had a prevalence rate of 19.48% [8].

A comparison between the main data gathered from the two provinces is indicated in Table 1.

Optic Neuritis Risk Cases of MS

The etiology of acute retrobulbar optic neuritis is not yet clear. According to some authors, 45% of patients who have had an episode of optic neuritis will later develop MS, and according to Bradley and Whitty [7], 51% of optic neuritis cases can be interpreted as the first episode of MS.

Similar data are given by Hutchinson [15], Nikoskelainen [25], and Collis [12]. Leibowitz and Alter [17] state that in over 266 cases of MS studied, retrobulbar optic neuritis appeared as a first isolated symptom in 14% of the cases. Arnason [2] and Batchelor [3] studying the frequency of histocompatibility antigens (HLA) in MS patients and in subjects affected by optic neuritis, found different genetic substrata among the two groups; it follows that retrobulbar optic neuritis represents the first symptom of MS in relation to the presence of HLA-A3 and HLA-B7 antigenic determinants.

Platz et al. [27] found no differences between frequencies of antigenic determinants in the MS and in the optic neuritis groups and believe these are two different clinical manifestations of the same disease.

Materials and Methods

We studied 25 subjects (20 females and 5 males ranging in age from 14 to 40) who had suffered one or more episodes of retrobulbar optic neuritis. All subjects were given a complete ophthalmological examination and were subjected to an erythrocyte-unsaturated fatty acid (E-UFA) test [5, 14] and HLA typing [11].

Table 2. The E-UFA test in acute retrobulbar neuritis

Positive:	8 (32%)
Negative:	17 (68%)

Results

The results of the E-UFA test were positive in eight subjects (TAble 2) (32%).

The results of HLA typing are reported in Table 3. These data have been compared with a group of 121 MS patients typed in two studies by Cazzullo and Smeraldi [11] and with a group of healthy volunteers (384 subjects) tested by Mattiuz et al. [23].

A greater frequency of HLA-A9 and B7 antigens was found in the HLA typing: the difference, however, was not statistically significant.

Table 3. Frequency of HLA antigens in acute retrobulbar neuritis and multiple sclerosis

HLA antigens	Frequency (%) in controls (no. 386)	Frequency (%) in acute retrobulbar neuritis (no. 25)	Frequency (%) in multiple sclerosis (no. 121)
A 1	26.4	17.6	28.9
A 2	47.1	29.4	50.4
A 3	21.5	11.7	17.3
A 9	27.5	47.1	40.5
A 10	24.1	11.7	12.4
A 11	6.7	–	5.8
B 5	24.3	23.5	27.3
B 7	9.1	17.6	13.2
B 8	11.4	5.9	14.9
B 12	18.6	17.6	20.7
B 13	9.1	17.6	6.6
B 15	–	–	8.8

Discussion

HLA-A9 is the most frequent antigenic determinant in the Italian population affected by MS [11, 30]. These data, which differ from the results obtained by other foreign authors [4, 16] can be explained by the hypothesis of a different marker of MS susceptibility in this population as compared with others, where a greater frequency of HLA-A3, B7, and Dw2 antigens occurs.

It is well known that specific HLA antigens are nonrandomly distributed among the different populations of the world; some types are very common in some populations while rare in others [13].

The A3 and B7 antigenic determinants are very frequent in the Scandinavian population, where there is a high incidence of MS, but less frequent in Italy, where a lower incidence of the disease is found.

The higher frequency of antigens in the Scandinavian population, as well as other factors, may account for the fact that the MS susceptibility gene is more likely to be found on A3, B7, Dw2 than on other haplotypes [29].

Moreover, selective positive pressures might exist to maintain the gene associated with the same haplotype. Indeed, the "founder effect" claimed by Degos and

Dausset [13] and by Terasaki and Mickey [29], independently of any selective theory, may be the reason for the differences obtained when HLA distribution is investigated in different ethnic groups. The results obtained using the E-UFA test (positivity in 32% of cases) confirm the hypothesis that acute retrobulbar optic neuritis is the first symptom of MS.

Therefore we consider it very important, as already out by Cazzullo [10], to identify, through these tests, those subjects who are to be given an adequate pharmacological treatment; this is to be done not only for a therapeutic but also for a prognostic purpose. These results would be checked by following the patients for some years.

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Optic Neuritis in Hannover – An Epidemiologic and Serogenetic Study

P. HALLER [1], U. PATZOLD [1], and G. ECKERT [2]

The incidence of optic neuritis in Germany is unknown. Epidemiological investigations in other countries have revealed a geographical distribution comparable to that of multiple sclerosis (MS) [1, 4, 6, 7, 10]. Calculations of MS risk after isolated optic neuritis [3, 5], and HLA typing [2, 9] have been controversal. We started a prospective optic neuritis survey in Hannover, Lower Saxony, in September 1975.

Methodology

In the district of Hannover (555,589 inhabitants on January 1, 1977) all patients suffering from acute optic neuritis have been identified and included in a prospective epidemiological and clinical study since January 1, 1976.

The examinations carried out include clinical and social history, general and neurological examination, kinetic and static perimetry, fundus photography, and HLA typing.

The course of the disease is followed by regular ophthalmological and neurological investigation and repeated static perimetry.

Results

From January 1, 1976 to January 1, 1978, 36 patients were presented to us by their ophthalmologists immediately after having been examined for the first time. Patients with known MS are not included in this number. Six of the 36 had vascular or toxic optic nerve damage. Thirty patients had a so-called isolated optic neuritis at the time of the first investigation.

The incidence rate of optic neuritis was 2.69 per 100,000 (Table 1). The female-male ratio was 2 : 1. The age-adjusted incidence rate had its maximum between 21 to 44 years.

Patients with more education (grammar school, university degree) were affected more often than others. This difference was statistically significant (Table 1). Twenty-four of the 30 patients affected have by now developed symptoms of MS. No other causes of optic neuritis could be found. Patients with optic neuritis in this study and in a retrospective investigation of optic neuritis (n = 96) more often showed an association with HLA-A3 and HLA-B7 (34.3%, 34.3%) than control groups of healthy

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Table 1. Incidence of acute optic neuritis (retrobulbar neuritis and papillitis) by age, sex, and social class. Hannover, Lower Saxony (Jan. 1, 1976 – Jan. 1, 1978, 555,589 residents)

	Number	Annual incidence/100,000 population	
		Rate	95% confidence interval
Both sexes	30	2.7	1,458 – 4,276
Male	10	1.9	0,782 – 4,367
Female	20	3.3	1,776 – 5,884
6 – 20 years	2	1.1	
21 – 44 years	27	7.4	
Male	9	4.9	
Female	18	10.1	
45 – 59 years	1	0.2	
Higher education	16	5.5	
No higher education	14	2.3	
Isolated after follow-up of 6 months to 2.5 years	6	0.8	

inhabitants of Hannover (n = 124, HLA-A3 29.0%; HLA-B7 26.6%). This difference is not statistically (chi-square test) significant.

Conclusions

Our investigation revealed that Hannover is a district of high optic neuritis risk. Similarly high incidence rates have been reported for Carlisle [4] and Rochester [7]; lower rates have been reported in Israel [6], Hawaii [1], and Finland [10].

Follow-up examination demonstrated that an unexpected high number of patients had definite or suspected MS. The careful exclusion of other optic nerve damage explains the high MS risk. HLA typing confirms the same association of A3 and B7 as in patients with MS [9]. Because it is generally accepted that more than 40% of MS starts with optic neuritis, the incidence rate of MS in Hannover must be more than 5.0 per 100,000 per year, an incidence rate which has been found in another district of Lower Saxony by Poser [8]. Therefore the incidence rate of optic neuritis is a good marker for MS risk in a certain area. Carrying on this study might be of great value for detecting environmental factors which enhance or diminish MS in a distinct area.

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Some Features of MS in Pécs, Hungary

G. PÁLFFY [1]

During the past 30 years our department has collected data on 35 cases of proven, 87 cases of definite, 54 cases of probable, and 43 cases of questionable multiple sclerosis (MS), according to the diagnostic criteria of Bauer [2].

The histologically proven cases were investigated under the light microscope using the usual cell, axon, myelin, glia, and lipid staining methods.

The definite, probable, and questionable MS material consists of patients examined by myself.

The cerebrospinal fluid (CSF) of each patient was studied at least once but in most cases also during every exacerbation. The cell count, cytology, protein content, mastix reaction, and IgG/albumin index were also determined. Using the silver staining method described by Kerényi and Gallyas [3] we succeeded in demonstrating the gamma globulin subfractions characteristic of MS in a small sample of unconcentrated CSF [4].

To identify every slight optic lesion, a very sophisticated neuro-ophthalmological and electrophysiological examination, including flicker fusion frequency and visual-evoked response (VER), was performed in each case.

To demonstrate possible cerebral cortical lesions, first of all, those of the parietal cortex, gnostic and neuropsychological examinations were done.

To exclude diseases other than MS with as much certainty as possible, neuroradiological examinations were carried out, viz. – plain X-ray of the skull and spine, pneumoencephalography (PEG), pneumomyelography (PMG), and myodil myelography, if necessary.

For 3 years now we have been investigating HLA types and immunological parameters of our patients [1]. This investigation permits the preliminary conclusion that a patient's cellular immunologic state is depressed during exacerbations and is normal during remission and slow progression. In our material the relative risk for HLA-A28, -B7 and -Bw16 was 2.8, 2.42, and 2.4, respectively.

In Baranya County, which is situated in Southern Hungary, every neurologist in the Outpatient Departments has worked in our department, and knowing of my interest in MS sends all his MS patients to our department not only at the first bout but also during later exacerbations.

In Hungary the Hempt lyssa vaccine containing sheep brain, i.e., a heterologous nervous tissue, is in general use. Every year thousands of people are inoculated with this vaccine.

Two fatal cases of disseminated encephalomyelitis (one hyperacute and one subacute) following the use of lyssa vaccine have been observed in our department

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during the past 2 years. In the first case the lesion was localized in the pontine, spinal and cerebral white matter, the foci were hemorrhagic-inflammatory. The hypergic character of the lesion was indicated by eosinophilic infiltration. The histological examination of the second case is in progress.

Several cases of CNS complications following the use of various sera and vaccines have been observed in our Department [5, 7]. Some of these cases ran a course which was indistinguishable from MS [6].

In Baranya County there are several gipsy settlements throughout the county around the villages, sometimes also in the neighborhood of towns and in forests. The number of gipsies in Hungary is more than 500,000 at this time. The hygienic situation of the gipsies is very poor and differs greatly from that of the other ethnic groups in Hungary (Hungarians, Germans, Slavish). Until two decades ago they lived in tents not only in the summer but also in the winter, without water supply, electricity or gas.

Intermarriage between relatives is common even today and it is exceptional that gipsies mix with Hungarians or other ethnic groups in Hungary.

In the 30 years of my medical career I have never seen a gipsy patient suffering from MS.

The HLA types of the gipsies are now being investigated.

Summary

This is report on some features of 219 MS patients in Pécs, southwestern Hungary, during the last 30 years. The CSF was examined in each case; the gamma globulin subfractions characteristic of MS were demonstrated using a silver staining method. The relative risk for HLA-A28, -B7 and -Bw16 was 2.8, 2.42, and 2.4, respectively. No gipsies with MS have been found. Two fatal cases of disseminated encephalomyelitis following the use of lyssa vaccine were observed.

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Discussion

H. J. Bauer: I would like to ask Dr. Broman a question. You mentioned that 15% of your observations had no reccurences; is that correct? Does that mean that there were 15% monocyclic forms in your series?

T. Broman: I think that the 9% of lowest probability correspond fairly well with monofocal and mono-bout forms of MS.

H. J. Bauer: I apologize for putting this in a lighter vein, but this very interesting thing about the gypsies gives me an idea. Every year at Pentecost a great number of gypsies from many parts of Europe assemble in St. Marie-sur-la-Mere in the Camargue for the festival of the three Marys' going across the sea. They take their sick with them. If some clever epidemiologist could go there he might be able to pick up some MS data among them.

M. Alter: I would like to mention Dr. Pálffy's observations regarding the gypsies way of living. This confirms our observations in Israel, where we compared the various ethnic groups, particularly the Arabs, the Sephardic population, and the Ashkenazi population. It was very interesting for me to hear that SSPE is common in gypsies, the very group that has so little, if any, MS. This is true among the Arabs too. Despite what Yaffa Leibovitz may have shown, Prof. Field, the drawers in Israel are very shallow. In fact when one relates the number of Arabs to the number at risk the rate of MS in Arab populations is very low.

Clinical Definition and Assessment of the Course of MS

IMAB-Enquête Concerning the Diagnostic Criteria for MS

H. J. BAUER [1]

The criteria for MS diagnosis formulated by the Schumacher Committee in 1965 were a part of a system of guidelines for the evaluation of MS therapy [3]. While there is general agreement as to the fundamental importance and validity of these criteria, the need to revise and supplement them has become increasingly apparent for a number of reasons:

1. In the original formulation, the Schumacher criteria demand a clear "yes-no" decision in diagnosing MS. While this is legitimate for therapeutic trials, it poses serious difficulties in epidemiological studies, particularly in view of one of their basic aims: to find exogenous factors relevant to the etiology and pathogenesis of MS.
2. The exclusion of first bouts and the first period of 6 months in MS with initial chronic manifestation from evaluation in epidemiological studies precludes the possibility of optimally exploring the factors operative in the earliest stages of manifest MS. Incidence studies necessarily become inaccurate because of the restriction to retrospective information.
3. The age limits of onset are too restrictive: the recommendations of the Schumacher Committee in their original form exclude childhood and adolescent forms of MS, as well as manifestations late in life, from the diagnosis of MS.
4. Although there is still no specific diagnostic test for MS, modern laboratory methods, especially the examination of cerebrospinal fluid (CSF) when evaluated in conjunction with clinical data, make the diagnosis of MS possible with a high degree of reliability in a larger percentage of cases than using "clinical criteria" alone. Basically, an antithetical view of clinical/laboratory data appears to be unfortunate, since the latter are de facto also clinical data obtained with special examination techniques.

The problem of a revision of the recommendations of the Schumacher Committee was discussed in detail at the Second International Symposium on MS in Göteborg in 1972, which was organized and conducted by T. Broman and his co-workers [1]. The fruits of this meeting were the formulation of extremely valuable guidelines regarding the validity of diagnosis, the course of the disease,the definition of important clinical manifestations, scoring systems, and principles of epidemiological investigation of MS. But a revised text of the Schumacher criteria was not drafted. A slightly revised version was presented by Kurtzke, in which the questions listed under 1–4 were still left open, however.

A decisive improvement in the criteria formulated by Rose et al. at the US-Japan Conference on MS in 1976 is the grading of diagnosis as clinically definite, prob-

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able, or possible [2]. This takes into account some of the most important problems listed above, especially by including first bouts in the probable group and omitting restrictions with regard to age of onset. There still remains the integration of laboratory findings into the diagnostic criteria.

The purpose of this investigation was to obtain a survey of opinions on problems relating to the diagnostic classification of MS from the large group of clinicians and other scientists experienced in dealing with the practical and scientific problems related to MS who belong to the International Medical Advisory Board (IMAB) of the International Federation of Multiple Sclerosis Societies. Although it would be unreasonable to expect a rigid and internationally recognized system of standardization, a greater degree of coordination in the presentation of diagnostic data and the assay of the course of MS would seem highly desirable for many areas of work.

Results

Of 120 questionaires sent out, 58 answers from 25 countries [Argentina, Austria, Australia, Belgium, Denmark, France, East Germany (GDR), West Germany (FRG), Hungary Ireland, Norway, Poland, Romania, Sweden, Switzerland, UK, USA, USSR, USSR-Lithuania, Venezuela] were received. These included the majority of clinical neurologists whose active work and commitment to MS research is internationally known.

The following answers and comments were received

Question I. Do you consider

1. The diagnostic criteria recommended by the Schumacher Committee 1965 (11 replies),
2. The revision presented at the Göteborg symposium in 1972 by J. Kurtzke (14 replies),
3. The modification suggested by Rose et al. at the US-Japan Conference on Multiple Sclerosis in February 1976, (26 replies), or
4. Some other system of diagnostic criteria employed or recommended by you as the most useful guideline for establishing a diagnosis of MS? (24 replies).

No single set of criteria 1–4 was favored by the majority of responders. Approximately an equal number gave preference to the modification of the criteria of the Schumacher Committee as proposed at the US-Japan Conference on Multiple Sclerosis, or to a further modification which, as the commentaries clearly show, should include the evaluation of cerebrospinal fluid (CSF) findings. This point is stressed in several comments (Amaducci, Clausen, Lhermitte, Minderhout, Pálffy, Schuller, Wuethrich, Verdes) (laboratory criteria, especially CSF findings meriting consideration, are discussed under V). Minderhout considers CSF abnormalities necessary for a definite clinical diagnosis of MS. In this connection, Schuller states that CSF analysis is essential for diagnosis, even if only one part of the central nervous system (CNS) is involved. Amaducci considers "clinical diagnostic criteria very useful for a guideline, but at this point not significant after the introduction of laboratory and other diagnostic tests, such as CSF, visual-evoked response (VER), CAT, etc.

A further reservation regarding the criteria recommended by the Schumacher Committee and the modifications of Kurtzke was that "the age restriction (Alter,

Schuller), which, if handled strictly, bars the childhood and adolescent forms on the one hand, and the MS forms with late onset – which may be forms in which an unrecognized very bland first bout, is followed by many years of latency, an inapparcept, subclinical, nonprogressive course – from the diagnosis of MS."

Fog does not agree with the criterion in the recommendations of the Schumacher Committee: "The involvement of the neuraxis must have occurred temporally in one or the other of the following patterns: (1) In two or more episodes of worsening, separated by a period of one month or more, each episode lasting at least 24 h; (2) Slow or stepwise progression of signs and symptoms, over a period of at least 6 months."

He points out that first bouts may often last less than 6 months, and yet there are disseminated symptoms in the first bout, combined with a spinal profile – excluding other disease – which make a diagnosis of probable MS logical, even if the remission is complete. With respect to the decision that "slow or stepweise progressive course extending over at least 6 months" may be classified in the definite category, Fog points out that this is the usual course in the so-called spinal cases, but that these must be considered as only probable, even if a typical CSF profile is found and VERs are positive. He raises the question of whether pure spinal MS exists at all, pointing out that in autopsy studies Zimmermann was not able to find such cases; even in "spinal cases" diagnosed neuropathologically the question remains whether this must be attributed to an insufficient exploration of the brain.

Other criteria suggested as preferable or useful were those of Allison and Millar (Alter, Presthus), of the Research Committee of the Japanese Ministry of Health and Welfare (Iwashita, Kuroiwa), McDonalds classification (Martin), Müller's own classification of 1949, and the grading system used by the Research Group of the Deutsche Forschungsgemeinschaft (Pálffy). E. V. Schmidt and O. A. Khondkarian favor a modification of the Schumacher criteria including doubtful cases. A simple classification including only the two forms, definite and doubtful, is preferred by den Hartog-Jager. Wuethrich considers "so-called first bouts with characteristic CSF findings as clinically definite cases".

Kahana favors a modification of the criteria of Rose et al. from the US-Japan Conference on MS, and formulates four categories: (1) definite MS proved by autopsy; (2) clinical definite MS based on the criteria of Rose et al.; (3) probable; and (4) possible.

The probable classification is based on several criteria:

1. Cases clinically fit the clinical definite category, but with onset of the disease before age 10 or after age 50.
2. History of relapsing and remitting symptoms without documentation of signs; however, patient's report is reliable or is substantiated by other family members or friends: documentation of one neurologic sign typical of MS.
3. Cases with progressive monofocal neurologic disease of the white matter of the CNS where VER or other electrophysiologic tests suggest dissemination or elevated gamma globulin in CSF.
4. No better neurological explanation.

The possible category is based on criteria 1, 2, and 3 as in Rose et al. or on a documented single bout of symptoms with multifocal signs: no fever, no pleocytosis in

CSF and no viral disease prior to the neurological attack suggesting acute disseminated encephalitis (ADE).

Kahana reports that of 281 cases collected in the Israel survey of 1960 (30% possible cases), 121 have died thus far. In six the diagnosis of MS was disproved by autopsy and in nine by further clinical reevaluation (previous classification: five probable and ten possible).

Referring to his own work, Broman points out that the choice of system depends on the aims of a study and the method used in collecting material.

Question II. Would you agree that the characterization of MS as "clinically definite – probable – questionable – verified by autopsy" is preferable to an unconditional diagnosis of MS or its negation, and that a proability scale of this type should be advocated as a prerequisite in clinical studies?

There was practically unanimity (55–2) with respect to the desirability of grading diagnostic validity – definite/probable/possible or some variant of these terms – in preference to a yes–no diagnosis. Schumacher remarked that "in view of the vagaries of the disease (a wide range of patterns in the longitudinal history and in the cross-sectional picture of neurologic findings), and in the absence of a specific laboratory test for MS, an unconditional diagnosis is possible in only a proportion of patients with MS".

There were differences of opinion with respect to the criteria of grading and the terminology. Kurtzke proposed the classification of MS cases as clinically definite if there was full compliance with the Schumacher criteria, as probable if they were "not quite clinically definite", and a third group as "possible/questionable". Brown prefers "possible" to questionable; Knight suggests "MS suspect" for the third group.

Alter and Wolman as well as Kuroiwa consider only autopsy-proven cases as definite and restrict positive classification to "probable" and "possible".

Lhermitte considers it difficult to distinguish probable and questionable cases.

Shibasaki advocates including Devic's disease as "MS probable", despite its monophasic clinical course.

Schuller objects to clinical categories "because they might lead to a misinterpretation of the diagnostic value of biological investigations and consequently to an inexact interpretation of different courses of MS."

Question III. The recommendations of the Schumacher Committee do not sanction a diagnosis of MS during the first bout, even if its symptomatology is characteristically disseminated and CSF findings are typical. The drawback of this recommendation is that the earliest and possibly most highly informative cases with respect to the etiology and pathogenesis of MS are lost for systematic and prospective study – which is also possible with early therapy. Would you consider an inclusion of these cases into a "MS probable" (first bout) category?

A 4–1 majority of answers (42–12) favored revising the Schumacher criteria to include first bouts under a diagnosis of MS, if clinical and laboratory criteria are characteristic. Commentaries on this recommend the grading of diagnostic validity as "probable or possible MS".

Suggestions made for the designation of first bouts include:

1. Early probable or first bout if there is evidence of scattered lesions (Alter),
2. MS probable, first bout (Sutherland),

3. MS probable (Dassel, Kelly – "but any study that includes these cases should probably be confined to these cases only. They are not very common").
4. MS possible (Kahana, Koetsier),
5. Possible MS (acute disseminated encephalomyelitis) (Kurtzke), and
6. MS based on first bout (Tourtellotte).

Schumacher considers a diagnosis of MS during the first bout undesirable. He would sanction characterization of this condition as "possible" or "suspected MS" in any clinical study. However, all such cases should be earmarked for later withdrawal, should the patient show evidence of another disease. He further comments: "Since the relation of a single, widespread attack of ADE to 'acute MS' remains unresolved, and since there is no way of separating a patient with ADE who recovers, partially or fully, and will never have another attack, from one who has subsequent recurrence (MS), it is essential to maintain the hard and fast criterion that a single, first episode of scattered neurologic dysfunction as in MS does make not a diagnosis of MS until a new episode or further progression occurs."

Rose points out that first bouts are important for the follow-up studies, but that unless there is concrete evidence for dissemination, first bout cases are not appropriate for therapeutic trials.

Kuroiwa considers MS very likely in typical neuromyelitis optica. Japanese criteria include first bouts as "possible MS."

Question IV. Are you conducting a study or systematically collecting and following up cases of optic neuritis (ON) in view of the fact that a high percentage progresses to typical MS?

Forty percent of the answers (22–33) indicated that studies on ON and MS are being undertaken. In a number of ansers, it was pointed out that the percentage of ON progressing to MS is low (Kurland, Amaducci – less than 10%; Kuroiwa, Shibasaki – 10% in 8 years; Schumacher – 30–35% in 25 years).

On the other hand, Caceres found that 35 of 50 of his cases of ON had progressed to MS. He pointed out, however, that one autoptically proven case had demyelinating foci in the spinal cord and medulla. In their study on optic neuritis in relation to MS, Kahana, Alter, and Feldmann remark that available estimates of the frequency with which patients with ON develop MS range from 13 to 87%. Their own follow-up study in 105 patients indicated that after 10 years $32.3 \pm 5.6\%$ of patients with ON would develop MS and, after 14 years, about one-half would develop MS.

Knight and Kurtzke mentioned their study in progress on a 30-year follow-up of US Army men with ON. Dean reported that he is conducting a study on Devic's syndrome. Fog deplores the fact that in Copenhagen there is an agreement *not* to admit pure ON into hospitals, but to see them in ambulatory ophthalmological clinics, because "remissions are mostly complete".

Question V: Eighty-three percent of those surveyed (49–10) indicated that laboratory tests are being used in establishing the diagnosis of MS. In 48% (28–30), laboratory tests were found useful in the estimation of process activity.

Fifty-five percent (32 of 58) of the answers state that lumbar puncture is always performed, when possible; 41% (24 of 55) say it is performed in the majority of cases. This implies that lumbar puncture is a routine procedure in 96% of cases, and

indicates that physicians experienced in the handling of MS give a high rating to diagnostic information obtained using this procedure. The divergence with respect to the repetition of lumbar puncture is exemplified by the comments of Minderhout and Schumacher on the one hand, who limit the procedure to "only once to support diagnosis," with repetition only if the CSF was normal on first puncture, and the statement of Tourtellotte, who performs lumbar punctures in some patients as often as three times per week in an effort to assess process activity from CSF findings.

Cell counts and differentiation of CSF cells are regular diagnostic procedures according to 94% and 84% of all answers, respectively. Special cytological methods (33%) included rosette tests for B and T lymphocytes (Brage, Clausen, Fog, Iwashita, Jellinger, Minderhout, Pálffy), lymphocyte transformation to MBP and other CNS constituents (Clausen, Kahana, Pálffy), electron-microscopic studies (Iwashita), and various special sedimentation techniques. In relation to special serological and laboratory tests listed in the following parts of this survey, it appears that many of the special methods mentioned are not permanent routine procedures.

Only 76% of the answers indicated the use of quantitative determinations of CSF protein, an unexpectedly low figure that may require rechecking. The most frequent method mentioned was Lowry's method (Amaducci, Broman, Frick, Gautier, Iwashita, Lhermitte, Pálffy, Scheller, Bauer). Other methods were turbidimetric procedures with sulfosalicylic acid (Knight), trichloroacetic acid (Minderhout), the biuret method (Presthus), Rieder's method (modification of Cu-Folin reaction, and Heller's method for albumin (nitric acid turbidimetric test – Verdes).

A number of clinicians have apparently abandoned quantitative protein determinations in CSF, limiting their assays to the determination of albumin and various globulin fractions using electrophoresis or immunoprecipitation. Tourtellotte has repeatedly pointed out that albumin, which is synthesized exclusively in the liver and never in the CNS, more accurately reflects abnormalities of CSF protein components than total protein determination.

It appears that colloidal reactions are being abandoned in routine CSF diagnosis, with only 45% still using the goldsol or mastix reaction. A micromethod requiring only 0.55 ml of CSF developed by Reiber is employed in the Göttingen Neurology Department as a "second scanning parameter" in addition to immunodiffusion techniques.

CSF electrophoresis is routinely used according to 77% of the answers. Most of these did not indicate the method used. Procedures mentioned were agar gel (Amaducci) agarose (Iwashita), cellogel (Koetsier), polyacrylamide gel (Brage, Schmidt/Moscow), and the Kerenyi-Hegedüs Method (Pálffy).

Broman mentioned the occasional use of immunoelectrophoresis by means of Dencker's method.

Immunodiffusion (ID) for the selective quantitation of various protein components was reported in 59%, of the answers with 43% using the Mancini technique of radial immunodiffusion and 18% using electroimmunodiffusion (EID). The protein components quantitatively assayed in most cases included albumin, IgG, IgM, IgA, but also IgD, IgE, C3 (Brage), C3, C4, B-factor, and CRP (Schuller).

Clinicians are gradually becoming aware of the importance of the simultaneous assay of protein components in blood and CSF. Thirty-five percent reported simultaneous electrophoretic analysis; 43% reported simultaneous electrophoresis or

EID. These methods are finding increasing interest, since according to the formula of Ganrod-Laurell, Link's quotient or Schuller's modification of the simultaneous assay of albumin and IgG in CSF and blood presents a more accurate possibility for the differentiation of IgG synthesis within the CNS and a disturbance of blood/CSF-barrier function than procedures previously available.

An important place in CSF diagnosis of MS must be granted to the demonstration of oligoclonal bands (components with restricted heterogeneity) in the gamma globulin range (Lowenthal), with 52% of answers indicating a visual evaluation or quantitative analysis of these subfractions using agar gel, electrophoresis, agarose electrophoresis, or isoelectric focusing (IEF) (Amaducci, Broman, Delmotte, Knight, Koetsier, Tourtellotte).

A large number of serological reactions and other laboratory tests (40% of all answers) were mentioned: antigen-antibody reactions; tests against antigenic components and a cytotoxic test against measles, rubella, herpes simplex, polio, influenza, parainfluenza, and varicella zoster (Broman, Clausen, Iwashita, Schuller, Verdes); a toxoplasmosis test as an indicator of process activity (Wuethrich). Studies on complement and immune complexes were mentioned by Fog and Knight; immunofluorescent assay of IgM was mentioned by den Hartog-Jager.

Frick has developed an antibody-dependent lymphocyte cytotoxicity test against myelin basic protein (MBP). In routine CSF analysis of MS patients, the demonstration of MBP in CSF described by Whitaker, Cohen, and McKhann was reported by Carnegie and from the Göttingen Neurology Department.

The assay of nucleic antibodies (DNA and RNA) using counter immunoelectrophoresis (Schuller, personal method), antinuclear, anti-DNA, antimitochondrial-, antithyroid, antithymic, antiparietal, anti-interstitial substance, and antimuscle (smooth and striated) antibodies and other very special tests were mentioned by Brage, Minderhout, and others. Caceres mentioned systematic platelet recounts.

From the Soviet Union Schmidt reported on the determination of acute phase proteins, xanthine-hypoxanthine, and cyclic AMP; Visockas reported on cold autohemagglutinins, seromucoid concentrations in blood, quantitative mucoprotein determinations in 24-h urine, C-reactive protein, and alcohol determinations.

Cazzullo mentioned the use of Field's EUFA-test and the TEEM test. Koetsier could find no difference between MS and non-MS-patients using Field's MEM test. A study presently being carried out by Field in Göttingen on coded material from MS patients and controls is still incomplete.

In a number of answers reference was made to special tests such as the measurement of visual-evoked responses and computer scans in the diagnosis of MS. The determination of histocompatibility antigens was also mentioned, but only in one answer (Schuller) were these considered to be diagnostic value in atypical cases.

Conclusions

The enquête has shown

1. That there is a strong motivation for endeavoring to maintain a series of standards for the diagnosis of MS and to keep them in line with the present level of

knowledge concerning MS and problems relevant to the practical management, clinical research, and the continuous development taking place.
2. That the diagnosis of MS should be graded with respect to its validity on the basis of clinical symptoms, course, and laboratory criteria.
3. A large majority of clinicians and researchers favors the inclusion of first bouts under a diagnosis of MS if the criteria of disseminated symptomatology and characteristic CSF findings are fulfilled.

Since this poses the problem of distinguishing ADE as a disease different from the nosological group of MS, any accepted classification should provide a system for earmarking first bout cases for later withdrawal. However, in this connection there often remains, the enigmatic question of whether monocyclic cases of disseminated encephalomyelitis are first manifestations of a nonrecurring form of MS, or postvaccinal, para-, and postinfectious disseminated encephalomyelitides requiring strict differentiation from MS. Since the number of the latter conditions is small compared to the large number of cases of MS, which would all be missed in the initial phase if the original criteria of the Schumacher Commission were strictly observed, the balance is definitely in favor of including them as probable or possible cases of MS, with the additional designation as first bouts.

4. In view of the great divergency with respect to the percentage of cases of ON, which later develop the disseminated symptomatology and course of MS, the great interest taken in this question, and the considerable number of studies in progress, it would seem fortuitous to try to coordinate these efforts and concentrate on the prospective investigation of this problem.
5. There is fairly good agreement concerning the laboratory criteria, especially the CSF investigations, which are important for supporting a diagnosis of MS. These are mononuclear pleocytosis, increase of gamma globulin and the appearance of oligoclonal bands (components with restricted hererogeneity) in the gamma globulin range, identification of the gamma globulin increase as IgG, and evidence of IgG synthesis within the CNS.

With respect to CSF cells, a considerable number of studies on B and T cells are under way. This, as well as numerous serological studies and a broad spectrum of spinal laboratory investigations on the CSF and blood of MS patients, reflects the efforts to find criteria for a better estimation of the course of MS and to identify pathogenic mechanisms.

It would seem useful to differentiate clearly between laboratory studies aimed at finding new and better diagnostic tests, laboratory criteria for the assay of the course of MS, and, on the other hand, investigations that promise to shed light on immunological or biochemical particularities relevant to the pathogenesis of the disease, but not informative with respect to its diagnosis or course. This would prevent many clinical research groups from starting investigations not correlating with their primary aims and their actual possibilities for successful work.

It is not within the scope of this meeting to arrive at an official decision regarding a reformulation of criteria for the diagnosis of MS, and the lack of time within the program of our symposium leaves room for only a limited discussion. Based on the results of the enquête, an attempt has been made to formulate a draft which

could possibly serve as the groundwork for updating the criteria for the clinical diagnosis of MS:

I. MS proved by autopsy.
II. Clinically definite MS.

1. Relapsing and remitting course with at least two bouts separated by no less than 1 month, or
2. Slow or stepwise progression extending over at least 6 months.
3. Documented neurological signs attributable to more than one site of predominantly white matter central nervous system pathology.
4. Characteristic CSF findings:
   a) Mononuclear pleocytosis.
   b) Increase of gamma globulin and demonstration of oligoclonal bands (components with restricted heterogeneity) in the gamma-globulin range.
   c) Identification of the gamma globulin increase as IgG and evidence of IgG synthesis within the central nervous system.
5. Onset of symptoms, usually between ages 10 and 50.
6. No better neurological explanation.

III. Clinically probable MS (subgroup: "probable – first bout")

1. History of relapsing and remitting symptoms, signs suggestive of MS, but without documentation of unequivocal signs of multifocal white matter disease.
2. A documented single bout of symptoms with signs of multifocal white matter disease with good recovery, and followed by variable symptoms and signs.
3. Pathological CSF alterations suggestive of MS, but full-blown MS profile as specified under II 4 not obligatory.
4. No better neurological explanation.

IV. Clinically possible MS (subgroup "possible – first bout")

1. History of relapsing and remitting symptoms without documentation of signs.
2. Objective neurological signs but insufficient to establish more than one site of central nervous system pathology.
3. Pathological CSF alterations not obligatory.
4. No better neurological explanations.

Monosymptomatic retrobulbar neuritis is located here.

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The Role of Evoked Potentials in the Diagnosis of Multiple Sclerosis

W. I. McDonald [1]

Electrophysiological tests are now widely used in the assessment of patients suspected of having multiple sclerosis (MS), and it is appropriate to review their role in diagnosis. A more detailed review has been published recently [8]. Here I shall consider four questions.

What Do Evoked Potential Tests Reveal?

When abnormal, evoked potentials provide evidence for the existence of a lesion in the system being tested – visual, auditory, or somatosensory. Additional information about localization within the system can often be gained from close study of the potentials themselves or their distribution, or by comparing the results obtained from two procedures, for example, the electrospinogram and the somatosensory cortical potential.

How Efficient Are Evoked Potential Tests in Detecting Lesions?

In all three systems, electrical abnormalities are found in virtually all patients who have abnormal physical signs. The incidence in cases without abnormal physical signs is shown in Table 1. From this it can be seen that the electrical procedures are exquisitely sensitive in detecting abnormalities. In relation to the visual system, it is important to stress that the incidence of abnormalities in the pattern-evoked poten-

Table 1. Incidence of abnormal evoked potentials in patients with clinically definite MS but no abnormal signs related to the system being tested

System	% Abnormal	Reference
Visual	47 (84) [a]	Hennerici et al. [6]
Auditory	51	Robinson and Rudge [9]
Spinal	56	Small et al. [10]

[a] The two different percentages derive from the use of two different methods of pattern stimulation in the same group of patients

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tial varies in different series and even within the same series [6] when stimulus parameters (e.g., check size, field subtended) are changed. It is likely that variations in patient groups also contribute to the variation in incidence of abnormalities, although it is noteworthy that similar figures have been obtained in two laboratories in different countries using identical techniques [4, 11]. A similarly wide range in the incidence of abnormalities is found in clinically definite and in less definite cases (Table 2).

Overall, evoked potential abnormalities are found in at least 50% of MS cases, but the incidence may rise to over 80% in the visual system.

Table 2. Incidence of abnormal visual-(pattern) evoked potentials in clinically definite MS

Reference	No. of patients	% Abnormal
Halliday et al. [4]	34	97
Asselman et al. [1]	31	84
Hennerici et al. [6]	16	81 (94) [a]
Matthews et al. [7]	61	75
Zeese [10]	26	92

[a] The two different percentages derive from the use of two different methods of pattern stimulation in the same group of patients

How Specific Are the Changes?

Four classes of abnormality may be seen in evoked potentials – changes in latency, amplitude, wave form, and distribution of the potentials. In the auditory- and spinal-evoked potentials, changes in amplitude appear to be more important than changes in latency, whereas the reverse is true for the visual system. Because the visual system has been more widely investigated than the other two, it is appropriate to consider it in more detail.

Types of Abnormality in Multiple Sclerosis

Although a delay with a relatively well-preserved wave form is characteristic of MS, other abnormalities occur. A reduction in amplitude is striking in the acute stages of optic neuritis (ON) and when there is persistent impairment of visual acuity in the established disease. Changes in wave form may occur, particularly a broadening in the response. Occasionally, a change occurs in the distribution of the potential (Fig. 1). Normally, the visual-evoked potential attenuates symmetrically on either side of the midline. Lesions in the optic chiasm and tract, however, produce asymmetries in distribution which are characteristic of the location of the lesion and independent of their pathological nature [2]. Figure 1 shows records from a patient with MS. The pattern is similar to that shown in Figure 2 (except that the asymmetry is to the op-

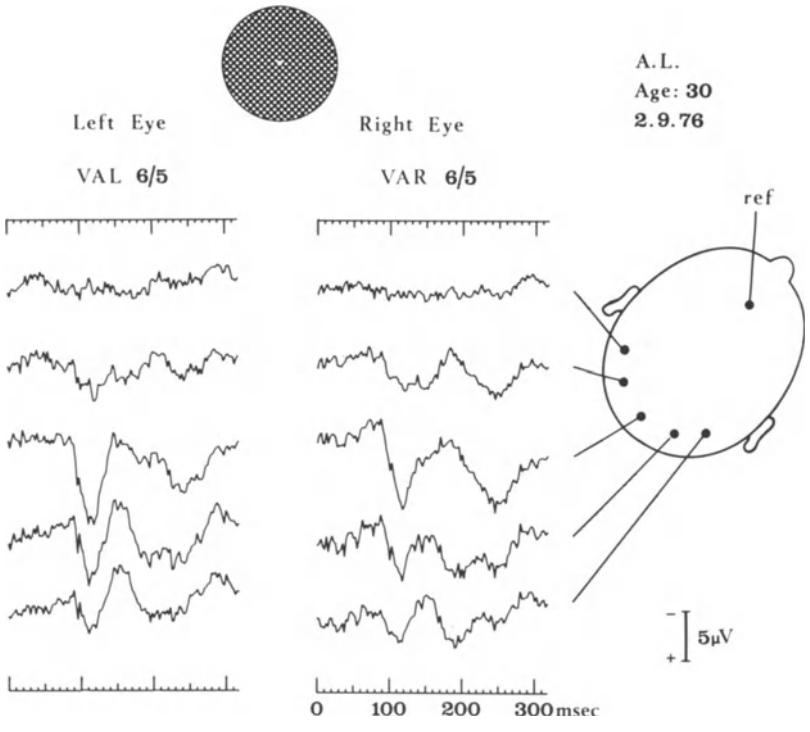

Fig. 1. Visual-evoked potentials after stimulation by pattern reversal in a patient with MS. Note that the response recorded from the midline is of normal amplitude and latency but that there is a marked asymmetry in the lateral distribution of the potential. The asymmetry is the same from each eye, indicating that the lesion is in the optic tract. (Reproduced through the courtesy of Dr. A. M. Halliday)

posite side) recorded from a patient with operatively proved craniopharyngioma. It is important to note that had only the midline response been recorded, no abnormality would have been detected. These observations emphasize the desirability of using routinely laterally placed recording electrodes in addition to the standard midline lead.

Specificity to Multiple Sclerosis

Although a substantial delay in the first major positive wave is characteristic of MS, it is not diagnostic of it. We have seen delays in ischaemic optic neuropathy, compression of the optic nerve (although here the delays do not usually exceed 25 ms), in some (but not all) spinocerebellar degenerations, and in toxic and tropical amblyopia [5].

The evoked potential tests are thus merely a sensitive extension of the physical examination. They can provide solid evidence for the existence of abnormality and for its localization, but not for its pathological nature. The latter may be suspected, but the real significance of evoked potential abnormalities must be interpreted in the light of the clinical picture.

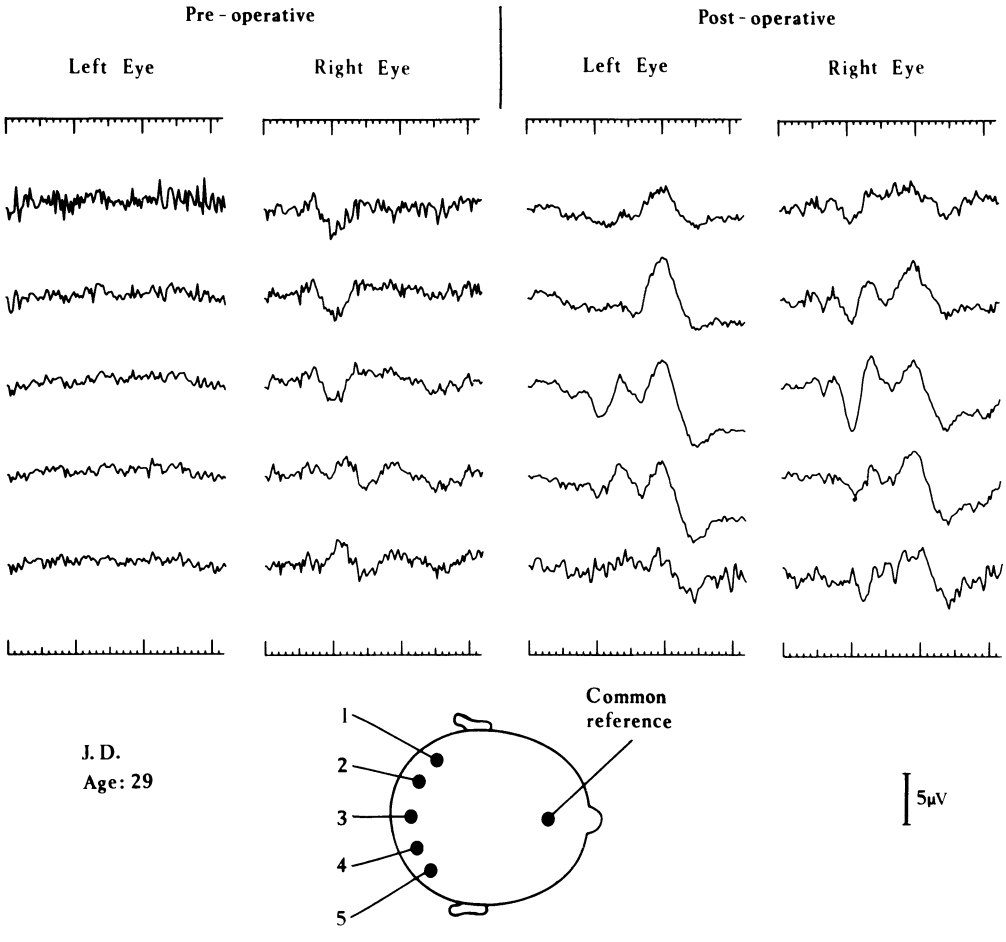

Fig. 2. Records obtained as in Figure 1 but from a patient with craniopharyngioma. Again the midline response is normal but the distribution of potentials asymmetrical. Preoperatively, there was no response from the left eye, in which the acuity was profoundly reduced. Note that the asymmetry was reversed when the tumor was removed [5]

What is the Clinical Role of Evoked Potential Studies?

The clinical role of evoked potential studies is essentially that of detecting subclinical second lesions in patients in whom the diagnosis is not yet clinically definite. The procedures are time consuming and nothing is gained by carrying them out where there is objective clinical evidence of abnormality in the tract concerned. The particular examination needed will be determined by the clinical problem – the visual evoked potential, for example, is the procedure of choice in progressive spastic paraplegia, which is abnormal in one-quarter to three-quarters of patients (Table 3).

We are currently investigating the auditory-evoked potential in isolated ON to determine whether abnormalities in this response are of predictive value in assessing the risk of subsequent developments of MS and how this investigation relates to other prognostic factors [3].

Table 3. Incidence of visual-(pattern) evoked potential abnormalities in patients with progressive spastic paraplegia

Reference	No. of patients	No. abnormal
Halliday et al. [4]	13	5
Asselman et al. [1]	22	5
Hennerici et al. [6]	14	5 (6) [a]
Matthews et al. [7]	9	7

[a] Different incidences were obtained with different methods of pattern stimulation in the same group of patients

Conclusion

Evoked potential tests should be carefully selected according to the clinical picture in the individual patient. They provide a very sensitive means of detecting abnormality, even in the absence of clinical signs. Although the abnormalities are not specific to any single desease process, when interpreted in the light of the rest of the clinical picture, they are often useful in making an early diagnosis of clinically definite MS.

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Value of the Evoked Potential Examination in Diagnosis of MS

P. KETELAER [1]

A complete survey of current knowledge and performance of the various neurophysiological techniques in objectifying demyelinating lesions has been published by McDonald and Halliday [5]. They insist on a high incidence of pathological responses using the different evoked-potential examinations in definite multiple sclerosis (MS):

1. Visual-evoked response (Asselman: 84%; Halliday: 96%).
2. Brain stem-auditory-evoked response (Robinson and Rudge: 77%).
3. Blink reflex (Kimura: 66%).
4. Electrospinogram (Small: 89%).
5. Somatosensory-evoked responses (Baker: 76%).

In a study of somatosensory-evoked responses in 250 MS patients, we were able to demonstrate a very high incidence of pathological responses, namely, 93% with increase in latency and wave-form abnormalities [3].

Of special interest is the presence of subclinical evoked potential abnormalities, which has been reported extensively. As a diagnostic tool, my colleagues and I decided to look for the incidence of these subclinical modifications by applying a multimodal evoked-potential examination.

In a preliminary study, although performed on a small series, it was possible to point out the efficiency of this procedure for detecting clinically silent lesions in slightly handicapped and full ambulatory patients [1].

A more extensive study of 50 patients was recently presented [2]. According to McDonald and Halliday's criteria for the classification of MS, 24 patients who were classified as definite MS and 26 suspected probable and possible MS patients were

Table 1. Multimodal evoked potential examination in MS [a]

	Deltenre et al. [1]		Khoshbin et al. [4]	
	Definite MS $n=24$	Suspected MS $n=26$	Definite MS $n=25$	Suspected MS $n=25$
VEP	72.3%	11.5%	80%	48%
SEP	72.3%	34.6%	84%	64%
BAEP	67.9%	31.5%	52%	30%
BR	50%	25%	33%	18%

[a] VEP = visual-evoked potential; SEP = somatosensory-evoked potential; BAEP = brain stem auditory-evoked potential; BR = blink reflex

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examined. The pattern reversal visual-evoked response, the somatosensory-evoked potentials, the brain-stem-auditory-evoked response, and the blink reflex examinations were performed using standard techniques. The results (Table 1) were compared with those of Khoshbin et al. [4] and prove the highest incidence of abnormalities for the visual-evoked response and somatosensory-evoked potentials and the lowest rate for the blink reflex examination. The most important feature of this multimodal evoked-potential study (Table 2) was the occurrence of subclinical lesions in 62.5% of the definite MS population studied. In this respect, the visual-evoked response examination was most valuable, since 50% of the patients presented subclinical visual-evoked response abnormalities.

Table 2. Multimodal evoked potential examination in MS

	Subclinical abnormal evoked potential results
Definite MS	62.5% in at least one test 50% in visual-evoked potential only
Suspected MS	31% in at least one test

We believe that multimodal evoked-potential examination in slightly handicapped MS patients can be most valuable in demonstrating clinical silent lesions. As a diagnostic tool, this examination leads to the establishment of a clinically probable or possible MS diagnosis.

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Pattern-Evoked Visual Potentials in 251 MS Patients in Relation to Ophthalmological Findings and Diagnostic Classification

K. Lowitzsch [1]

Introduction

One of the main problems in multiple sclerosis (MS) research is to set up diagnostic criteria for clinical classification. Since the Göteborg MS Symposium in 1972, many European neurologists have become familiar with the criteria given by Bauer [4] including the clinical course of the disease, the dissemination of the demyelinating process in time and space, and cerebrospinal fluid (CSF) findings. The most critical point, however, is to prove a multitopical site of the lesions. Because of the discrepancy between autopsy findings [20, 22, 31, 33] and clinical symptoms [1, 16–19, 25, 28] several attempts have been carried out to verify so-called silent lesions using neurophysiological methods.

According to the visual system in the first series, flash-evoked cortical potentials were used [11, 26, 30]. Later on Halliday et al. [12, 13] introduced the pattern-reversal evoked response. They were able to demonstrate the advantages of this method in relation to flash-evoked responses by means of high inter- and intrasubject stability of visual-evoked response (VER) shape and peak latencies. However, up to now only a few pattern reversal studies have been done on sufficiently large and clinically defined series [2, 8, 13, 21, 23, 29]. Lowitzsch et al. [21] used the documentation and classification system developed by Bauer [4] and Poser et al. [27] and were prepared to demonstrate an improvement of MS classification due to delay of the VER latency. In the present study of a more extensive MS series consisting of 251 patients, the reliability and diagnostic value of the test as well as the relationship to ophthalmological findings and recovery processes in terms of remyelination shall be discussed.

Methods

Based on the method of Cobb et al. [7] as modified by Halliday et al. [12, 13], pattern reversal was used as a stimulus to evoke visual-evoked responses (VERs). A mirror system triggered by a digital stimulation unit displaced a checkerboard pattern twice per second. The reversal time was about 5 ms. Single squares measured $50' \times 50'$; a 30° field of vision was stimulated. Luminosity and contrast data as well as the recording and averaging arrangements are described in detail in a previous paper [21].

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MS Patients

Two hundred and fifty-one MS patients were examined. Concerning clinical and ophthalmological examinations, we refer to our previous paper mentioned above. The criteria devised by Bauer [4] are listed in this paper and serve as a basis for the diagnostic classification. The "possible" MS group comprised 88 patients, the "probable" MS group 82 patients, and the "definite" MS group comprised 81 patients.

Results

Control Subjects

The control group consisted of 48 healthy subjects of both sexes and various ages [15]. It was possible to obtain VERs from all control subjects. A positive wave with a peak latency of around 100 ms (P_2 according to Harding [14]) could be recognized regularly. The mean value from all VERs derived by monocular stimulation was 104.3 ± 4.5 ms. Comparing the latencies of P_2 after monocular stimulation in the same subject, a difference of 2.05 ± 2.1 ms was obtained. P_2 latencies as well as differences between right and left eye stimulation have been classified as abnormally delayed if the normal mean plus 2.5 SD was exceeded (115.5 ms, 7.5 ms, respectively).

MS Patients

Ophthalmological Findings. Extensive ophthalmological examinations including static perimetry have been carried out in 135 MS patients [21]. A history of retrobulbar neuritis was present in 52% of the total of 269 eyes (Fig. 1). Visual acuity < 5/7 was

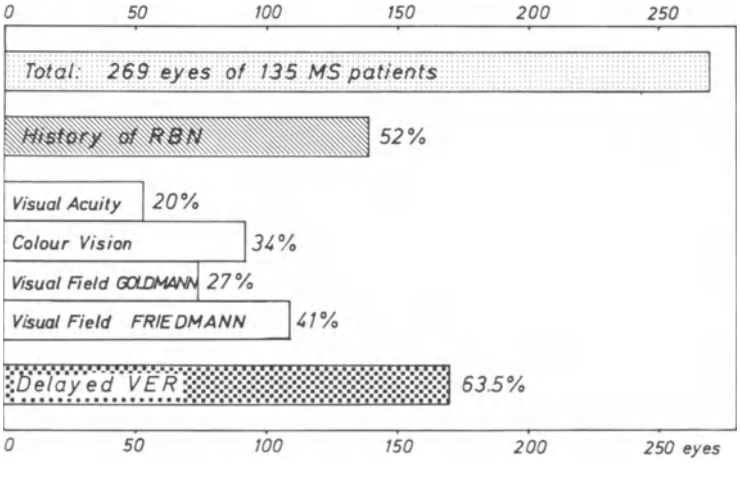

Fig. 1. History of retrobulbar neuritis (RBN), ophthalmological signs, and delayed pattern VERs in 269 MS eyes

found in 20% of the eyes and disturbances of color vision (Ishiara, Haitz) in 34% of them. Visual field defects could be detected using dynamic perimetry in 27% and by using static perimetry in 41% of the eyes. The VER, however, was delayed in 63.5% of the eyes (Fig. 1).

VER's by Pattern Reversal and Flash Stimulation. The superiority of pattern-reversal over flash VERs in diagnosing retrobulbar neuritis (RBN) could be demonstrated in 101 eyes with ophthalmologically proven RBN: 42% showed a delayed flash response, while 92% had delayed responses after pattern reversal stimulation.

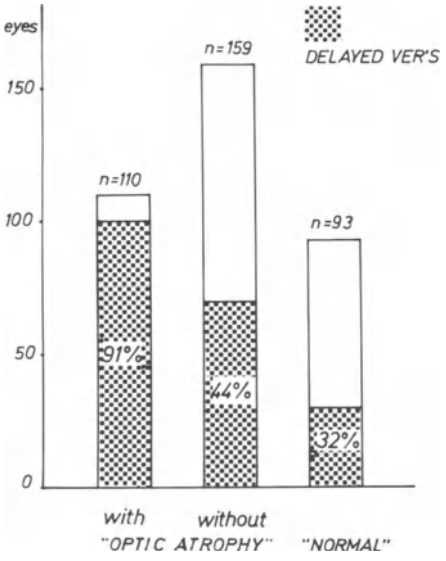

Fig. 2. Delayed VERs in 269 MS eyes in relation to "optic atrophy" and clinically "normal" ophthalmological findings

VER and "Optic Atrophy". The number of eyes with "optic atrophy" based on the criteria of Ballantyne and Michaelson [3] as a functional deficit in vision and field and possibly pallor of the disc is shown in Figure 2. We found this condition in 110 eyes out of a total of 269. The VER was delayed in 91% of these, while 44% of the remaining 159 eyes without optic atrophy showed a VER delay. Within this group the ophthalmological assessment proved to be normal in 93 eyes; however, 32% of these had abnormally delayed VERs (Fig. 2).

VER and MS Classification. VERs were delayed in one or in both eyes in 73% of the total 251 MS patients. In the "possible" MS group ($n = 88$) we found delayed VERs in 60%, in the "probable" MS group ($n = 82$) in 77%, and in the "definite" MS group ($n = 81$) in 83% of the patients (Table 1; Fig. 3).

Discussion

The mean P_2 latency of normal pattern VER was found to be in good agreement with the values given by Halliday et al. [13] (103.8 ± 4.3 ms) using a similar tech-

Table 1. Delayed VERs (in percentage) of MS patients in relation to the clinical MS classification in different clinical series

Author	Total group	MS certainty		
		"Possible"	"Probable"	"Definite"
Halliday et al. [13] [a]	51 (96%)	12 (91%)	5 (100%)	34 (97%)
Asselmann et al. [2] [a]	51 (67%)	14 (21%)	6 (83%)	31 (84%)
Lowitzsch et al. [21] [c]	135 (73%)	20 (65%)	42 (60%)	73 (82%)
Matthews et al. [23] [a]	113 (62%)	28 (38%)	24 (58%)	61 (75%)
Collins et al. [8] [b]	98 (52%)	31 (23%)	30 (50%)	37 (78%)
Lowitzsch (1978) [c]	251 (73%)	88 (60%)	82 (77%)	81 (83%)

[a] Classification criteria: McAlpine et al. [24 a]
[b] Classification criteria: McDonald et al. [24 b]
[c] Classification criteria: Bauer [4]

nique. However, in other studies performed using a mirror system, too, different means and SDs have been calculated: some have found shorter values such as 90.5 ± 4.3 [2], 99.1 ± 5.5 [8], and 100.5 ± 4.4 [23]; others, however, have measured longer latencies, e.g., 120.0 ± 12.0 ms [29]. These differences are probably due to minor variations in technique such as smaller check sizes and stimulation fields or higher reversal frequencies and prolonged reversal times.

Most authors stipulate an upper range of the mean plus 2.5 SD [8, 13, 23], while Asselman et al. [2] were more stringent and required the mean plus 3 SD. We accepted the mean plus 2.5 SD as the limit of normality (116 ms).

In all 251 patients diagnosed as MS cases, 73% had delayed VERs (Table 1). In our earlier series of 135 MS patients in 1976, the same proportion could be observed [21]. This value corresponds remarkably well with the results reported by Asselman et al. [2], who found 67% with delayed responses. While Matthews et al. [23] saw similar values (62%), Halliday et al. [13] gave a figure of 96%. Up to now this extremely high rate of delayed VERs has not been confirmed.

According to the subgroup of different certainty, the highest rate of VER abnormalities was found in the "definite" group (Table 1): 83% of 81 patients had abnormal delayed VERs. This agrees with the other clinical series: Halliday et al. [13] calculated 97%, Asselman et al. [2] 84%, Lowitzsch et al. [21] 82%, Matthews et al. [23] 75%, and Collins et al. [8] 78%.

In the "probable" group ($n = 82$), delayed VERs were observed in 77% of the cases, and in "possible" MS ($n = 88$) in 60% of the patients. Considering only subgroups containing more than 20 patients, there seems to be a sufficient coincidence in "probable" MS but striking differences in the "possible" groups (Table 1). These groups are in any case very heterogeneous; moreover, because of the various classi-

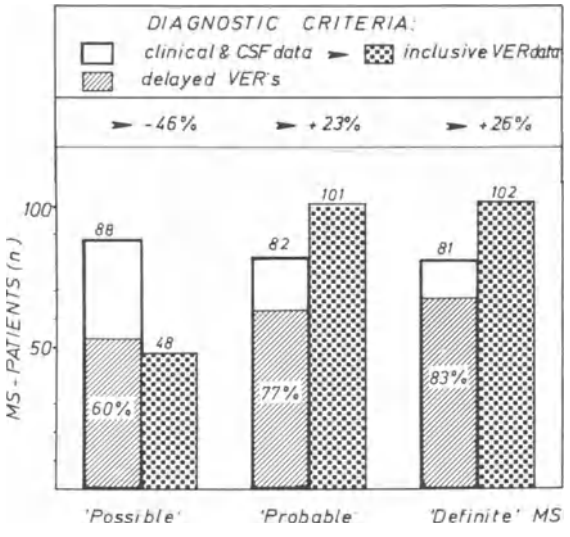

Fig. 3. Improvement of clinical classification in 251 MS patients with regard to delayed VERs

fication criteria indicated in Table 1, the composition of these groups differs widely, especially in regard to non-MS spinal cases. However, in our series the percentage of delayed VERs in this group was very high, indicating a lesion in the anterior visual pathways.

Thus, in 46% of the "possible" MS patients, a reclassification could be performed due to the proven dissemination of the demyelinating process (Fig. 3). Likewise in the "probable" group, 23% could be reclassified, too. Hence, in a total of 61 patients (24%), the clinical classification could be improved using VER findings as an additional tool to verify the dissemination of the process. Although it is well known that the VER test is not MS specific, delayed VERs in MS patients are of high reliability. VER findings, therefore, should be included into the diagnostic criteria for clinical MS classification.

Although a detailed ophthalmological examination measuring visual acuity, color vision as well as visual fields by dynamic and static perimetry was performed, the pattern-reversal VER was the most sensitive test for optic nerve dysfunction [21]. In a smaller MS series, this was confirmed by Ellenberger et al. [10], although only flash VERs were used.

Further, it has to be pointed out that static Friedmann perimetry can detect visual field defects at a higher rate than dynamic Goldmann perimetry (Fig. 1) as has been demonstrated in other neuro-ophthalmological disturbances as well [5, 6, 9].

However, despite the high reliability of delayed pattern reversal VERs in most cases of an acute or old attack of optic neuritis, there are a few cases with ophthalmologically proven "optic atrophy" but normal VERs (Fig. 2, left column: 10 eyes). One must consider whether the test might be too rough in cases with very dispersed or eccentrically localized plaques.

On the other hand, Waxman and Brill [32] recently have argued that reduction in internode distances along remyelinated fibers might play a role in promoting functional recovery as a result of facilitation of uninterrupted transmission along af-

fected axons. VER normalization during the course of the disease, therefore, might be an indicator for ongoing remyelination. Further VER studies must be done during the course of the disease to get more information on the dynamic development of demyelination and remyelination.

Summary

1. Pattern-reversal visual evoked potentials (VER) have been recorded in 251 MS patients.
2. The VER test was more powerful in diagnosing even clinically silent lesions of the anterior visual pathways than a sophisticated ophthalmological examination measuring visual acuity, color vision, and visual fields.
3. Visual field defects could be detected by static Friedmann perimetry in a higher rate than by dynamic perimetry.
4. The clinical classification of the 251 MS patients according to the criteria given by Bauer [4] with 81 patients in the "definite" group, 82 patients in the "probable" group, and 88 in the "possible" group could be improved in 23% of the cases when delayed VERs were taken in account.
5. It is suggested that one should include the pattern VER test in the diagnostic criteria for MS classification.

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Pattern and Flash Visual-Evoked Responses in Multiple Sclerosis

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Introduction

Until a few years ago, the diagnosis of a demyelinating disease as multiple sclerosis (MS) was finally decided only according to the clinical symptomatology and classified in probable, possible, and established cases [13].

Electrophoresis of the cerebrospinal fluid (CSF) disclosed an important rise of gamma globulins in about 60% of MS patients. Agar gel electrophoresis of the CSF permitted the detection of increased gamma globulins, with the appearance of slow cathodic fractions [12].

This was a significant step toward improved diagnosis of MS because up to 75% of the MS patients could be labeled "established diagnosis."

Delmotte [4] could confirm this conclusion with thin layer isofocusing and claimed to find an oligoclonal reaction in 95% of the cases. This oligoclonal reaction is hitherto the only biological test that confirms the clinical diagnosis of MS.

Nevertheless, this fractionation by agar electrophoresis of the raised gamma globulins seemed not to be specific for MS alone, but could also be found in other neurological disorders.

It is the purpose of this study to determine whether electro-ophthalmography, a noninvasive method of recording visually evoked responses (VER), is a reliable method to determine with certitude the diagnosis of demyelinating diseases (MS), if it is possible to establish this diagnosis in a more accurate manner than with biochemical techniques, and if it confirms the latter.

If VER can give information about subclinical visual symptoms in doubtful cases of demyelinating disease, research in electrophysiology should be emphasized with the aim of refining an important objective diagnostic tool.

Materials

Although histopathologic examination of toxic optic neuropathy cases demonstrates a demyelination that may be considered as characteristic of toxic neuropathies, we will not consider exogenous optic nerve disease in this paper.

Among factors of endogenous origin, we considered:

1. Demyelinating diseases of unknown origin with heterogeneous involvement of the visual pathways (45 cases of MS), of which 14 were established using agar

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electrophoresis, and of which 31 cases were diagnosed based on a clinical symptomatology (14 suspected: probable or possible, 17 established cases);
2. Diseases causing axonal damage to the optic nerve which were of local vascular origin or were dependent on a rise in intraocular pressure. The topography of the lesions is defined to the optic disc and lamina cribrosa (six cases of ischemic sectorial neuropathy; ten cases of open-angle glaucoma). It is of note that in both conditions disturbances of axonal flow exist and that normally the involved axons do not have a myelin sheath at the level of the optic disc and retina.

Thirteen recently graduated healthy volunteer medical doctors served as control cases, to be considered as having normal visual perception and pathways after full examination and correction of mild refractive errors.

Technique

The instrumentation of stimulation (flash and pattern), amplification, and registration has been described in previous papers [16–18].

Methodology

From the neuro-ophthalmological standpoint we must stress important conditions greatly influencing shape and size of the wave, rather than increasing delay.

Most studies discuss VER registrations as normal or abnormal, without considering amplitude. No distinction is made between pattern and flash [20], or only amplitude studied [19]. Evaluation of amplitude is important [5], together with interocular difference in latency [9].

We insist that the variability of the answers depends on modifications of the technique: it is of prime importance to relate the applied methodology and instrumentation.

Before and during VER registration:

1. Ambient room temperature should be normal (about 20 °C);
2. Amplitude should be increased after dark adaption [6];
3. Complete macular examination should be made (Amsler grid, angiofluorography): macular disease affects the VER amplitude;
4. We should note the place of the active midline electrode: moving progressively towards the nasion, a steady decrease of amplitude is obvious, going to nearly complete extinction;
5. We should observe fixation: in conditions of released foveal fixation, away from the checkerboard, the amplitude during pattern VER greatly decreases;
6. We should observe muscular strain and activity: especially the muscles of the neck, depending often upon the condition of the patient, may significantly alter the wave shape of the VER tracing;
7. We should be sure that always flash and pattern stimulation are applied to every patient.

Results

Although we obtain six waves [three positive ones (P) and three negative deflexions (N)], we considered only N-1 and P-1, because all other deflexions N-2, P-2, N-3, and P-3, to our feeling, are to be considered at present as equivocal. Differential analyses underway in our laboratory now suggest that P-3 corresponds to saturation of cell activity. We considered N-1 and P-1 as primary responses and all other components of the wave as secondary responses of doubtful origin in the present state of knowledge. In our recording the negative deflection N-1 is followed by P-1, a positive one.

Control Cases

For the mean normal values we refer to Table 1. *P* values between normal and MS cases are less than 0.001 for amplitude and vary between 0.025 and 0.05 for delay, which means that for both delay and amplitude, the results are highly significant.

It must be noted that our P-positive amplitude is the mirror image of the N-negative amplitude obtained by other authors [8].

In the series of control cases, we registered two out of 13 with a reversal record. In our series of 31 objectively established MS patients, the registration of five of them showed the same reversal phenomenon, corresponding to the percentage obtained in the control group (15.3 to 16.1).

MS Cases

It appears from Table 2 that the mean values indicate an increase of delay and a lowering of amplitude.

Characteristic is the change of the wave shape showing many notchings together with lowering of amplitude (Fig. 1).

Table 1. Mean normal values of control cases

Stimulation	Delay (ms)		Amplitude (μV)	
	$\bar{x}$	σ	$\bar{x}$	σ
Flash	108.9	15.75	14.4	6.0
Pattern	102.0	5.35	9.3	1.98

Table 2. Mean values of MS cases

Stimulation	Delay (ms)		Amplitude (μV)	
	$\bar{x}$	σ	$\bar{x}$	σ
Flash	133.9	44.5	5.9	3.0
Pattern	122.3	37.0	4.8	2.4

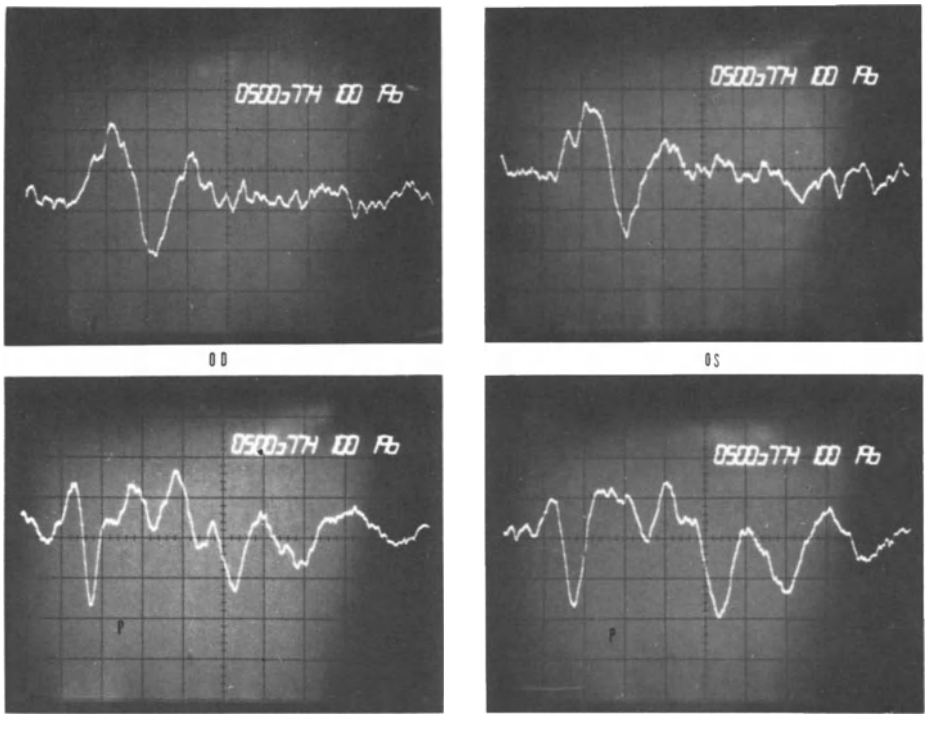

Fig. 1. Typical pattern VER (P) and flash VER (F) in sclerosis multiplex. After both pattern and flash stimulation, N-1 and P-1 exhibit changes of shape; notchings occur and their summation reduces the amplitude. Probably these changes are more reliable than increase of latency because they reproduce the summated answer of all fibers

In Table 3 we list our cases after the eye symptoms and CSF examination. The table illustrates that of 45 suspected or established cases, 34 have a change of wave shape (amplitude) and size. In the suspected group, this is three of 14, and in the established group 31/31. The 11 normal VER cases with normal delay in the suspected group proved not to be MS cases, yet one of them had positive CSF biochemistry.

Of the three MS cases, only one showed an increase of delay and no eye symptoms (circled in Table 3); of the two with disturbed amplitude, one had optic neuropathy and the other no eye symptom.

Of the established cases, seven had normal delays: two with diplopia and CSF +, one with optic neuritis and CSF –, and four without any eye symptoms, of which two had normal CSF.

Table 4 shows that in some cases the pattern answers were normal and the flash answers lowered. This might suggest that foveal axonal fibers are not involved, but the fibers representing the perifoveal and in a larger sense the posterior macular pole region are affected.

In our series, MS patients more consistently show disturbed amplitude than changes in delay, which conflicts with published results [1–3, 7, 8, 10, 11, 14, 20], but

Table 3. Number of suspected and established MS cases presenting changes of amplitude and/or delay [a]

Eye symptoms	VER (45 cases)						
	Suspected (14)			Established (31)			
				Change of amplit		Normal delay	
	CSF +	CSF −	No CSF	CSF +	CSF −	CSF +	CSF −
Nystagmus	−	−	−	4	3	−	−
Diplopia	−	1 N	3 N	2	3	2	−
ON	−	−	1	2	4	−	1
Nystagmus + ON	−	−	−	1	1	−	−
Diplopia + ON	−	−	−	−	1	−	−
Absence	1 N	1 1 N	5 N 1	5	5	2	2
Total	1	3	10	14	17	4	3

[a] N = normal; ON: optic neuropathy. Normal delay of 31 established cases, 24 presented increase of delay

Table 4. Thirty-one established cases

Stimulation	Delay		Amplitude	
	Increase	Normal	Change	Normal
Flash	20	11	29	2
Pattern	23	8	27	4

is consistent with the data of Feinsod and Hoyt [5] and Hoeppner and Lolas [9]. Van Lith [20], although considering the need for reevaluation of latency, depending on reversal time, does not distinguish responses after pattern from those after flash stimulation, and in his opinion, amplitude does not need to be considered.

On the contrary, we strongly recommend consideration of wave shape and size after both flash and pattern stimulation, which makes the method more reliable in detecting MS patients.

We did not meet a single case with only disturbed delay, but the contrary was observed: this means low amplitude and normal delay.

It should be emphasized that about 30% of the established MS cases showed a disturbed VER in the absence of any clinical abnormality of the eyes (10/31). Of these ten cases, all showed a change in wave shape with low amplitude, but four of them did not have an increase of delay.

From these results we may conclude that even in the absence of visual symptoms, VER is suggestive for axonal damage.

Is the VER Method Specific for MS?

We compared our results to those with diseases causing axonal damage at the level of the optic nerve.

Regarding open angle glaucoma (OAGD, Table 5, Fig. 2), central visual acuity is normal, delay is increased with nasal defects of the visual field, but amplitude is always low, with beginning Bjerrum's zone involvement.

Sectorial ischemic discopathy is characterized by inferior nasal field defects, joining an arcuate scotoma at the lower pole of the blind spot corresponding to absolute filling defects of the deep optic disc on angiofluorography. VERs show decisive low amplitude and increased delays in the affected eye, if both flash and pattern stimulation have been done. In this latter group it must be emphasized that the nasal defects proceed from the lower pole of the optic disc, while in glaucoma, Bjerrum's scotoma usually proceeds from the upper pole of the blind spot [15].

Although central visual acuity is always good in OAGD, the same changes appear as in MS patients.

We may conclude that the VER changes as observed in MS patients are not specific but may be observed in every case where axonal damage to the optic pathway exists. It is not indicative of any topographic relationship.

Table 5. Changes of VER in cases of OAGD and sectorial ischemic neuropathy

Disease	Delay (ms)				Amplitude (μV)			
	Flash		Pattern		Flash		Pattern	
	$\bar{X}$	σ	$\bar{X}$	σ	$\bar{X}$	σ	$\bar{X}$	σ
OAGD (10 cases)	95	19	109	34	8.6	2.3	7.7	3
Sectorial ischemic neuropathy (6 cases)	103	23	99.0	25	4.8	1.3	4.6	2.6

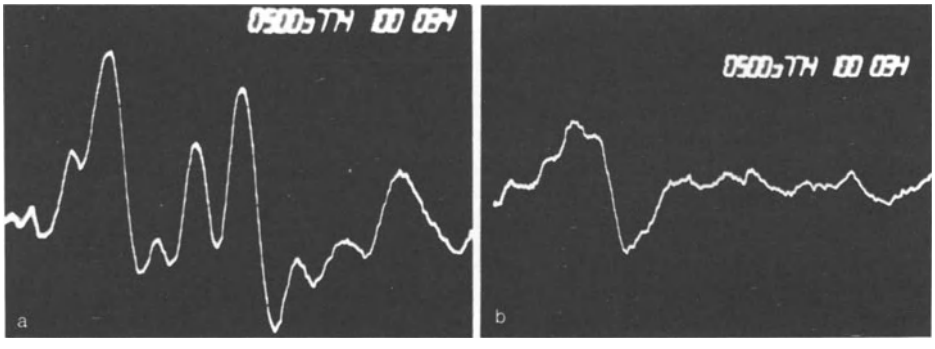

Fig. 2. Typical pattern VER (a) and flash (b) in glaucoma: increased intraocular pressure; no loss of central visual field; only beginning Bjerrum's scotoma, no peripheral field loss

Moreover, the wave shape of the first component is not only invariably lowered to a degree corresponding to the amount of affected fibers, but the wave shape shows indentations at the onset of the disease which are an expression of the axonal damage. Again, if more axons are involved, more frequent indentations appear, each time with a simultaneous decrease of amplitude.

Finally, if many fibers are affected, the indentations are countless and the amplitude decreases at the baseline.

Furthermore, it must be emphasized that changes in VER may not be considered as selective for damage to the papillomacular bundle, because in OAGD, where this bundle is preserved, VER changes are similar to those of optic neuropathy.

Summary

In MS, increase of delay may be important, but the earlier change is in the behavior of the wave shape; amplitude is always lower, even in MS patients without visual subjective symptomatology, provided not only flash but also pattern stimulation has been done. Normal amplitude with pattern stimulation does not necessarily mean normal amplitude with flash. Combining both stimulation methods, we obtained lowering of the amplitude and change of wave shape in 100% of cases, while latency was increased in only 77% of cases. Flash and pattern VER might be even more pathognomonic in MS than oligoclonal gamma globulin fractionation on agar, which is positive in 75% in cases. It is suggested that subclinical MS cases should be submitted to full electro-ophthalmographic investigation.

Sectorial ischemic neuropathy and OAGD patients show the same findings, showing that the method is not specific for demyelinating diseases, nor selective for papillomacular bundle involvement, but that VER, considering only its first components, reflects axonal damage somewhere in the visual pathway.

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Quantitative Evaluation of Visual Disturbance in Optic Neuritis. A Follow-up Study of 30 Cases by Static Perimetry

P. HALLER, U. PATZOLD, and H. KOLBE [1]

Acute optic neuritis is a clinical model for the study of the dysfunction of multiple sclerosis (MS) lesions [3]. Pathophysiological approaches have been made by visual evoked potentials [1]. Examination of the size and shape of central scotoma may give a more distinct picture of progression and remission of the lesion.

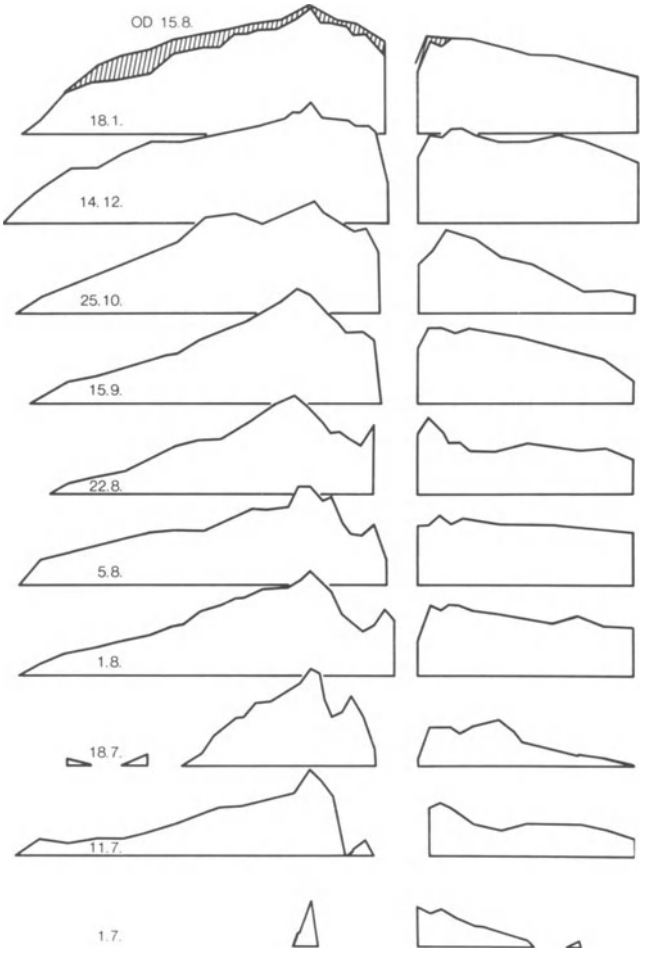

Fig. 1. Profile perimetry curves (0–180 degree meridian representing quantitative and qualitative measurement of optic nerve dysfunction in a case of optic neuritis). Complete recovery of visual and kinetic perimetry, slight diffuse dysfunction in static perimetry

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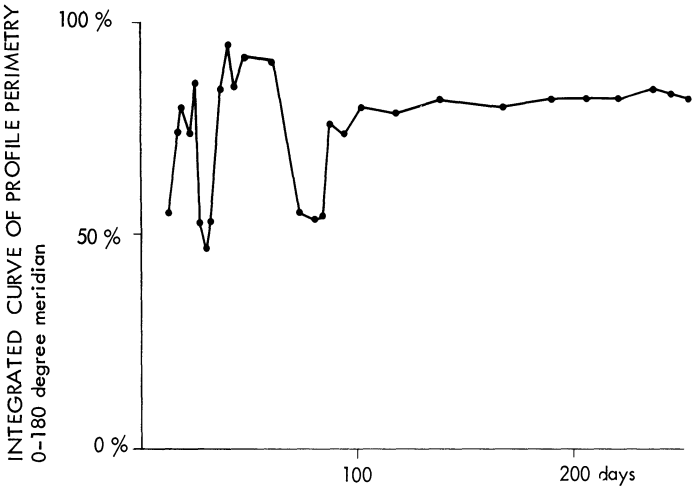

Fig. 2. Quantitative measurement of visual dysfunction in optic neuritis. Acute stage with recovery and relapse; chronic stage with persistent dysfunction

The aim of the following study was to determine whether static perimetry is appropriate for quantitative and qualitative evaluation of dysfunction and whether it can be used in isolated unilateral lesions in the optic nerve.

Methodology

In connection with our prospective epidemiological optic neuritis survey in Hannover, we investigated periodically for 6 months to 2 years, 30 patients using the "Tübinger Perimeter" [2]. More than 2000 investigations were performed by one of us. The exact central fixation was obtained by a special fixation help, so it was possible to perform repeated investigations in 0 degree meridian through the blind spot 60° temporally to 60° nasally, which represents nearly the whole cross section of the optic nerve (Fig. 1). Additional chiasmal or retrochiasmal lesions could be excluded by horizontal static perimetry of the other eye. The amount of dysfunction was calculated by integration of the so-obtained profile perimetry curves (Fig. 2).

Results

Static perimetry makes it possible to determine the localization of the lesion; the degree of visual disturbance can be calculated; it is possible to study the spread of the lesion and the following improvement qualitatively and quantitatively (Figs. 1, 2); and the influence of several factors, such as body temperature, exercise, and drugs can be measured.

The lesion begins near the central optic vein, producing a centroparacecal scotoma. The spread is centrifugal, with a sharp demarcation line from unaffected pe-

ripheral optic nerve fibers. Later an absolute scotoma becomes a relative one with no sharply defined edges.

Finally, during the next 6 months, a diffuse lesion of the optic nerve remains. Remission can occur even after 1 year, and a persistent scotoma is rare.

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Evaluation of Motor Deficits in Patients Suffering from Multiple Sclerosis

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Introduction

It is generally accepted that motor deficits in the lower extremities are among the most important symptoms of multiple sclerosis (MS). An objective analysis of these deficits would seem to be essential for the following reasons: (1) Pathophysiological research has yet to clarify the nature and localization of lesions in different types of muscular hypertonia (i.e., spinal and supraspinal spasticity) or in other motor disturbances like spinal and supraspinal ataxia; (2) Detection of discrete motor deficits (e.g., weak monoparesis, obscure gait disturbances) and specification of abnormalities using qualitative and quantitative methods can be helpful in establishing the diagnosis; and (3) quantitative measurements in motor disturbances are necessary to evaluate the effectiveness of the drugs used in the treatment of motor disturbances.

Numerous investigations on patients with spastic disorders have dealt with the observation and description of reflex phenomena such as the H reflex, recruiting curves, dynamic and phasic stretch reflexes, and tonic vibration reflex [1, 5]. Voluntary movements have so far been investigated only in very restricted numbers. In these cases, polygraphic recordings of the electrical activity from many muscles during active as well as passive movements in spastic patients have revealed some interesting facts [2, 3], even though these results were not expressed in quantitative terms.

There is much evidence for assuming that abnormal or distorted movements may be due not only to abnormal muscle tone, but also to abnormal afferent and efferent transmission via long loop pathways, indicating that motor deficits may be the result of a complex interaction of abnormal voluntary innervation and exaggerated responses to passive muscle lengthening.

In order to meet the research demands outlined above, "bicycling" at different speeds and loads was chosen as an experimental model of a highly stereotyped and reproducible type of movement. This offered the possibility of differentiating between interacting motor abnormalities of active voluntary innervation, postural control, and responses to passive change in muscle length. This paper deals with the qualitative and quantitative analysis of activity patterns in normal subjects and MS patients suffering from lesions in descending motor pathways.

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Methods

A total of ten women and eight men, all in good health, volunteered as normal subjects; their ages ranged from 19 to 59 years. The second experimental group comprised 21 MS patients (12 women and nine men; age–range 21–61 years) suffering mainly from motor disturbances, which, from a clinical point of view, were obviously attributable to lesions of the descending pyramidal and extrapyramidal systems at the spinal level. Five patients showed a positive Babinski response alone and slightly increased dynamic stretch reflexes in the extensor muscles of the lower limbs; ten patients exhibited Babinski responses combined with exaggerated reflexes and sustained cloni; in addition six patients manifested a spastic paraparesis.

In each subject the electrical activity of four muscles – tibialis anterior, medial gastrocnemius, rectus femoris, and biceps femoris – was recorded with bipolar surface electrodes (Ag-AgCl) placed 3–5 cm apart over the belly of each muscle. The surface electromyograms (EMGs) were fed through an amplifier with a frequency response 3 dB down at 60 Hz and 700 Hz and registered on a 16-channel EEG (Siemens, Mingograf). Electromyographic (EMG) data, pedal position, and torque were also stored on a 8-channel tape recorder (HP-3968 A).

The activity patterns of the various muscles were studied during active bicycling on a common ergometer (Siemens, ergometry system 380B) at various loads [1, 2, 4, and 6 kilopoundmeter (Kpm)] and rotation rates (20, 30, and 40 cpm). Prior to each recording session the height of the saddle was adjusted to the length of the subject's legs so that with the knee joint maximally stretched the foot, in the rectangular position, could just reach the pedal. Visual feedback from a tachometer enabled the subjects to maintain a given rotation rate. The lowest position of the right pedal was set to 0°.

The EMG activity was analyzed in the following manner: (1) evaluations of start and end of muscle recruitment, (2) descriptions of activity profiles, and (3) calculations of activity integrals (arbitrary units).

In order to denote the start and end of activity, the original EMGs were full-wave rectified and then voltage-to-frequency converted. The frequency of pulse output was 0–100 Hz (0–1 mV/s). The start of activity was defined as the point where the pulse frequency exceeded 5 Hz and the end as the point where the frequency fell below this value. The EMG activity profiles were analyzed by means of "integral (number of pulses) – versus-pedal angle histograms." The bin width was 20°; each histogram represented the integrals of four rotations (see Fig. 3).

Results

In order to decide whether an observed activity pattern was pathological, the extent of inter- and intra-individual differences in normal subjects had to be determined.

A normal activity pattern during bicycling (Fig. 1) may be characterized as follows: (1) Each muscle is recruited within a distinct range of pedal positions and shows similar profiles in consecutive rotations. (2) In the case of the rectus femoris (see also Figs. 2, 3) and gastrocnemius muscles, an alternating recruitment of both sides

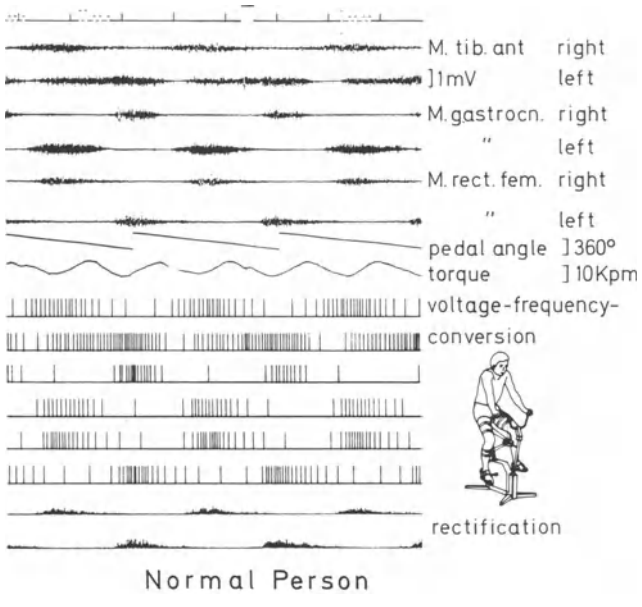

Fig. 1. EMG activity patterns in a normal subject during bicycling at a rotation rate of 20 c/min. Channels 1–6: surface EMGs of various leg muscles. Channel 7: pedal position; the angle at which the right pedal is in the lowest position is set to 0° (top of the triangular signal). Channel 8: torque. Channels 9–14: the pulse frequency conversion of the full wave rectified original surface EMGs. *Lower two rows:* examples of full wave rectified EMGs of both rectus femoris muscles. *Upper row:* time calibration of 1 s (*small vertical lines*)

occurs, whereas the tibialis anterior muscle exhibits a constant level of overlapping activity. (3) In the main, these characteristics are independent not only of load and rotation rate but also of training effects and length of the leg. Stable rotation rates can be maintained at the loads applied in these experiments.

The inter-individual variation (mean and SD) of start and end of muscle activity in 18 normal subjects is presented in Table 1 and Figure 2.

As can be seen, the duration of rectus femoris muscle recruitment is subject to a very small inter-individual variation, being the smallest of all the muscles investigated. This muscle would therefore seem to be most suitable for elucidating pathological phenomena.

Table 1. Start and end of muscle recruitment in normal subjects

Muscle	Start of recruitment (±SD)	End of recruitment (±SD)
Right tibialis anterior	140±126°	281±46°
Left tibialis anterior	227± 44°	85±43°
Right gastrocnemius	275± 31°	88±31°
Left gastrocnemius	79± 28°	259±37°
Right rectus femoris	124± 11°	309±14°
Left rectus femoris	310± 12°	125±12°

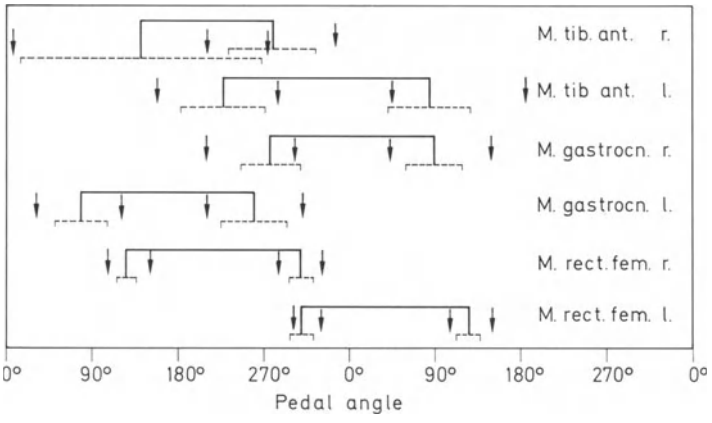

Fig. 2. Start and end of muscle activities in 18 normal subjects during bicycling. *Solid vertical lines:* mean values of pedal angle at which muscle activity starts and ends. *Solid horizontal lines:* mean duration of muscle recruitment. *Broken lines:* SD. *Arrows:* extreme values

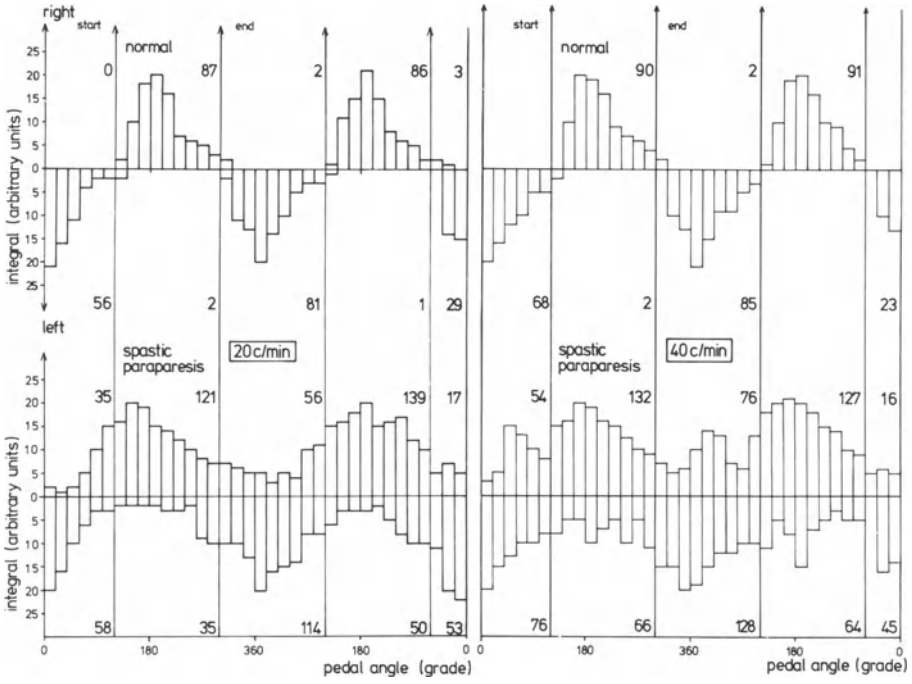

Fig. 3. Integral-versus-pedal angle histograms in a normal subject (*above*) and in an MS patient with spastic paraparesis (*below*). The histograms show the alternating activity of the right (upwards) and left (downwards) rectus femoris muscles at rotation rates of 20 (left) and 40 c/min (right). The bin width is 20° and each column represents the total number of pulses in four rotations. *Long vertical lines* with *arrows* represent the mean values of start and end of activity as calculated from the data obtained with the normal subjects. The numbers represent the total integral in "recruitment" and "rest" periods

Illustrations of activity profiles and calculations of activity integrals are shown in Figure 3. Integral-versus-pedal angle histograms are presented for a normal subject and a patient suffering from spastic paraparesis. Both subjects worked at a constant load of 4 kpm at either 20 or 40 c/min. In the normal subject it is evident that the right and left rectus femoris muscles are recruited alternately; this alternating activity is not dependent on the rotation rate.

In order to make a quantitative evaluation of the extent of the motor disturbances, the integral of the EMG activity during the "rest period" was expressed as a percentage of the integral in the "recruitment period". Use of such percentages has the advantage that sources of variation introduced by the recording conditions can be largely excluded, so that comparisons of the EMG activities can be made both within and between individuals. In the patient with spastic paraparesis the typical premature and prolonged muscle recruitment can be observed. In the two rotations demonstrated in Figure 3, at 20 cpm the percentages in the rest period were 30% and 46% in the right leg and 30% and 43% in the left; at a rotation rate of 40 cpm the percentages increased to 47% and 57%, and 51% and 52%, respectively.

The activity profiles in the spastic patient also showed changes when the rotation rate was increased to 40 c/min. At passive lengthening of the rectus femoris muscle, a peak in activity became apparent, especially on the right side. This phenomenon was only observed in cases with a markedly increased muscle tone.

In patients with only a Babinski response the low percentage (5%–15%) in the rest period was indicative of a pathological situation, although with common clinical methods deficits in posture and locomotion could not be detected. In patients with exaggerated dynamic reflexes and slightly increased muscle tone the percentages ranged from 10% to 32%. In all cases the values were measured at a rotation rate of 20 c/min.

Discussion

Quantification of motor disturbances is a prerequisite for following the course of a disease and evaluating therapeutic effects in individual patients. In practice, however, the assessments of muscle tone performed with current methods often display considerable variability from patient to patient, despite the number of precautions taken.

The present experiments in normal subjects indicate that an analysis of the activity pattern during bicycling is a suitable method for evaluating deficits in descending motor pathways. This kind of locomotion-like movement reveals only small intra-individual differences irrespective of whether consecutive rotations are analyzed in a single experiment or in a number of experiments. The innervation pattern of the rectus femoris muscle in particular is characterized by small inter-individual differences. Training effects and leg length have a more or less negligible influence on muscle activity patterns. A further advantage offered by the present method is the possibility of detecting subclinical motor disturbances (e.g., Babinski response alone). In spastic patients the "rest-period integral-recruitment-period integral" percentages correspond extremely well with the clinically evaluated severity of the motor

deficits. In contrast to a gait analysis, bicycling can also be employed with patients who are unable to walk; only patients with a markedly increased muscle tone had to be excluded.

It must be emphasized, however, that the advantages of movement pattern analysis during bicycling are more apparent in the analysis of knee extensors and flexors than in the case of the gastrocnemius muscles. Moreover, the tibialis anterior muscle, with its wide inter-individual range in normal subjects, does not appear to be suitable for the elucidation of pathological states. These deliberations are substantiated by the physiological roles of the various muscles observed during bicycling: the precise innervation of the rectus femoris muscles is essential for load compensation and maintenance of the required rotation rate, whereas the tibialis anterior sets the ankle joint in such a position as to guarantee optimal transfer of the leg's total muscular strength. The latter function, however, is not absolutely essential for bicycling, as can be shown from observations in patients with lesions of the peroneal nerve.

The main findings in these experiments were the premature recruitment and late relaxation of the extensor muscles during active stereotyped bicycling in patients with lesions of the descending motor pathways. This phenomenon can best be discussed in the light of the accepted pathophysiological mechanisms underlying spasticity. On the one hand, it is common to assign a key position in the production of spasticity to the combined release of the α- and γ-systems. Evidence from animal experiments [4], on the other hand, suggests that in the course of locomotion the α-motoneurons are under supraspinal control not only during muscle recruitment, but also during muscle relaxation. This supraspinal control results in subthreshold excitation or inhibitory action of the α-motoneurons in distinct phases of a movement. These modulations, in turn, are a prerequisite for the compensatory action of segmental and long loop reflexes. In accordance with these considerations the phenomenon of the prolonged recruitment period in spastic patients can be interpreted in terms of hyperactivity in the α- and γ-systems. The increased bombardment via the muscle spindles and the enhanced excitability of the α-motoneurons obviously elicit premature recruitment of motor units in the phases in which these units would normally be in a subthreshold activated state.

The activity observed at maximal muscle lengthening and the higher rotation rate (Fig. 3) most probably represents a dynamic reflex component typical of spastic states.

In summary, it seems reasonable to suggest that the qualitative and quantitative analysis presented here might be of use in the commonly occurring diagnostic and therapeutic problems in MS patients. In cases where only a single lesion can be detected by the usual clinical methods, the elucidation of subclinical motor disturbances would almost definitely confirm the polytopic nature of the disease. Drugs with suggested positive actions can be tested in an objective manner and symptomatic treatment in spasticity can be optimally adjusted.

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CT Scan in the Diagnosis and Assessment of the Course of MS

R. WÜTHRICH [1]

It was originally hoped that computer tomography (CT) would greatly facilitate the diagnosis of multiple sclerosis (MS), the assessment of its course, and research into its pathogeny. Experience has not up till now fulfilled this hope [1–5]. This is chiefly because MS foci only very rarely differ from the surrounding normal brain tissue in their atomic density. The resolution of existing apparatus is not yet fine enough to distinguish between tissues with only small differences in density, and there are also difficulties in recognizing small or very irregularly formed pathologic regions. However, it will be shown in the following paper that CT can also lead to a new insight into demyelinating diseases, despite the above-mentioned difficulties.

Our Investigations

Between 1973 and 1978, 150 cases of MS have been examined in the CT department of the Cantonal Hospital, Basel (95 women and 55 men). The average duration of illness at the time of examination was 9½ years, the average age, 42 years. Twenty patients were examined more than once, not including those re-examined very shortly after the first scan. An injection of contrast medium was used in 49 cases to supplement the native scan procedure.

The diagnosis of MS was "clinically highly probable to certain" in 96 of the 150 cases. The other cases were mostly suffering from the early stages of the disease; in these the diagnosis could not be definitely accepted, despite thorough investigations.

The object of the CT scan was scientific interest only in a third of the cases, while in two-thirds we hoped to obtain more diagnostic information.

Our figures do not therefore represent a systematic series of examinations. They can be considered as giving more or less the picture that results in a population of MS patients who are under observation in a neurological department. The later stages of the disease are under represented. The investigations were carried on with an EMI scanner with the matrix 160/160.

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Results

The following table summarizes results in the 150 patients:

Normal finding	68
Pathological finding	82
External hydrocephalus	14
Internal hydrocephalus	15
Ext. and int. hydrocephalus	33
Foci detected (including 14 foci + hydrocephalus)	30
Findings independent of MS	4

It can be seen that although brain atrophy is found in about half the cases, MS foci are visible only in about one-fifth.

In the first 100 cases examined we tried to correlate CT results with clinical findings. We found satisfactory relationships between degree of disablement and pathological change, between psychic alterations and dilatation of the ventricles, and between atrophy and duration of illness. In a few cases, however, there were large discrepancies between the clinical and radiological results. Surprisingly meager CT findings were combined with marked clinical deficiencies, and there were considerable atrophies which were not suspected from the clinical data. Certain patients with first bouts already showed definite dilatation of the ventricles.

Localized atrophies in the pons, for instance, in the cerebellum, or unilaterally in the hemispheres could be satisfactorily correlated with clinical findings. Only exceptionally did purely spinal forms also show atrophic cerebral lesions.

Very often the foci were not definitely recognizable even after a long duration of the illness. In the 30 cases where foci were found, there were 14 with perventricular low-density zones taken to be foci of demyelination; but these foci could not always be satisfactorily differentiated from artifacts. In the remaining 16 cases there were marked low-density zones without ventricular contact, which in our opinion can be definitely classified as MS foci. Clinically these included eight cases of acute "encephalitic" MS with large bilateral foci in the white matter; in two of these, multifocal zones taking up contrast medium could also be seen. Isolated foci were recognizable in the parenchyma in eight cases, four of which were patients with severe bouts. Only one of these unilateral lesions took up contrast medium. In four patients who were not having bouts there were clearly defined low-density zones, but all these cases had a history of severe bouts combined with cerebral symptoms. Thus in the 49 cases with an injection of contrast medium, only three showed uptake of the medium, all of these being in an acute stage of the illness. The acute cases provided the most information about the course of MS. The formation of foci could be followed; in certain cases they became smaller in circumference, more clearly marked, or disappeared completely.

In some cases the scans were repeated after intervals of up to 4 years. We could see an increase in atrophy in only one case, and this was only to a small extent.

There were relevant secondary findings only twice in 150 cases, both in the form of tumors. In one case of clinically certain MS a meningioma of the convexity of the brain led to focal epilepsy. In another a dermoid of the cerebellopontine angle was discovered by CT scan. An autopsy later confirmed its coexistence with MS, which had been diagnosed before the discovery of the tumor.

Discussion

The incompleteness of our series, and of others published up to now, must be admitted. In fact, experience is lacking in the systematic assessment of the course of the illness and in the regular use of contrast medium. Despite these limitations, there are certain valuable conclusions which can now be reached about CT investigations in MS patients. Extended use of the present methods and the development of new apparatus will doubtless greatly improve the results. We can now draw the following conclusions:

1. Only in exceptional cases can the diagnosis of MS be made directly on grounds of CT investigation. The MS foci are visible in only about one-fifth of all cases and they cannot be distinguished unambiguously from other pathologies of the white matter. The lesions sometimes take up contrast medium and sometimes do not.
2. Atrophic changes are recognizable in the CT picture in about half the cases. They can mostly be correlated with clinical findings, as is also the case with foci.
3. Patients with acute severe bouts are most suitable for investigations into the course of the disease to give insight into the dynamics of its progress. In a course with chronic progression, the time intervals have to be long in order to see changes in the CT picture.
4. CT investigation is an extremely useful tool for the differential diagnosis of MS.
5. Better results can be expected in the near future using new apparatus and techniques.

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Quantitation and Assessment of the Course of Multiple Sclerosis. Some Remarks

T. Fog [1]

In his excellent book about multiple sclerosis (MS) Dr. Bryan Matthews [9] writes that in trying to evaluate any treatment a number of schemes have been proposed, but "these methods are usually extremely time-consuming ..." and he concludes that "there is a serious need for simple and more objective methods of assessment." Science, even clinical science, is often time-consuming, and as long as we have no specific laboratory method, as we have not, we are forced to use clinical registrations, and only these in our research. MS is a chronic disease, and any therapeutic trial in chronic diseases must be a long-term study, and therefore time-consuming; but it must be as precise as possible.

Which method is to be recommended? We must make clear that it is necessary to differentiate between scoring methods based upon neurological signs and demonstrable in a neurological examination, and a disability scale, which must be divided, according to Tore Broman, into two different types [1, 2]. One is a rough estimate of the patient's capacity in daily activity (walking, skill of hands, vision, etc.), and the second is the patient's own reaction to his deficit, his capacity during daily life, based especially upon character and mood. Broman [1] has given a beautiful example of a patient who from a clinical point of view was severely handicapped (a high score) but was able to do full-time, advanced work.

Some of the many different suggestions for schemes do not differentiate between neurological and ability deficits as defined above. Therefore I should like to recommend the following schemes as useful, even if other schemes may exist:

1. Kurtzke's [7] two schemes: (a) neurological impairment; and (b) the disability status scale (ten steps).
2. Those of Broman [2] and his group. They differentiate between the following three types: (a) *Scoring of neurologic deficit and dysfunction*, i.e., rating of neurological deficit according to neuron systems and CNS regions, reminding one of Kurtzke's [7] scheme; (b) *Scales for scoring of regional activity of daily living (ADL) impairment* – This is defined as the extent of the patient's illness as it affects his personal efficiency in the activity of daily living after maximal rehabilitation has been achieved, including the use of technical aids (braces, crutches, etc.). Impairment can be measured for the separate topical regions or body systems and the reduction of performance expressed in percentage value for the region in question or related to the whole man; (c) *Scales for scoring of ability and dependence* – Here ability is defined as social ability, and dependence as the amount of help from other persons.

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The scales are constructed in percentages: Ability from 0 to 100%, dependence from 0 to 100%, i.e., patients are registrated on the plus or/and minus side of the *y* axis, which is zero.

Any method may be computer-designed, but until now only two systems of this type have been published.

1. Poser and Bauer's system [10] comprises a basic documentation form based on a combination of disability and functional, i.e., neurological impairment. This basic documentation sheet is supplemented with a special registration consisting of two optical reader sheets which permit a quantification in three grades. This is then coupled with special studies. Using this system almost 3000 MS patients were registered within a reasonably short time. It has been used in studies of cerebrospinal fluid (CSF) findings, in information on environmental and social factors concerning MS patients, and in other epidemiological studies, follow-up studies, studies in prognosis, studies of clinical types according to course, and in clinical findings, etc. [10].

2. My own system [3] is based on a scoring of neurological signs and symptoms according to a 3–5 point scale for all demonstrable neurological signs, and a total deficit is registered by adding up all signs present. This is a very simple procedure, and it seems useful in the hands of any competent neurologist. We have now modified this to a system [5] based on two forms: (1) a *G form* which involves the patient's data and anamnestic information, to be filled out at the first examination and only once, even if corrections later may be made and (2) a *A form* used for the actual clinical status every time a new neurological examination is made. These two sets of information are transferred to a computer either by means of punched cards or entered on a data-display terminal. In our department, computer programs are available for entering, editing, storing, and printing out these forms (Fortran V). We have just implemented a new system containing a rearrangement of the same information, whereby data are entered directly in a mask at the CRT display terminal with no use of forms. We have written a set of detailed instructions to each form [5], and this may be acquired from us.

When these computer-designed systems are used, a neurologic examination must be performed at regular intervals, for example, in studying therapy. This is a condition when a statistical analysis is to be made. It is also advantageous if only a limited number of people accustomed to this type of work are used, thus minimizing the time-consuming problems. I am using 20–25 min per patient to make our A form.

The selection of patients is, of course, decisive. How many patients and which type of patient is necessary, for example, in a therapeutic trial?

I shall not discuss the diagnostic problem here. We must assume that, as far as possible, definite MS cases must be the basis for the material.

Two different ways to register the course are well known. One is by comparison of the number of relapses before, during, and perhaps after the trial. This is, according to my experience, the most frequent method used and the worse. There are several reasons for this postulate. One is the difficulty in definition of a relapse. According to the definition of the word "relapse," the term must be reserved for a flare-up of earlier existing signs and symptoms. But if so, many relapses will only be exacerbations due to some endogenous or exogenous circumstances which have nothing to do with real activity of the MS in itself. It may be a consequence of the disease and

not due to the activity of some unknown virus or antigen-antibody reaction. No doubt some relapses are a real expression of an increase in the number of fresh plaques localized near earlier plaques, but to differentiate between these two perhaps almost identical clinical manifestations may be extremely difficult. The judgement may often depend on the critical ability and experience of the examiner. I can assure you that I have often had great trouble in making a decision, which must be done, if you are to use the number of relapses as a basis for evaluation.

There is, of course, no doubt if you are confronting a fresh retrobulbar neuritis, a brain stem attack, or some other new combination of signs and symptoms; but if you limit the calculation to these newly emerging signs and symptoms, the demand arises for a convincing study covering several years, many patients, and several examiners. Lhermitte et al. [8] have analyzed the course of 240 MS cases. The mean annual frequency of attacks (i.e., both relapses and bouts) is 0.66 for all patients. They conclude that it should be necessary to follow 590 patients for 1 year or 190 patients for 2 years before being able to attest to the effectiveness of a treatment decreasing the frequency of attacks by 25%! In most cases the relapse rate decreases spontaneously over a period of time, but this was not found in Lhermitte's study. If this decrease in relapse rate is correct, which is my impression – but I never made an analysis – the difficulty in using this rate is only greater.

Therefore, in my opinion, the best clinical method is to select patients with a moderate or more malignant course. You will always be able to find this type of patient (see Raun, Fog et al. sect. xxx). I have called this category "progressive cases". By this term I do not mean cases showing steady progress, but cases with progress due to a more or less steady activity (relapses, attacks, and progress without sudden episodes) demonstrable by frequent neurological examinations. I have no doubt that if some substance had any influence upon the course of MS, this would be measurable in these active cases. They are not so difficult to find if you have some information about the disease's course during the first 3–5 years. If it was more or less steady during these first years, and if the patients have reached a stage with a real handicap, this activity will continue during the following years [4].

By using our scheme [6] we have been able, by regression analysis, to say something about the negative effect of the transfer factor in 12 patients, followed for 2 years, with each patient as his own control; and in a double-blind study on 16 other MS cases treated with transfer factor and compared to 16 other cases treated with a placebo in a 1-year study. We were able to conclude that the disease activity continued during this transfer factor period and that no difference was demonstrable in the rate of activity in the placebo group measured against the treated group.

Of course, other types of registration may be useful in studying isolated functions, i.e., spasticity, paresis, or tremor, as described by Tourtelotte [11] or, as previously mentioned, by using Poser and Bauer's system [10]. My remarks are especially directed at the therapeutic trials in our effort to control the course of MS.

We must face the fact that MS is a chronic disease, and that our efforts to influence any chronic disease will be time-consuming and must be long lasting.

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Multiple Sclerosis Clinical Trials: A Comprehensive System for the Measurement and Evaluation of Neurologic Function

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Abstract

Although many systems have been developed to measure the cognitive, sensory, and motor functions of multiple sclerosis (MS) patients, few are as comprehensive as the Neurofunction Laboratory. Under development since 1960, the Neurofunction Laboratory presently consists of six different qualitative and quantitative evaluation systems for measuring and evaluating neurologic function. The Neurofunction Laboratory includes: (1) a quantitative neuropsychological examination; (2) an instrumented clinical quantitative neurologic examination; (3) a quantitative simulated activities of daily living examination; (4) a cerebral evoked response examination; (5) a video-neurologic assessment; and (6) a neurologist's qualitative examination of neurologic function. In addition, a battery of clinical laboratory tests is included to evaluate organ systems and adversities. The instrumented tests have been evaluated for reliability and validity when administered by trained technicians, and for effects of motivation, learning, handedness, age, and sex. The Neurofunction Laboratory has been used in MS clinical trials to evaluate putative therapies. Methods have been developed to reduce data from clinical trials into composite neurologic functions vs time to facilitate the neurologist's task of determining how near normal function a treatment brings a MS patient. In these ways, the Neurofunction Laboratory offers the neurologist a quantification of the nervous system that is not available with ordinal scale data and hence enables the clinician to assess more objectively the value of a therapeutic treatment.

Measurement Systems in MS

Multiple sclerosis is a human neurologic disease of unknown etiology and variable course for which there is no known effective treatment. Damage to myelin throughout the central nervous system (CNS) produces impairment of sensory, motor, and other neural functions. Although many treatment regimens have been explored over the years, the quality of clinical trials and the objectivity of analysis have varied widely, leading to waste, false hopes, and equivocable scientific information [3].

To improve the evaluation of putative therapy in MS, several measurement systems have been developed. Among the time-tested systems are those developed by

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Kurtzke [8], Fog [5], Bauer [2], Anderson et al. [1], Rose et al. [21, 22], Potvin and Tourtellotte [11], and Potvin et al. [17]. These scoring systems typically utilize a broad variety of tests or observations and thereby reflect the fact that no single test procedure or clinical observation is sufficient to measure or characterize MS. The comprehensive evaluation system developed by Tourtellotte and his group is described in this paper.

This comprehensive evaluation system consists of six different modalities for measurement and evaluation of neurologic functions, some of which are unique and some of which incorporate features of other scoring systems cited above. The evaluation system, known collectively as the Neurofunction Laboratory, includes (1) a quantitative neuropsychological examination; (2) an instrumented clinical quantitative neurologic examination; (3) a quantitative simulated activities of daily living examination; (4) a cerebral evoked response examination; (5) a video-neurologic assessment; and (6) a neurologist's qualitative examination of neurologic function. In addition, a battery of clinical laboratory tests is included to evaluate organ systems and adversities. In the Neurofunction Laboratory, instrumented tests are used whenever possible to detect small but clinically significant changes in neurologic function over time. Concurrent extensive qualitative assessments are also included in order to evaluate functions for which no instrumented tests exist, to provide direct estimates of concordance with quantitative data, and to provide a basis for comparisons with other studies utilizing the more traditional qualitative scoring systems.

The development of the comprehensive instrumented Neurofunction Laboratory to quantify nervous system function has been an important research goal of our group of neurologists, biomedical engineers, neuropsychologists and biostatisticians since the early 1960s. Part of our long-term goal is to bring to clinical neurology a type of quantification of neurologic function which does not now exist. The Neurofunction Laboratory tests have been evaluated for learning, reliability, and validity and have been used in clinical drug trials with MS patients.

Description of the Neurofunction Laboratory

Each of the six evaluations which presently constitute the Neurofunction Laboratory is described in turn below.

The Neuro-Psychological Examination

The Neuro-Psychological Examination consists of standardized tests to evaluate specific higher cerebral functions. Tests used in clinical trials are shown in Table 1.

The Clinical Quantitative Neurological Examination

The Clinical Quantitative Neurological Examination consists of tests to measure vision and specific functions of the upper and lower extramities that are most relevant

Table 1. The neuropsychological examination

Test Name	Task Description	Ability Measured
Peabody picture vocabulary	Identify item in picture	Vocabulary capacity
Eisenson test for aphasia, color naming, and recognition	Name colors and recognize words	Motor aphasia
Double simultaneous (face-hand) stimulation	Sensory function based on ability to perceive simultaneous stimulations	More sensitive and discriminating measure of sensory stimulation than single sensory stimulation tests
Discrimination of right-left and body parts	Identify right from left and body parts	Spatial-verbal capacity
Raven colored progressive matrices	Pattern completion using visual systems of thought	Higher nonverbal reasoning capacity
Hooper visual organization	Relate parts of objects and organize into a whole	Visuo-constructional capacities
Symbol-Digit modalities	Substitution of numbers for geometric symbols (written and oral)	Compare efficiencies of two modalities for the same visuo-perceptial and mental functions
WAIS Subtests: [a]		
Digit symbol	Written substitution of symbols for digits	Oculomotor coordination; sensitive to changes in cerebral efficiency
Picture completion	Perception of small missing details	Perceptual and visual conceptual capacities; sensitive to cerebral function and not efficiency
Picture arrangement	Organizing randomly placed pictures in a logical story sequence	Comprehend a total social or practical situation
Object assembly	Assembling cut-up pieces of 4 objects	Visuo-constructional ability involving common objects
Digits forward	Repeating numbers	Immediate auditory memory
Digits backward	Repeating numbers backwards	Immediate auditory memory
Similarities	Classification of two different items into a single category	Formation of abstract concepts involving verbal reasoning

[a] WAIS denotes the Wechsler Adult Intelligence Scale

to the broad range of clinically established neurologic deficits found in MS patients. In a study conducted by Ivers and Goldstein [7] the most frequent symptoms of MS were found to be retrobulbar neuritis, diplopia, paresthesia, weakness or incoordination of the upper and lower extremities, and gait difficulty. Impairment of the upper or lower extremities is often the result of disabilities such as contractures, weakness, excessive or premature fatigue, incoordination, or paresthesia. These disabilities may act alone or in combination. Prehensility (the grasping function), representing the most important action of the upper extremities, requires adequate strength

Table 2. The clinical quantitative neurological examination tests of vision [11]

Test Name	Measure	Units	Instruments
Corrected near vision	% central visual efficiency	Percent normal	Jaeger chart with glasses
Uncorrected near vision	% central visual efficiency	Percent normal	Jaeger chart without glasses
Corrected distance vision	% central visual efficiency	Percent normal	Snellen chart with glasses
Uncorrected distance vision	% central visual efficiency	Percent normal	Snellen chart without glasses
Near and distance vision	% central visual efficiency	Percent normal	Jaeger and Snellen charts with glasses
Pinhole vision	% central visual efficiency	Percent normal	Pinhole glasses and Snellen chart

of not only the grip, wrist, and shoulder but also requires stamina, reaction time, speed, coordination of movements, proprioception, tracking, dexterity, and protective sensation (touch, pain, temperature). Ambulation, representing the most important function of the lower extremities, requires adequate strength, stamina, reaction time, speed and coordination of movements, balance sense, proprioception, and protective sensation. These functions were emphasized in the selection of tests and the development of the Clinical Quantitative Neurological Examination. To date, 57 tests, conducted on each body side, have been developed to quantitatively evaluate many of the neurologic functions found in the standard neurologic examination [4].

Tests of vision, which use Snellen and Jaeger charts, are listed in Table 2. Vision tests are conducted with and without regular glasses, and with multi-pinhole glasses to help compensate partially for visual defects due to astigmatism and scotomas, when the best corrected vision has not been obtained.

Tests of upper and lower extremity neurologic function are shown in Tables 3 and 4, respectively. One or more tests are used to measure each of the major neurologic functions of the upper extremities (strength, steadiness, reactions, speed, coordination, sensation, and fatigue) and of the lower extremities (strength, speed, coordination, station, gait, sensation, and fatigue).

The Simulated Activities of Daily Living Examination

A battery of timed tests was developed to measure simple activities of daily living (Table 5). Such tasks are of great practical importance to MS patients in putative trials. In one sense, many of these tests differ from those listed in Table 3. The tests focus on a patient's capability to perform simple sensory-motor skills and do not measure separate neurologic functions. For example, one timed test, which requires the patient to unbutton and button a garment as rapidly as possible, reflects a compound measure of the patient's reaction time, speed, and bimanual coordination in

Table 3. The clinical quantitative neurological examination tests of the upper extremities [11]

Test Name	Measure	Units	Instruments
Strength			
Grip	Average maxima of two 3-s trials	Pounds of force	Jamar hand dynamometer
Wrist dorsiflexion	Average maxima of two 3-s trials	Pounds of force	Modified Newman myometer
Extended arm abduction	Average maxima of two 3-s trials	Pounds of force	Modified Newman myometer
Steadiness			
Hand resting tremor	Average absolute movement of three 10-s trials	0.01 G · s/s	Tracking task battery with accelerometer
Arm sustention tremor	Average absolute movement of three 10-s trials	0.01 G · s/s	Tracking task battery with accelerometer
Hand aiming control	Hole diameter minus stylus diameter	In./32	23 hole steadiness apparatus
Arm aiming control	Hole diameter minus stylus diameter	In./32	23 hole steadiness apparatus
Hand force control	Average absolute error of three 20-s trials	Gram · s/s	Tracking task battery with force stick
Arm force control	Average absolute error of three 20-s trials	Gram · s/s	Tracking task battery with force stick
Reactions			
Simple hand reaction time	Average of best 4 of 5 trials	Milliseconds	Reaction-time apparatus with finger release button
Controlled arm reaction time	Average of best 4 of 5 trials	Milliseconds	Tracking task battery with position stick
Hand vigilance	Correct minus incorrect taps for a 7½ min trial	Percent correct	Vigilance lamp apparatus with finger tap board
Speed			
Hand tapping	Number of taps in one 10-s trial	Taps/10 s	Hand tapping board with interval counters
Hand-arm tapping	Number of taps in one 10-s trial	Taps/10 s	Hand tapping board with interval counters
Lower arm-sweep	Time to sweep arm from 10% to 90% of a 40 degree movement. Average of best 4 of 5 trials.	Milliseconds	Tracking task battery with position stick

Table 3. Continued

Test Name	Measure	Units	Instruments
Coordination			
Finger grasping, placing	Number of inserted pins in one 30-s trial	Pins/30 s	Purdue pegboard and timer
Finger grasping, rotating, placing	Time to grasp, rotate, and reinsert 8 pegs	Seconds	Large pegs, pegboard, and timer
Interfinger manipulation	Average number of rotations in two 10-s trials	Rotations/10 s	Eight inch pencil and timer
Lateral reaching, tapping	Corrected index of difficulty/movement time in one 10-s trial	Bits/s	Hand tapping board with interval counters
Random arm tracking	Average absolute error of three 20-s trials	Degree · s/s	Tracking task battery with position stick
Progressive arm tracking	Average reciprocal of effective time delay of 5 trials	Radians/s	Tracking task battery with position stick
Arm-shoulder pursuit tracking	Average of three 20-s trials	Percent time on target	Lafayette rotary pursuit apparatus
Sensation			
Distal touch, finger	Length of filament for which 2 of 3 strokes are felt	Centimeters	Cochet and Bonnet esthesiometer
Proximal touch, upper arm	Length of filament for which 2 of 3 strokes are felt	Centimeters	Cochet and Bonnet esthesiometer
Finger pad vibration	Vibratory movement at threshold averaged for 3 trials	Microns/10	Biothesiometer
Elbow vibration	Vibratory movement at threshold averaged for 3 trials	Microns/10	Biothesiometer
Finger two-point discrimination	Threshold distance for detection of two points	Millimeters	Sweet two-point compass
Distal to proximal joint position	Joint location where position is correctly stated in 4 of 4 trials	Joint number	None
Thermal pain, dorsum of hand	Temperature tolerance for average of 3 trials	Degress centigrade	Thermal pain apparatus
Fatigue			
Grip strength	100 (5th trial)/(1st trial)	Percent fatigue	Jamar hand dynamometer
Hand tapping speed	100 (taps in last 10 s)/(taps in 1st 10 s)	Percent fatigue	Hand tapping board with interval counters
Lateral reaching, tapping coordination	100 (bits/s in last 10 s)/(bits/s in 1st 10 s)	Percent fatigue	Hand tapping board with interval counters

Table 4. The clinical quantitative neurological examination tests of the lower extremities [11]

Test Name	Measure	Units	Instruments
Strength			
Foot dorsiflexion	Average of maxima of two trials	Pounds of force	Modified Newman myometer
Extended leg flexion	Average of maxima of two trials	Pounds of force	Modified Newman myometer
Speed			
Foot tapping	No. of taps in one 10-s trial	Taps/10s	Foot tapping board with interval counters
Coordination			
Lateral movement, tapping	Corrected index of difficulty/ movement time for one 10-s trial	Bits/s	Foot tapping board with interval counters
Station			
Two leg standing, eyes open	Maximum time of three 30-s trials	Seconds	Timer
One leg standing, eyes open	Maximum time of three 30-s trials	Seconds	Timer
Two leg standing, eyes closed	Maximum time of three 30-s trials	Seconds	Timer
One leg standing, eyes closed	Maximum time of three 30-s trials	Seconds	Timer
Gait			
Tandem stepping with handarm aid	Time to take 6 heel-to-toe steps	Steps/s	Parallel bars and timer
Tandem stepping	Time to take 6 heel-to-toe steps	Steps/s	Parallel bars and timer
Sensation			
Distal touch toe	Length of filament for which 3 of 3 strokes are felt	Centimeters	Cochet and Bonnet esthesiometer
Proximal touch, prepatellar	Length of filament for which 3 of 3 strokes are felt	Centimeters	Cochet and Bonnet esthesiometer
Toe vibration	Vibratory movement at threshold averaged for 3 trials	Micron/10	biothesiometer
Tibial tuberosity vibration	Vibratory movement at threshold averaged for 3 trials	Micron/10	biothesiometer
Distal to proximal joint position	Joint location where position is correctly stated in 4 of 4 trials	Joint number	None
Thermal pain, dorsum of foot	Temperature tolerance for average of 3 trials	Degrees centigrade	Thermal pain apparatus

Table 4. Continued

Test Name	Measure	Units	Instruments
Fatigue			
Extended leg flexion	100 (5th trial)/(1st trial)	Percent fatigue	Modified Newman myometer
Foot tapping speed	100 (taps in last 10-s)/(taps in 1st 10s)	Percent fatigue	Foot tapping board with interval counters
Lateral movement, tapping coordination	100 (bits/s in last 10-s)/(bits/s in 1st 10s)	Percent fatigue	Foot tapping board with interval counters

moving to the button, steadiness, and fine finger manipulative activities. In clinical trials, these tests show the largest and most consistent improvements [17].

In another sense, the simulated activities of daily living tests complement tests in the Clinical Quantitative Neurological Examination. The former measure a patient's capability to perform skills and activities relevant to daily living. If this capability is altered in a clinical trial, the latter tests help to delineate which basic components underlie the changes.

Selected tests from Tables 3 and 5 can be organized to focus on the primary purpose of the upper extremities, namely, prehensility. Two important components of prehensility function are tracking and dexterity. Accordingly, the tracking test group includes lateral reaching and tapping, random arm tracking, progressive arm tracking and arm-shoulder pursuit tracking; and the dexterity group includes finger grasping and placing, finger grasping, rotating, and placing, interfinger manipulation, cutting with a knife, using a fork, picking up coins, managing large/small buttons, zipping a garment, typing a bow, manipulating safety pins, putting on gloves, speed of handwriting, dialing a telephone, unwrapping a Band-Aid, squeezing toothpaste, and threading a needle.

Use of the Simulated Activities of Daily Living Examination is especially important for assessing MS therapeutic trials. These simple timed tests are directly correlated to the sensory-motor problems that often cause MS patients to consult a physician. These represent the simple skills that the MS patient is most interested in improving while participating in a putative treatment. Therefore, we feel that all MS putative trials should include at least the Simulated Activities of Daily Living Examination for evaluation of neurologic function.

The Cerebral Evoked Response Examination

The measurement of changes in electric potentials over the cortex caused by sensory stimulation has proved useful for quantifying abnormalities and detecting clinical silent lesions. Since the first visual evoked response study in MS [20], many investigators have alluded to the usefulness of visual, auditory, and somatosensory evoked response testing for the objective evaluation of MS patients in clinical trials.

Table 5. The simulated activities of daily living examination

Test[a] Name	Instruments
Eating	
Cutting with a knife	Timer, plate, fork, knife, and permoplast
Using a fork	Timer, plate, fork, and permoplast
Upper Extremities	
Dressing	
Putting on a shirt	Timer and shirt
Managing large button	Timer and cloth with one large button mounted on a board
Managing small button	Timer and cloth with one small button mounted on a board
Zipping a garment	Timer and cloth with one zipper mounted on a board
Tying a bow	Timer and large shoelace mounted on a board
Manipulating safety pins	Timer and two safety pins
Putting on gloves	Timer and two garden gloves
Communication	
Speed of handwriting	Timer, paper, and pencil
Phonation	Timer
Dialing a telephone	Timer and telephone
Miscellaneous	
Picking up coins	Timer and four coins placed on a marked plastic sheet
Unwrapping a Band-Aid	Timer and Band-Aid
Squeezing toothpaste	Timer, tube of toothpaste, and a board
Threading a needle	Timer, thread, and large-eyed needle
Lower Extremities	
Arising from a chair	
with hand-armchair aid	Timer and chair with arm supports
without hand-armchair aid	Timer and chair with arm supports
Tandem stepping[b]	
with hand-arm aid	Timer and 10 ft parallel bars
without hand-arm aid	Timer and 10 ft parallel bars

[a] All tests measures are the average of two trials. Tests durations are measured using a timer recording to the nearest 0.1 s

[b] Tandem stepping tests are included from the Clinical Quantitative Examination of Neurological Function for completeness

Tests and equipment used to measure cerebral evoked responses in the Neurofunction Laboratory are shown in Table 6. To increase the probability of detecting clinically silent lesions, some of our current studies evaluate MS patients while seated in a sauna with the head exposed. Core temperature is raised + 1° C and a battery of tests is conducted in 30–45 min.

Table 6. Cerebral evoked response examination[a]

Sensory Mode	Type of Stimulus	Equipment
Visual	Single	TV screen with phase-reversing checkerboard display, PDP 11/34[b]
	Repetitive (critical flicker photic driving)	Frequency oscillator and flash, PDP 11/34
Auditory	Single	Pulse generator or gated voltage controlled oscillator, headset, PDP 11/34
	Repetitive (brain stem)	Same
Somatosensory (median and peroneal nerve)	Single	Electric stimulator, SIU[c], PDP 11/34
	Repetitive	Same
Multimodal cognitive	Single	T scope or Graphics display, audiotape recorder, PDP 11/34

[a] Tests are done at room temperature and also with subject in sauna to raise body core temperature +1° C
[b] Programmable data processor
[c] Stimulus isolation unit

Table 7. The cineneurological examination

A. Overview		
1.	Use the red chair. Photograph entire body sitting in red armchair. Forearms are resting on thighs, with hands hanging freely between thighs.	(10 s)
B. Cranial Nerves		
2.	Extraocular movement – Commands to patient are as follows: (close-up) a. "Look left as far as possible, and hold." (2.5 s) b. "Look right as far as possible, and hold." (2.5 s) c. "Look up as far as possible, and hold." (2.5 s) d. "Look down as far as possible, and hold." (2.5 s)	(10 s)
3.[a]	Rapid eye blink – Patient looks at camera. (close-up)	(5 s)
4.	Showing teeth – Patient pulls corners of mouth back (laterally) as far as possible while showing teeth maximally. (close-up)	(2 s)
5.	Putting out tongue – as far as possible, downward over chin. (close-up)	(2 s)
C. Upper Extremities		
7.[a]	Holding out both upper extremities horizontally −90° arm flexion, forearms pronated in extension, hands and fingers in extension. Position is held as steadily as possible. Start film with the patient's hands in his lap.	(10 s)
8.[a] and 9.[a]	Supination – pronation of forearm (with hand in extension) as fast as possible on ipsilateral thigh. Right upper extremity (RUE) for 5 s; left upper extremity (LUE) for 5 s.	(10 s)
10.[a] and 11.[a]	Finger to nose to knee (ipsilateral) as fast as possible – Index finger is used for pointing; other fingers are in flexion. RUE for 5 s; LUE for 5 s.	(10 s)

Table 7. Continued

12.[a] and 13.[a]	Holding out hand, tapping thumb and index finger (opposition) as fast as possible. (close-up). RUE for 5 s; LUE for 5 s.	(10 s)
14.[a]	Pencil rotation on a table – Unsharpened pencil is held vertically with thumb and first and second fingers. Top of pencil is moved away from patients's body and end is tapped on the table after every 180° of rotation, as fast as possible. (close-up). RUE for 5 s; LUE for 5 s	(10 s)
15.[a]	Managing a large button on a table – (Cut off at 10 s). Beginning with hands resting on table, patient unbuttons button, opens the two fabric flaps laterally to touch table, rebuttons button, and rests hands on table – all as fast as possible. (close-up)	
16.	Writing name, address, and date as fast as possible with a pencil, using normal-sized handwriting. (close-up)	(10 s)
	Close-up of the writing on paper, held up by assistant.	(2 s)
17.	Putting on shirt. – Puts on shirt handed to him by assistant as fast as possible. Patient is seated in armchair but sits toward edge of chair seat.	
D. Lower Extremities		
18.[a]	Rising from chair as fast as possible, using hands to push off from chair arms – Patients stands to full height, sits, stands again, etc., as many times as possible in the allotted time.	(10 s)
19.[a] and 20.	Tapping foot on floor (with heel as stationary pivot point) as fast as possible with shoes on feet and patient seated. Right lower extremity (RLE) for 5 s; left lower extremity (LLE) for 5 s.	
21.[a] and 22.	Heel-knee-ankle as fast as possible with shoes on and patient seated, heel of one foot moves all the way up and down anterior aspect of contralateral tibia as accurately as possible. RLE for 5 s; LLE for 5 s.	(10 s)
23.[a]	Walking back and forth six ft across the room as fast as possible – Patient keeps an equal distance from the camera, moving across its field and turning as fast as possible.	(19 s)
Total – 175 s		

[a] Trained technician demonstrates normal performance in the same frame. With proper camera delay-timing device, only one technician is needed in shooting a film

The Cineneurological Examination

A video film is taken of MS patients as they perform simple tasks of daily living (Table 7). To better compare their performance, the asymptomatic technician performs the same tasks at maximum speed next to the patient. The video films provide a permanent visual record of selected neurologic disabilities at crucial points in the course of a clinical trial and are available as an unbiased, open representation of changes, or lack of changes, in neurologic function.

The Qualitative Examination of Neurological Function

Of the six evaluation systems, only the Qualitative Examination of Neurological Function is carried out by a neurologist. All others are administered by technicians who are trained to administer tests and collect data in completely standardized fashion [6].

Table 8. The qualitative examination of neurological function for MS

Assessment	Number of Tests
1. Mental deficiency (general mental status, ability to follow instructions, disturbance of wakefulness and sleep regulation, mental symptoms, global rating)	19
2. Neurologic signs and symptoms (general neurologic symptoms, special neurologic symptoms)	34
3. Evaluation of activities of daily living by inquiry	6
4. Visual system	7
5. Hearing	4
6. Speech, swallowing, and respiration	3
7. Sensation of face and upper extremities	38
8. Reflexes of jaw and upper extremities	5
9. Motor (gait and station, face, neck and upper extremities, trunk and lower extremities)	127
10. Reflexes of the lower extremities and a global assessment	11
11. Sensation of neck, trunk, and lower extremities	33
12. Bauer's qualitative neurological exam	22
13. Fog's qualitative neurological exam	4
14. New symptoms and phase of MS disease course	3
15. Patient's opinion of treatment	4
16. Physician's opinion of treatment and assessment of therapeutic index	6
17. Sibley's 7-day symptom summary (modified)	32
18. Special signs and symptoms and adverse side effects and significance of adverse side effects	48
19. Kurtzke rating examination	9

Table 9. Assessment of therapeutic index [25]

		Efficacy			
		Complete relief of symptoms	Satisfactory relief but not complete	Unsatisfactory relief but not worse	Worsening of manifestations
Adverse Reaction	None	1	3	5	10
	Minor or Acceptable	2	4	6	11
	Major	7	8	9	12
	Unacceptable	13	14	15	16

The Qualitative Examination of Neurological Function is one part of the MS Protocol Book maintained for each patient in a putative clinical trial [24]. A description of components of this examination is found in Table 8. The first 16 sections are performed in the order shown to minimize time spent in moving a patient with severe MS. Most of the tests are scored using 6-point ordinal scales. In addition to including the scoring systems of Bauer, Fog, Kurtzke, and Sibley, the Qualitative Examina-

tion of Neurological Function includes a number of tests modified from Broman and Lorenz [1].

An important part of the physician's opinion of a treatment is the assessment of therapeutic risks vs benefits. A therapeutic index score with a range of 1 to 16 has been developed (Table 9). A numeric grid is utilized in which row categories reflect increasing adverse reactions and column categories reflect efficacy of treatment. For example, a patient who had a major but acceptable adverse reaction and had unsatisfactory relief but did not worsen would obtain a therapeutic index score of 9. The therapeutic index gives a numeric score for assessing the risks vs benefits of a therapy. It also provides a means by which to compare directly the relative efficacy vs risk of more than one therapeutic agent for a given patient in a clinical trial utilizing the crossover design.

Clinical Laboratory Tests

In addition to quantitative and qualitative tests of neurologic function, a battery of clinical laboratory tests is carried out to evaluate organ systems and adversities. The following are evaluated: vital signs (pulse, respiration, temperature, blood pressure, weight); hematology; endocrine; renal; hepatic; gastrointestinal; muscle; immunologic; cardiac; pulmonary (X-ray); opthalmology (visual acuity, tonometry, visual fields); nervous system (X-ray, electroencephalograph, brain scan, computerized tomography of the head, cisternography, electromyograph, neuropsychiatric, CSF).

Evaluation of the Neurofunction Laboratory for MS

During the past decade, major studies have been designed to improve test measures and data displays as well as to evaluate tests in the Neurofunction Laboratory. Normal subjects and MS and Parkinson's disease patients have participated in experiments to evaluate the instrumented tests utilized in the Neuro-Psychological Examination, the Clinical Quantitative Neurological Examination, and the Simulated Activities of Daily Living Examination. Evaluation of tests in the Cerebral Evoked Response Examination is currently underway.

Tests have been evaluated for reliability [9, 12, 15, 23], validity [16], learning [12–15, 19], motivation [9], age [14, 19], handedness [9, 12, 14, 15, 19, 23], and sex [14, 23]. In this way, our group of neurologists, biomedical engineers, neuropsychologists, statisticians, and computer programmers learns more about instruments, test measures, level of training of test administrators, and test instructions. Test are modified, redesigned, or eliminated as necessary. A synopsis of evaluation studies follows.

Data Displays

Over the years, considerable effort has been expanded to obtain more meaningful measures and data displays from instrumented tests. One tracking test display has

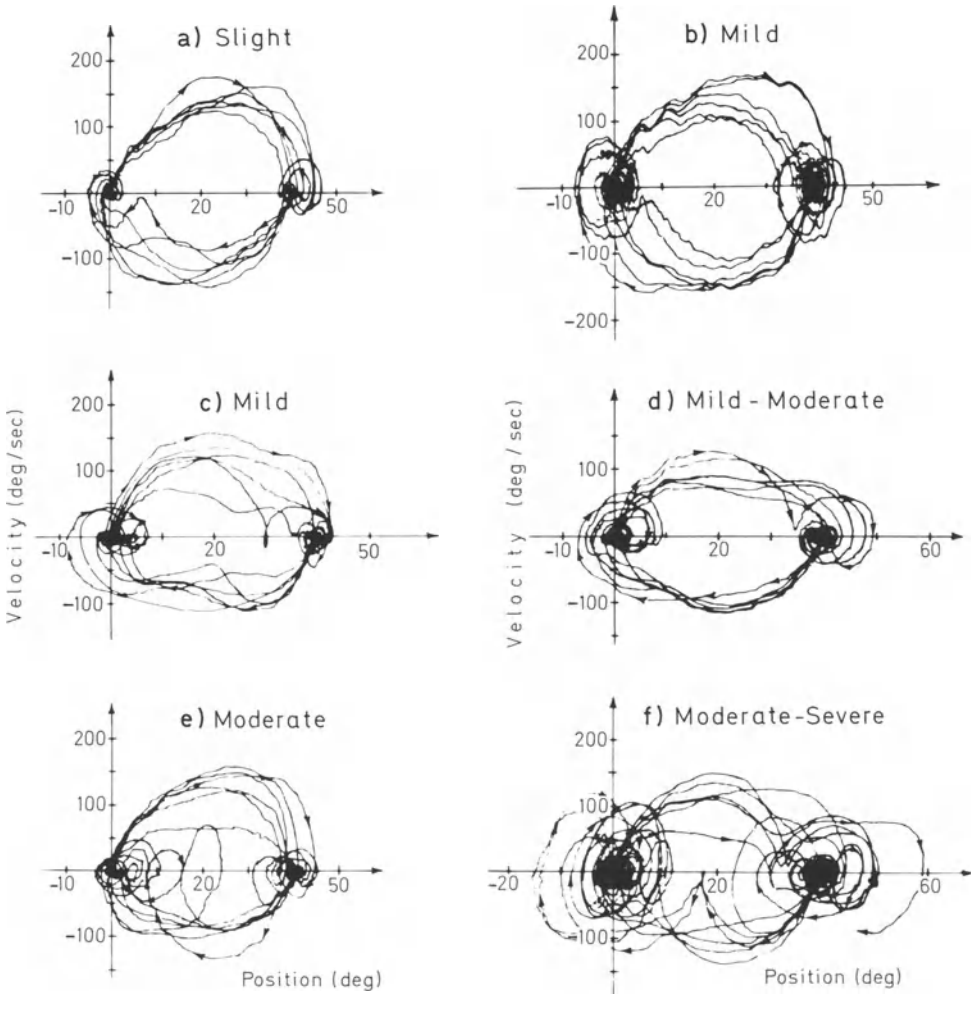

Fig. 1. Phase plane trajectories of six MS patients performing a step tracking task and comparison to a neurologist's judgment in rating the finger-nose test

been developed that is particularly useful in MS, namely, phase-plane trajectories of a step-tracking test. In step tracking, the patient grasps a position stick and makes hand-eye coordinated movements as dictated by instantaneous movements of a line on a display screen [18]. Although different from the neurologist's finger-nose test (for observing intention tremors and precision of coordinated movements), this test successfully elicits many of these hand-eye movement characteristics [10]. Application of a mathematical technique called phase-plane analysis preserves in graphic form the interesting movement characteristics (see Fig. 1). As can be seen, phase-plane features appear to be in close agreement with the neurologist's judgment in rating the finger-nose test. From the display the neurologist can almost visualize an MS patient doing the finger-nose test.

Table 10. Neurologic function of MS patients and normal subjects in the simulated activities of daily living exam expressed as a percentage of younger adult normal function [14]

Test	Younger adult normal function[a]						Percentage of younger adult normal function			
	20 males		20 males + 20 females		20 females		10 Matched normals for MS patients		10 MS patients	
	Mean	±2 SD	Mean	±2 SD	Mean	±2 SD	%	SD	%	SD
Two leg standing, eyes open			30.0	0.0			100.0	0.0	55.0	48.5
One leg standing, eyes open			30.0	0.0			100.0	0.0	4.5	9.6
Two leg standing, eyes closed			30.0	0.0			100.0	0.0	21.7	41.6
One leg standing, eyes closed			30.0	0.0			91.8	18.7	0.0	0.0
Tandem walking with supports			3.3	0.8			87.6	16.1	19.6	18.1
Tandem walking without supports			3.2	0.9			87.5	17.1	7.4	15.8
Putting on a shirt			7.4	3.9			105.0	36.3	41.4	25.9
Managing three visible buttons			11.7	3.9			114.0	16.3	40.3	17.0
Zipping a garment			2.2	0.9			94.9	14.6	41.6	17.8
Putting on gloves	5.4	2.5			6.4	3.0	93.0	34.7	57.2	26.5
Dialing a telephone			8.7	1.4			92.5	7.3	59.0	15.8
Tying a bow			6.4	2.4			112.4	20.4	47.6	18.9
Manipulating safety pins			5.0	1.9			115.0	22.3	42.5	17.5
Picking up coins			5.2	1.2			92.5	13.0	34.9	16.3
Threading a needle			3.3	2.5			122.0	40.2	39.1	20.2
Unwrapping a Band-Aid	8.8	3.0			10.3	4.1	83.4	25.9	30.5	18.5
Squeezing toothpaste			2.8	1.4			107.0	25.4	36.4	11.3
Cutting with a knife	5.9	2.4			7.4	2.4	110.4	21.9	45.6	20.2
Using a fork			1.4	0.5			100.0	23.6	42.2	12.7

[a] The scores for younger adult males and for younger adult females are not combined when the difference in means is at least 10% and $p < 0.05$. Test units are in seconds

Test Measures

Methods for expressing neurologic function of patients and for reducing data so that other investigators can readily interpret a study are of great importance. A first step is to eliminate the need for test units and normative ranges by expressing the neurologic function of patients as a percentage of function of normal controls matched for age and sex. In analyzing the results of a neuropharmacologic trial, the clinician is

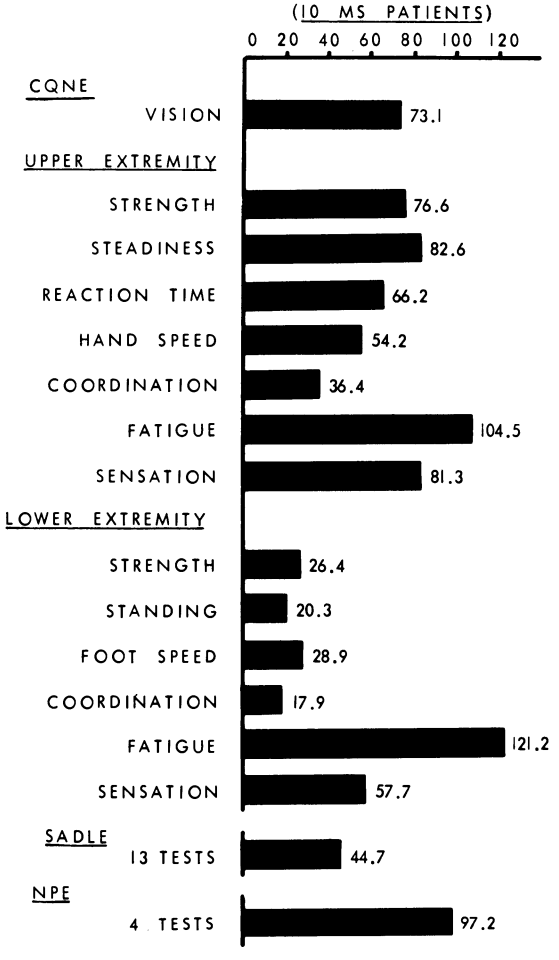

Fig. 2. Neurologic function of 10 ambulatory MS patients expressed in functional categories or composites as a percentage of normal function matched for age and sex. Abbreviations include: CQNE – Clinical Quantitative Neurological Examination; SADLE – Simulated Activities of Daily Living Examination; and NPE – Neuropsychological Examination [14]

interested in knowing how close to normal the treatment has brought the patient and in knowing that the functional level of the patient is double or triple his functional level on entry to the trial. Therefore it is reasonable to express the neurologic function of patients in all tasks as a percentage of normal function. Many tests show significant differences in function of males and females and, therefore, require that function be matched by sex. As discussed below, many tests show significant effects of age on function and therefore require that neurologic function be matched by age. Once the function of patients in neuropharmacologic trials is expressed as a percentage of age- and sex-matched normal function, data may be reduced by combining individual tests into meaningful indexes of neurologic function.

An example of MS data expressed as a percent of normal function is illustrated in Table 10. For tests of dexterity, MS patients perform at 30%–60% of young adult normal function. In Fig. 2, the function of MS patients is displayed in a bar graph for simple evaluation. The percent normal function shown for each neurologic function was obtained by expressing each relevant test measure as a percent of normal function and averaging. Casual inspection of the data reveal that lower extremity function is considerably less than upper extremity function, as expected for MS.

Reproducibility

Reproducibility of test measures has been evaluated utilizing a 4×4 Greco-Latin square experimental design. Eight normal subjects were tested under various conditions (different examiners, different times of the day, different days, and different weeks). The results indicated good inter- and intra-examiner reproducibility once the examiners (two clinicians and two physical therapists) were carefully trained [9, 23].

The evaluation of the test administration training program is very important. The quality of training received by the physical therapists who administer the tests and collect raw data reflects directly the degree of precision that can be obtained in assessing neurologic function. That is to say, data are no better than the method used to obtain them. It is of interest to cite that after several years of test development, an experiment designed to evaluate our training program revealed the training was in some ways inadequate [6, 23], indicating that appearance of meticulous training is insufficient. Following precise retraining, the evaluation studies were conducted successfully [6], and later, training techniques were extended for use in a multicenter clinical trial. These results indicate that technicians are as capable of administering the tests as clinicians.

The results of this evaluation of the test battery had important implications. The clinician's time with his patients is not significantly increased, since technicians administer tests and analyze data. Administration of tests by technicians ensures a more perfect "blinding" of a study since they are less likely to detect telltale effects of a treatment than a clinician.

Motivation and Short-Term Learning

A study was designed (1) to investigate the success that clinicians and technicians achieve in uniformly and adequately motivating subjects, (2) to compare two different kinds of normal control groups that are often used for evaluating therapeutic trials, and (3) to assess the effects of short-term learning (0.5 h test–retest interval) among patients and normal subjects on successive examinations [13].

Twenty normal younger adult subjects, 20 normal adult subjects, and 20 patients (10 with MS and 10 with Parkinson's disease) participated in the study. Each subject group was divided into two subgroups, a control subgroup and an incentive subgroup. The substantial monetary incentives and verbal encouragement, in addition to that ordinarily given by the examiner, that were given to the incentive subgroups (but not to the control subgroups) were found insufficient to improve differentially the function of the matched subgroups on a subset of the tests in the battery. For data relevant to MS, see Table 11. These results indicate that clinicians and technicians are capable of motivating subjects uniformly and adequately.

Analysis of the control subgroup data for short-term learning effects revealed that younger adult normal subjects showed the largest improvement in function and older adult normal subjects showed less improvement. However, patients with MS and Parkinson's disease showed essentially no improvement. From these results, it is doubtful that appreciable improvements in the function of MS patients in clinical trials can be attributed to learning.

Table 11. Comparison of amount of improvement made between younger adult normal subjects and MS patients when offered financial incentives [13]

Test[a]	20 Younger adult normals			10 Patients with MS		
	Mean Exam 1	% improvement	Paired |t| value	Mean Exam 1	% improvement	Paired |t| value
Grip strength, D	74.3	15.3	4.79[e]	65.0	0.9	0.40
Grip strength, N	67.0	12.3	4.76[e]	58.0	8.6	1.52
Simple reaction time	8.4	12.3	5.06[e]	13.4	9.1	3.20[c]
Hand speed, D	70.8	2.7[b]	1.52	43.3	26.7[b]	4.21[e]
Hand speed, N	62.2	3.4[b]	1.77[d]	40.3	23.6[b]	3.46[e]
Hand coordination, D	36.9	8.5	3.20[e]	14.6	11.0	0.96
Hand coordination, N	30.7	9.4	5.65[e]	15.7	10.2	0.45
Hand coordination errors, D	1.4	41.7[b]	1.35	0.4	45.0[b]	1.92[d]
Hand coordination errors, N	1.4	0.4[b]	1.10[e]	0.3	30.0[b]	1.41
Rotary pursuit, 60 rpm-D	13.7	253.8	5.21[e]	0.9	21.5[b]	0.80
Rotary pursuit, 60 rpm-N	8.9	188.8	4.28[e]	1.1	43.4	1.31
Purdue pegboard	14.8	7.6	3.49[e]	5.1	3.0	1.00
Small peg rotation	11.5	8.2	4.43[e]	29.7	5.1[b]	1.10
Vibration sense of finger	0.3	15.8[b]	0.44	11.7	55.0[b]	1.36
Managing 3 visible buttons	11.3	7.5	2.70[c]	35.9	6.0	0.26
Putting on gloves	5.7	0.3[b]	0.50	13.4	4.7[b]	0.45
Manipulating safety pins	5.1	9.4	1.89[d]	14.6	5.3[b]	1.15
Picking up coins	5.2	8.2	3.23[e]	18.5	10.9	1.24
Threading a needle	2.9	20.9	2.65[c]	18.0	5.4[b]	0.62

[a] Scores for the D (dominant) and N (nondominant) body sides are not combined for any group of subjects when, for any group, the difference in means is at least 10% and $p \leqq 0.05$. All subjects declared themselves right-handed

[b] Indicates worse performance in examination 2

[c] $p \leqq 0.05$

[d] $p \leqq 0.10$

[e] $p \leqq 0.01$

Reliability, Long-Term and Short-Term Learning Effects

A study [15] was designed to evaluate the reliability of test measures in a manner consistent with the administration of the tests in clinical trials. A 1-month test–retest interval was chosen. Normal younger adult subjects were used since large variations in patients' performance could justifiably be attributed to disease progression and to exacerbations. Alternatively, the large variation in patient function could artificially inflate reliability coefficients. Twenty normal younger adult subjects were evaluated once in all tests and 1 month later were contacted for reevaluation. At the completion of the second examination, the subjects repeated a subset of tests to evaluate differences between long-term (1 month test–retest interval) and short-term learning (0.5 h test–retest interval).

The results of the study indicate that: (1) most test measures are reliable, and (2) there are no important differences between short-term and long-term learning effects. Of 72 tests evaluated, the tests for fatigue, error, vibration, using a fork, and

zipping a garment were found to be unreliable. With respect to learning effects, the largest improvements were found for rotary pursuit followed by many of the tests of coordination, particularly those requiring manual or finger dexterity. Only slight changes were found for tests of vision, strength, and speed of hand and foot. Since this study, unreliable tests have been modified or eliminated from the Neurofunction Laboratory.

Age Effects

Two studies were designed to examine the effects of age on neurologic function and to assess the importance of selecting proper control groups for assessing clinical trials. In the first study [14], 40 young adult normal subjects, 10 patients with MS and their 10 normal spouses, and 10 patients with Parkinson's disease and their 10 normal spouses participated in this study. Each subject group had equal numbers of males and females and differences due to sex were taken into account prior to analysis for age effects. Significant decreases in neurologic function with increasing age were found for: (1) tests requiring fine coordinated movements of the dominant hand, (2) tests of steadiness performed with the arm in a supported position, and (3) tests of reaction time, tandem gait, and sensation. Nonsignificant decreases in neurologic function with increasing age were found for: (1) most motor tests performed by the nondominant hand, (2) tests of steadiness performed with the arm in an unsupported position, and (3) tests of vision, strength, and speed. Where there were significant differences among the three normal subject groups, it was the oldest normal subject group that differed from the two younger subject groups. Normal younger adult neurologic function was not significantly better than that of normal subjects in the age range of patients with MS; but, normal younger adult neurologic function was significantly better than that of normal subjects in the age range of patients with Parkinson's disease, especially on tasks requiring fine skilled movements of the dominant hand and coordinated activities of the lower extremities. These results indicated that the neurologic function of young patients with MS could be expressed as a percentage of the function of either age-matched normal controls or normal younger adult controls. The neurologic function of patients with Parkinson's disease and the older MS patients could be expressed only as a percentage of the function of age-matched controls.

In the second and more recent study [19], 61 normal male subjects from 20 to 80 years of age were evaluated on two occasions using a comprehensive battery of 138 instrumented tests of neurologic function. The reliability of each test measure was determined and any measure found unreliable ($r < 0.41$) was not further analyzed. Significant age-related linear decreases were found for almost all neurologic functions. Percent decline over the age span varied from less than 10 to more than 90 percent for different functions. For the upper extremities, the largest declines ($> 50\%$) were associated with hand-force steadiness, speed of hand-arm movements, and vibration sense; for the lower extremities, the largest declines were found for one-legged balance with eyes closed and for vibration sense. For 13 of 14 tests where significant dominant body side-effects were found, larger losses in function with aging were observed on the dominant body side. Subjects improved their scores on

retest 7–10 days later by more than 5% on only 17 tests, 9 of which were activities of daily living tasks. No significant differential learning effects were found across age groups. The results again point to the importance of developing an age-based neurologic function data bank so that patient therapy can be evaluated in terms of age- and sex-matched normal function.

Clinical Applications of the Neurofunction Laboratory for MS

From 1965 to 1968 a multicenter (USA) clinical trial was conducted to compare ACTH with placebo for the treatment of MS patients in a stage of acute exacerbation. Our quantitative neurologic examination was one of the seven evaluation systems used in the multicenter trial. The quantitative neurologic data from the study have recently been reevaluated [6a] using a transformation of the data which expresses patient scores in terms of a percentage of age- and sex-matched normal function followed by a reduction of the data into composite neurologic functions vs time as seen in Fig. 3. Remarkable data reduction was accomplished. In the original report of the study, data presentation consumed seven full pages of numeric data (three tables with 1,134 numeric entries). Visualization of the overall treatment effect was difficult and exceedingly tedious and time-consuming. Since data for each test was entered in raw form (such as pounds of force for strength tests or taps per 10 s for speed of hand), the maze of numbers made it very difficult to determine if a change from 42.5 to 47.0 hand taps in 10 s was clinically important. Further, the numbers did not relate to the clinician's goal of returning a patient toward 100% normal function.

The results displayed in Fig. 3 support the positive findings of previous reports without loss of clinically important statistical information. In addition, the methodology helps to simplify the task of interpreting results by enabling the clinician to determine how near normal function the patient came following the treatment trial.

Our group visualizes a day when the clinician will make a diagnosis and send the patient to the Neurofunction Laboratory for a workup in much the same way that he now sends the patient to the EEG or EMG Laboratory or to the Stress Test Laboratory in Cardiology. Precise measures of neurologic function will be obtained and stored in a computer system for ready retrieval. If the patient returns for a follow-up examination several months later, the patient will again be sent to the Neurofunction Laboratory. It will be possible to readily assess changes in neurologic function in terms of percent normal function, which over time will help the physician to assess accurately disease progression, the efficacy of a treatment, or toxic effects. If the patient subsequently suffers a stroke, or is given a drug, or is given rehabilitation therapy, etc., precise records of the amount of change in neurologic function will be available. If the patient begins to take drugs prescribed for other medical reasons, such as birth control pills, any adverse effects on the nervous system can be readily established, and on an individual patient basis. Alternatively, if the patient changes jobs, the effect of an on-the-job toxicity could be detected. In short, the Neurofunction Laboratory can be used to maintain records of standardized tests of neurologic function on an individual patient basis as well as data for large-scale therapeutic trials or toxicosis.

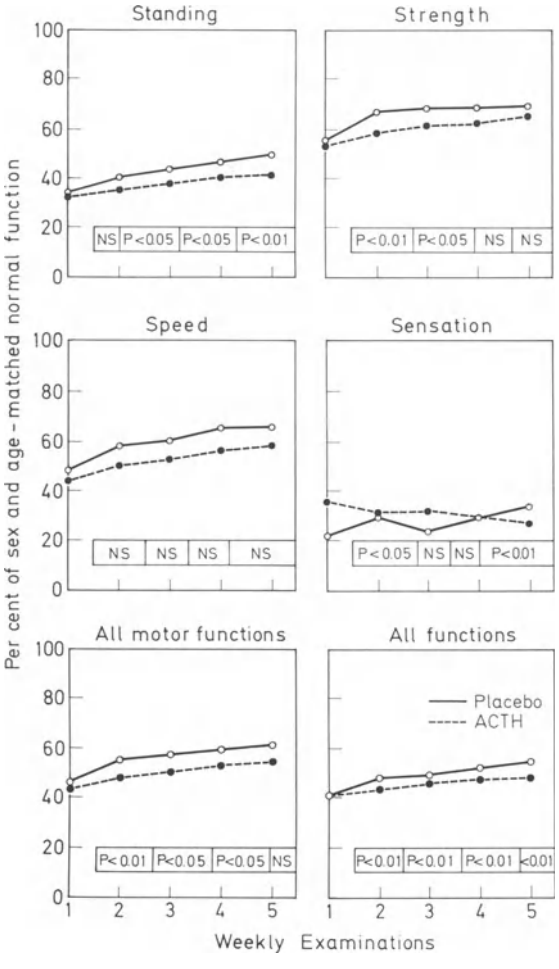

Fig. 3. Lower extremity percent of normal function for similar rests combined at each examination period for the placebo (n = 94) and ACTH (n = 103) groups. Statistical significances are given for the comparison of the placebo vs ACTH groups with respect to mean changes from the pretreatment exam No. 1 to each of the following exams [6 a]

Summary

Our group has developed a comprehensive Neurofunction Laboratory that has been evaluated to ascertain reliability and validity, and to assess the effects of age, handedness, sex, learning, motivation, and training of technicians. Numerous data analyses and reduction schemata have been investigated; however, the most meaningful appears to be to relate the function of patients in terms of age- and sex-matched normal function. With this method, the data display has a direct relationship to the physician's and patient's goal of returning to 100% of age- and sex-matched normal function. Comparison of the clinical impression of treatment with the quantitative test battery in clinicial trials has given consensual validity to our procedures.

In this report, a type of model Neurofunction Laboratory to quantitate and serially evaluate neurologic function has been presented. The laboratory can be used by

a clinical neuropharmacologist to evaluate precisely the effect of putative treatment in MS.

Acknowledgments. Supported in part by The National Multiple Sclerosis Society, the Kroc Foundation for the Advancement of Medical Science, VA Medical Research Funds, and The University of Texas at Arlington Organized Research Funds. The authors gratefully acknowledge permission to reproduce material from the following: Arch Phys Med Rehabil 54:432–440; Arch Phys Med Rehabil 56/10:425–547; Clin Pharmacol Ther 24/2:146–153, copyrighted by the C. V. Mosby Company, St. Louis, Missouri, USA; J Chron Dis 26:699–717, copyrighted by Pergamon Press; and Prog NeuroPsychopharmacol 2:533–578, copyrighted by Pergamon Press.

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Clinicopathological and Epidemiological Documentation of Patients with Multiple Sclerosis

S. Poser [1], H. J. Bauer [1], and C. Th. Ehlers [2]

For the collection of basic information on multiple sclerosis (MS) patients a standardized documentation system was developed within the framework of the Research Program of the DFG (Deutsche Forschungsgemeinschaft) "Etiology and Pathogenesis of Multiple Sclerosis and Related Diseases" [4].

The clinical data are recorded on two optical reader sheets during examination by the physician, read by an optical reader, and analysed by an IBM 370/158 computer. Different programs allow for the statistical analysis of data on all patients recorded and for individual follow-up in patients of special interest.

Of the 2300 cases recorded, data on 1271 patients from 21 different hospitals have been analyzed thus far. 41% were classified as clinically definite, 50% as probable, and 9% as possible cases [2]. The course of the disease was primary remitting in 31%, remitting and chronic progressive in 50%, and progressive from onset in 19%; 59% of the patients were females, and 41% males.

Table 1. Symptomatology of 1271 MS patients

Disturbance	At onset	Course	Present condition	During total course (mean duration 11 years)
Spasticity (Babinski)	19%	77%	78%	85%
Paresis	43%	83%	77%	88%
Sensitivity	41%	78%	73%	87%
Optic nerve	36%	51%	52%	66%
Eye motility	13%	25%	15%	34%
V/VII	7%	16%	13%	23%
Brain stem/cerebellum	23%	69%	75%	82%
Cerebral	4%	31%	36%	39%
Vegetative	10%	58%	56%	63%

The symptomatology of these 1271 cases at the beginning, during the further course, at present examination, and during the total course of the disease is given in Table 1. The reversibility of signs and symptoms is reflected by the difference between "during total course" and "at present examination." The rate of reversibility was high for diplopia, optic nerve involvement, and other cranial nerve disturbances, whereas spastic pareses showed the lowest degree of reversibility.

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For more detailed study the following groups of patients were selected from the total series:

1. Patients with an exclusively spinal symptomatology throughout the course of their disease ($N = 109$, [5]).
2. Patients with early and severe involvement of brain stem functions ($N = 110$).
3. Patients with optic neuritis at onset ($N = 441$).
4. Patients with a benign course (disability of not more than grade 3 according to Kurtzke [3] after a duration of the disease of more than 14 years, $N = 64$).
5. Patients with a malignant course (disability grade of seven or more after a duration of less than five years, $N = 35$).
6. Families with more than one member with MS ($N = 83$).
7. Patients of the epidemiological area of southern Lower Saxony ($N = 195$).
8. Female patients with a history of pregnancy and/or intake of oral contraceptives.

The last group is analyzed in more detail in Figure 1. To test for a possible influence of oral contraceptives on the disease, the present disability of patients with similar duration of the disease and similar gestation history is compared for "takers" and "non-takers". Three categories are registered: no pregnancy, pregnancy before disease onset, and pregnancy during the disease. Figure 1 shows that the level of disability of patients who had never been pregnant was lower for "takers" compared to "non-takers", whereas in patients with a previous pregnancy the disability level did not differ for the both groups.

These results may indicate that oral contraceptives are not used by severely disabled patients as often as by the less disabled, but a positive effect of oral contraceptives on the early course of the disease – the patients without pregnancy were younger than the patients with previous pregnancies – is not ruled out. Arnason and

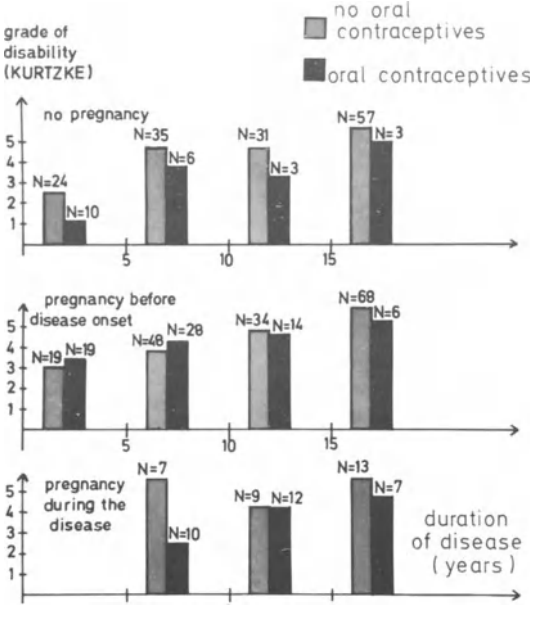

Fig. 1. Disability of women with MS. Comparison of "takers" ($N = 118$) of oral contraceptives and "non-takers" ($N = 345$)

Richman [1] could demonstrate that oral contraceptives suppressed experimental allergic encephalomyelitis (EAE) in animals. An analysis of the different components showed that ethinyl estradiol inhibited EAE, while medroxyprogesterone acetate failed to do so.

Whether this effect of estrogens on delayed hypersensitive responses is also exerted in MS, remains to be determined by further study. At present we can reassure patients with MS that oral contraceptives do not have an unfavorable effect on their disease.

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Taxonomy and Multiple Sclerosis

G. W. ELLISON and L. W. MYERS [1]

Professor Bauer has presented the results of the International Medical Advisory Board (IMAB) Enquete concerning the Diagnostic Criteria for Multiple Sclerosis (MS). On page 16 of the Enquete he outlined a classification scheme for MS. We wish to address some of the problems the scheme approaches.

In the Enquete, Professor Bauer makes several references to the efforts of Rose et al. [4]. Their classification scheme was based upon the experience of Drs. Rose and Tourtellotte and upon the efforts of Myers and Ellison to classify their patients in a busy MS research clinic. We are still thinking about the taxonomy and nosology of MS.

The logical approach to nosology of disease proposed by Alvan Feinstein seems particularly appropriate to the problems we face in MS [3]. Feinstein also has wrestled with the use of "details of categories used in the classification" and has even provided us with a name for the effort – Taxonorics [2, 3]. Professor Bauer has been responsible for introducing ideas about the relative certainty (probability) of the clinical diagnosis [1]. The Enquete reflects his and our continuing concern about accurate diagnosis and classification.

The division of the taxonomy of MS with which we must deal is the "denomination". Our efforts in this direction are guided by the purpose to which we will use our classification. By denomination we summarize our definitions of what we mean by MS. Denomination is divided into several categories to help organize our thoughts. These categories are –

1. The focal "essence"
2. The type of reasoning
3. The scope of inclusion
4. The method of aggregation
5. The assignment of weights
6. The consequences of exclusion [3].

The focal essence of MS in the clinic is multiplicity. There are multiple signs and multiple symptoms serving as sets which can be represented by Venn diagrams (Fig. 1).

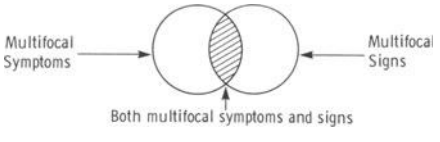

Fig. 1

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The type of reasoning employed is empirical. That is, we classify patients by using observed evidence – either plaques under the microscope or signs and symptoms of disseminated dysfunction in the central nervous system (CNS).

We also try to use inferential reasoning. We infer from the presence of multiple signs and symptoms that there will be multiple lesions in the CNS. Also, we might observe that oligoclones are present, but we infer that these clones indicate an aberration in the immune response of the CNS.

The scope of inclusion for most medical problems and diseases has been based on "selective exclusion". We use neurologic signs and symptoms and we might also employ certain supportive laboratory signs to define the person with multiple sclerosis. For our purposes the color of hair or eyes or the weight and height are not useful and are excluded. If we used all the data available the scope of inclusion would be characterized as "encompassing inclusion" and would overwhelm us in detail. However, it is the large amount of detail which forces us to select and which provides leads for research upon MS.

The method of aggregation appears to be conjunction rather than addition. If we use multiple signs as one set and multiple symptoms as another set, where these sets overlap is called a conjunction of sets according to Boolean algebra. But the conjunction of multiple signs and multiple symptoms is not sufficient to describe multiple sclerosis. Another dimension is needed – time. As time passes, the multiple signs and symptoms must occur several times.

There is great importance attached to the assignment of weights. Although we have not used the additive method of aggregation, we have to deal with the relative importance of the various factors used to diagnose and classify MS. Clinically we recognize certain signs as especially good indicators of MS (e.g., median longitudinal fasciculus syndrome, or the combination of optic neuritis and transverse myelitis). Other signs (e.g., weakness, loss of vibration sense) are less selective for MS. If in the future it should be decided that laboratory criteria will be used to indicate classes of patients, we would expect that oligoclones in the CSF would weight heavily toward the diagnosis of MS whereas an elevated CSF protein would be less specific.

And, in the overall assessment of the patient, weighting is also implied. The IMAB has suggested the use of probablistic qualifying statements, e.g., clinically definite MS, clinically probable MS, and clinically possible MS, to characterize the people we study. We agree with the principle of asserting the probability of MS in a patient but take issue with the criteria for clinically probable MS. Perhaps we might recast the definitions of clinically probable MS to:

A) History of relapsing and remitting symptoms with signs only of unifocal white matter disease.
B) A documented single bout of signs and symptoms of white matter disease of the type often encountered in MS, e.g., median longitudinal fasciculus syndrome.
C) Either A or B with a spinal fluid examination in which oligoclonal bands or increased IgG production within the CNS is lacking. (Parenthetically, we might call these observations "laboratory signs" for MS.) Mononuclear pleocytosis may or may not be present.

It is apparent that subclasses of MS patients exist. We must characterize the course. Thus we can classify a person as having MS of the progressive type, of the

relapsing type, of the conjunction relapsing, and the progressive type, and we can even use subsets and classify some patients as benign (Fig. 2).

Finally, we must deal with the consequences of exclusion. As time passes and advances occur in the laboratory or in the field, we find our classification inadequate to the task. Perhaps, just such a situation exists now. It appears that the IMAB is trying to bring laboratory "signs" into our consideration. Perhaps we should also

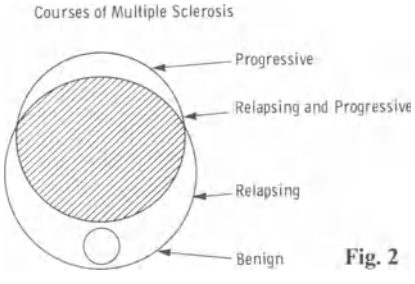

Fig. 2

use abnormalities found with newer diagnostic techniques (e.g., visual-evoked potential aberrations) as "signs" of white matter dysfunction. Under the rules of denomination, we are permitted to develop and use supplementary taxonomies to take into account these new findings. Then, we draw closer to the goal of a classification is "all inclusive and mutually exclusive."

In summary, the goal of the IMAB to achieve a universally acceptable classification scheme of MS seems most desirable to us. Using the rules of taxonomy, we can achieve such a classification. But when new techniques increase our understanding of the etiology and pathogenesis of the disease, we must be prepared to again revise our theories.

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Discussion

T. Broman: I would like to make some comments concerning the assessment of the course of MS. In my opinion the purpose of the study should always determine the methodology in the collection of data on a category of patients. The methodology must be different in special studies on possible effects of treatment, in geomedical studies on the prevalence of the disease, or in a study of the natural history of the disease. For special studies on therapy you will always have to rely on selected material, as you have to make sure that the patients are willing to cooperate over an appropriate period of time, that they have symptoms and signs of lesion which can be well documentated as the result of MS, and that you and your co-workers are prepared to do all the necessary work according to plan. In epidemiological studies the aim is different. You want to get an idea concerning what you call a prevalence rate and you have to direct your studies toward diagnostic evaluations of all registered cases in a particular geographical area, irrespective of whether the symptoms and signs are easy to evaluate or not (e.g., if the patients have other diseases as well), irrespective of whether the patients are willing to cooperate or not, and the study is usually terminated when you have the impression that you have collected as many cases as it is possible to find during a limited period of time. Provided different epidemiological studies are carried out with the same care, the figures obtained can be compared, although the data may be incomplete, particularly concerning benign cases. Even if a prevalence study is made with the utmost exactitude over a long period of time, the material cannot be considered as reflecting the real average situation for MS patients. Thus the sex rate and the variation in the age at onset will be influenced by the selection that is unavoidable for all cases with long duration of the disease. If the purpose of the study is to describe the course of the disease not only in a selected material but as an attempt to contribute to the description of the natural history of the disease, there remains only one way: the hard work of searching for every patient who may have symptoms or signs consistent with a diagnosis of possible MS for a sufficiently long period of time, and a follow-up study of all such cases for decades as thoroughly as possible. Perhaps one should be retired (as I am) in order to carry out such studies. There are many difficulties with such a project, since one must deal not only with patients who have manifest and perhaps severe symptoms and who are more than ready to cooperate, but also with those who may be anxious and negativistic or patients who feel quite well and are unaware of a suspected diagnosis of MS.

In this way, over several decades one will get something which could be called incidence material, and I think that is what I am most interested in.

J. F. Kurtzke: Now what is the purpose of having diagnostic criteria in MS? After all, as physicans this is one to one: you and the patient. His disease is either present or absent. His probability of having MS is zero or 100%. The probabilities are

based on how good a fortune teller you are in terms of how likely you are to be correct when you apply the label. Now as to the matter of how we do this, the purpose of it, other than direct patient care, is to provide a group of *X* patients that is uniform and will be usable for clinical, laboratory, and epidermiological purposes.

Now without going into detail and not using any of the charged words, there are really three minimum groups into which patients should or could be classified – Class 1, 2, and 3. Class 1 would be those meeting the most rigid criteria. What one means is that every competent neurologist you know (and by definition all neurologists are competent) would make the same diagnosis as you, and your prediction is that, if and when he comes to autopsy, he will have MS.

The Schumacher criteria were really designed for a treatment trial and they were therefore purposely the most rigid set of criteria possible. That is class 1. Now there are certainly patients that you, I, or most of us would be equally comfortable with calling MS. But they don't meet the most rigid criteria of Schumacher or any of the other groups. These would be class 2. Then you should have a much lower chance of showing that class 3 patients have MS. These are the patients who you feel will come down with MS. But right now they are not MS. One doesn't want to lose track of them. The first group you would call definite, because I don't feel that splitting them up into autopsy proved and nonautopsy proved is really necessary. Clinically, we call a patient myasthenia gravis. Why can't we just as positively, even though incorrectly, call him MS? The second group we would call probable and for the most purposes I would be very happy putting together the first two classes, the definite and the probable. The third class I would call possible. Now what should this include? The essence of MS, clinically, so far as we know, is that it is a scattered disease and it occurs over time. The "over time" is the reason that I don't think that first bouts should be included in the top category that you will use for all purposes. How else you would like to classify them I don't know. Most optic neuritis does not progress to MS. Whether most means 80% or 60% is an argument.

Most cases with acute disseminated encephalomyelitis do not progress to MS. If they don't, then why put them in your top classes? Beyond that, there is not much to say on the clinical criteria. For a number of reasons I do not think that laboratory criteria should be included as an essential to diagnosis, no matter what they are.

If we make the spinal fluid findings obligatory, this means that nobody can be diagnosed as having MS except at a center or at least a hospital. At least in our country we don't do spinal fluids in the office. Secondly, if you are sure a patient has MS, why do a spinal tap? It costs somebody money (unless you have another purpose for the spinal tap). It costs money and in our case it is the patient who pays. It costs time and it is not a totally risk-free procedure. Also which tests of the many that we have are you going to take as the criterion? For example, if clinically you have a patient that fulfills all the Schumacher criteria and you are sure that he has MS and the spinal fluid is normal, are you going to change? Of course not!

Finally, an even more pronounced comment on the evoked response work: there are only a few centers that can do what is considered adequate evoked potentials. Some years back we were doing some work with rheoencephalography and we were getting beautiful traces of things that were wrong in the carotid system until we started doing normals and coding them blind and found that the normals had all these assymetries and these deferred wave forms and there was no correlation whatsoever

with anything going on in the head. Specificity to me is not specificity in terms of "is there a lesion," but "is there a pathological lesion of a definite sort?" I have yet to see a good series which meets, for one thing, in the visual area, all the criteria that Dr. Neetens pointed out. In these series it is essential to make sure you're doing the right things in the right way, wherein the assessment is done blind and large numbers of normals are studied. We have been trying to get evoked response off the ground for 5 years. I've gone through three neurologists to try to get it set, but they've all left. I'd love to have a tool that would tell me that I have something wrong in the optic system in my spinal cord cases, but would also like to know that it is correct. Now by definition all neurologists are paranoid, we don't trust anybody else's diagnosis. I'm not going to trust a little wiggle unless I know that that little wiggle is indeed meaningful. And so far, unless somebody can present me with the adequate data that normals don't show this at all – I don't mean five normals, I mean a goodly number – then I find it difficult to believe that when the only evidence of lesion is what happens to the little wave, this patient's diagnosis is then going to be changed from spinal cord tumor to MS.

Dr. Tourtellotte: John Kurztke, it's like old times.

My plan is to direct your attention to yet another scoring system. We refer to this scoring system as QENF, or quantitative examination of the neurological function. We utilize bioengineering instruments to get real measuring units. We can quantitate most every clinical neurological test that you and I do at the bedside.

The data can be expressed as a percentage of normal function and the data can be easily computerized for analysis and display. A paramedic can be trained to do these examinations.

We consider our neurological function laboratory to be the equivalent of a cardiopulmonary testing lab, where one has the percentage of normal function of heart rate, heart function, pulmonary function, and our method is as useful for evaluation of total maximum physical condition. I thought I might illustrate my point by two studies. One of them is not mine.

(Slide) This is, in its most simplified parameters, a test of acute retrobulbar neuropathy using the Schnellen test in order to quantitate visual function. This is a study by Rawson, Liversedge, and Goldfarb and I'm sure it is so classical that you're all aware of it. They took 25 patients, 50 patients, randomized them, gave them ACTH for a month, and scored their visual acuity over a 30-day period and of the end of 2 weeks 76% of patients who received ACTH had normal vision, with contrast to 20% in controls, and after 30 days there was still a treatment comparison difference here. Again, this kind of neat and quantitative neurological exam, the ability to delegate the precise test itself to a paramedic, who can be totally blinded, seems to me to be needed in some of our clinical trials, perhaps for adjudication, and maybe even perhaps for practical clinical usage.

(Slide) We've reworked the national cooperative ACTH study again and I'm very sorry that I didn't have a chance to bring the reprint that has just arrived. What we did is one of the five systems that we used in the national cooperative ACTH study – what we referred to as a quantitative examination of the neurological systems. We had ten centers; each one of these centers sent a physical therapist to my lab in Ann Arbor, whom I trained to perform the tests. We also furnished all the bioengineering instruments. These instruments were then taken back to these various

centers. Every 3 months I sent my physical therapist to the various laboratories to see that this instrument was still standardized, that is, that the individual was performing to the standard that they had learned at the University of Michigan. For example, in testing the function of the upper extremity, the purpose is really prehensility, but you must do it with a certain amount of speed, you must have a certain amount of coordination, you must have a certain amount of strength, it musn't tire, you must not burn it off, and you must know where it is. It is possible with the type of data that we collet, these real numbers, that these data can then be converted into the percent of normal value for an age-matched group. Then it is possible to consider composites of all these different factors, or each one of the factors can be looked at individually. For example, in our national cooperative ACTH study, it turned out that we had 100 patients in each one of our groups. A pretreatment examination was done on all these patients and it turns out that each one of these groups is randomized, with no pattern among the patients other than that they had all had a bout 8 weeks before admission to the study, that they were still in a worsening phase or not an improving phase, and that not one of the patients had been treated with the treatment we were using, which was ACTH. And it turns out that these patients start out with 60% of normal function of their upper extremity. During the course of the treatment trial, we examined the patients again at 2 weeks, at 3 weeks, which was the end of the treatment period, and administered no medicine at the time of the fourth and fifth exams. Then we could plot the data in such a way that you can see here that the placebo had an effect and the treatment was weak, because we only improved the 100 patients on ACTH by about 8 or 9%. You can see that most of the improvement took place during the time of the treatment. Then there was a tapering-off period and the patients were able to maintain that function over the said period of time of tests.

Discussion Synopsis

The ensuing discussion centered on the question of using laboratory criteria, especially CSF findings, in the diagnosis of MS. J. C. Kurtzke very emphatically denied the necessity and desirability of including CSF findings in a set of criteria for MS diagnosis. He reinforced his previous statement by pointing out that laboratory confirmation in making a diagnosis of MS required that the patient be in a hospital or clinic. This would to a large extent rule out the possibility of doing epidemiological studies, especially in areas without neurological centers. He emphasized that in epidemiological work there are great difficulties in getting patients to cooperate, and that this could be achieved maximally to the extent of a blood test, but certainly not by a spinal tap to obtain CSF. He said that he did not consider CSF findings as essential for the diagnosis of MS.

K. Johnson stressed the importance of precise diagnostic classification in clinical care of patients, and that this should probably include CSF studies. He added that these are very useful for clinical research aimed at determining more about the disease, from its cause to its epidemiology. On the other hand, he pointed out that in view of the lack of standardization of laboratory tests, the incorporation of laborato-

ry criteria as prerequisites of diagnosis might be dangerous; since most of the laboratory parameters which have been found useful in the MS diagnosis (e.g., IgG, oligoclonal bands) relate to immunological reactions, so study them as diagnostic aids and therefore as the primary way of looking for aspects in the immunology and pathogenesis of MS. This might be one type of clinical criteria to be used in care of patients which would include CSF alterations and electrodiagnostic changes. But in terms of future investigations of patients, diagnostic criteria should be clinically derived and not include CSF.

Torben Fog remarked that there are patients in whom CSF findings are of great importance for diagnosis. He mentioned hereditary ataxia patients as an example in which pathological visual evoked responses are found in many cases but in which no pathological CSF findings are found. Ten percent of the patients in his study on hereditary ataxia had been diagnosed as MS cases. He advocated a differentiation between the information required in research and practical routine work.

W. Tourtellotte discussed the possibility of having, especially for the cases classified as clinically definite MS, two sets of criteria: one with the support of CSF findings and one without CSF.

T. Broman remarked that in his group they had tried to solve the dilemma of CSF analysis and diagnosis by using two figures for diagnostic probability: one with and one without CSF.

Pointing out that there are MS patients with very characteristic symptoms and signs and a typical course but with normal CSF findings, Tourtellotte said that he certainly would not change his diagnosis of MS in view of the lack of pathological alterations in CSF. On the other hand, and relevant to cases of this type, he recalled reports given at this symposium with certain HLA patterns and no oligoclonal bands, which could be benign or less severe types of MS. This could possibly be of great utility. Referring to the investigations of Tore Broman and his group in the Göteborg area over a period of about 30 years, he considered Dr. Lorenz Bergmann's results using agarose gel electrophoresis as probably some of the most important yet attained. By analyzing oligoclonal bands in CSF, Bergmann feels that he can diagnose MS about 5 years earlier.

H. J. Bauer pointet out that CSF findings are not basically different from clinical criteria found by taking the case history and doing a physical examination of the patient; in fact, they are also clinical signs identified using laboratory procedures. The value of CSF findings as diagnostic aids should be assessed according to the degree of reliability which they offer when confronted with clinicopathological criteria. Since in the early stages of MS many cases do not present a characteristic symptomatology and course, CSF alterations typical for MS can be of genuine value in arriving at an earlier and more reliable diagnosis in such cases. To this, W. A. Sibley remarked that in every clinic there are a number of MS cases who do not yet fit the strict Schumacher criteria. A large number of these patients have a picture of chronic myelopathy, with progressive paraplegia, a negative myelogram, and increase of polyclonal gamma globulin in CSF. He sees no reasonable reason for excluding them from the MS diagnosis.

Whenever possible, Bauer considered it desirable to refer MS patients to clinical centers, remarking that in practice many cases are earmarked as MS by cursory ex-

amination and left permanently with a diagnosis generating serious psychological and social problems, and possibly requiring revision.

He emphasized that the aim was not to make CSF examination an invariable prerequisite for the diagnosis of MS, and that the purpose of this discussion was not to definitely arrive at a final verdict as to whether CSF findings or other laboratory results should be incorporated into a set of diagnostic criteria for MS. The reports and discussions of this problem should convey to the International Medical Advisory Board (IMAB) of the IFMSS the opinions of qualified clinical researchers as to whether a reassessment and possibly an updating of the clinical criteria for MS is desirable, especially in view of the progress made in laboratory investigations, or as to whether a consideration of this problem is viewed as superfluous.

In line with the results of the enquête, those present appeared fairly unaminous in the opinion that the IMAB should be encouraged to establish a working group for this purpose.

Ketelaer objected to Kurtzke's comparison of the value of measuring evoked potentials (EPs) using rheoencephalography. Alterations of EPs definitely indicate demyelination, the cause of which must be determined by other criteria; however, Kurtzke replied that he was not equating rheoencephalography with EPs, but only cautioning against overinterpretations without adequate normal controls and evaluating asymmetrical findings as an indication of existing pathology.

O. R. Hommes, refering to a statement made by Fog in his report that the definition of a relapse is in some way a matter of experience, emphasized the need for a precise definition of a relapse – progress of symptoms already present or new symptoms, indicating a plaque in a different area of the central nervous system?

S. Poser saw a problem in Tourtellotte's quantitation tests in view of the fact that training might lead to improved results, although there was no improvement in the pathological process.

Tourtellotte explained that there are two kinds of tests for motor function: one relating to abilities, the other to skills. He stated that his quantitative examination program includes certain basic neuropsychological examinations – cognitive function tests which, unless one has an intensive training period, do not change appreciably. As to skill tests, these have more to do with putting activities of daily living into numerical terms. In addition, there are tests for strength, speed, and coordination, and these really cannot be changed much. Essentially, the tests developed over the years test abilities, and if a patient is properly motivated, they do not change unless the pathological process improves or deteriorates, for it is hard to change your ability.

The neurofunction laboratory, started at Ann Arbor, Michigan, has been maintained and developed for 15 years, and the 85th paper relevant to research there has just been written. There is a great interest in using this laboratory's methods for comparative studies, for double-blind studies, and for control tests. A lot depends on the training of the paramedics engaged in this work, and upon their enthusiasm and ability to teach the patients about what they are to do.

Multiple Sclerosis: Generating Effective Human Services

R. J. SLATER [1]

Genesis of the MS Human Service Problem

Over the years, voluntary agencies have been organized to help individuals whose illness or problems were not adequately met by existing professional activities and community service institutions. This paper makes the point that for a voluntary agency to function effectively, it must first make decisions based on adequate information about needs and resources; second, it must relate closely to the evolving governmental and community organization of human services; and third, it must function as a coordinator of numerous services, acting mainly as a communications link.

Among the chronic illnesses for which better medical and social services must be organized, multiple sclerosis (MS) provides unique problems. Because of the unpredictable waxing and waning of the physical disability of each patient and the resultant complications involving the integrity and economic stability of family life, coping with the disease over a period as long as 40 to 50 years requires a broad range of supportive services. In brief, the critical problems to which services need to be addressed are:

1. Unpredictability and severity of medical and psychosocial needs,
2. Fragmentation of the mix of human services available to meet those needs,
3. Lack of coherence among those fragmented services to meet individual needs of patients and families,
4. Difficulty of access to those services,
5. Economic instability resulting from unstable employment and income.

The significant question is: How can a voluntary agency address such complex problems of human services organization? In the United States the National Multiple Sclerosis Society is comprised of 140 chapters, each of which is responsible for a catchment area. Funds are raised for two purposes – to support a research program dealing with the pathogenesis of the disease, and to provide local human services for patients and their families. Against a background of rising costs of human services and increasing governmental intervention, the Society is examining how its chapters can play a significant role in addressing the above five problems in provision of human services.

In the search for better ways to deploy services required by individual patients from among the network of publicly and privately sponsored programs in each community, there are two requirements basic to any strategy. The first is a system of ongoing *information retrieval* in order to make operational decisions – the develop-

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ment of a simple, workable data base. The second is a system to assure *organizational linkage* between services provided by various medical and social work professionals and the other agencies including activities considered in the context of self help and mutual aid. Only if these requirements are fulfilled is it possible for chapter personnel to develop priorities for the service budget and also to make decisions on the best use of professional and lay volunteer time and effort.

What Is the Basis of an Effective Information System?

Five inventories of information are required to develop adequate planning of services:

1. The range of needs of individual patients and families,
2. The mix of services available in each community or region to meet those needs,
3. The qualifications and availability of personnel to provide the range of services,
4. The settings in which patients will require services,
5. The economic reimbursement arrangements available for the varying services.

Such information required by a voluntary agency must take into consideration the data systems implemented by the government. In the United States, the federal government is generating a marked increase in regulations to implement its national health policy which is being designed to assure access, contain costs and improve quality of care [6]. The incremental move toward a scheme of national health insurance is described [5] as being built on a health care regulatory system comprised of two components. One component requires annotated information for review by professionals of procedures performed in hospitals and is designed to regulate and ultimately limit the *demand* for inpatient services. This national program monitors the delivery of institutional care for patients covered by numerous governmental reimbursement laws, many of which impinge on the care of persons with chronic illnesses such as MS.

The second regulatory component is developing from the recent implementation of over 200 Health Systems Agencies (HSA) throughout the United States charged with the responsibility for monitoring the planning and delivery of services. The HSA legislation was drafted in coherence with the above-mentioned professional auditing laws in order to regulate the *supply* of facilities, equipment, personnel, and services. The expected long term outcome of these demand/supply regulations will be a decrease in utilization of inpatient facilities and an increase in ambulatory programs. The significance of these laws to those involved with MS and other chronically handicapping neurological diseases will be how best to improve ambulatory and home care services and what role voluntary activity will play in this expanding highly regulated and costly system.

One of the relevant features of the HSA regulations is that the law requires that data be assembled and analyzed concerning (a) the health status of the residents in each agency area, and (b) the effect the area's health care delivery system has on the health of the residents. While this is a laudable objective, there is concern in the United States [4] that there is virtually no information on the correlation between health

services and health status. In this regard, Martini et al. [9] have recently reported that traditional health indices are more sensitive to sociodemographic characteristics of the community, whereas monitoring the effectiveness of medical care requires indices relevant to hospital and medical procedures for which outcomes measures can be more readily developed.

To meet this need for more information, the National Center for Health Statistics of the Department of Health, Education and Welfare (HEW) has recently established a Cooperative Health Statistics System which collates and analyzes information gained in large part from existing data systems and vital statistics generated by local jurisdictions. Relevant to this plan, the HEW Health Statistics Plan 1978–1982 [7] notes that available data on health status usually applies to classes of conditions, of disabilities, of impairments, and of related measures but not to persons and populations. Most data sources employ a cross-sectional sampling approach in focussing on the general population or activities in specific health care settings. Consequently, the information is incomplete because not all segments of the population or settings are covered. This is an important problem as it pertains to the population of neurologically handicapped and in particular to the smaller number of high risk, high cost, multi-problem MS patients. In considering these limitations, Spencer et al. [13], made special reference to the need for more selective description of information vital to the determination of health status and management needs of physically handicapped patients requiring various forms of rehabilitation.

In the United States, another current approach to improving information for planning is the development of "minimum essential data sets" [10]. These are designed to permit comparisons of information on persons interfacing with different human service agencies as well as broadening the collection of vital information on psychosocial components of dysfunction. Thus far, two data sets have been developed – a Hospital Discharge Data Set and an Ambulatory Care Encounter Set. A proposed Long-Term Care Data Set is under review. Each of these requires analysis by voluntary agencies such as the National Multiple Sclerosis Society to determine what information these sets contain which can be useful and what further information may be necessary for each voluntary agency to gather in order to determine its optimal service and expenditure decisions.

A number of MS chapters in the United States have individually undertaken "needs surveys" and other health statistic samplings, and it is important that such endeavors mounted under the Society auspices be coherent with the afore-mentioned minimum data sets being generated under federal and state government sponsorship. Given the extensive nature of such interview instruments as the Activities of Daily Living Index [8] and the Sickness Impact Profile [2], the question remains as to how much information is required to be included in minimum data sets to provide a basis for specific improvement in organization of services by voluntary agencies.

It is important to recall that the special role of a voluntary agency is to bring together services to meet specific needs of its own patients which are not being met by other means. Consequently, the approach to information collection might well be limited to a periodic inventory of patients' needs, available community resources and reimbursement systems, of specialized and voluntary personnel, and of the manner by which these are relevant to the various settings in which the patients are living

and working. Such information would be an essential adjunct to governmentally organized data systems and could likely extend the usefulness of information collected by the government for purposes of planning and regulation alluded to above.

Based on these considerations, it can be anticipated that new and expanded approaches to information analysis by both government and voluntary agencies can play a critical role in how chapter service programming and expenditures are planned. In addition, such information can highlight weaknesses and inequities in national and local laws, regulations and services for which concerned individuals can play an advocacy or ombudsman-like role in the continuing quest to improve the quality of care for the chronically handicapped.

Establishing Linkages Among Human Services

Despite the wide range of organizational patterns of health and human service programs which have historically evolved in different countries, the main concern of citizens with medical and social problems is the difficulty of ultimate coordination of the various resources at the *point of encounter* in their own community. What can be characterized as the patient care continuum ranges across a series of professionals, employers, agency personnel, family members, and friends who individually and collectively identify with the patient in a variety of settings. These settings vary from the research medical center or general hospital and clinic to various ambulatory service locations including the doctor's office, the home and family, the place of work, and ultimately a nursing or retirement institution.

Within the framework of the hospital inpatient and outpatient programs, services are usually organized within the component disciplines such as neurology, urology, physiatry, etc. Interdisciplinary communication to provide coordinated, convenient, and effective multispecialty coverage may be difficult to implement without special efforts at scheduling and supportive organization. This multidisciplinary "medical model" of care is at variance with the experience of patients in ambulatory settings of the community. Here psychosocial concerns are addressed by professionals such as visiting nurses, social workers, home health aides, and a wide range of others based on the activities of daily living or a "functional model". Bringing coherence to these classically different medical and psychosocial support systems is a long-standing problem which gains importance as increasing numbers of people are afflicted with some form of chronic illness and additional specialists are trained to provide different kinds of services.

To assure operational integrity within this complex of personnel and services, certain critical linkage components to monitor access to appropriate services and to assist coordination among required multi-service responses are needed. Within the context of the relatively uncoordinated mix of community human services agencies one perceives the primary role of the voluntary service agency as a catalyst and coordinator to meet the specific needs of people.

In the United States, where medical and social services are not as well organized and coordinated as in certain other countries, the above problems are the cause of much of the high cost of the social-response mechanisms increasingly ordained by

the government. Albeit the ability of individuals to cope in times of trouble and crisis varies widely, the fact is that more effectively organized helping systems, be they medical or social, could alleviate much anguish and optimize conditions for productive living and quality of life. In this sense, voluntary agencies such as the MS chapters, as prototypes of coordinators of service for chronic illness management, must represent the holistic concerns of MS persons and families.

This indicates that to bridge the gap between the functional model of care in the community setting where the Patient Services Coordinator is operative and the professional multidisciplinary setting of the medical model, there may be a need for a comparable individual such as a nurse specialist coordinator operating in the medical setting. These roles of linking hospital services to the community services need not be limited to specific professional stereotypes. What is essential is that there be an open dialogue between the institutionally based programs and the community-based programs to provide an orderly coping process for MS persons and their families and others, such as employers influenced by their illness.

In looking to the future development and role of voluntary agencies in the scheme of more highly regulated national health and social services, it is helpful to work from conceptual models of organization. The American experience has been that, despite well-intentioned programs by governmental agencies and various community institutions, such as hospitals and social welfare organizations, many of which have special personnel ordained to monitor medical and social problems of handicapped patients, there frequently remain major obstacles to addressing the specific needs of individual patients in a coherent and optimal manner.

A Model of Organization for Voluntary Services

For planning purposes, the National Multiple Sclerosis Society in the united States is adapting a conceptual model devised by Curtis and Neuhauser [3]. This scheme links the activities of voluntary agencies to the political framework of both the community care-giving agencies and institutions and the governmentally sponsored departments addressing various human services. Figure 1 depicts this scheme, in which the MS Patient Services Coordinator plays a central coordinating role by receiving advice and assistance from various governmental and community agencies as well as citizen volunteers on matters of concern to patients requiring particular services or combination of services. What is not yet worked out in the evolving scheme of health services organization in the United States is how a voluntary agency such as an MS chapter might relate in a predictable and integral manner to the politically mandated health systems structure of the government. Some form of information and accountability for services rendered should be provided, and in Figure 1 this is depicted as a dotted line connecting the MS professional program to the Health Systems Agency of the region or state. The heavy, solid arrow indicates the clearly defined program responsibility to the MS chapter and its voluntary board of directors. In the United States, chapters may or may not work in cooperation with other voluntary associations, depending largely upon the local history of voluntary cooperativeness, which varies widely.

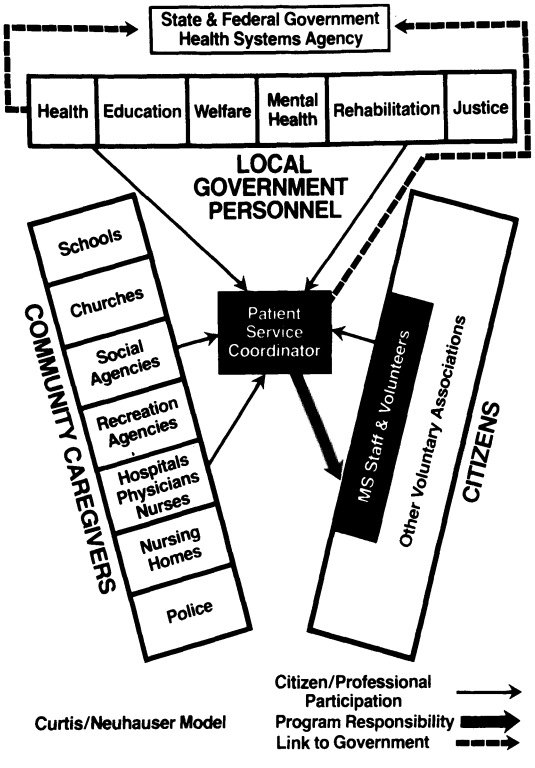

Fig. 1. Schematic representation of three groupings of individuals with whom a Patient Service Coordinator (PSC) of a voluntary agency must work to integrate services. The box on the right represents citizens and identifies the MS agency. The box on the left represents organized human service professionals or institutions of the community and the upper side represents the local offices of state government service departments. At the top, the Health Systems Agency is the monitoring and planning agent for the State and Federal Government and must have consumer (citizen) representation on its governing board as indicated by the dotted line connecting it to the Patient Service Coordinator. Success of the Coordinator is dependent on integration of appropriate personnel to solve specific combinations of problems of MS patients

The manner by which such a chapter-coordinating operation is focussed on the problems of individual MS persons and families is depicted in Figure 2. In this configuration, the Patient Services Coordinator acts as the organizer in the development of problem-solving teams targetted to meet the multiple needs in complex situations affecting a patient. Such problem solving may require variable input from different agency or institutional personnel and may require shorter or longer periods of attention. Frequently the Chapter Coordinator acts as an ongoing guidance counsellor within the context of the target teams.

Figure 2 depicts four examples of problem-solving teams. Patient 1 may represent a young mother afflicted with MS, complicated by depression and inability to cope. Under these circumstances, coordinated assistance might be effected by a mental health worker, social service worker, volunteer friends, and monitoring by the MS Patient Services Coordinator.

Patient 2 may represent an older MS patient in circumstances for which family home care is no longer possible. The combined efforts of the public health nurse, the nursing or retirement home staff, and one or more volunteers may form a basic team. Monitoring by the MS Coordinator will make possible additional assistance as necessary.

Patient 3 may represent a patient recuperating from a relapse and undergoing physical and vocational rehabilitation. The combined and coherent efforts of rehabilitation personnel, social workers, medical professionals, and appropriate volunteers can greatly enhance coping and return to useful activity.

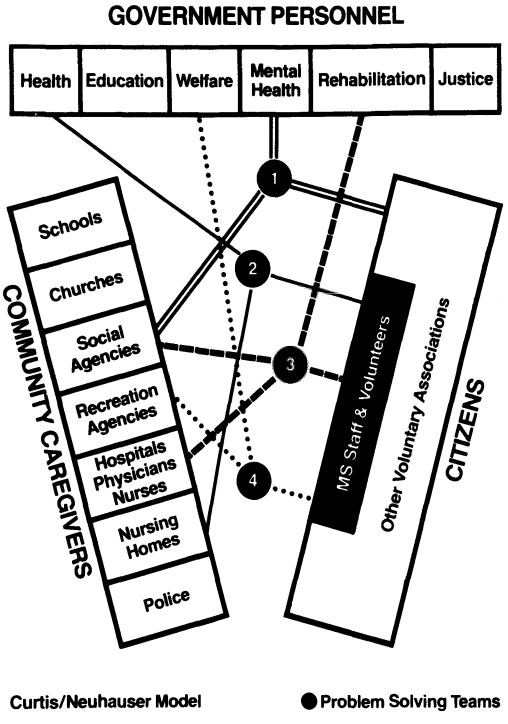

Fig. 2. Four hypothetical multiproblem patients are identified with connecting links to professional and volunteer individuals organized from various human service agencies by the Patient Services Coordinator (PSC) as problem-solving teams. The role of the PSC of the MS staff is to monitor the needs, responses, and outcomes of high risk, multiproblem patients requesting assistance

Patient 4 may represent a moderate to severely afflicted patient confined to home for whom the quality of life is very limited. In these circumstances, the coordination of welfare programming with a recreational agency and assistance from volunteers can open up the outlook on life from one of endless confinement for many of the chronically ill.

It is apparent that this operational concept must be limited to a manageable population. Under current operational circumstances in the United States, the implementation of this model may be difficult, on the one hand, because of staff limitations and the complexity of service arrangements in large urban areas and, on the other hand, because of distances separating patients and resources in more remote rural areas. However, much can be accomplished by voluntary effort, given the will to achieve even if there is only a minimal staff to help coordinate communication and monitor outcomes. To this end, some system of adapting the organizational framework illustrated above is being explored in different areas of the United States.

Curtis and Neuhauser [3] considered such cooperative arrangements to be operable within a population size where a clearly defined *sense of community* among the agencies, professionals, and a volunteer self-help/mutual aid network could prevail. In MS planning in the United States, it may be possible for the MS chapter to work out various contractural arrangements with other health agencies, such as the Visiting Nurse Association, to achieve the coordinating influence necessary for coherence of needed human services in manageably defined geographic areas which are encompassed within the chapter's overall catchment area. More information is needed about what types of local arrangements have been successful in different circumstances in other countries.

Future Directions

Apart from a central orientation toward research on the pathogenesis and management of MS, the health program objective of the Society in the United States is to play a vital role as a linkage and catalytic mechanism to foster and coordinate an optimal array of services. The dissemination of information as well as the development of formal linkage mechanisms would have to be performed even if there were a massive expansion of governmentally supported services for long term disability, as has occurred in many other countries. Indeed, experience has demonstrated that as services proliferate there is an even greater need for these linkage mechanisms. This point was emphasized in the report of Simkins and Tickner [12] which delineated the continuing problem of fragmentation of service access and reimbursement systems in Great Britain despite major efforts to create an integrated health care system since 1946.

The International Federation of Multiple Sclerosis Societies represents widely divergent social and governmental systems each of which operate with the objective of providing an optimal set of health and social services. Probably the most effective manner of transferring experience from one system to another will be to compare modes of adapting services to people in the context of local community pattners, the point of service encounter, where flexibility for volunteer/professional interface occurs. To this end, there is increasing interest in comparing systems of information retrieval and analysis in different social systems which can help generate more effective understanding of how these human services objectives can be achieved. Armitage [1] outlined how the countries of the European Economic Community have formed a working party to compare various forms of health survey. He emphasized that health interview surveys offer the principle opportunity for action and that it may be possible to incorporate health questions into various types of multipurpose international surveys.

In designing techniques of information gathering and analysis, it is important to heed the concerns expressed by Sackett et al. [11]. They addressed the problems inherent in the conceptualization and cost of implementing individual aspects of health index questionnaires, to be administered by trained lay interviewers, which would provide valid biological, clinical and psychosocial information for evaluation. It follows that to achieve results meaningful both to a voluntary agency and to other community and governmental institutions, planning for the development of data sets should be conducted as a cooperative venture among the relevant agencies and professionals.

At the heart of the role of any voluntary agency, no matter what the nature of the political system in which it is operative, is the focus on unmet needs for a special group of people. New emphasis must therefore be placed on the fact that their success will largely depend on the nature of information on personal needs which they can determine and evaluate in coordination with governmental data. This will provide maximal potential for sharing of resources such as equipment and professionals or individuals willing to perform special tasks. In assisting chronically handicapped individuals and their families, resource sharing to deal with classes or problems, for instance the requirements for transportation, home care, rehabilitation,

recreation, psychosocial counselling etc., will likewise limit the costs and increase the effectiveness in provision of services.

Ultimately, voluntary agencies like the Multiple Sclerosis Societies must husband their scarce resources so as to maximize their impact on patients and families at highest risk. While every effort to focus international scientific research on the pathogenesis and prevention of MS must continue, there persists a major challenge for volunteer individuals and groups to explore and advocate new patterns of cooperative activity with medical and social planners and providers. Only then will it be possible to link individual human initiative, expressed through voluntary activities, with the functions of community institutions and agencies to improve the effectiveness of our various human services.

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Summary and Concluding Remarks

H. J. BAUER [1]

Experimental Models in Demyelinating Disease

At the Locarno symposium on the pathogenesis and etiology of demyelinating diseases in 1967, Seitelberger stressed that on neuropathologic grounds human MS is a process and has features of its own which simply cannot be compared to the changes found in experimental models. This statement is still valid today. Meanwhile, however, at least one of the animal models of experimental demyelination, chronic relapsing EAE, has come closer to MS in the human. The undeniable value of experimental models, even though they clearly represent a pathologic process different from MS, is the possibility of focusing on phenomena that are, or may be, key reactions in the chain of pathogenetic events leading to demyelination and its recurrence in the human disease.

Raine pointed out the similarities of Stone and Lerner's model of chronic relapsing EAE in strain 13 guinea pigs, produced by a single injection of isogeneic spinal cord in complete Freund's adjuvant. They reported on the permanent suppression of the experimental disease by injections of myelin basic protein (MBP) and incomplete Freund's adjuvant. Wisniewski and Glassman were able to improve the model and to produce a chronic disease with predictable remissions and relapses in 80% of strain 13 guinea pigs. They were not able to induce chronic EAE with plaquelike demyelination by sensitization with MBP, however, and they found a sensitization to MBP only in the acute episode of the experimental disease. They concluded that antigens other than MBP play an important role in recurrence of EAE and the formation of large demyelinated plaques similar to those in MS. These observations may be relevant to current therapeutic trials with MBP and copolymers in MS. Considering the fact that no adjuvants are being used in these trials, and furthermore that MBP is subject to rapid proteolysis in the blood stream (Alvord and co-workers), Wisniewski's findings could question the chances for success in the treatment of recurrent chronic MS with these substances. The report of Nagai and co-workers on the prevention of EAE by complete Freund's adjuvant alone should also be mentioned in connection with this question.

Lipton and Dalcanto consider Theiler's virus infection of the central nervous system of mice an excellent model for MS because there is selective demyelination in the chronic phase of the infection, with simultaneously occurring active and inactive lesions, and because a chronic progressive disease, with immune-mediated myelin breakdown, can be produced using tissue-culture adapted virus. In their report they also point out, however, that in the Theiler virus infection there is often severe leptomeningitis, and that immunosuppression produces an overall potentiation of the

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infection, with increased microglial proliferation and neuronal necrosis and an increase in mortality.

Acute and recurrent demyelination due to virus-induced destruction of oligodendrocytes caused by mutants of mouse hepatitis virus was described by Lampert and co-workers. Nagashima et al. reported on subacute demyelinating encephalomyelitis in rats due to infection with murine corona virus JHM. Corona virus JHM was also used in an in vitro carrier culture system of persistently infected mouse neuroblastoma cells by Stohlman, Sakguchi, and Weiner to study the question of defective viral infection, in which viral antigens are undetectable by conventional techniques even in the presence of complete viral genome. One of the intriguing aspects of this work is the possibility that latent virus genome, not interfering with cell function under ordinary conditions, could produce cell damage and even death under stress. Referring to MS, a mechanism of this type could serve to explain how an oligodendrocyte, subject to infection by an unidentified defective virus, could suffer damage and how demyelination could be triggered by unspecific and transient stress. Of interest in relation to this experimental approach is the possible isolation of corona virus from autopsy material in two cases of MS by Burks and co-workers. A laboratory contamination appeared unlikely in view of the positive serologic findings in body fluids of the cases from which the virus was isolated. The authors were cautious with respect to a causal relationship, and further confirmation appears necessary.

Chronic demyelination due to visna-virus remains an interesting experimental approach: since the visna-virus possesses a reverse transcriptase and is capable of producing a provirus not recognized by the immune defense mechanism of the body, latent infection can persist in a small proportion of brain cells. The possible significance of similar mechanisms in MS requires further study.

A possible biochemical approach to the question of virus infection in MS may be the development of techniques to detect viral genomic information in organ material. An example of this was presented by Dörries and ter Meulen with the examination of MS brain material for genomic information of herpes simplex type 1 and adenovirus type 2 by DNA–DNA reassociation kinetics. This technique, which proved useful in herpes encephalitis, did not detect any signs of herpes or adenovirus in the brain material of ten cases of MS.

Chronic relapsing EAE appears to be the most noteworthy recent advance in the study of models of experimental demyelination. However, the use of different virus models in research on the cause of MS testifies to the fact that, in addition to the pathogenetic mechanisms operative in EAE, other possibilities demand consideration. The impression could arise that increasing knowledge in the field of experimental demyelination is not narrowing the possibilities of developing one valid concept, but leading to further diversification. Naturally occurring viruses and mutants provide us with a variety of models for demyelination, each of which may have a lesson to offer in the study of the cause or causes of demyelination in MS, but none providing the precise experimental counterpart to the human disease.

In line with this is the situation with respect to the epidemiologic and virologic investigations on common viruses and MS: while myxoviruses, especially measles, appear as prime candidates, increased antibody titers have also been found in some MS cases against herpes simplex, varicella, rubella, and cytomegalovirus. Of the va-

rious isolates that have been reported, none have been sufficiently confirmed to date. With this background, the tenuous situation of laboratory investigations in relation to the etiology and pathogenesis of MS is pinpointed. The relevance of laboratory findings to MS thus far is predominantly based on their casuistic or statistical correlation to the clinicopathologic condition, insofar as this is identifiable on the basis of clinical findings and, in a comparatively minute proportion of cases, on histopathologic confirmation.

Laboratory Investigations in Relation to Etiology and Pathogenesis of MS

The broad spectrum of laboratory methods used in the examination of body fluids and tissue material from MS cases encompasses three objectives:

1. Improvement of reliability of the diagnosis of MS;
2. Assay of process activity;
3. Investigations relevant to the etiology and pathogenesis of MS.

The last of the three objectives was the topic of the second section of our symposium.

In his survey on IgG, Tourtellotte points out that de novo IgG synthesis in the nervous system can be measured by the use of an empiric formula (his own, Ganrod-Laurell's, or modification of these), an IgG isotopic test, or the demonstration of oligoclonal bands. He presents available evidence and accepted arguments for the hypothesis that CNS IgG synthesis is related to the production of MS lesions, and he also proposes that its quantification may serve as a useful parameter in assessing the value of putative therapies in MS. Numerous studies have shown that local IgG-synthesis, though of considerable diagnostic value, is not a practicable parameter for assessing process activity. However, in their studies on immunosuppressive therapy with cytostatic drugs, Hommes et al. found that the IgG concentration in CSF responds to immunosuppression.

Oligoclonal bands are found in 80%–90% of CSF specimens from MS patients, with the figures in the literature ranging from 45% (Clausen) to 90%–95% (R. M. Schmidt et al.). In a comparison of the agar gel technique, the most widely used method, with isoelectric focusing (IEF), Delmotte found the latter to be the more sensitive method. IEF also showed a 100% correlation with capillary isotachophoresis in the detection of local IgG synthesis; both methods present a more accurate methodological basis for the differentiation of locally synthesized IgG and a disturbance of blood-CSF barrier function than other procedures do.

An approach different from the analysis of oligoclonal bands is the quantitative determination of IgG subclasses in CSF and serum of MS patients by a radioimmunoassay described by Morell and Skvaril. By this technique, Kaschka and associates found Ig1 significantly higher in MS patients, with correspondingly lower values for the three other IgG subclasses.

One of the important results emerging from recent work on the analysis of IgG components of CSF is the possibility of differentiating an increase of IgG in CSF

due to local synthesis within the CNS from an increase that results from damage to the blood-CSF barrier. The demonstration of oligoclonal bands is a valuable technique supporting the diagnosis of MS. In addition to the possibility of correlating antigens to oligoclonal bands, the identification of antibodies composing these subfractions promises to become an important area of laboratory research on pathogenetic problems of MS.

This of course poses the problem of antigens relevant to the demyelinating process. Wajgt et al. found increased titers of measles and parainfluenza type 3 specific antibodies in serum and CSF, and a correlation to the IgG response. Sindic and coworkers found an association of oligoclonal bands with elevated IgM concentration in CSF, and they considered this as a useful index of immunologic disorders affecting the CNS. Analyzing IgG oligoclonal bands in CSF, a predominance of light chains type kappa was found, with kappa and lambda components presented in the same band in 27 of 39 MS patients. Studying CSF lipids in demyelinating disease, Seidel, Heipertz, and Buck proposed that the cerebral origin of sphingomyelin and linoleic acid is unlikely. This work is in line with the fact that many previous attempts to correlate the concentration of lipid constituents in CSF to a demyelinating process have not been useful in diagnosis. With respect to MBP the situation appears to be different: the results of several investigators in different parts of the world (Cohen and McKhann, Whitaker, Hempel, Kohlschütter and others) all indicate that the concentration of MBP in CSF is a useful criterion of process activity. Panitch and K. Johnson demonstrated antibodies against MBP more frequently in active stages of MS, but they found no correlation to IgG or oligoclonal bands.

Clausen et al. found two "MS-specific" antigens in brain tissue, one of which caused antibody formation in experimental animals, the nature of which (microbial, brain cell protein or complexes) is still unknown. Measles antigen was found in three of seven brains, but not considered as specific for MS.

The reports mentioned exemplify only in a fragmentary manner various approaches endeavoring to elucidate the nature of oligoclonal bands found in a high percentage of CSF specimens from MS patients. This is a field of work still in its beginnings. A critical prerequisite in all of these studies is the reproducibility of bands. Since it is accepted that oligoclonal bands consist of antibodies with restricted heterogeneity, reliable quantitation is important for the identification of antibodies involved. But even with these problems solved, there still remains the question of whether we are dealing with etiologic and/or pathogenetic determinants, or only with epiphenomena.

A most instructive contribution to this question was the report by Vandvik and Norrby on viral antibody responses in the CNS of patients with MS. Whereas in a number of diseases (SSPE, chronic-progressive rubella encephalitis, mumps, meningitis, and neurosyphilis) the bulk of oligoclonal bands consists of antibodies relevant to the causal agents, this is not the case in MS. With the "imprint electroimmunofixation" technique, they found that local synthesis was restricted to antibodies against measles, mumps, rubella, and herpes simplex in nine of ten MS and in five of ten optic neuritis patients. Later varicella zoster antibody was found (10 of 12 cases) to be even more frequent than measles (6 of 12 cases). However, no relationship was established between the locally synthesized antibodies identified thus far and the major fractions of oligoclonal IgG demonstrable by agarose electrophoresis of

the CSF. As yet the question remains unanswered, whether nonspecific activation could explain all of the locally synthesized oligoclonal IgG, or whether the bulk of oligoclonal IgG in the CSF of MS patients is due to antibody against a still unknown agent. The study of antibodies that result from nonspecific coactivation of cells in diseases of known etiology is in progress. In this connection it should be recalled that there are rapidly progressing MS cases with special symptomatology, but devoid of characteristic CSF alterations. A careful study of these cases with respect to antibody patterns in their oligoclonal fractions might provide new leads.

By the use of skin tests, delayed hypersensitivity to measles antigen – an anergy indicating a selective defect in cell-mediated immunity – was shown to be less in active MS than in patients with stable disease or in healthy adults (Valdimarsson and Agnarsdottir). These authors also observed an impaired release of infectious measles virus by MS lymphocytes. They point out that these results suggest the importance of investigations on the cellular immune response to other enveloped RNA viruses as well. During the past years, the relationship of measles antibody titers to MS has been the subject of numerous studies; in most cases, however, without precise relationship to the clinical stage, i.e., process activity of the disease. Höher and associates found peak titers against subunits of measles virus, hemolysin and nucleoprotein within 1–3 weeks during the acute phase. No titer increases were seen with parainfluenza and poliomyelitis. By radioimmunoassay of antibodies against measles, rubella, and respiratory syncytial virus antigens, Salmi and associates demonstrated significant changes in intrathecal synthesis of antibodies against one or more of the viruses during the course of the disease in 8 of 20 patients, but no correlation to the clinical stage was observed. Salmi and his group are in the process of studying further viruses, and their results indicate that the intracerebral production of antibodies varies from one individual to another, suggesting no single virus as the responsible agent for MS.

An experimental approach to the problems is dicussed in the study by Sandberg-Wollheim, Koprowski and associates on hybridomas of mouse myeloma cells versus human lymphocytes. Since antibodies against viruses account at best for only a very small fraction of the CSF-IgG in MS, it is hoped that the hybrids produced by CSF cells of MS patients may help to characterize some of the antibodies involved.

With the rapid advances in knowledge concerning lymphocytes and cell-related immune phenomena, studies on serum and CSF cells – one of the oldest laboratory methods in the diagnosis of MS – have become one of the "hot areas" in research on the immunology of MS.

The findings of Antel and Arnason, that suppressor cell function and mitogenic reactivity vary with disease activity, have pointed the way to an informative laboratory parameter for assessing process activity. The reduction in suppressor cell activity in the increment stages of MS bouts and the sharp rise in the period of recovery is an impressive finding, with further development perhaps a practicable tool for assessing process activity and monitoring of therapeutic studies.

An interesting aspect of this work is the relationship between suppressor cell activity and immune complex levels. Jans et al. detected immune complexes in 25% of sera and 47% of CSF specimens from MS patients. They found a positive correlation to CSF pleocytosis, but none to IgG, duration, severity, and disease activity.

Cuzners study on proteinase inhibitors touches on a problem long recognized, but still in dire need of more systematic study: the proteinase activity of CSF in relation to clinical activity. Cuzner points out that proteolytic activity and increased fragments of MBP appear in periods of exacerbation of MS, with a corresponding decrease of proteinase inhibitors.

Several symposium reports were devoted to studies on the distribution of B and T cells in various stages of MS and in the course of immunosuppressive therapy, as well as to basic investigations such as receptor functions of T cells (Eder and co-workers). The response of T lymphocytes from MS patients to antigens in MS brain material in a high percentage of cases and the pathognomonic nature of the reaction seen by Offner et al. may be of practical diagnostic value and should be analyzed for its potential use as a criterion of process activity.The fact that a drop in E rosette-forming lymphocytes under ACTH therapy was transitory in the investigations of Kaschka and co-workers could reflect limitations in the immunosuppressive effect of the therapy used. The question whether persisting T cell depression is obligatory for positive results of ACTH or steroid therapy merits further study.

Logically, stress on cellular immune reactions in MS has placed chief emphasis on investigations of T cells and their functions. Sayk's reminder, that in the initial stage of an MS bout plasma cells and monocytes are important components of the CSF-cytogram, and his reference to the "blast-like" cells described by Lumsden, should not be forgotten in the overall analysis of cellular interactions in the course of MS and during therapeutic programs. A survey of electrophoretic mobility techniques and the assay of lymphokine activity was given by Meyer-Rienecker et al. They propose a diagnostic program that includes CSF cell count and CSF-cytogram, protein analysis with electrophoretic subfraction, lymphokine assay, cytopherometric blood cell studies, cytotoxicity test, and genetic typing. All-inclusive diagnostic regimes of this type would be desirable and could be proposed in many variants. In practice, there always remains the inevitable compromise reconciling available technical facilities, know-how, costs involved, and the limit of what a patient will concede in the way of diagnostic manipulations.

Weiner and Stohlman's remark that immunopathologic and immunosuppressive states are two sides of the same coin should be borne in mind. Although recognized many decades ago, it still is difficult for some investigators to accept the idea that immune incompetence (or defect) is not irreconcilable with immunosuppression as a principle of therapy. Experimental evidence is presented that viruses interfere with cellular immune responses, producing an autoaggressive response to host protein, a host antigen cross-reacting with a virus antigen, or an altered host antigen derived from a previous CNS viral infection. Such an impairment of immune regulatory mechanisms may result in an autoimmune disease. Considerations of this type are important as basic concepts for the broad spectrum of laboratory approaches, probing for key mechanisms in the pathogenicity of MS. That evidence for many assumptions still remains incomplete, despite extensive and noteworthy experimental work and laboratory studies, is reflected by the investigations of Silberberg and associates on the role of antibodies in MS. Their findings show that not only protein components like MBP, but also lipid haptens, can induce central and peripheral nervous system demyelination, and that it remains unclear whether one or

more, or varying antigen-antibody mechanisms are operative in producing demyelination in MS.

In dealing with laboratory methods of diagnosis, it has become a cliché to set morphology apart from the biochemical, serologic, and virologic studies. With the exception of some CSF and T cell cytology this is somehow also reflected by the program of this symposium. It is all the more noteworthy that the morphological contributions deal with questions of basic importance in diagnosis.

Adams' characterization of the active lesion drives home a simple, but extremely important, lesson to the clinician and laboratory expert: since modern methodology provides practicable in vivo approaches, we must endeavor to recognize signs of myelin breakdown, neurologlial hyperplasia, edema and special features of meningeal inflammation. It would be advantageous to interpret laboratory results on the basis of what the morphologist teaches us concerning the features of the active lesion.

Ingrid Allen gives a positive answer to the old question whether macroscopically normal white matter in MS is different from normal tissue, pointing out that 72% of her histologic samples were abnormal. Since this is an expression of microlesions in only 13% of them, there is obviously more to look for in the morphogenesis of the demyelinating process in MS than the perivascular lesion. The demonstration by Prineas of lymphatic capillaries and lymphoid tissue in the brain and spinal cord pinpoints what is still almost a terra incognita of CNS morphology. Confirmation and more detailed study of the barrier surfaces between the lymphatic system and CNS tissue is urgently necessary because it may well provide key information on the morphogenesis of the demyelinating focus. Electron microscopy is producing a wealth of morphological data where the prime challenge, as indicated by Argyrakis, is that of correct interpretation.

Recent Trends in the Therapy of MS

In the discussions on recent trends in ("cause-related") therapy of MS, consideration was given to three topics: cytostatic drugs, MBP and copolymers, and polyunsaturated fatty acids. All three approaches are based on hypotheses concerning the pathogenesis of MS. The variability in the natural history of this disease precludes any deductions not based on careful documentation and statistical analysis. Thus far, only the treatment of MS with cytostatic drugs has progressed to the point where statistical evaluations are beginning to emerge.

The two alternatives of cytostatic therapy of MS are long-term therapy with azathioprine and courses of intensive immunosuppression with cyclophosphamide with or without corticosteroids.

Aimard and associates reported on 77 MS patients treated with azathioprine for longer periods of time (mean duration, 5 years). In 77 patients treated during the "progressive phase" there was no improvement, and deterioration continued. Of 38 cases treated in the "remittent phase" 23.5% became worse, as compared with 52.5% in 78 controls. In 12 cases, treatment had to be discontinued because of hematologic complications (4), gastrointestinal disturbances (4), thrombophlebitis (1), cause unknown (3).

Long-term treatment was carried out by Frick et al. in 88 patients. In 64 patients treated for at least 1 year, the relapse rate dropped from 0.42 to 0.16 cases per year during treatment. After discontinuation the relapse rate in 47 patients was 0.33. In the total group of 73, 8 patients improved, 15 deteriorated, and 50 remained unaltered. Of 24 cases with chronic-progressive course, 13 deteriorated and 10 remained unchanged. The authors recommend azathioprine treatment as early as possible and administration over a period of at least 2 years. In spite of their relatively favorable figures, they summarize their experience in the statement: "immunosuppressive therapy in its present form is problematic and unsatisfactory; it should be understood as a precursor of a specific intervention in the immunopathogenesis of MS."

Dommasch and co-workers also reported on fewer relapses during azathioprine treatment of relapsing-remitting cases (n = 8). Secondary chronic progression was apparently not influenced at all (n = 8), and there was also no change in the course of 7 patients with primary chronic progression. Effectiveness of azathioprine therapy did not last beyond the period of treatment. These authors also considered azathioprine therapy as unsatisfactory in a large number of cases, and further trials with intensive immunosuppression necessary.

Patzold et al. reported on 52 patients treated with a dose of 2 mg azathioprine/kg body wt. and 45 controls. The two groups were of comparable age (mean age, 29.6 and 32.8 years) and mean duration of disease (5.4 and 5.0 years). The mean duration of treatment was 433 days. The relapse rate in the azathioprine group was 1.08 and 1.2 per year for the control group, with a mean degree of progression of 0.016 and 0.026 respectively. These preliminary results were not considered statistically significant.

Gonsette and co-workers of the Belgian National Center for MS have by far the greatest experience with immunosuppressive therapy using cyclophosphamide. Reporting on 201 patients treated with a dose of cyclophosphamide sufficient to maintain a lymphopenia of <1000 cells/mm^3 during 2–3 weeks, a marked reduction of annual relapse rate was observed in 70% and an improvement of neurologic signs in 60%. A stabilization period of 2–3 years was achieved, and relapses occurring after treatment appeared less severe and easier to control with corticosteroids. However there was no influence in acute cases and in advanced chronic forms.

Eighty-six patients with chronic-progressive MS were treated with 400 mg cyclophosphamide + 100 mg prednisone per day for a period of 3 weeks by Hommes et al. They saw "strong and long lasting improvement" and a decrease of CSF-IgG% persisting for 3 months after treatment. DR_{W2} homozygous MS patients with high progression rate responded best to treatment. This raises the question whether identifiable genetic determinants can prognosticate the result of this type of immunosuppressive therapy.

Summarizing results thus far on the cytostatic therapy of MS, it can be said that there appears to be a reduction of relapse rates especially in the early cases, that there is no benefit in chronic-progressive forms, and that the long-term benefit, while definitely outlasting the effect of corticosteroids and corticotrophins, is transient. In severe relapses, combination therapy with corticosteroids is warranted. On the basis of present knowledge and experience it may be said that there exists a rationale for treatment with cytostatics insofar as this type of therapy may limit the amount of ir-

reversible damage produced by the acute demyelinating episodes, which seem to be less frequent and less severe at least for a certain period in the course of the disease. The question of complications requires further study and a longer period of experience, especially with respect to oncogenicity and the influence on gestation processes.

Double-blind studies have repeatedly been brought into the discussion. The great difficulties in any such attempt are quite apparent, and the consensus appears to be that in view of grave technical problems, but also on ethical grounds, double-blind studies with cytostatics presently available are impracticable.

Therapeutic trials with myelin basic protein must be considered logical if the analogy of EAE and MS, i.e., of similar basic mechanisms with respect to the demyelinating process, is accepted. On the other hand, it may be said that therapeutic trials in MS with MBP can help to clarify the question whether this analogy is valid or not. Obviously, the first prerequisite in a speculative trial of this type must be that the administration of MBP is innocuous. This question was meticulously considered in the study by Salk and associates, and the absence of adverse effects provided the basis for a dose of 0.5–3.0 mg/kg body wt. per day for protracted periods of time. Atypical patterns of anti-MBP IgG, and IgG were found in MS patients, indicating defective immunoregulation in patients with MS. The preliminary results by Romine do not permit any conclusions concerning the question whether MBP therapy is beneficial in MS or not. Both presentations were reports on work in progress, and considerable time will still be required before definite results can be presented. Regardless of whether they are positive or negative, they should be an important contribution, aiding our comprehension of pathogenetic mechanisms in MS.

It is generally known that minute doses (1–10 μg) of MBP with Freund's adjuvants induce EAE, whereas large doses (antigen excess) suppress EAE. This effect can also be produced by peptide fragments of the MBP molecule. In these fragments encephalitogenity depends on the presence of tryptophan, but suppression is possible with MBP peptide residues lacking any tryptophan. Following these findings, Arnon, Sela, and Teitelbaum synthesized nonencephalitogenic, randomized basic polymers, which proved effective in suppressing EAE. One of these a copolymer with molecular weight of 23,000 designated COP1, consisting of L-alanine, L-glutamic acid, L-lysin and L-tyrosin, is being used in clinical trials analogous to those carried out by Salk and co-workers with MBP. Arnon reported on three patients with acute disseminated encephalitis who recovered completely under COP1 treatment, but in four patients with advanced chronic MS results were inconclusive. This was also the case in short term trials in 16 patients of the Göttingen University Neurologic Department. In both trials a small daily dose of 2–3 mg COP1 was injected IM. This dose may have been too small; further trials and informative tests for monitoring the response are necessary.

That a number of polyunsaturated fatty acids or essential fatty acids (PUFA = EFA) essential to body growth and function cannot be synthesized by the body and that recent studies have indicated an altered composition of myelin and/or a deficiency of these substances in persons with MS has led to the use of diets rich in PUFAs and to dietary supplements with substances like sunflower oil and preparations such as naudicelle (an extract of evening primrose containing a high percentage of linoleic acid and γ-linoleate). Publications on these problems have come up with di-

vergent results, and the question is still open whether and to what extent they are pathogenetically relevant factors in MS.

In an experimental study Mertin found that PUFAs suppressed EAE in Lewis rats and discussed the concept that, in addition to physicochemical effects on cell membranes, the immunosuppressive effect of EFAs may be determined by the action of prostaglandins.

Field pointed out that in the human the immunosuppressive effect of PUFAs is transient, being lost after some months. His own hypothesis is based on the existence of an inborn metabolic defect in the handling of PUFAs. He attributes a prophylactic value to the administration of γ-linoleate early in life. Results of cytopherometric tests (MEM-LAD, E-UFA, PGE_2-test) are presented and the hypothesis of a metabolic defect in handling PUFAs in the production and maintenance of myelin is stressed on the evidence of results in family studies (Field and Joyce).

In studies on patients receiving sunflower oil and naudicelle capsules as dietary supplements, Seidel and Heipertz found no differences in the serum linoleic acid levels in comparison with controls. The loss of long-chained fatty acids of sphingolipids, with a corresponding increase of short-chained fatty acids was identified as a pathogenetic factor responsible for myelin instability in an experimental study by Heipertz and Seidel.

Both possibilities, the role of PUFAs in cell-mediated immunity and the metabolic defect of myelin, require further basic study. This also applies to the therapeutic or prophylactic value of PUFAs in MS and the diagnostic role that can be attributed to Field's cytopherometric tests, since results in the literature are still controversial.

A double-blind study of amantadine treatment of MS by Ellison et al. revealed no differences between this substance and placebo. Brage reported on 166 patients treated with thymectomy, in 33,7% of which he saw a favorable effect.

In all of the procedures discussed in this section, the intention was "cause-related" therapy. Without exception they are characterized by a more or less tenuous hypothetical basis. To a certain extent results seen thus far appear encouraging and justify further therapeutic trials, but without exception more ground work and critical controls are required.

Epidemiology of MS

Studies of histocompatibility antigens in MS patients in various parts of the world have established an increased frequency of the tissue types A3, B7, and especially Dw2 in Eurocaucasians, but a predominance for other tissue types in Japanese, Israeli, and Arab MS patients. A survey of these differences and of possible correlations to immune reactions is given by Oger and Arnason. Raun, Fog et al. pointed out that the relative MS risk for Eurocaucasians with type Dw2 is 4 : 1. They also found a statistically higher rate of progression of the disease in Dw2-positive MS patients. This was also found by Hommes to be the case in the Netherlands. The particular importance of the HLA-D system in MS was borne out in a study of 300 MS patients by Grosse-Wilde et al.

An indication that the predominance of certain histocompatibility types in populations may have a bearing on MS prevalence rates is seen by Dean in that B7 is found in an unusually high percentage of the inhabitants of northeastern Scotland (34.4%), where MS prevalence is also high.

According to Zander, who analyzed the correlation between HLA-A, B-haplotypes, and MS in 34 pairs of siblings, a dominant type of inheritance with low penetrance may be operative with respect to MS susceptibility. He also considers the possibility, however, that additional genetic factors are codeterminant. Oger and Arnason, reviewing the literature on family studies, could not find adequate evidence of a single gene in the HLA complex as the susceptibility-determinant for MS. Alter asserts that efforts to establish the existence of an MS susceptibility gene by tracing inheritance of a shared HLA haplotype in multiplex MS families have failed.

Though representing only a brief section of the agenda, the reports and discussions on population genetics and MS clearly show the present state of our knowledge in this field, as well as the necessity to correlate investigations on histocompatibility types in MS not only with geographic and racial data, but also with specific exogenic conditions and the clinical situation. It must also be considered that, even with respect to methodology, the science of histocompatibility antigens is still in an early stage of development, and that new techniques may necessitate the revision of some of our present data.

Regarding geographic distribution, Alter pointed out discrepancies in the relationship of latitude and MS frequency, the local variations on examination of the "fine structure" of MS epidemiology, the gaps in our knowledge concerning the MS frequency at the extremes of latitude, in large areas of South America, the oriental countries, central and northern Africa. Referring to the multiplicity of environmental factors, the information on MS in migrant populations and the genetic aspects, Alter considers it necessary to supersede the phase of descriptive epidemiology, which has supplied a large body of information but no final answers regarding the cause and pathogenesis of MS, with an "experimental epidemiology" of combined field and laboratory studies.

Reporting on prevalence rates in Japan, Kuroiwa stressed that, in spite of great environmental changes, there has not been an increase in the last 20 years. He also pointed out that, compared to MS in the western countries, there were differences in clinical features, with more severe spinal lesions and visual loss, more intensive inflammatory reactions in the CSF, as well as no comparable HLA patterns and no increase in measles antibodies. Iwashita described the CSF alterations in 66 consecutive cases. Several patients with acute exacerbations had pleocytosis with polymorphonuclear cells predominating. The percentage of IgG increase (44%–51%) and the presence of oligoclonal bands (45%) was lower than that observed in most western case series. There were no differences in measles antibody titers in serum and CSF by hemagglutination inhibition and radioimmunoassay in MS and non-MS patients.

Dean examined the frequency of "hospitalized prevalence" of MS among immigrants to the United Kingdom. He found MS to be as common or nearly as common among immigrants from Europe and the old Commonwealth countries (Australia, Canada, New Zealand) as in people born in the United Kingdom. Immigrants from Italy had almost the same risk, from Ireland and Cyprus ⅔ of the risk, and

from Spain about ½ of the risk of the British. As for the new Commonwalth countries (West Indies, Africa, Malta, India, Pakistan) the risk of being hospitalized with MS was very small. A prevalence study in Malta and parts of Sicily revealed remarkable differences: in the city of Enna on the island of Sicily a prevalence of 53 per 100,000 was found. Malta had a prevalence of only 4 per 100,000. These remarkable differences are being examined more carefully to analyze ethnic, genetic, and exogenous factors involved.

Cazullo and co-workers presented prevalence rates from various areas in Italy, ranging from 5–7 per 100,000 in Messina and Naples to 20 in Aosta. A more detailed study in Novara and Varese provinces produced prevalence rates of 19.5 and 24, respectively.

Epidemiologists have constantly been on the search for MS clusters and the epidemic occurrence of this disease; results thus far have been meager and controversial. Within the high frequency areas of Europe Kurtzke identified spatial clustering in Norway, Sweden, Denmark, Switzerland, Finland, northern Scotland, and possibly the Netherlands. In a careful survey of all MS patients on the Faroe Islands, Kurtzke and Hyllested found 24 patients who met all criteria for a point source epidemic. They consider this to be the result of an infectious agent brought in by British occupation forces during World War II.

Palffy reviewed the clinical features in 219 MS cases observed in southwest Hungary (Pecs district) during the past 30 years. A remarkable observation was the complete nonexistence of MS in the gypsy settlements of the area.

These studies reflect two aspects that appear noteworthy: the global picture that is gradually emerging follows the accepted latitudinal pattern, and the observation that in far eastern countries of comparable latitude MS is rare, but that there are conspicuously abrupt differences in rather narrowly defined areas that do not fit into the large scale pattern. One of these is the abrupt drop of prevalence within the area of the Swiss canton of Wallis described by Bärtschi-Rochaix. Such differences may provide essential pathogenetic clues. Unquestionably, the validity of such figures is not only a statistical problem, but also one of the meticulous exploration of the areas in question, and of comparable methodology. The potential information involved underlines the necessity of what Alter calls experimental epidemiology. One of the approaches is the thorough and long-term epidemiologic study of pilot areas.

Two studies of this type were briefly described: a unique long-term study by Broman and associates has been under way for almost 30 years in the Göteborg area in Sweden. Some of the data on 312 patients, to be published in a detailed monograph, were presented at the symposium. An incidence rate of 5.3 per 100,000 was given, and in discussion Broman mentioned a prevalence rate of 130 per 100,000. A long-term epidemiologic study is also under way in the Göttingen area, where a prevalence rate of 76 per 100,000 was found. The 195 MS patients of the area have been carefully documented, and follow-up now covers a period of 12 years.

In studies of this type, the inclusion of optic neuritis is desirable. Haller et al., studying the epidemiology of optic neuritis in Hannover, found the same association of HLA types A3 and B7 as in MS; they estimated an annual incidence rate of 5 per 100,000.

One of the chief problems in bringing the relation of optic neuritis and MS into proper perspective is the great difference in data concerning the percentage of cases of optic neuritis as an initial symptom of MS. Figures range from 10% to 87%. The figures calculated by Poser et al. from our own data pool are 16% optic neuritis as the sole initial symptom and 39% as an initial manifestation along with other neurologic symptoms. It would therefore be a major error to equate optic neuritis with MS. On the other hand, Kuroiwa's observation is noteworthy that optic neuritis is an especially severe disturbance in the Japanese cases.

The Clinical Definition and Assessment of the Course of MS

The validity of epidemiologic studies depends on the thoroughness of survey of a given area and on the quality of the clinical data registered. Standardized diagnostic criteria are prerequisites for the comparability of results. Progress in clinical research, especially the development of laboratory techniques, which, though nonspecific, are of great value in supporting the diagnosis of MS, have motivated the International Medical Advisory Board of the International Federation of MS Societies (IFMSS) to carry out an investigation on the question of updating the diagnostic criteria of the Schumacher Committee of the US National MS Society. The results of the investigation were presented at the Göttingen symposium and provided the basis for a subsequent decision at the 1978 meeting of the IFMSS to establish a working group that should make recommendations. The most crucial questions, which led to considerable diversity of opinion in the discussion, were (1) whether the results of laboratory methods should be considered as criteria for establishing a definite, probable, or possible diagnosis of MS and (2) whether first bouts should be included as probable or possible MS. In the discussions it became apparent that the chief difficulties are encountered in defining the purpose that a set of diagnostic criteria is to serve: either to establish precise diagnostic standards for therapeutic studies (as originally intended by the Schumacher Committee) and for sophisticated clinicopathologic studies in relation to etiology and pathogenesis, or to provide, in a more limited way, general directives for diagnostic classification on the practical level and in epidemiologic field work where the facilities for laboratory studies are not available. The proceedings of the symposium could do no more than to provide a stimulus by raising questions. It may appear curious that after more than 100 years of clinicopathologic experience and research, the diagnosis of MS is still a problem; on the other hand, the influx of virologic, immunologic, biochemical, morphological, and neurophysiologic data obtained with modern research technology confronts us with baffling questions: for example, do we really know what MS is and is it a specific disease entity? To remain on solid ground in the face of such questions we need a well-defined clinical basis of description and classification, reconsidered from time to time to meet the challenge of changing concepts and new knowledge.

In addition to the refinement of CSF cytology, the measurement of IgG synthesis in the CNS and the demonstration of oligoclonal bands, a number of neurophysiologic methods and computer axial tomography are valuable new additions to the diagnostic armamentarium in MS.

McDonald reviewed the essential features of visual, auditory, and somatosensory evoked reactions and their clinical value in diagnosis. Although not specific for a demyelinating lesion, they can be determinants in establishing the polytopic nature of lesions and thereby the probability of MS, even when clinical features and laboratory tests are not typical. Ketelaer presented a multimodal evoked potentials program for the detection of clinically silent lesions, consisting of visual, brain-stem auditory, somatosensory evoked reactions and the blink reflex. He found subclinical evoked potentials in at least one of the tests in 62.5% of cases with clinically definite MS. In a study of 251 MS patients Lowitzsch found that diagnosis could be improved by 23% if visual evoked reactions were taken into account. Neetens et al. found the change in wave-shape even more reliable than latency in establishing a lesion in the optic system. They also stressed the generally accepted fact that visual evoked reactions are not specific for MS, and that sectional ischemic neuropathy and open-angle glaucoma give the same findings. The value of static perimetry for the precise quantitative evaluation and follow-up of visual disturbances in optic neuritis was described by Haller and co-workers.

By its very nature computer axial tomography cannot convey specific information concerning the demyelinating character of a lesion, but it has taught us two important things concerning MS: (1) that extensive transient focal edematous lesions (hypodense zones) can appear in the hemispheres in an early stage of acute MS, and (2) that a moderate degree of brain atrophy is a frequent finding even in cases not presenting conspicuous signs of functional impairment. Wüthrich presented data on 150 consecutive cases in which he found focal hypodense areas in about 20% and atropic changes in 50% of the cases. This method can be a most useful tool in following the course of acute exacerbations of MS and the effect of therapeutic procedures.

In a discourse on the effort to update the diagnostic criteria of MS, Ellison and Myers recommended a systematic approach according to the rules of taxonomy, presenting brief examples for their concept.

Basic rules for the quantitation and assessment of the course of MS were summarized by Fog, and a number of systems for diagnostic evaluation were briefly described. He pointed out particularly that the assessment of the course of MS, with its polytopic, variable, and transient symptomatology presents intricate problems. A standardized documentation system was described by Poser et al. and was used by the MS Research Program of the Deutsche Forschungsgemeinschaft, registering on optical mark reader sheets and providing computer analysis with printouts.

Diagnostic evaluation comprises (1) the establishment of diagnosis, (2) the assessment of the course of the disease, and (3) the evaluation of functional impairment. Careful case histories with meticulous registration of symptoms often convey little or no information on what the patient can still do. Evaluation of function is difficult because correct assessment requires numerous parameters and their correlation; factors like motivation and fatigue – one of the important, early, and often neglected symptoms in MS – are nonmeasurable variables.

Potvin et al., who have spent almost 20 years developing their neurofunction laboratory as an instrumentarium for the evaluation of neurologic function, gave a description of methods employed, the analysis of reproducibility, the role of learning, and other influences that must be considered in evaluation and clinical trials. Benecke and Conrad described the evaluation of motor deficits by the neurophysio-

logic analysis of stereotyped motions, using the model of bicycling for the study of spasticity and paresis of the lower extremities.

The reports and discussions of the Göttingen Symposium covered a wide range of topics, undertook a realistic, honest inspection of many unsolved problems and accepted no euphemistic explanations for them. It is gratifying that the sum total of this endeavor is on the positive side: the assurance that we are forging ahead, recognizing lines of advance that offer reasonable promise of success, and identifying approaches that may be mere blind alleys. One of the noteworthy features of this meeting was its organizational basis: it was a joint effort of many scientists, research funding organizations, National MS Societies, and the Medical Advisory Board of the International Federation of MS Societies. Our sincere thanks to all is the most pertinent concluding remark that can be made. May the cooperation that became visible in making the Göttingen meeting possible be a continuum, serving to intensify the international effort in the search for the cause and cure of MS.

The following addendum to the "scientific" program may seem to some almost irrelevant in view of the highly specialized reports that fill this monograph – all too far away from the interests of the scientists from many countries whose contributions sum up to an informative survey of our present knowledge concerning MS. However, there is one central reality in the complicated topography of MS research bringing us all together: the MS patient.

The panel discussion that concluded the symposium program was devoted to a brief survey of organizational concepts for the long-term care of MS patients. In reports from various countries, the experience with existing institutions was summarized and discussed in relation to the prerequisites for clinical research – careful assessment of the patient's condition and continuity in observations on the course of MS – but also beyond that and independent of the research aspect in relation to the everyday practical needs, the medical, psychological, and socioeconomic problems of the individual patient. The discussion was no more than a fragmentary entrée, but it strengthened the motivation and provided the groundwork for a forthcoming conference of the International Federation of MS Societies on this important topic.

Panel Discussion

The panel discussion which concluded the symposium program was devoted to a brief survey of organizational concepts regarding the long-term care of MS patients. In reports from various countries, experience with existing institutions was summarized and discussed in relation to the prerequisites for clinical research – careful clinical assessment and continuity in observations on the course of MS. Moreover, MS was discussed independent of the research aspect in relation to practical needs, and medical, psychological, and socioeconomic problems of the individual patient.

R. M. Schmidt: The discussion this afternoon in this portion is entirely open from the standpoint of members of the panel, with no prearranged or rehearsed plan of what we wish to say. In our different countries we think somewhat differently; in some health care and research are more controlled than in others. And even in countries with national schemes of developing clinical care or clinical research programs things have been done somewhat differently. In this context, the discussion will deal with (1) the advisability of one or more national centers, which might include an MS hospital; (2) clinics in terms of specialized clinics, perhaps associated with a medical/neurological center, such as the one here in Göttingen; and (3) the advantages and disadvantages of other concepts.

When I was a member of the US National Advisory Commission on MS, we rather closely examined the need to have comprehensive clinical programs. These were considered for research support if they were designed in connection with teams of competent researchers, not only clinical researchers but also those engaged in neuroscientific research relevant to MS. An example of this in the US is the University of Pennsylvania, where expert clinicians and superior scientists are working on multiple aspects of MS. Of course the management and organization of long-term therapy has a more general aim, namely, the humane and good care of patients who have MS or other diseases, the provision of well-studied groups of patients when we have new and experimental therapies to test, and new scientific approaches that may further our understanding of the disease.

The panel discussion is given in an abridged verbatim version, with reports and discussions from the following countries:

Denmark – T. Fog
Sweden – T. Broman
USA – R. M. Schmidt, R. J. Slater, W. A. Sibley, G. W. Ellison, W. Tourtellotte
West Germany – H. J. Bauer, S. Poser
Belgium – R. E. Gonsette
Netherlands – J. M. Minderhoud
Switzerland – R. Wüthrich
Canada – W. T. McIlroy

Great Britain – R. Kelly
Austria – K. Summer
Australia – P. Coleville
Japan – Y. Kuroiwa.

T. Fog: At Haslev, a former tuberculosis sanitarium 60 km from Copenhagen (58 beds) and in a smaller unit close to Aarhus (28 beds) there are special institutions where MS patients can be treated for 2–3 months. On the average they are admitted to these institutions once a year, but also oftener if the necessity arises (acute episodes, exacerbations).

In addition to therapy, it is the purpose of these units to educate students, practitioners, nurses, paranurses, physiotherapists, and social workers concerning special aspects of the treatment of MS patients, such as the alleviation of spasticity, how to treat bladder troubles, decubital ulcers, etc.

One of the disadvantages of this organizational concept is this: when you treat patients very intensely as in Haslev, they must continue treatment after dismissal. Even though the patients are informed as to how this is to be done, they don't do it. Returning to their old milieu, with the reversion from the pleasant conditions at the institution to the realities at home, psychological problems may arise.

T. Broman: In Sweden the situation is not ideal, but it works. The organization for MS patients varies in different parts of Sweden. At Göteborg there has been a special outpatient department for 10–15 years. A part of this is open to Ms patients and cooperates with the local MS Society. Patients can contact the doctor they know; they can write or telephone to him. There is close cooperation with a department for neurological rehabilitation, where physiotherapy is carried out and the resources for the home and work situation are worked out. The doctors cooperate with the local MS Society by going to meetings once or twice a year to answer questions. These can also be asked by MS patients anonymously in writing.

R. M. Schmidt: What happens if it turns out that the patient does not have MS?

H. J. Bauer: Our experience in West Germany is probably comparable to that in other countries. If a patient is receiving compensation for MS, which he will probably lose if it is established that he does not have it, he may be very indignant. It is often difficult to convince patients with spinal cord or brain tumors of the necessity of a myelography, angiography, and/or operation. It is very important – subject of course to this consent – to inform family members and to discuss the resulting problems with them and the patient.

The problem has another aspect: the first thing that MS centers to which patients are sent should do is check the diagnosis as carefully as possible or make sure that such a checkup has been made at a competent neurological center and at a recent date. If there is any doubt, the patient should be referred back to such a center for this purpose.

T. Fog: I had the following experience with two MS patients who thought they had MS and then were told that they did not have it: they became depressed because they had to leave the whole setup and atmopshere relating to the disease.

R. E. Gonsette: The Belgian National MS center at Melsbroek was founded 20 years ago. During this time, files of approximately 3000 patients, many of them relapsing cases, were accumulated. The hospital capacity is 140 beds.

We think that such centers are important for MS research because there a large patient pool is available. Most of our patients are chronic cases, some having had the disease for almost 20 years. One particular problem at our center is that it is far away from the nearest general hospital. Another drawback is its designation as a "MS center", which produces psychological problems in MS patients who don't know their diagnosis, but also in patients hospitalized with incipient MS who meet patients with more serious handicaps. Despite these problems, we are deeply convinced that such monovalent clinics with a permanent pool of Ms patients and careful follow-ups are a valuable tool for progress in diagnosis and treatment.

W. A. Sibley: Clinical centers have a real place in the study of MS for several reasons. One is that all research involves some measurement, and we don't have an adequate animal model. It is therefore of vital importance to gather accurate data. The late Henry Miller was asked if he could prove that trauma produced exacerbation. He believed that it did in some instances, but felt that this was not subject to proof, but rather a matter for experts. I think we are beyond that now, for by collecting and computerizing data in a prospective way we might simply answer a lot of questions. For instance, by asking whether MS is a uniform disease in all parts of the world at any given time, we might find out whether exacerbation rates are the same in the north as in the south. We might determine the effects of stress, exertion, immunization, etc. – for which we all have different answers at the moment. We really cannot advise patients in a rational way – we all think we can, we have our own schemes, but none of them are based on proof. We need centers in order to gather this kind of data and get more information about the disease which cannot be derived from animal models.

Dr. Minderhout: I agree with the experience gained in Melsbroek. Psychological factors may be serious disadvantages for such centers. There are other points: the education of students in other hospitals may suffer, because they will not see any MS patients there because these have all been hospitalized at a center. For research in MS centers, and even for diagnosis, it is important to have the possibility of comparison with other neurological diseases. Also, the facilities at an MS center may not include the care of MS patients with regard to socioeconomic factors, rehabilitation at home, and other problems prevailing after dismissal.

R. Wüthrich: Switzerland is small and prosperous and there are no financial problems relating to the care of MS patients. This was one of the first answers obtained from a survey conducted by the MS Societies. There are two rehabilitation centers, one in Montana (50 beds) and one in Walmstadtberg (30 beds). Very little research work is done there; however, scientific work on MS takes place in the universities. One of the main problems is what to do with patients not in a rehabilitation or treatment center, and this refers especially to the young handicapped. We are presently surveying the opinions of MS patients, for it is important not to plan without asking the patients. If you ask the patients, you may get answers you don't expect. All patients indicated that they did not want to go too far away from home. Therefore centers should not be hundreds of kilometers away from the families of the majority of MS patients. Patients also say that if they have to stay somewhere for a longer period of time, it shouldn't be at a hospital. Centers should therefore avoid having the character of a hospital as far as possible. Always ask the patient first; you may have to change some of your own ideas.

T. Fog: With respect to the education of medical students, all patients in Denmark pass through neurological departments. Patients are admitted to the special MS sanitaria only after assessment in a neurological clinic, so no neurological department loses MS patients. Very early MS cases, where the patients do not know their diagnosis, are not admitted to MS sanitaria. The most frequent are the patients with moderate to severe MS. Initially, I feared that by putting people with MS together emotional problems would result. After 20 years of experience I can say that, surprisingly, this is not the case.

H. J. Bauer: It is quite a general experience that the majority of MS patients welcome the opportunity to talk with fellow patients about their troubles. We all could cite notable examples of how they help each other, form clubs, enjoy common social doings, and work at common tasks, such as a welfare bazaars.

In West Germany, MS hospitals totalling over 400 beds have been established in Asbach, Falkenstein, Hachen, Langscheid, and Berlin-Wannsee, where patients can be treated for an average period of 6–8 weeks. This is unquestionably beneficial, especially for patients with MS of moderate severity. However, our German MS hospitals have not fulfilled the hopes placed in them for a number of reasons: they do not provide adequate facilities for the severe cases and possibilities for permanent care on the one hand, and they are not adequately equipped and staffed for adequate rehabilitation of MS patients on the other; this also makes it impossible to carry on scientific investigations in these hospitals with continuity. They are also rather far away from medical centers, with the exception of Dietenbronn, which is closely affiliated with the University of Ulm. The most serious question remains where, with the exception of nursing homes, can we send advanced cases who require rather intensive care and have trouble getting along at home?

What we lack are chronic hospitals, and in this connection two divergent opinions exist: institutions specialized for a particular disease, i.e., MS, or caring for a variety of chronic disease. There is something to be said for and against either concept. The chief argument against the special hospital is the psychological stress that might be generated by putting MS patients together. Personally, I would favor the special hospital, because for the severely ill it is of primary importance to have competent treatment and care for bed sores, bladder trouble, locomotor disturbances, etc. I think that the best possible relief in such conditions by far outweighs the fear concerning the psychological stress of being in a special MS hospital.

Another shortcoming of our German MS hospitals is that they can't take adequate care of these patients and the majority of our patients who have trouble getting along at home, who get into conflict with their families, end up in nursing homes not properly equipped and staffed to take care of their special needs.

G. W. Ellison: Taking up on Dr. Wüthrichs comment, one of the main reasons for a national center or regional center is the hope that it brings the patients. If you follow the course of patients, they have to go through a difficult diagnostic period, then they have a label placed upon them, they come to the doctor, and the first question they ask is "Doctor, what is the cure?" And you say, "I have no cure, but I will take care of you in the best way I know until the cure comes along." Then the next question is going to be: what life style will they follow? Loneliness sets in, anger with the physicians, fear for the future, uncertainly about their course, and guilt about having the disease. Finally, they get together with somebody else who has

MS. We began this type of program and labeled it a psychosocial support group, based on the model of anonymous alcoholics. And we found out, as Dr. Bauer has found, that these people are tough – they are really strong. And they get together – Dr. Wüthrich said we should ask the patients, but in Los Angeles they tell us, we don't ask. But they tell us the things that they have strong feelings about. And then a change comes over them; as they deal with more people who have MS, they become reliant upon themselves and they rely upon their friends who have MS, and our telephone stops ringing so often. We've transferred some of the responsibility for their life to them. And after all it is their life to live. We are interested in their medical problems, and we as researchers have the intellectual and empathic challenge of trying to do something about MS. These are some of the comments that I would make concerning the need for MS centers.

Dr. McIlroy: The Canadian Society until now has always felt that its primary purpose was the support of MS research. But there has been considerable pressure in last 3–4 years to get involved with MS clinics. Until recently the only MS clinic in Canada was the one at the Montreal Neurological Institute, and that has been in existence for 15–20 years. The Canadian MS Society decided to provide some starting financial support for three further MS research clinics. These would be clinics where patients would be carefully documented and used for research purposes. And before the clinics were to be funded, the character of the research going on at that particular university had to be assessed. So we now have clinics at the Montreal Neurological Institute, at the University of Montreal with Dr. Pierre Duquette, who worked in Los Angeles a couple of years ago, at London, Ontario with Dr. Donald Paty, and a further clinic in Calgary. Now these clinics are just getting underway. I think they are helping to stimulate and promote MS research in these areas, and there is a certain fringe benefit in that patients are probably getting better care. A greater problem for us to handle, and one we haven't come to grips with yet, is the problem of service clinics. Of course it has been said already this afternoon that we should listen to the patients and their families. But if we did that, certainly in our country anyway, we would be establishing service clinics in every province and every area of the country. I'm not sure that this is desirable. I'm also not sure whether it would be beneficial for the MS Society to get involved with patient service clinics. I've always wondered about the wisdom of lay volunteers becoming involved with patient care. This is a very controversial area. I appreciate that the American MS Society is going to move very slowly and that it is thinking about this matter. We will be interested to hear what your further thoughts are on that.

R. J. Slater: I would just like to add that I'm from Toronto, and a fellow Canadian, and they exported me to the States with some of these socialized ideas that I have about patient care.

T. Broman: Dr. Slater reminded me of a very important problem that I forgot to mention. The doctors in our department working with MS are not working alone on the problem. They are doing it together with physiotherapists, and particularly with a social worker. And most of the patients, who know us for years, make contact first and most often with the social worker. She then comes to us with the papers and problems and we discuss the necessities.

The most important element in the care and organization of MS patients is not clinical care, but the outpatient department with home service.

S. Poser: That is the model which we also consider most important. Patients who were examined by the same doctor came back; those who had a new doctor missed the dates. Therefore continuity in medical care is the most important point in keeping them attached to the hospital and to research activities.

Kelly: I agree that is important to have national centers, but it is equally important that MS patients should be within the context of a neurological clinic with wide interests and connected with general hospitals or academic centers. In special hospitals, multidisciplinary research is much more difficult. Special hospitals are overloaded with long-term cases. You tend not to get the acute cases, who go to the ordinary neurological clinics. Of course the situation is different from country to country. But as far as England is concerned, the staffing of hospitals that are devoted to one particular sort of disorder, MS, proves to be almost impossible. You can't get nurses of the right caliber to work in them, and you can't get proper junior hospital staff. It improves the whole patient care situation if this is a part of a larger department rather than a separate institution devoted exclusively to MS.

Referring to Dr. Slater, he felt that his framework wouldn't work in countries with different sorts of social service setups. This is perfectly true. Really we have in existence a framework just as you put up on the board. And the reason why it doesn't work is purely and simply because the governments which are supposed to provide the money don't understand that frameworks like that don't work unless you have sufficient staff. It's a perfectly workable framework. In some of the better organized districts, e.g., in London, just that sort of framework works extremely well in managing geriatrics. But it will work in one district and it won't work in 20 others. Because the other 19 districts will not provide the money to provide the physiotherapists and the district nurses. And one of the great problems, of course, is that in socialized medicine every general practitioner has to look after 3500 patients or else he can't pay his milk bill. And that is too many patients for one doctor to do and look after MS patients correctly. Its just a question of money.

Dr. Summer: Austria is also a small country, but unlike Switzerland, it has little money. So our point of view is somewhat different. In Austria we have a number of ambulatory centers to which MS patients can come weekly. We consider it best to first get from the patient a list of his or her needs, and not to proceed on the basis of what we want to do. The list may perhaps begin with mobility, holidays, family handling, job, etc. When we have learned what the patient needs, our team discusses the most efficient things to do for this patient with the limited money available. The aim in this assistance should be that the patient is not rigidly guided by the organization. We are very happy if the patient does not come to us after we have helped him, and becomes more self reliant.

Furthermore, we have founded a special hospital for the rehabilitation of MS patients.

Dr. Coleville: (Slides presented to illustrate examination principles.) We have been trying to establish what did these people need, and what the areas of difficulty are. We got some basic data about the patient and we classified this as "intact, limited, helper required (which was critical), and totally dependent." A helper is required if the patient has to be *cued* to do the activity – if he doesn't do it on cue, he doesn't do it. So we had a group of self-care activities, a group of mobility activities, a group which referred to communication, and a group which referred to

intellectual function and perception of their environment. Then we had a socio-economic group and a few funny things we were just interested in looking at.

Here's one with good scoring at the top, but starting to decay at the bottom, in a lot of financial difficulty, but with good family support. The next one: a patient quite able to dress herself, who can manage everything but the bath, and that's because her vision isn't good enough. An absolute disaster in this area, family support which collapses. And that patient, who is in effect totally independent, scores 99 out of 100 for dependence, and in actual fact is a nursing home patient. And that is because she has no regard for other people, is totally demanding, and the family support has collapsed. Using this sort of thing we end up with the preposition that if you are teaching people a new skill, compared with rheumatoid arthritis patients, for example, they take twice as many trials to learn the skill, and the next day they've forgotten twice as much as the normal individual. And this is the case in about 50%.

Y. Kuroiwa: I would like to just briefly describe patient service status in Japan. We have an MS research committee with members from all over Japan – about 20 people comprising a kind of consultation center.

Next comes the financial problem. The MS committee is supported by the government. MS is a kind of special disease, to be helped by the local as well as the federal goverment. If a patient is sent to the local health center with the diagnosis of MS, then further medical care will automatically be supported fifty-fifty by the local and the federal government. This solves the financial problem.

Our MS Society has been established only recently. We had a patient society established much earlier, at least 5 years ago. They have a sort of self-encouraging, self-supporting party group, not too aggressive, but which stimulates government quite a lot and urges the promotion of research.

As Dr. Kelly remarked, to establish an institute for a single disease exclusively is sometimes a waste of personnel. And a disease requiring a multidisciplinary approach sometimes is not suitable for a monovalent institution. In the Kiushu area I am trying to establish a center for chronic neurological disease.

T. Fog: Dr. Kelly raised the question of getting nurses and doctors for MS centers. In Denmark, of course, we also have this problem. But there have been no difficulties at all during the 20 years that Haslev has been in operation, there we have always had enough nurses. As for the doctors, we have two neurologists; once a week we go down to the institute and do practical work there. If something happens to the patient, for instance, an accident, the general practitioner living near Haslev is called. The economic problem is the budget for our institution (58 beds = 7 million Kroner per year).

R. J. Slater: Referring to Dr. Bauer's remark on the management of chronic handicapped patients, we have a very difficult problem in the US: because there is no place to send these people, they cannot be maintained in an acute phase hospital. So they have to go to a nursing home. In our system the nursing homes are within the reimbursable system of government payments, set up for senile people. And consequently one finds fairly young patients with MS, who are simply out of touch with life by being placed there. I think that the type of system that Dr. Fog has at Haslev Hospital, if that could be reproduced, let's say within rehabilitation centers that are attached to general hospitals throughout the country and where there would be social life built up for the MS patients, would operate as a rest center where

they could go for a weekend, a week, or a month at a rather lower rate of charge. But the problem of course is to build that into the reimbursement system of the country. I think that's the direction we would probably think of going, if we were as an MS society able to lobby with the government.

W. Tourtellotte: With the issue before us, I would like to give a few of my own comments. First, I think that such an operation should have an outpatient clinic attached to it. This means that the patients can then live at home and work at home. With this outpatient clinic should come a nurse who is actually taught and trained by the doctor, so that home care can be given through this doctor in his way.

I'd like to also emphasize the point that Dr. Kelly made. I have the feeling that these operations come off better in a full hospital, where you can have an acute neurology ward, perhaps next to a medical intensive care unit with psychiatrists and social workers.

In the Veterans Administration we have opportunities to give this "total care"; we not only have intermediate care for some of our severe MS patients, but the VA is also forward-looking enough to have given me a special MS treatment ward in which every one of the patients in one way or another is on either a pharmacological protocol or we're evaluating things in a serial fashion.

I also look forward to the success of the Canadian research clinics. I'd rather have them called MS telephone and treatment clinics, with the idea that you have hope before you, and that the patients don't feel like they are coming into a type of experimental setup. Because not all your patients are going to be in this situation and I don't think any patients should be excluded from the little we can offer them. I hope this summarizes an operation that, you know, has all these things, but still is kind of shooting for the stars.

T. Fog: There's an enormous difference between the atmosphere of a hospital and an institution like Haslev, where they are free to go about as they please. The climate is quite different from a hospital, and if you're spending 2 months, it is not good to be in a hospital for so long a time.

Editorial note: Without question, the organization of long-term care for MS patients, the operational forms, will continue to vary from place to place, depending on the structure of the respective society and the traditions and practical possibilities from which care has grown. Regardless of the organizational differences, however, the vital problems relating to the MS patient remain the same. This warrants a comparison and an exchange of the experiences gained in coping with these problems.

All of the participants in the panel realized that the discussion had been only a fragmentary approach to basic questions concerning the long-term therapy and prospective care of MS patients, but that so many important points had been brought up, that a continuation at the next meeting of the IMAB with more precise questions was necessary.

Subject Index

S. Poser

Multiple Sclerosis

An Analysis of 812 Cases by Means of Electronic Data Processing

1978. 28 figures, 16 tables. VIII, 93 pages
(Schriftenreihe Neurologie, Band 20)
ISBN 3-540-08644-7

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Berlin
Heidelberg
New York

The concept behind and techniques involved in basic documentation procedures are described, and show how a patient-oriented data pool can be constructed to establish the etiology and pathogenesis of multiple sclerosis and related diseases.
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T. Tsuboi, W. Christian

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Band 18

E. Esslen

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