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SEARCH FOR BIOMARKERS IN INFLAMMATORY DEMYELINATING DISEASES OF THE NERVOUS SYSTEM

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Current concepts on Multiple Sclerosis

Multiple sclerosis (MS), the most common cause of chronic neurologic disability beginning in early to middle adult life, has in recent years emerged as a subject of considerable interest to the neuroscience community. The global burden of MS has a number of truly remarkable characteristics, including an increasing incidence over the past century, an influence of latitude on risk, and increased risk in both females and white populations, especially those of northern European ancestry. Tracing the historical roots of MS has proven to be difficult, given the lack of knowledge of clinical-anatomic localization in neurology prior to the late 19th century. Notable is the paucity of convincing MS-like illnesses in the historical record prior to this time. Two reports from the late 13th century described women afflicted with chronic, multifocal, and partially remitting neurologic illnesses that conceivably might have been MS; these were the cases of a woman named Halla contained in the Icelandic Saga of St. Thorklar (Poser, 1994) and of a Dutch woman named Lidwina van Schiedam (Medaer, 1979). These uncertain cases excepted, MS makes its first clear appearance in 1822 in the diaries of Augustus D'Este, the illegitimate grandson of King George III (Firth, 1948).

Although it is not known whether MS is indeed a "new" disease, studies of the prevalence of MS conducted in multiple regions of the world suggest that the incidence increased steadily during the 20th century. It is possible that some of this increase is an artifact of improved case ascertainment, but most investigators believe that it is, at least in part, real, suggesting the presence of new environmental triggers. For many years multiple sclerosis was considered an immune-mediated disorder, primarily of interest to immunologists. However, the situation changed in the mid-1990s with the recognition that a neurodegenerative process, unresponsive to immunosuppression, was responsible for progressive neurological impairment. This new information, largely derived from results of clinical trials and reassessment of the neuropathology of MS, has brought myelin biology to the forefront of MS research. It emphasizes the need to understand, in the context of this disease, the axonal

changes that follow demyelination, axon-myelin interactions essential to normal neuronal function and survival, and oligodendrocyte differentiation and remyelination. This review builds on both well-established and emerging concepts of pathogenesis and their relationship to therapy, and the focus is on three core themes underlying the disease process: genes, inflammation, and neurodegeneration. No brief review of MS can fully address the breadth of issues related to this complex and multifactorial disease, and for more detail a number of outstanding sources exist (see for example Waxman, 2005 and Compston et al., 2006). The current challenges for the MS research community are to integrate large volumes of data from multiple sources to develop improved models of pathogenesis, and to translate these into effective therapies for the two million affected individuals worldwide.

Clinical Features

Symptoms of MS result from interruption of myelinated tracts in the central nervous system (CNS); the peripheral nervous system is spared. Initial symptoms are commonly one or more of the following: weakness or diminished dexterity in one or more limbs, a sensory disturbance, monocular visual loss (optic neuritis), double vision (diplopia), gait instability, and ataxia. Onset may be abrupt or insidious, and early symptoms may be severe or seem so trivial that a patient may not seek medical attention for months or years. As the disease worsens, bladder dysfunction, fatigue, and heat sensitivity occurs in most patients. Ancillary symptoms include Lhermitte's symptom (an electric shock-like sensation down the spine and into the limbs evoked by neck flexion), hemifacial weakness or pain, vertigo, and brief tonic spasms and other paroxysmal symptoms (thought to represent discharges originating along demyelinated axons). Cognitive deficits are common, especially in advanced cases, and include memory loss, impaired attention, problem-solving difficulties, slowed information processing, and difficulties in shifting between cognitive tasks. Depression is experienced by 60% of patients during the course of the illness, and suicide is 7.5-fold more common than in age-matched controls. The diagnosis of MS

has been revolutionized by magnetic resonance imaging (MRI) technology, which reveals multiple, asymmetrically located white matter lesions distributed throughout the white matter of the CNS, with a predilection for the corpus callosum and deep periventricular regions (Figure 1). New lesions are heralded by breakdown of the blood-brain barrier associated with perivenous inflammation and detected by extrusion of the heavy metal gadolinium across the blood-brain barrier. Spinal cord lesions are frequently present and can be detected with high sensitivity using high-field MRI. Ancillary tests, used primarily in uncertain or problematic cases, consist of cerebrospinal fluid (CSF) studies revealing low levels of inflammation with mononuclear cells (generally <50 cells/mm3) and raised levels of immunoglobulin, including antibodies with restricted clonotypes (oligoclonal bands). Evoked potentials in the visual, auditory, or sensory pathways may also be helpful in identifying additional, silent lesions (Hauser and Goodin, 2005).

Although early microscopic studies reported demyelination in the cerebral cortex of affected brains (Brownell and Hughes, 1962), MS is generally perceived as a white matter disease. There is, however, an increasing interest in the involvement of gray matter in MS, and some observers believe that cortical plaques are important contributors to motor, sensory, and cognitive disability in MS (Kidd et al., 1999; Peterson et al., 2001). Cortical demyelination appears to be very rare in acute or early relapsing MS and reduction of cortical thickness is seen primarily in patients with long-standing disease (Bozzali et al., 2002), suggesting that cortical pathology follows the white matter disease (Kutzelnigg et al., 2005).

Microglia activation and diffuse axonal injury are typically associated with active lesions in the cortex, but the classical perivascular lymphocytic infiltration characteristic of the white matter plaques is absent (Bo et al., 2003; Peterson et al., 2001; Kutzelnigg et al., 2005). Overall, there is an emerging consensus that cortical lesions and atrophy are major contributors to disease burden in patients with MS. The development of novel animal models displaying demyelinating lesions in the cortex (Merkler et al., 2006) will be of great value to fully assess the role of cortical pathology in MS.

The Basis of Neurological Disability

Although MS can vary from a benign illness to a rapidly evolving and incapacitating disease, most patients with MS ultimately experience progressive disability. Approximately 85% of all MS patients manifest initially as recurrent attacks of neurologic dysfunction (relapses); these occur 1–2 times annually and, especially in the early phases, are typically followed by gradual improvement (remissions) over several months. Women are affected with relapsing-remitting MS twice as frequently as men. Approximately 15% of patients begin with a purely progressive course, termed primary progressive MS; interestingly, these patients tend to be older than relapsing-onset patients and are male as often as female. MS relapses are associated with the development of new, focal, and usually permanent MRI lesions. These areas of altered signal intensity, once established, reflect fluid shifts due to a combination of demyelination and gliosis. Serial studies in relapsing MS indicate that approximately six out of seven newly formed MRI lesions are clinically silent. Furthermore, even symptomatic lesions do not in most cases produce permanent disability, as remissions are the rule, especially early in the disease course, and the correlation between the total lesion load detectible by MRI and concurrent disability is weak (Goodin, 2006). Nevertheless, the development of a large number and volume of lesions during the initial years of MS is strongly associated with a greater risk of disability occurring years later (Brex et al., 2002; Rudick et al., 2006). There is a widely circulated aphorism in the field that a relapse in 2006 may produce disability in 2016. This thinking has led to the widespread application of anti-inflammatory disease modifying therapies for MS, largely in the hope that reduction in the accumulation of focal lesions not only decreases relapses but might also lessen late neurodegeneration and ultimate neurologic disability. Disability in MS is not due primarily to the effects of relapses, but results from chronic progressive worsening, usually manifested as progressive spastic weakness of the limbs. For patients with relapsing-onset MS, the risk of transitioning from a relapsing to a progressive course

is relatively linear and approximately 2.5% annually. Fifteen years after diagnosis, fewer than 20% of patients with MS have no functional limitation, 50% to 60% require assistance when ambulating, 70% are limited or unable to perform major activities of daily living, and 75% are not employed. In a recently published natural history study, females reached disability endpoints at an older age than males (Confavreux and Vukusic, 2006a). However, the most important clinical factor influencing progression to disability was age at clinical onset: the younger the onset, the younger the age achieving the disability milestones. In contrast, no other variables, including relapsing or progressive course, substantially affected the time to reach severe disability (Confavreux et al., 2003; Confavreux and Vukusic, 2006b). Furthermore, times from clinical onset to a Kurtzke Expanded Disability Status Scale (EDSS) score of 6 (walking with unilateral aid) and 7 (wheelchair bound) were primarily influenced by the time the patient reached a score of 4 (limited walking but without aid). In contrast, no other variables had a measurable influence on the time elapsed from a score of 4 to a score of 6 or 7, or from a score of 6 to a score of 7. These observations suggest that once a certain pathologic threshold is reached, most patients with MS progress along a common irreversible neurodegenerative pathway. Despite important advances in therapeutics for MS, none of the currently available disease-modifying drugs have yet been shown to significantly alter the long-term natural history of the disease (Feldmann and Steinman, 2005; Hohlfeld and Wekerle, 2004; Noseworthy, 2003). Further, the partial, negligible, or deleterious effects that some approaches have yielded in the clinic, despite being successful at the bench, reflect the complex molecular interactions operating in MS and the limitations of current working hypotheses as faithful models of disease pathogenesis. These models support the occurrence of two overlapping and connected arms, inflammatory and neurodegenerative (Figure 2). Any satisfactory understanding of the biology of MS must explain the temporal relationship between these two phases of MS (Figure 3).

Genetic Susceptibility

MS clusters with the so-called complex genetic diseases, a group of common disorders characterized by modest disease risk heritability and multifaceted geneenvironment interactions. The genetic component in MS is primarily suggested by familial aggregation of cases and the high incidence in some ethnic populations (particularly those of northern European origin) compared with others (African and Asian groups), irrespective of geographic location. High frequency rates are found in Scandinavia, Iceland, the British Isles, and North America (about 1–2 in 1,000). Lower frequencies are found among southern Europeans. The disease is uncommon among Samis, Turkmen, Uzbeks, Kazakhs, Kyrgyzis, native Siberians, North and South Amerindians, Chinese, Japanese, African blacks, and New Zealand Maori (Rosati, 2001). According to some observers, this characteristic geographical distribution implicates a pathogen that is not ubiquitously distributed (see below).

However, this prevalence pattern can also be explained, at least in part, by the geographical clustering of northern Europeans and their descendants (Sotgiu et al., 2003).

Evidence of risk heritability in the form of familial recurrence has long been known. The degree of familial aggregation can be determined by estimating the ratio of the prevalence in siblings versus the population prevalence of the disease (ls). For MS, the ls ranges between 20 (0.02/0.001) and 40 (0.04/0.001). Half-sibling (Sadovnick et al., 1996), adoptee (Ebers et al., 1995), spouse (Ebers et al., 2000), and risk assessment studies performed in Canada appear to confirm that genetic, and not environmental, factors are primarily responsible for the familial clustering of cases. However, an intriguing association with month of birth was observed in the Canadian familial cases, reflecting perhaps an interaction between genes and an environmental factor operating during gestation or shortly after birth (Willer et al., 2005). Concordant sibs tend to share age of symptom onset rather than year of onset, supporting a genetic effect on the familial recurrence. Concordance in families for

early and late clinical features has been observed as well, suggesting that in addition to susceptibility, genes may influence disease severity or other aspects of the clinical phenotype (Barcellos et al., 2002; Brassat et al., 1999; Kantarci et al., 2002). Twin studies from different populations consistently indicate pairwise concordance (20%–30% in identical twin pairs compared to 2%–5% in like-sex fraternal twin pairs), providing additional evidence for a genetic etiology in MS. Overall, neither the recurrence familial rate nor the twin concordance supports the presence of a Mendelian trait. Modeling the available data predicts that the MS-prone genotype results from multiple interacting polymorphic genes, each exerting a small or at most a moderate effect to the overall risk. Their incomplete penetrance and moderate individual effect probably reflects epistatic interactions and postgenomic events; these may include genes that rearrange somatically to encode a vast variety of immune receptors, posttranscriptional regulatory mechanisms, and incorporation of retroviral sequences. An additional layer of difficulty is encountered when genetic heterogeneity is considered, whereby specific genes or alleles influence susceptibility and pathogenesis in some affected individuals, but not in others.

The HLA-DRB1 gene on chromosome 6p21 is the strongest genetic factor identified as influencing MS susceptibility (Figure 4). The association of MS with HLA genes, specifically the DRB1*1501 allele, has been a consistent finding across nearly all populations (Oksenberg and Barcellos, 2005). Recent studies suggest the possibility that complex trans HLA-DRB1 allelic interactions may determine the balance between susceptibility and resistance (Barcellos et al., 2003, 2006; Dyment et al., 2005). For example, there is a DRB1*15 dose effect on susceptibility, and the DRB1*15/08 and DRB1*15/14 genotypes are high risk and protective, respectively. The exact mechanism(s) by which the DRB1 gene influences susceptibility to MS remain undefined, but are likely related to the physiological function of HLA molecules in immune responses, including antigen binding and presentation, and T cell repertoire determination by negative selection of high-avidity autoreactive T cells within the embryonic thymic microenvironment (Stratmann et al., 2003; Wucherpfennig, 2005). Although a recent high-density single nucletoide

polymorphism (SNP) study covering the MHC region assigns the entire association signal to the HLA class II region (Lincoln et al., 2005), the debate concerning the role of non-HLA class II genes mapping to this region continues, with some data suggesting that additional disease genes lie within the central class III and/or telomeric to the class I HLA regions. The identification of the true predisposing gene or genes within the HLA region has been held back by the extensive linkage disequilibrium (LD) across the locus (Miretti et al., 2005). LD refers to the presence of alleles at neighboring loci segregating together at the population level more frequently than would be expected according to the genetic distance that separates them. LD generates long-range correlations among allelic variations in the human genome, the so-called "haplotype-blocks." The maintenance of these blocks is most likely due to the nonuniform distribution of recombination, which tends to occur at "hot spots" demarcating one block from the next. The extended haplotypes are the result of recent population history and, if common in a population, may indicate recent positive selection events. Available data show that LD extends over greater physical distances in the MHC region than elsewhere in the genome as a result of reduced recombination rate in the region, 0.44 cM/Mb, compared with a genome-wide average of 1.2 cM/Mb. Because LD patterns differ between populations, a powerful approach to resolving this complex genetic obstacle will be to scrutinize and compare a large number of MS haplotypes in well-characterized datasets from distinct populations. In the case of MS, this can be accomplished by comparing high-risk groups of northern European descent versus low-risk nonwhite populations. African Americans are at a lower risk for MS when compared with northern Europeans and white Americans (Kurtzke et al., 1979; Wallin et al., 2004), but tend to have a more aggressive disease course (Cree et al., 2004). In a recent study of HLA-DRB1 and -DQB1 alleles and haplotypes in an African-American MS cohort, a selective association with HLA-DRB1*15 was revealed, indicating a primary role for the DRB1 gene in MS independent of DQB1*0602 (Oksenberg et al., 2004). It is then likely that HLADRB1 constitutes the centromeric boundary of the class II DR-DQ association in MS. African-American patients also exhibited a high degree of allelic

heterogenity as disease association at the DRB1 locus was found for DRB1*1501, DRB1*1503, and DRB1*0301 alleles. The haplotypic features of the DRB1*1501-DQB1*X (non 0602) and DRB1*1503-positive chromosomes indicated an older African origin for the HLA-associated MS susceptibility gene(s), predating the divergence of human ethnic groups, rather than being solely due to genetic admixture with people of European descent. HLADRB1* 1501 has a relative low frequency in Africa. Positive selection for this allele appears to have occurred in Europeans, but not in Africans, and although the factors which drove this selection, presumably some infectious pathogen, are unknown, one possible consequence was a heightened susceptibility to MS in Europe, a disorder almost nonexistent in Africa. As indicated before, compared to European Americans, African Americans are at low risk for MS, supporting the presence of genetic risk factors that occur at higher frequency in Europeans. Because the sections of the genome in African Americans inherited from their European or African ancestors have only had an average of six generations of recombination, extended LD is present in these segments, and non-MHC disease genes are potentially amenable to identification through admixture mapping using relatively low numbers of ancestry-informative genetic markers (Patterson et al., 2004; Seldin et al., 2004). Admixture mapping is based on the observation that, on average, 80% of the ancestry of African Americans is West African and about 20% is European, and works by searching through the genome for sections with an unusually high proportion of European or African ancestry compared with the average. African Americans affected with MS will inherit a higherthan-average proportion of African or European ancestry, depending on which population has a higher risk for disease at the genetic level. We have already had successes with admixture mapping and just recently identified a locus on chromosome 1 where there is a significantly high proportion of European ancestry in MS chromosomes compared with the genome-wide average and controls; followup should identify the exact gene responsible for the admixture signal associated with MS (Reich et al., 2005). Unequivocal genetic linkage and association was repeatedly demonstrated in the HLA region. Using the elegant formulation of Risch (1990), it is possible to use

the values of HLA allele sharing by descent in sibships and estimate the proportion of λ_s that is explained by the HLA-DRB1 locus. Using data from 98 multicase MS families, we estimated that the HLA region accounts for 17%–60% of the genetic susceptibility in MS (Haines et al., 1998). Yet even at the upper bound of this estimate, much of the inheritance of MS remains unexplained. The lack of an obvious and homogeneous mode of transmission has slowed progress by preventing the full exploitation of classical genetic epidemiologic techniques for gene discovery. Nevertheless, an approach that dominated MS genetics until recently involved first determining the chromosomal region of the genetic effect by linkage analysis, which has been extremely productive for mapping genes responsible for monogenic diseases. The establishment of genetic linkage requires the collection of family pedigrees with more than one affected member to track the inheritance of discrete chromosomal segments that deviate from independent segregation and cosegregate with the disease. The potential of linkage mapping for gene identification in complex diseases was highlighted in studies of type 2 diabetes, Crohn's disease, and schizophrenia (Stefansson et al., 2002). To assess the full power of the linkage approach and upgrade the MS genetic map, one of the largest linkage screens ever performed in an autoimmune disease was recently completed by an international consortium (Sawcer et al., 2005). Unequivocal linkage was demonstrated in the MHC region (as expected) and suggestive linkage was identified on broad regions of chromosomes 17, 19, and 5; fine mapping is in progress. However, the statistical scrutiny of this map indicates that the next generation of studies attempting to identify the genes influencing the development of this disease will need to rely on association methods and large DNA collections. Genome-wide association studies harbor great potential for complex disorders, but a number of very important challenges, including how to interpret results obtained from large numbers of statistical tests, and how to detect biologically meaningful interactions between polymorphisms that confer disease risk, will need to be overcome. Progress in developing affordable high-throughput genotyping technology and a better understanding of the complex functional structure embedded in the human genome

suggest that the tools may finally be at hand to achieve the elusive goal of whole-genome association studies. A powerful trio-based study using 500,000 SNP arrays is currently in progress by this consortium, and results are expected by early fall. This screen will provide a more definitive MS genetic map, but all candidate loci will require stringent independent replication. Furthermore, interpretation of genomic data must take into account that variants of interest may result from segmental duplications (Fredman et al., 2004), inversions (Stefansson et al., 2005), loss of imprinting (Mummert et al., 2005), paralogous or other regions with polymorphic genomic imbalances (Iafrate et al., 2004), or genes resistant to X-inactivation (Carrel and Willard, 2005). It is also important to consider that preferential allelic expression provides an additional source of variability (Lo et al., 2003b; Yan et al., 2002), and copy number polymorphisms (CNPs) contribute substantially to normal human genomic variation for numerous genes involved in neurological function, regulation of cell growth, and regulation of metabolism (Sebat et al., 2004).

Role of the Environment

Epidemiological, clusters or outbreaks, and migration studies have been widely used to illustrate potential environmental influences on MS. Although the interpretation of most of these studies has been difficult, in part due to the small number of study participants in the individual reports, the results have been influential and do suggest a role for environmental factors in MS, and in some cases, they suggest the existence of critical time periods for exposure to putative environmental disease agents. A large number of environmental exposures have been investigated. Those include viral and bacterial infections, nutritional and dietary factors, well water consumption, exposure to animals, minerals, trauma due to accident or surgery, pollution, solar radiation, temperature, rainfall, humidity, chemical agents, metals, organic solvents, and various occupational hazards. Common viruses are among the most frequently studied and biologically plausible putative infectious agents related to MS pathogenesis, and many have been proposed at one time or another to be the

causative MS agent. Prominent candidates have included measles, rubella, mumps, and the herpes viruses, including Epstein Barr virus (EBV), herpes simplex virus (HSV) 1 and 2, varicella zoster virus, and HHV6. Strong evidence for a role of EBV in particular, a ubiquitous herpesvirus with a worldwide distribution, has been indicated by epidemiologic (Ascherio et al., 2001) and laboratory studies (Cepok et al., 2005; Levin et al., 2005). A higher risk of infectious mononucleosis (associated with relatively late EBV infection) and higher antibody titers to the latency-associated antigen EBNA1 are associated with MS, and conversely, individuals never infected by EBV are at lowMSrisk. No compelling mechanistic explanation to account for the EBV-MS relationship is known. Attempts to isolate the causative environmental trigger(s) in MS have been largely unproductive and have failed to provide breakthrough insights into mechanisms of disease susceptibility and pathogenesis. This may be due to heterogeneity operating also at the level of causative factors. Whether the genotype dictates different forms of the same disease in response to a common causative agent or other trigger or whether the genotype reflects different diseases with completely separate environmental causes is not known. The expectation that any single agent would have enough specificity and universality to account for all cases of this disease seems unlikely. However, it would be premature to dismiss the possibility that a previously undetected agent could be responsible for MS, acting either as a trigger for an autoimmune response, or even as a chronic active CNS infection directly responsible for symptoms.

Triggering and Persistence of Inflammation

Early in the 1930s, Rivers and others defined the laboratory autoimmune disease experimental allergic (or autoimmune) encephalomyelitis (EAE) (Rivers and Schwenker, 1935). EAE can be induced in a variety of animal species, including nonhuman primates, by immunization with myelin proteins or their peptide derivatives. When studied in genetically susceptible animals, immunization induces brain inflammation accompanied by varied signs of neurologic disease. EAE and MS

share common clinical, histologic, immunologic, and genetic features; hence EAE is widely considered to be a relevant model for the human disease (Steinman and Zamvil, 2006). Demonstration in the early 1960s that EAE could be adoptively transferred by myelin-sensitized T cells inaugurated the era of T cell immunology in MS research, an approach that in many respects dominates the field to this day. Although the inflammatory changes that occur in MS may ultimately be shown to be secondary rather than primary (inflammation and selective destruction of brain elements may also occur in non autoimmune conditions, including genetic disorders such as adrenoleukodystrophy or chronic virus infections such as HTLV-1), numerous studies in blood, cerebrospinal fluid, and brain tissues of individuals with MS have exposed cellular and humoral immune responses against CNS antigens that are not as prominent in non-MS controls (Hafler et al., 2005). These studies provide the rationale for a disease model driven by the loss of immune homeostasis and uncontrolled immune responses against structural CNS components.

During the initial state of the inflammatory response, lymphocytes with encephalitogenic potential are activated in the periphery and home to the CNS, become attached to receptors on endothelial cells, and then proceed to pass across the blood-brain barrier (BBB), through the endothelium and the subendothelial basal lamina into the interstitial matrix. Remarkably, the presence of immunocompetent cells with autoimmune potential appears to be an embedded characteristic of the (healthy) immune system in vertebrates (Genain et al., 1994; Hohlfeld et al., 2000). These cells may provide important inflammatory signals necessary for wound healing, angiogenesis, neuroprotection, and other maintenance functions. Furthermore, CNS-specific T cells can provide neurotrophic factors such as BDNF and contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood (Ziv et al., 2006). The transition from physiological to pathological autoimmunity involves at least two factors: (1) the loss of immune homeostasis, normally maintained through the induction of anergy or apoptosis, receptor downregulation, editing, and anti-idiotypic/cellular regulatory networks, and (2) the engagement and activation of lymphocytes by adjuvant signals including,

conceivably, recurrent exposures to exogenous pathogens. This could occur via nonspecific polyclonal activation of T and B cells by bacterial or viral antigens, or, alternatively, as a consequence of structural homology between a self protein and a protein in the pathogen, a process commonly referred as molecular mimicry (Steinman et al., 2002). It is notable, for example, that components of the myelin sheath share amino acid homologies with proteins of measles, influenza, herpes, papilloma, and other viruses. These pathogens acquired sufficient homology to engage myelin-specific T cells and drive a misguided response (Lang et al., 2002). Amino acid identity may not even be required for cross-reactivity to occur between the autoantigen and the mimic, as long as they share chemical properties at critical residues that allow anchoring to antigen-presenting molecules and interaction with the T cell antigen receptor. Once activated, T cells express surface molecules called integrins, which mediate binding to the specialized capillary endothelial cells of the BBB. One such integrin, VLA-4, binds the vascular cell adhesion molecule (VCAM) expressed on capillary endothelial cells following induction by TNF-a and IFN-g during an inflammatory response. Building on successful modulation of demyelination in animal models with anti a4b1 integrin antibodies (Yednock et al., 1992), clinical trials with Natalizumab, a humanized monoclonal antibody directed against the a4 integrin of the adhesion molecule VLA-4, were conducted, and very encouraging results were reported in both MS (O'Connor et al., 2004) and Crohn's disease (Ghosh et al., 2003). Unfortunately, this modality of therapy resulted in an unexpected compromise of CNS immune surveillance mechanisms in a small number of individuals who were concomitantly treated with immunomodulatory or immunosuppressive therapies, resulting in progressive multifocal leukoencephalopathy (PML) due to JC virus infection (Sheridan, 2005). At least one affected patient was on Natalizumab monotherapy at the time PML developed. Ransohoff (2005) proposed that Natalizumab may activate bone marrow B cells, which appear to serve as natural reservoir for PML virus in the healthy adult and promote viral replication and passage of the virus to the CNS. As the activated T cells migrate across the BBB to reach the CNS parenchyma, they express gelatinases

(matrix metalloproteinases, MMP) responsible for lysis of the dense subendothelial basal lamina. The clinical relevance of metalloproteinases is underscored by the observation that some members of this family of molecules are present in the cerebrospinal fluid of patients with MS, but not in normal controls (Leppert et al., 1998). TNF-a-converting enzyme (TACE, ADAM 17), a member of the ADAM (a disintegrin and metalloproteinase) family of enzymes, releases TNF-a from its cell membrane-bound precursor by proteolytic cleavage. In a longitudinal study of 11 relapsing-remitting MS patients, TACE mRNA expression in peripheral blood mononuclear cells showed a significant correlation with the number of lesions (Seifert et al., 2002). Thus, metalloproteinases may act not only as mediators of cell traffic across the BBB, but may also increase the inflammatory reaction through TNF processing. Furthermore, a direct neurotoxic effect for metalloproteinases has been proposed as well; microinjection of activated MMPs into the cortical white matter of experimental animals results in axonal injury, even in the absence of local inflammation (Newman et al., 2001). Beta interferon, currently the most frequently employed treatment for MS, works in part by reducing expression of MMP by activated T lymphocytes and interfering with their passage across the BBB (Leppert et al., 1996; Stuve et al., 1996). After traversing the BBB, pathogenic T cells are reactivated by fragments of myelin antigens. Recent data suggest that this is a two-step process (Platten and Steinman, 2005). Primed CD4+ T cells are first engaged by CD11c-expressing antigen presenting cells (APCs) in the perivascular space before moving into the parenchyma. Reactivation induces additional release of proinflammatory cytokines that stimulate CD11b microglia, further open the BBB, and stimulate chemotaxis, resulting in additional waves of inflammatory cell recruitment and leakage of antibody and other plasma proteins into the nervous system. Both CD11c and CD11b are members of the integrin family expressed on neutrophils, monocytes, NK cells, and activated T and B cells. The activated C11b+ cells also contribute to the inflammatory milieu by secreting T cell-activating factors such as IL-12, IL-23, and toxic mediators such as nitric oxide (NO) and oxygen radicals. Pathogenic T cells may not be capable of producing or inducing tissue injury

in the absence of the secondary leukocyte recruitment. For example, in EAE mediated by adoptive transfer of myelin-reactive encephalitogenic T lymphocytes, these cells are among the first to infiltrate the CNS, but constitute only a minor component of the total infiltrate in the full-blown lesion. Despite being very effective in preventing EAE, anti-CD4 antibody therapy has not been successfully translated to humans (Lindsey et al., 1994). CAMPATH-1H, which targets the CD52 antigen present on lymphocytes and monocytes and causes prolonged T cell depletion, showed substantial reduction in disability at 6 months in relapsing, but not secondary progressive, MS patients, perhaps due to the suppression of ongoing inflammation in these patients with active inflammatory disease (Coles et al., 2006). The dramatic appearance of thyroid autoimmunity in up to one-third of treated patients may be related to the relaxation of T cell-mediated homeostatic mechanisms induced by the anti-CD52 therapy (Goodnow et al., 2005). B cell activation and antibody responses appear to be necessary for the full development of demyelination, both in humans and in experimentally induced diseases in animals. In most MS patients, an elevated level of immunoglobulins synthesized intrathecally can be detected in the cerebrospinal fluid. Although the specificity of these antibodies is mostly unknown, anti-MBP specificities have been detected. Myelin-specific autoantibodies have been detected bound to the vesiculated myelin fragments, at least in some patients (Genain et al., 1999; O'Connor et al., 2005). Recent data suggests that antibodies specific for aquaporin-4 water channel, a component of the dystroglycan protein complex located in astrocytic foot processes at the blood-brain barrier, appear to be a biomarker of neuromyelitis optica, an demyelinating disease that selectively affects optic nerves and spinal cord and is considered an inflammatory condition that phenotypically resembles a severe variant of MS (Lennon et al., 2005). Myelin-specific infiltrating B cells have been detected in the MS brain, and in the CSF and brain parenchyma of affected individuals there is an elevated frequency of clonally expanded B cells with properties of postgerminal center memory or antibody-forming lymphocytes (Baranzini et al., 1999; Colombo et al., 2000; Corcione et al., 2004; Owens et al., 1998; Smith-Jensen et al., 2000; Zhang et al., 2005). Limited

histological data suggests that B cell differentiation, affinity maturation, and antibody secretion occur primarily in the Virchow-Robin and meningeal spaces in MS, which may assume secondary lymph node-like characteristics. Antibodies may participate in myelin and axonal destruction through different mechanisms such as opsonization, which facilitates phagocytosis by macrophages; complement fixation; or stimulation of antibody-dependent cell-mediated cytotoxicity (ADCC) by binding to natural killer cells. The systemic administration of B cell-depleting antibodies, such as the humanized anti-CD20 monoclonal antibody Rituximab, is currently under evaluation in MS (Cree et al., 2005; Cross and Stark, 2005), and positive results from a phase 2 trial of Rituximab in relapsing-remitting MS were recently reported. Rituximab causes transient depletion of CD20+ pre-B and mature B cells, but not stem or plasma cells. B cell depletion affects antibody production, as well as Bcell-mediated antigen presentation and activation of T cells and macrophages. Antagonism of Bcell-activating factor of the tumor necrosis factor family (BAFF; also known as BLyS—B lymphocyte stimulator), an important survival factor for peripheral B cells, may be an alternative or an adjunct to Rituximab therapy to enhance autoimmune B cell sensitivity to intrinsic tolerance mechanisms (Goodnow et al., 2005). BAFF is primarily produced by radioresistant lymphoid stromal cells, but a recent report suggests that it is also produced in the brain by astrocytes and is upregulated in MS lesions (Krumbholz et al., 2005). An anti-BAFF antibody-based therapy is currently being tested in systemic lupus erythematosus.

Modulating the Immune Response

Interferon β (IFNβ) is a member of the type I interferon family and, as a recombinant reagent, is the most frequently prescribed therapeutic to control exacerbations in relapsing-remitting MS. IFNβ has pleiotropic effects, including antagonism of IFN-γ-mediated MHC upregulation on antigen-presenting cells, alteration of the profile of cytokine expression, modulation of apoptotic pathways, and blockade of migration across endothelia. Overall, IFNβ has been shown to decrease clinical relapses, reduce brain MRI activity, and possibly slow progression of disability. However, the effect of

this treatment is partial, and a substantial proportion of patients are not responders. Therapy has been associated with a number of adverse reactions, including flu-like symptoms, transient laboratory abnormalities, menstrual disorders, increased spasticity, and dermal reaction. Furthermore, the significance of long-term effects and impact on disease progression has not been determined. Baranzini and collaborators applied predictive modeling tools to a longitudinal gene expression dataset generated from MS patients treated with IFNβ (Baranzini et al., 2005). The analysis identified sets of gene triplets whose expression, when tested before the initiation of therapy, can predict favorable response with up to 87% accuracy. It is noteworthy that the genes in the top-scoring triplet were Caspase-2, Caspase-10, and FLIP, three apoptosis-related molecules. Despite the relatively high predictive accuracy of these models, the functional link between genes and therapeutic effects of this drug is still unclear.

Glatiramer Acetate, a polymer molecular mimic of a region of myelin basic protein, is another popular FDA-approved drug for MS. It affects the cytokine expression pattern, but it also may saturate MHC molecules, preventing efficient presentation of autoantigens by monocytes and dendritic cells, and may induce active T cell suppression against MBP (Farina et al., 2005). A new generation of therapeutic synthetic peptides developed based upon a better understanding of the molecular structure of HLA molecules may be available for clinical trials in the near future (Stern et al., 2005). Glucocorticoids are also potent inhibitors of antigen presentation function. The chemotherapeutic drug cyclophosphamide is lympholytic and stimulates production of anti-inflammatory Th2 cytokines. Additional experimental therapies focus on interference with antigen presentation to encephalitogenic T cells (altered peptide ligands, intravenous antigens), induction of a Th2 response, T cell depletion (anti-CD52 or anti TCRVβ), blockade of adhesion molecules, administration of anti-inflammatory cytokines (IL-10, TGF-β), or neutralization of proinflammatory cytokines (type IV phosphodiesterase inhibitors, nerve growth factor, TNFR p55 Ig fusion protein, anti TNF-αIgG1). Anti-TNF biologicals (monoclonal antibodies and soluble receptor) have been extensively and successfully used in rheumatoid arthritis,

ankylosing spondylitis, Crohn's disease, and psoriasis. However, treatment of MS using lenercept, a TNFRp55-FC construct, appears to have increased the frequency of relapses. N-(3,4,-Dimethoxycinnamoyl) anthranilic acid (3,4-DAA), an orally active synthetic derivative of the amino acid tryptophan metabolite anthranilic acid, suppressed proliferating of myelin specific T cells, inhibited production of proinflammatory cytokines, and reversed EAE paralysis; tryptophan metabolites may represent a novel class of drugs to control inflammation in MS (Platten et al., 2005).

Other approaches that have also proven effective in blocking EAE, such as the use of statins, which inhibit LFA-1, modulate MHC expression, and induce immune-deviation, and antihistamines, which engage H1 receptors found in MS brain, may provide a new therapeutic strategy in MS for previously approved drugs. A preliminary trial with statins has shown some degree of efficacy (Vollmer et al., 2004). Similarly agonists for the peroxisome proliferator-activated receptor-α and angiotensin blockers may prove to be effective in MS by inducing a cytokine shift from the proinflammatory to the anti-inflammatory type (Feldmann and Steinman, 2005). Finally, it is possible to reverse ongoing paralysis in the EAE model by using vectors encoding regulatory cytokines (or inflammatory cytokine inhibitors) or by tolerizing the immune system via injection of naked DNA encoding myelin antigens together with DNA encoding the cytokine IL-4. DNA vaccination has been taken into the clinic for infectious disease and cancer, and trials are now being organized to apply this approach to autoimmune diseases, including MS (Feldmann and Steinman, 2005).

A key but unresolved question is whether a single mechanism of tissue damage is operative in MS, or whether fundamentally distinct pathologies are present in different cases. Heterogeneity has been observed in terms of whether the inflammatory infiltrate is associated with the deposition of antibody and activation of complement, and whether the target of the pathology is the myelin sheath or the oligodendrocyte (Lucchinetti et al., 2004). A recent study of very early MS lesions describes extensive apoptosis of oligodendrocytes as the earliest structural change observed (Barnett and Prineas, 2004). These changes, associated with microglial

activation but without other evident inflammatory infiltrates, resembled ischemic changes. One critically important question is whether this heterogeneity reflects fundamentally distinct mechanistic processes responsible for axonal damage, which may require overlapping restorative strategies.

Neurodegeneration

The traditional neuropathological view of MS highlights myelin loss as the key event leading to impaired propagation of action potentials across the exposed region of the axon and ensuing neurological deficits. However, the early literature on MS already described substantial axonal damage in active lesions (for an historical review see Kornek and Lassmann, 1999). Contemporary high-resolution histopathological studies reveal abundant transected and dystrophic axons in sites of active inflammation and demyelination, and confirm that partial or total axonal transection begins early in the disease process (Bitsch et al., 2000; Ferguson et al., 1997; Trapp et al., 1998). Axonal damage appears to take place in every newly formed lesion, and the cumulative axonal loss is considered now to be the reason for progressive and irreversible neurological disability in MS (Neumann, 2003). Furthermore, reduced axonal density is also observed in inactive and remyelinating lesions, cortical tissue, and the normal-appearing white matter in the brain and spinal cord. Pathological studies indicate that as many as 70% of axons are lost from lateral corticospinal tracts in patients with advanced paraparesis (Bjartmar et al., 2000). Advanced MRI techniques increasingly provide a clinically useful and quantitative window into the dynamic processes that result in progressive axonal and neuronal loss over time. Examples include accelerated rates of whole brain atrophy (Miller et al., 2002); gradual worsening of focal (T1) lesions over time (Bagnato et al., 2003); reductions in the predominantly neuronal/axonal marker n-acetyl aspartate (NAA) as assessed by spectroscopy (Gadea et al., 2004); disruption of individual white matter tracts, measured by diffusion tensor imaging (Pagani et al., 2005); and evidence of plasticity and altered functional connectivity by functional MRI (Cader et al., 2006). Taken together, these in vivo methods lend further support to the concept that axonal and

neuronal loss is responsible for the persistent neurological dysfunction that occurs in patients with MS (Filippi and Rocca, 2005).

Knowledge of the mechanisms that trigger axonal injury is far from complete and it is unclear whether demyelination is a prerequisite for axonal injury in MS. Mice null for the glial cyclic nucleotide phosphodiesterase (Cnp1) gene developed axonal swellings and neurodegeneration throughout the brain, leading to hydrocephalus and premature death, but the ultrastructure, periodicity, and physical stability of myelin are apparently not altered (Lappe-Siefke et al., 2003). On the other hand, mice deficient for myelin associatge glycoprotein (MAG) show late-onset axonal disease preceded by paranodal axon atrophy with reduced neurofilament spacing, suggesting that an underlying disruption of myelin can lead to a delayed and progressive axonal loss (Li et al., 1994). Late-onset degeneration and disability also occurs in proteolipid protein (PLP) null mice (Griffiths et al., 1998). This also may be the case in human MS (Garbern et al., 2002). Demyelination results in reduced support for the axons as well as redistribution of ion channels, destabilization of axonal membrane potentials, reduced excitability, and conduction block. Axons can initially adapt and restore conduction, explaining remissions, but eventually distal and retrograde degeneration occurs. Therefore, the early promotion of remyelination and preservation of oligodendrocytes remains an important therapeutic goal in MS. Another source of functional CNS plasticity is the potential for axonal remodeling independent of myelin integrity. In a modified EAE model, spinal cord inflammation resulted in extensive interneuron sprouting and connectivity associated with neurological recovery (Kerschensteiner et al., 2004).

In apparent contrast to the model of primary oligodendrocyte disease, some evidence suggests that axonal damage is mediated directly by resident and invading inflammatory cells and their toxic soluble products, in particular microglia, macrophages, and CD8+ T lymphocytes. Indeed, neurons and axons express class I MHC molecules, leaving them vulnerable to cytotoxic T cells (Hoftberger et al., 2004). Axon-specific antibodies and complement may also mediate axonal injury (Zhang

et al., 2005). A recent study in EAE provided convincing evidence that axonal damage is associated with tau phosphorylation and aggregation (Schneider et al., 2004); the tau pathology was dependent on inflammation and could be partially prevented by early prednisolone treatment.

Liu et al. (2006) add to the growing body of evidence in support of CNS-immune system cross-talk by demonstrating a role for neurons in controlling the function of inflammatory T cells within their immediate microenvironment. Neuron-T cell contact interaction results in the local differentiation of T cells with a CD15+TGF-β1+CTLA-4+Foxp3+ phenotype that suppresses proliferation of encephalitogenic CD4+ T cells and progression of EAE. Transforming growth factor-β (TGF-β) is a critical differentiation factor for the generation of these regulatory T(reg) cells, through upregulation of Smad3 and IL-9. Remarkably, the T(reg) cells appear to be converted from already-committed and activated encephalitogenic T cells in the CNS environment. Interleukin-6 (IL-6) completely inhibits the generation of Foxp3(+) T(reg) cells induced by TGF-β in peripheral tissues, but together, IL-6 and TGF-β induce the differentiation of pathogenic IL-17-producing cells from naive T cells. T-helper-17 is a recently described CD4+ lineage distinct from classical Th1/Th2 cells implicated in a growing list of autoimmune diseases. TGF-β acts to upregulate the IL-23 receptor, providing a positive autocrine signal for expansion of Th17 cells (Bettelli et al., 2006; Mangan et al., 2006). The overall cytokine milieu leads to the development of mutually exclusive effector or regulatory inflammatory pathways, which may be key to understanding how inflammation and infection is linked to the development of CNS autoimmunity and neurodegeneration.

Resident microglia activated in the neuroinflammatory process are likely to cause CNS tissue injury through the release of mediators such as NO and oxygen radicals, vasoactive amines, complement, proteases, cytokines, and eicosanoids. Chronic "inactive" (e.g., noninflammatory) MS lesions, and even normal-appearing white matter in MS cases, are also characterized by activated microglia and raised concentrations of NO (Lassmann, 2003). Excessive amounts of NO have been linked

to neurological symptoms as a result of direct injury to oligodendrocytes and axons (Acar et al., 2003; Smith, 2005). For example, demyelinated axons exposed in vitro to NO experience significant conduction block, whereas myelinated axons were affected only at higher concentrations (Redford et al., 1997). Possible mechanisms include the direct nitration of Na channels and inhibition of mitochondrial respiration, leading to reduced ATP production. Aminoguanidine, a selective inhibitor of NO, prevents the clinical development of EAE and reduces inflammation and demyelination (Cross et al., 1994). On the other hand, mice genetically deficient for NOS2A were shown to be highly susceptible to EAE induced by immunization with myelin oligodendrocyte glycoprotein (MOG) (Sahrbacher et al., 1998), consistent with the presence of complex and systemic regulatory networks in autoimmune demyelination.

NO also mediates the excitatory effect of glutamate, and the excess of glutamate released by microglia and macrophages, accompanied by a decrease in glutamate intake and metabolism, activates α-amino-3-hydroxy-5-methil-4-isoxazolp-propionic acid (AMPA), which is highly toxic to oligodendroglial cells and neurons. Blockade of AMPA-responsive glutamate receptors with AMPA antagonists ameliorates neurological sequelae in EAE, increases oligodendrocyte survival, and reduces dephosphorylation of neurofilament H, an indicator of axonal damage. Drugs affecting microglial function, such as hydroxymethyl-glutaryl coenzyme A (HMG-coA) reductase inhibitors, or agonists of peroxisome proliferator-activated receptor (PPAR) α, such as gemfibrozil, ameliorate EAE and are now being tested in MS (Platten and Steinman, 2005). Another source of glutamate release is the astrocyte, through a Ca-regulated exocytosis-like process (Bezzi et al., 2004). Interestingly, glutamate release appears to follow the activation of the CXCR4 by the chemokine stromal cell-derived factor 1 (SDF-1) and is followed by rapid release of TNF-α by the microglia, which can lead to neuronal death by apoptosis (Bezzi et al., 2001).

Recent work shows NMDA receptor (NMDAR) expression and function in oligodendrocytes (Salter and Fern, 2005; Karadottir et al., 2005). Expression was detected in several regions of the brain and at various developmental stages, but

appears limited to the oligodendrocyte processes, explaining why previous studies that have focused on the somata failed to detect these receptors. This represents an unexpected and novel finding since glutamate was thought to activate exclusively the AMPA and kainate receptors in oligodendrocytes. Under pathological conditions, NMDAR stimulation may provoke oligodendrocyte injury. Using an in vitro model mimicking ischemia, Salter and Fern (2005) demonstrate that glutamate activation of the NMDAR and the subsequent intracellular Ca^{2+} overload cause the loss of the oligodendrocyte processes. Moreover, the selective NMDAR antagonist MK801 was able to confer protection in this model. Karadottir et al. (2005) simulated ischemia in brain slices by energy deprivation, causing the development of an inward current in the oligodendrocytes, partly evoked by the NMDAR. Altogether, the data shows Ca^{2+} dependence in the NMDAR-mediated oligodendrocyte injury, and indicates that Ca^{2+} influx can affect the cytoskeletal element within the processes, which will determine their stabilization or retraction. The unusual subunit composition of these receptors, which include primarily the NR1, NR2C, and NR3A subunits, suggest that novel selective NMDA receptor antagonists can be developed devoid of the side effects characteristic of the existing ones.

Once the molecular mechanism of axonal damage is triggered, ion fluxes, mitochondrial dysfunction, and activation of proteases culminate in degradation of cytoskeletal proteins and axonal disintegration (Dutta et al., 2006). The early influx of Na^+ and Ca^{2+} ions into the axoplasm as a result of channel exposure or upregulation is highly excitotoxic and leads to interrupted axonal transport and accumulation of proteins, such as amyloid precursor protein, N-type voltage-gated Ca^{2+} channels, nonphosphorylated neurofilament proteins, and metabotropic glutamate receptors (Peterson et al., 2005). Limited data suggest that neuronal cell death by apoptosis could also have a significant contribution to neurodegeneration in MS (Cid et al., 2002).

Promoting Remyelination and Repair

A characteristic of many MS lesions is the presence of large numbers of NG2-positive oligodendrocyte precursor cells (OPC) as well as PLP-positive preoligodendrocytes that survive the acute inflammatory onslaught (Chang et al., 2000). These arrested oligodendrocytes extend processes to the vicinity of surviving axons, but fail to remyelinate. The development of practical strategies to promote reconstitution of functional myelin from this locally available precursor pool represents an obvious strategy for restorative therapy (for a recent review, see Dubois-Dalcq et al., 2005). Following injury, oligodendrocyte-mediated remyelination is dependent on the transcription factor Olig-1, which represents an excellent target for therapy (Arnett et al., 2004). Inflammatory signals derived from macrophages (Kotter et al., 2005) and lymphocytes (Bieber et al., 2003; Foote and Blakemore, 2005) also influence the capacity for remyelination. Remyelination is also enhanced by cytokines such as IL-1β (Vela et al., 2002), either directly or indirectly via stimulation of IGF-1 by astrocytes, macrophages, or monocytes (Mason et al., 2001). Growth factors, such as platelet-derived growth factor (PDGF) or FGF-2, have been shown to expand OPCs (Frost et al., 2003; Woodruff et al., 2004). Negative effects on remyelination can also result from the cytokine milieu present in a chronic inflammatory or highly gliotic environment (Diemel et al., 2004; Foote and Blakemore, 2005), highlighting the complexity of the interactions. Furthermore, it is possible that the demyelinated axon is unreceptive to remyelination due to intrinsic axolemmal changes, possibly including expression of inhibitory cell surface molecules such as polysialylated-neural cell adhesion molecule (Charles et al., 2000), changes in membrane caliber, neurofilament fragmentation, or energy failure (Dutta et al., 2006).

Human OPC engrafted in a myelin-deficient (shiverer) mouse were able to restore myelination (Windrem et al., 2004), raising hope that a similar strategy might be useful in MS. Schwann cells represent another potential source of cell replacement therapy (Bachelin et al., 2005). In EAE, systemically injected neurospheres protected animals from paralysis; however, these beneficial effects appeared to be mediated by

induction of immunoregulatory networks that reduced tissue damage, and not primarily by promotion of remyelination (Pluchino et al., 2005). The failure of adequate remyelination in MS appears to result from an inhospitable environment within the plaque or lack of sufficient signals for extensive myelination (Setzu et al., 2006), rather than a lack of myelin precursor cells. Therefore, it seems unlikely that therapy with nonengineered precursor cells will provide, on its own, a sufficient stimulus for remyelination. More likely, engineered cells could be used to deliver therapeutic molecules to areas of tissue damage.

The concept that progressive ("slow-burning") axonal loss occurring over time in demyelinated lesions is responsible for late chronic disability is supported by histologic studies (Bjartmar et al., 2000; Kornek et al., 2000) and by serial MRI studies revealing accelerated atrophy over time (Losseff et al., 1996). As summarized above, high concentrations of NO may contribute to mitochondrial dysfunction and raised intracellular Na^+ and Ca^{2+} (Smith et al., 2001). Consequent to demyelination, the coexpression of Na^+ channels with the Na^+/Ca^{2+} exchanger along the length of the naked axon could further contribute to Na^+ influx and raised intracellular Ca^{2+} (Craner et al., 2004). Axonal protection strategies using Na^+ channel inhibitors, either phenytoin (Lo et al., 2003a) or flecainide (Bechtold et al., 2004), have shown potential as neuroprotective agents in preclinical EAE models, and human trials are underway with phenytoin and lamotrigine.

With the aid of high-powered laboratory technologies, we are now in a position to define the full array of genes, molecules, and pathways operating in MS. This information will likely provide a reliable conceptual model of pathogenesis and lead to novel curative strategies. This goal can only be achieved if sufficient knowledge exists to distinguish disease variants, reliably classify therapeutic outcomes, and capture key individual genetic, medical, and molecular profiling variables.

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Current concepts on Chronic Inflammatory Demyelinating Polyradiculoneuropathy

Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) is a clinical syndrome based on a physiological and pathological concept (Dyck et al., 1975; Ad Hoc Subcommittee of the American Academy of Neurology AIDS Task Force, 1991; Burns, 2004; Köller et al., 2005). The core clinical features are a chronic progressive or relapsing and remitting, symmetrical, and sensory and motor polyradiculoneuropathy causing weakness of proximal and distal muscles. The cerebrospinal fluid (CSF) protein concentration is almost always increased. Electrophysiological evidence of demyelination is required for the diagnosis (Ad Hoc Subcommittee of the American Academy of Neurology AIDS Task Force, 1991; Nicolas et al., 2002; Van Den Bergh and Pieret, 2004). As the disease advances, axonal degeneration becomes superimposed. Histological examination of active lesions reveals lymphocytic infiltration and macrophage-associated demyelination (Bouchard et al., 1999). In addition to this core clinical picture, pure motor, pure sensory (Oh et al., 1992), multifocal sensory and motor (Lewis et al., 1982; Saperstein et al., 1999; Viala et al., 2004), and multifocal motor (Nobile-Orazio et al., 2005) forms have been described as subcategories or separate entities. Here we examine the evidence for the hypothesis that CIDP is auto-immune. In doing so, we have focused on the typical symmetrical sensory and motor core syndrome (Hughes et al., 2005) and taken into account recent reviews of the same topic (Kieseier et al., 2004; Rezania et al., 2004), presenting the evidence for each of the main proposals regarding etiology. Treatment of CIDP with immunomodulatory agents is only beneficial in two-thirds of patients, and even in these the treatment response is often unsatisfactory (Donofrio, 2003;Hughes, 2003). A better understanding of the disease mechanisms should guide the design of better treatments.

Pathology

Autopsy studies

Because the clinical definition requires that the neuropathy be chronic and death rarely occurs until many years have elapsed, it is not surprising that postmortem examinations predominantly show the resolution of damage rather than its initiation. A small number of studies have shown sparse multifocal inflammatory changes throughout the spinal roots and peripheral nerves with loss of myelin and axonal degeneration (Kimber et al., 2003; Hahn et al., 2005). Marked hypertrophy of spinal roots and nerve trunks sometimes occurs. This is due to onion bulb formation and the deposition of amorphous material consisting of acid mucopolysaccharides. In cases where there is no persistent inflammation (Matsuda et al., 1996), the presumption is that the inflammation had burned out or been suppressed by corticosteroid treatment. The central nervous system (CNS) has usually been spared apart from anterior horn cell chromatolysis and degeneration of the dorsal columns, which are sequelae to be expected after spinal root axonal damage (Hughes, 1990b). The distribution of lesions resembles that in chronic experimental autoimmune neuritis (EAN, discussed below).

Biopsy studies

Nerve biopsies have revealed that active lesions consist of endoneurial infiltrates of lymphocytes and macrophages. Deposition of immunoglobulin (Ig)M and complement or its membrane attack complex has been reported on the surface of myelin sheaths (Dalakas and Engel, 1980; Mazzeo et al., 2004). Macrophages can be observed traversing the Schwann cell basal lamina and penetrating and stripping the myelin lamellae as in acute inflammatory demyelinating polyradiculoneuropathy (AIDP) and EAN (Prineas, 1971). The occurrence of onion bulb formations of layers of Schwann cell cytoplasm interspersed with collagen fibers and loss of nerve fibers demonstrate the chronicity of the pathological process. Inflammatory cells are found in both the endoneurium and the epineurium but, in marked contrast to vasculitic neuropathy, are more abundant in the endoneurium in CIDP. The inflammatory lesions consist

predominantly of macrophages with variable numbers of T cells. In large series, sural nerve biopsy has been disappointing as a diagnostic instrument because inflammatory features are uncommon, being present in none of 23 biopsies in one series (Prineas and McLeod, 1976), only 11% of 56 biopsies in another (Barohn et al., 1989), and at the most 17% (with marked T-cell infiltration in only 4%) of 95 biopsies in the most recent large series (Bouchard et al., 1999). When they are seen, perivascular accumulations of T cells are often minor and may be predominantly epineurial (Vallat et al., 2003), which is against expectations and may reflect the rarity with which biopsies are obtained from sites containing early active lesions. The lack of inflammation in sural nerve biopsies is not surprising in view of the predominantly proximal and motor distribution of the lesions causing the neurological deficit. Although the pathological process is usually approximately symmetrical, occasional patients have initial involvement of a single nerve, which may persist or remit and then relapse for several years before other nerves become involved. The affected nerve may become markedly hypertrophic. Pathological studies of biopsies from such nerves have shown chronic inflammation in the perineurium and numerous onion bulbs in the endoneurium (Verma et al., 1990).

The occurrence of inflammatory foci is not pathognomonic of CIDP because they were also found in 12% of a series of 42 patients with Charcot-Marie-Tooth disease type 1: macrophage-associated demyelination was often present in nerves biopsies taken during childhood (Gabreels-Festen et al., 1993).

T cells

Immunohistochemical studies have shown that T cells are more frequent in the endoneurium of sural nerve biopsies from patients with CIDP than those in normal subjects or patients with chronic idiopathic axonal polyneuropathy (Cornblath et al., 1990;Schmidt et al., 1996;Khalili-Shirazi et al., 1998;Bosboom et al., 1999). The T cells predominantly bear T-cell αβ-receptor chains and consist of CD4 and CD8 cells in variable proportions, one sometimes exceeding the other. T cells in nerve biopsies

from patients with CIDP secrete matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, which will break down endoneurial proteins, a phenomenon which is not specific for CIDP, also occurring in vasculitic neuropathy (Leppert et al., 1999). The Vβ gene usage of the T cells in the sural nerve in CIDP is heterogeneous, indicating a broad spectrum rather than the restricted or clonal T-cell response expected if the cells were targeted to a limited number of antigens. This heterogeneous response could be caused by epitope spreading and may not represent the initial and presumably causative T-cell infiltrate (Bosboom et al., 2001). Activated T cells will enter the nerve irrespective of their antigenic specificity, recruited by chemokines and upregulation of endothelial adhesion molecules. For instance, endothelial cells in the nerves of CIDP patients contain the chemokine, interferon (IFN)-γ-inducible protein of 10 kDa (IP-10), and the CSF IP-10 concentration is increased. Cells resembling T cells in the endoneurium and perineurium express the IP-10 receptor CXCR-3 (Kieseier et al., 2002).

Macrophages

The cells expected to present protein antigens to T cells in the endoneurium are the macrophages, but there is also evidence that Schwann cells and endoneurial cells may play a role (discussed below). In conformity with this expectation, endoneurial macrophages, identified by their expression of the CD68 molecule, constitutively express human leukocyte antigen (HLA)-DR which is required for the presentation of conventional antigen to T cells (Van Rhijn et al., 2000). Macrophages contain nuclear factor (NF)-κB. This transcription factor is normally sequestered in the cytoplasm complexed to inhibitory κB (IκB) molecules. Upon receptor-mediated activation, IκB is phosphorylated and dissociates. Then NF-κB translocates to the nucleus and binds to the promoters of genes involved in macrophage activation and release of cytokines and enzymes. The low level of NF-κB in uninflamed nerves becomes much enhanced in active lesions in Guillain–Barré syndrome (GBS) and CIDP (Andorfer et al., 2001). In keeping with this finding, the expression of the inflammatory cytokines interleukin (IL)-1β and IL-6 is increased in the endoneurium in CIDP. Although the investigators

were uncertain of their localization, the responsible cells are likely to be the macrophages (Lindenlaub and Sommer, 2003). Increased numbers of cells in the endoneurium resembling CD68-positive macrophages express the chemokines CCR-2 and CCR-5, which are the receptors for monocyte chemoattractant proteins (MCPs) 1 and 2 (Kieseier et al., 2002).

The principal antigen-presenting cells in other tissues are the dendritic cells. Dendritic cells have the function of transporting processed antigen to lymph nodes and presenting it to naïve lymphocytes. Peripheral nerve, like brain, does not have many of these cells. Dendritic cells can be identified with antibodies to the CD83 molecule but could not be found even in inflamed nerves in one study (Van Rhijn et al., 2000). Dendritic cells express the CD1 family of receptors, which present non-protein antigens including glycolipids, to some T-cell subsets. Within the endoneurium, the macrophages do not express these constitutively but have the capacity to do so because CD1a, CD1b, and CD1c (but not CD1d) were seen on the endoneurial macrophages in biopsies from patients with vasculitis and CIDP (Khalili-Shirazi et al., 1998;Van Rhijn et al., 2000).

In addition to the engagement of the T-cell receptor (TCR) by antigen and major histocompatibility complex (MHC), stimulation of T cells requires co-stimulatory actions between accessory molecules on the antigen-presenting cell and ligands on the T cell. Thus, both CD80 (B7.1) and CD86 on macrophages and activated T and B cells engage with CD28 on the T cell and cause stimulation. They also engage with CD152 [cytotoxic T-lymphocyte antigen (CTLA-4)], which causes inhibition of T-cell activation. Because mRNA for CD80 and CD86 was increased in the endoneurium of sural nerve biopsies from some patients with CIDP (as well as GBS and neuroborreliosis), endoneurial cells (macrophages are the most likely candidates) have the potential to express this accessory molecule (Kiefer et al., 2000). So far, attempts to demonstrate CD80, CD86, and another co-stimulatory molecule, CD40, on endoneurial macrophages have been unsuccessful (Van Rhijn et al., 2000).

Endothelial cells

With the perineurium, the endoneurial endothelial cells normally contribute to the blood–nerve barrier, protecting the endoneurium from circulating inflammatory factors. The blood–nerve barrier is compromised in inflammatory neuropathy: loss of the tight junction proteins claudin-5 and ZO-1 from the endoneurial vessels occurs in CIDP and may contribute to the breakdown of the barrier (Kanda et al., 2004). Furthermore, cells in the endoneurial vessel walls secreting MMP-9, an enzyme involved in inflammatory processes, are abundant in sural nerve biopsies in CIDP but not in distal diabetic sensory neuropathy (Jann et al., 2003). The endothelial cells also have the potential to express cell adhesion and co-stimulatory molecules. In CIDP, endothelial leukocyte adhesion molecule (ELAM-1 or E-selectin, which is characteristically present on inflamed but not normal endothelium) was identified on the endothelial cells of the epineurium but not of the endoneurium (Oka et al., 1994). In both inflammatory and non-inflammatory neuropathy, MHC class II expression was present on endoneurial endothelial cells, although it was less abundant than on epineurial endothelium (Atkinson et al., 1993). In inflamed, but not normal, nerves, endothelial cells expressed CD58 [lymphocyte function-associated antigen-3 (LFA-3)], which interacts with CD2 on T cells and natural killer (NK) cells (Van Rhijn et al., 2000). This interaction can induce T-cell proliferation and T helper (Th)2-type cytokine production. In vasculitic neuropathy, endoneurial cells also expressed the co-stimulatory molecule CD86 (discussed above) (Van Rhijn et al., 2000).

Schwann cells

The Schwann cells, the most abundant cells in the endoneurium, express, or have the capacity to express, molecules which are able to contribute to the stimulation of T cells. There are increased amounts of mRNA for the cytokine IL-6 and growth factors nerve growth factor, glial cell line-derived neurotrophic factor, and possibly leukemia inhibitory factor and reduced amounts for ciliary neurotrophic factor in nerve biopsies from CIDP patients with corresponding alterations of their cognate receptors

(Yamamoto et al., 2002). The Schwann cells are the cells most likely to be responsible for these changes, which may be responsive to inflammation and nerve damage and involved in the repair process. In CIDP and vasculitic neuropathy, there was strong expression of CD58 (discussed above) by Schwann cells (Van Rhijn et al., 2000). If the Schwann cells were to be involved in presenting antigen to T cells, they would be expected to express the co-stimulatory ligands CD80 and CD86. There is conflicting evidence whether these molecules can be detected by immunohistochemistry (Murata and Dalakas, 2000;Van Rhijn et al., 2000) with the balance of evidence being that staining of Schwann cells with antibody to CD80 is rare (Kiefer et al., 2000). There is some experimental evidence to support a contribution from Schwann cells in presenting antigen to T cells. When stimulated in vitro and in inflamed nerve in vivo, they can express MHC class II molecules (Lilje and Armati, 1997;Mathey et al., 1999), although they do not constitutively do so (Atkinson et al., 1993). They can present myelin basic protein to myelin basic protein-responsive T-cell lines and stimulate the proliferation of P2-specific T-cell lines (Wekerle et al., 1986;Argall et al., 1992a;1992b). In the uninflamed nerve, Schwann cells express IκB which binds the excitatory NF-κB transcription factor and may therefore control inflammatory responses involving these cells (Andorfer et al., 2001)

Experimental Models

Classical experimental auto-immune neuritis

EAN can be induced in laboratory animals by immunization with peripheral nerve myelin or myelin proteins emulsified with Freund's adjuvant (Mäurer and Gold, 2002). Similar disease can be induced in Lewis rats with purified P2 basic protein, P0 glycoprotein, or PMP22 protein (Kadlubowski and Hughes, 1979;Milner et al., 1987;Gabriel et al., 1998). Immunization with antigens, such as myelin basic protein and myelin-associated glycoprotein (MAG), which are also present in CNS myelin, usually produces inflammatory responses predominantly in the spinal cord near the

root entry zones, but there may be some spill-over into the nerve roots (Weerth et al., 1999). The disease is predominantly T cell driven because it can be transferred with T cells alone directed against immunodominant peptides in the P2 or P0 molecules (Linington et al., 1984;1992). However, more severe disease with more demyelination is produced by the addition of antibody to myelin or myelin glycolipids at the same time as the T-cell transfer (Hahn et al., 1980;Spies et al., 1995). Administration of antibodies alone does not induce disease, probably because they do not reach the endoneurium. Intraneural injection of antibodies directed against myelin antigens, especially those such as galactocerebroside and P0 which are abundantly expressed at the myelin–Schwann cell surface, induces demyelination (Hughes et al., 1985). The models most studied have been acute, with disease developing after about 10 days, reaching a nadir after about 15 days and recovering substantially by 28 days. However, some animals develop a chronic relapsing course with histological appearances resembling CIDP including the formation of onion bulbs (Pollard et al., 1975;Harvey et al., 1987;Brosnan et al., 1988;Adam et al., 1989). In particular, a different rat strain, the Dark Agouti, has recently been shown to develop biphasic experimental neuritis in which the second attack was attributed to persistent T-cell activation with epitope spreading (Jung et al., 2004). EAN can be prevented or ameliorated by corticosteroids, intraperitoneal Ig, plasma exchange (PE), and a wide variety of immunosuppressive and immunomodulatory agents, some of which may also be effective in CIDP (Hughes, 1990a); one, leflunomide, is effective (Korn et al., 2001) but has been reported to cause peripheral neuropathy (Bonnel and Graham, 2004). EAN has also been used as a model for investigating the mechanisms involved in autoimmune demyelination. For instance, interference with the cell adhesion molecule intercellular adhesion molecule-1 and its ligand LFA-1, both important in T-cell activation, abrogates actively induced EAN in rats (Archelos et al., 1993;1994).

The histology of the acute disease is that of the more or less simultaneous breakdown of the blood–nerve barrier and migration of CD4 and CD8 T cells and macrophages into the endoneurial perivascular space and then more diffusely in the endoneurium (Powell et al., 1983). Macrophages invade the Schwann cell basement

membrane and insert themselves into the myelin lamellae, phagocytose myelin, and eventually denude the axons (Lampert, 1969). In areas of severe inflammation, the axons are damaged, collapse, and are replaced with ovoid clumps of myelin debris. The Schwann cells proliferate, and eventually some remyelinate the demyelinated axons, while others form redundant layers of cytoplasm which contribute to onion bulbs. These are especially marked in the dorsal root ganglia (Adam et al., 1989). This process is orchestrated by chemokines. In the rat model, mRNA for macrophage inflammatory protein-1α and -1β is upregulated first during the development of the disease followed by MCP-1, IFN-γ IP-10, and RANTES at the height of the disease (Kieseier et al., 2000). Because these cytokines are also upregulated during Wallerian degeneration, these changes are not specific for auto-immune inflammation.

It is more difficult to induce EAN in mice, but it has been done by immunization with peripheral myelin, P2 protein, and P0 peptide 180–199 (Taylor and Hughes, 1985;Calida et al., 2000). One group has induced EAN with the P0 peptide 180–199 in C57BL/6 mice and shown that the disease severity is reduced in mice genetically deficient in CD8 and especially CD4 cells but that CD4$^-$CD8$^-$ and B cells are not needed for disease induction (Zou et al., 2000). This model is particularly useful because of the ready availability of gene knockout mutants on the C57BL/6 background. Mice deficient for the CD28 molecule did not develop EAN showing the importance of this accessory molecule (Zhu et al., 2001). It was possible to show the much greater severity of EAN in apolipoprotein E-deficient mice, suggesting that this lipid transport protein has a role in protecting nerves from damage in inflammatory neuropathy (Yu et al., 2004). It also provides a model in which immunological manipulations can be undertaken to test possible treatments. Thus, soluble tumor necrosis factor (TNF) receptor type I and antibodies to IL-18 reduced the severity of EAN induced with P0 peptide 180–199 indicating the importance of these cytokines in enhancing the inflammatory process (Yu et al., 2002;Bao et al., 2003).

Spontaneous neuropathies in other animal models

The principal antigen-presenting cells in the peripheral nervous system are the resident endoneurial macrophages that are expected to constitutively express the CD86 co-stimulatory molecule required for T-cell stimulation. Transgenic mice, which over-express this molecule, develop spontaneous inflammatory demyelinating lesions in their CNS and spinal roots. This spontaneous autoimmune disease is T-cell mediated because it does not develop in mice which lack the TCR β chain gene (Zehntner et al., 2003). Non-obese diabetic (NOD) mice are widely used as a model for spontaneous diabetes. However, B7.2$^{-/-}$ (CD86) NOD mice are protected from diabetes but develop chronic inflammatory peripheral neuropathy instead (Salomon et al., 2001). Moreover, treatment of ordinary NOD mice with anti-B7.2 antibody also reduced diabetes and induced inflammatory neuropathy. Thus, the balance between co-stimulatory molecules and their CD28 and CTLA-4 receptors on T cells is crucial in the development of experimental autoimmune neuropathy. Because CD28 delivers a positive stimulatory signal and CTLA-4 an inhibitory signal to effector T cells, changing the levels of B.7 expression on antigen-presenting cell might deregulate a balance between the activating and inhibiting autoreactive T cells infiltrating the peripheral nervous system. In another NOD mouse model, depletion of the regulatory CD25^{+}CD4^{+} T cells by injection of anti-IL-2 monoclonal antibody induced autoimmune neuropathy as well as other T-cell-mediated diseases (Setoguchi et al., 2005). The antigens involved in these models are not known, but the observations are crucial to understanding autoimmune peripheral nerve disease. They suggest that a non-specific stimulus, such as an infection, might increase the expression of co-stimulatory molecules or alter the balance between activation and regulation in a susceptible individual and result in autoimmune disease of the nervous system. This broadens the focus from identifying individual antigens to include factors controlling the expression of the regulatory molecules in the nervous system.

Bystander Demyelination

An alternative hypothesis to auto-immunity as a cause of inflammatory demyelination is that immune responses to extraneous antigens reaching the endoneurium cause a

local non-specific inflammatory response and this in itself is sufficient to produce demyelination. It has not been possible to model this convincingly in laboratory animals. Inflammatory responses against tuberculin injected into the nerves of sensitized animals induce myelin breakdown, but this is the consequence of axonal degeneration and not primary demyelination (Wisniewski and Bloom, 1975;Powell et al., 1984;Powell and Hughes, 1987). Induction of an inflammatory response to ovalbumin by intraneural injection of antigen in rats which had received anti-ovalbumin T cells intravenously produced axonal degeneration but not demyelination: when antibodies to myelin were also given, then demyelination was produced, arguing for the necessity for an auto-immune response to induce primary demyelination (Harvey et al., 1995;Pollard et al., 1995). This experiment and the observations about over-expression of the B7-2 co-stimulatory molecule in the last paragraph show that both local inflammation and systemic auto-immunity are needed to generate autoimmune inflammatory demyelinating neuropathy.

Clinical Features

Epidemiology

The population prevalence of CIDP has been estimated as at least 1.2 per 100,000 and as much as 7.7 per 100,000 (Lunn et al., 1999;McLeod et al., 1999;Mygland and Monstad, 2001). It is consistently slightly more common in males than females, has an average age of onset in the 50s, and becomes more common with advancing years, although it also occurs in children. The disease has been reported from throughout the world without any suggestions of case clustering or racial predominance. In one series, the relapse rate was three times higher during a pregnancy than a non-pregnancy year with the relapses tending to occur in the last pregnancy trimester or in the puerperium (McCombe et al., 1987). Despite some suggestive case reports (Donaghy et al., 1989;Hayashi et al., 1993), there is no clear association between onset or relapses and the immediately previous occurrence of infection (Meléndez-Vásquez et al., 1997)[see Hughes (1990b) for review]. There are

a few reports describing an association between relapses and immunization, but these are insufficient to establish a significant pathogenetic role (Pollard and Selby, 1978;Pritchard et al., 2002).

Relationship to other diseases

It is principally the temporal course that distinguishes CIDP from GBS. The relationship between the acute inflammatory demyelinating form of GBS and CIDP is indicated by a group of patients with a subacute form of acquired neuropathy in whom the decision whether to classify them as GBS or CIDP is purely a matter of definition (Oh, 1978;Hughes et al., 1992;Oh et al., 2003).

The distribution of lesions distinguishes CIDP sharply from inflammatory diseases of the CNS, but there are rare reports of CIDP and multiple sclerosis occurring together (Di Trapani et al., 1996)[see Hughes (1990b) for a review of the earlier literature]. Central motor conduction and magnetic resonance imaging of the brain show subclinical lesions suggesting CNS involvement in one-third to one-half of CIDP patients (Thomas et al., 1987;Ormerod et al., 1990). Nevertheless, the overwhelming predominance of pathology in the peripheral nervous system directs the search for autoantigens to the peripheral nerves rather than the CNS.

It is possible to find cases of CIDP associated with other diseases of a possible autoimmune nature such as vasculitis and systemic lupus erythematosus (Rechthand et al., 1984), but it is difficult to interpret the meaning of these possible associations. On the one hand, the coincidence of two autoimmune diseases is more likely to have excited the interest of potential authors and editors so that the associations reported may not represent the true population association. On the other hand, most definitions of CIDP segregate patients with such associated disorders from those without (Ad Hoc Subcommittee of the American Academy of Neurology AIDS Task Force, 1991;Joint Task force of the EFNS and the PNS, 2005). So many small case series of the coincidence of diabetes mellitus and CIDP have been reported (Stewart et al., 1996;Uncini et al., 1999;Gorson et al., 2000;Sharma et al., 2002;Haq et al.,

2003) that it is likely that the association is due to more than chance. However, the association is with type 2 diabetes, which is probably a primary metabolic disorder, unlike type 1 which is due to the development of autoimmunity to pancreatic islet cell antigens, although there is evidence of anti-Schwann cell immunity (Saravia and Homo-Delarche, 2003). The reason for the association with type 2 diabetes is not clear.

Apart from paraproteinemia, which is considered in the next paragraph, the occasional occurrence of CIDP in association with neoplasia may be no more than coincidence in most cases. The coincidence with melanoma is probably an exception. Some patients with melanoma have developed CIDP at about the time of onset of the melanoma (Bird et al., 1996) or during immunotherapy with vaccinia myeloma cell lysates or following treatment with IFN-α(Fuller et al., 1994;Weiss et al., 1998;Anthoney et al., 2000). In one case, the serum contained antibodies to gangliosides which reacted with melanoma cells (Weiss et al., 1998). These observations raise the possibility that the tumor induces an immune response against tumor antigens that cross-react with Schwann cell or myelin antigens and hence induce an auto-immune neuropathy. Protein antigens have not been sought or at least not reported.

Significance of the association between CIDP and paraproteinemia

About 10% of patients with a CIDP-like neuropathy have a paraprotein, which is most often an IgM paraprotein. Of patients with IgM paraproteins, about half have antibodies against MAG or sulfated glucuronyl paragloboside (SGPG). Nerve biopsies from these patients characteristically show widely spaced myelin and deposition of IgM and complement (Nobile-Orazio et al., 2000). The absence of more obvious macrophage-associated demyelination in such cases is remarkable and suggests a very slow evolution of the pathological process consistent with the usual indolent clinical course of the disease. The pathogenesis of this subgroup is likely to be an antibody-mediated action interfering both with myelin formation or maintenance and

with Schwann cell–axonal signaling resulting in alteration of neurofilament spacing and axonal shrinkage (Lunn et al., 2002).

In patients with IgM paraproteins and no antibodies to MAG and those with IgG or IgA antibodies, the clinical course and pathological features are variable but probably more similar to those of CIDP in the absence of a paraprotein (Bleasel et al., 1993). Paraproteins do not occur in children and are rare in young adults. Unlike CIDP, paraproteins usually only occur in the elderly. The apparently greater sensory deficit and more indolent course in patients with a paraprotein in some earlier series might be accounted for by the inclusion of patients with anti-MAG antibodies (Bromberg et al., 1992; Simmons et al., 1995). Strong associations between antibodies to gangliosides of likely pathogenetic relevance have been described in axonal or predominantly axonal neuropathy syndromes, including chronic ataxic sensory neuropathy with anti-GD1b antibodies, chronic ataxic neuropathy with cold agglutinins and disialosyl antibodies (CANOMAD), and predominantly motor neuropathy with anti-GD1a antibodies [see Willison and Yuki (2002) for review]. There is also a strong association between multifocal motor neuropathy and anti-GM1 antibodies which is beyond the scope of this review (Nobile-Orazio et al., 2005). Attempts to discover antibodies to antigens other than MAG have rarely been successful in paraproteinemic demyelinating neuropathies. A neuropathy resembling CIDP is commonly associated with osteosclerotic or solitary myeloma, is part of the polyneuropathy organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes (POEMS) syndrome, and may respond to the treatment of the underlying condition. However, its pathogenesis remains unexplained and an association with characteristic antibodies has not been described (Dispenzieri et al., 2003).

Neurophysiology

Slowing and block of nerve conduction in CIDP occur because the normal focal concentration of sodium channels at nodes of Ranvier disappears and the internodal potassium channels become exposed at sites of demyelination. The neurological

deficit is increased by activity-dependent conduction block: activation of the electrogenic sodium–potassium pump and inactivation of the late K^+ channels cause hyperpolarization of the axonal membrane, increasing its threshold (Cappelen-Smith et al., 2000; Kaji, 2003;Sung et al., 2004). Hyperpolarization increases following ischemia (Cappelen-Smith et al., 2002). This activity-dependent hyperpolarization raises a possible neurophysiological explanation for at least part of the observed predominance of motor deficit in CIDP. Sensory axons are protected against membrane hyperpolarization by greater inward rectification and persistent sodium conductance. This will make them less susceptible to activity-dependent conduction block (Vagg et al., 1998).

Attempts have been made to use the effect of sera from CIDP patients to induce conduction block by applying them to experimental preparations. Sera from PE-responsive CIDP patients induced partially reversible slowing of conduction in marmosets following systemic injection, but no pathological changes were discovered and this model has not been pursued (Heininger et al., 1984). Yan and colleagues showed that IgG from four of 12 patients with CIDP responsive to PE induced demyelination in two experimental systems, direct injection into rat nerves and intravenous injection into rats which had been primed by previous induction of adoptive transfer T-cell EAN (Yan et al., 2000). In a subsequent study (Yan et al., 2001), they found that six sera from 21 similar CIDP patients contained antibodies to a P0-like band and that four of these six induced conduction block and demyelination upon intraneural injection in the rat. Absorption with P0 protein eliminated this activity. It has long been known that antibodies to galactocerebroside, the most abundant peripheral nerve glycolipid, induce conduction block and demyelination, but these antibodies are rarely found in CIDP. Although it has been proposed that antibodies to ganglioside GM1, which are commonly present in multifocal motor neuropathy and rarely in CIDP, might interact with sodium channels and induce conduction block, no such effect could be observed in experiments in which anti-ganglioside antibodies were placed on rat spinal roots (Hirota et al., 1997).

Sera and CSF from CIDP patients have been reported to have complex effects on the sodium channels of cultured cells (Würz et al., 1995). An endogenous pentapeptide QYNAD, which is increased in the CSF of multiple sclerosis and GBS patients, has been reported to inhibit sodium conductance reversibly. The same peptide has also been implicated in CIDP (Weber et al., 2002). However, others have been unable to reproduce the inhibition of sodium channels by QYNAD, and its role in contributing to the pathogenesis of inflammatory demyelinating disease is uncertain (Cummins et al., 2003; Quasthoff et al., 2003).

The extent to which cytokines and other inflammatory mediators produce demyelination in inflamed nerves is debatable. The fact that demyelination is not a prominent feature of non-specific inflammatory lesions in nerves speaks against them having a direct role (see section on bystander demyelination above). In keeping with this conclusion is the failure of TNF to induce demyelination immediately following injection into rat nerve (although limited demyelination does develop 3–7 days later, perhaps as a consequence of the cellular infiltration and subsequent release of other mediators) (Redford et al., 1995). A strong candidate as an agent directly responsible for causing acute conduction block is nitric oxide which is released by activated macrophages, possibly in sufficient quantities to induce conduction block in normal and especially demyelinated axons (Redford et al., 1997).

Immunogenetics

Weak associations were at first discovered between CIDP and HLA-8, CW7, and DR3 in three small studies (Stewart et al., 1978; Adams et al., 1979;Vaughan et al., 1990) but were not confirmed in two others (Feeney et al., 1990; Van Doorn et al., 1991). However, the largest study only contained 72 patients. A single study has shown that that the frequency of the M3 allele of α1-antitrypsin is increased from 11.5% in controls to 29% in 52 patients with CIDP (McCombe et al., 1985). Now that the clinical heterogeneity of CIDP is better appreciated, its immunogenetics would be worth restudying. Such studies should include a search for relevant mutations in the

genes encoding the relevant candidate antigens. Martini and colleagues have crossed (Schmid et al., 2000) P0 knockout mice with RAG knockout (TCR deficient) mice, and the resultant mice had less severe disease than those with normal TCRs. This demonstrates that T-cell responses play a role in hereditary demyelinating neuropathies, and it is possible that processing of mutated myelin proteins causes abnormal antigen presentation which leads to autoimmunity and the development of inflammatory neuropathy.

The Role of T cells

Two recent reviews conclude that T cells are likely to be involved in the pathogenesis of CIDP (Kieseier et al., 2004). If one accepts that the antibodies to P0 discovered in CIDP are important because they have belonged to the IgG class, T cells are expected to have been involved in the isotype switch. If conversely one takes the position that antibodies are so difficult to find that they are unlikely to be important and CIDP is a tissue-specific autoimmune disease, then T cells must be directly involved.

Circulating T cells expressing DR antigens are increased in CIDP patients compared with normal controls (Taylor and Hughes, 1989), and the serum concentrations of soluble IL-2R and IL-2 were increased compared with patients with other neurological diseases, both indicating T-cell activation (Hartung et al., 1990;1991). In addition, there are markedly increased frequencies of recently activated T cells in the circulation of patients with CIDP compared with normal controls, and most of these are CD4^{+} T cells (Van den Berg et al., 1995). Five of 20 CIDP patients had increased serum TNF-α concentrations, as in GBS, and there was a relationship with disease activity (Misawa et al., 2001). There was a small increase in the proportion of T cells in the blood containing IL-4 but not IFN-γ in CIDP patients (and also multiple mononeuropathy) compared with normal controls in one study (Horiuchi et al., 2001). By contrast in the CSF, which may mimic more closely events in the endoneurium, there was an increase in the concentrations of IL-6, IL-8, and IL-17 and also in the

percentage of IFN-γ$^+$ IL-4 T cells in untreated CIDP compared with other neurological diseases which suggest activation of the Th1 pathway (Mei et al., 2005).

There have been very few studies of the targets of T-cell activity in CIDP. Some of the first studies with a variety of tests showed reactivity with whole nerve homogenate or with P2 protein, but others did not [see Hughes (1990b) for review]. We found lymphocyte transformation responses to purified bovine P2 in five of 13 CIDP patients but not to purified human P0. There were responses to one of two synthetic rat P2 peptides in two of 13 patients (and four of 17 controls) and one of four synthetic rat P0 peptides in two of 13 patients (and one of 17 healthy controls) (Khalili-Shirazi et al., 1992). The peptides studied did not represent the human sequences and did not cover the full length of the molecule. Furthermore, other proteins such as PMP22 might be the targets (Gabriel et al., 1998). These limited data are insufficient to exclude the possibility that T-cell responses to myelin proteins are present and possibly important in CIDP.

The Role of Regulatory Cells

The emphasis so far has been on studying the cells that stimulate or mediate autoimmunity in the nerves. The cells that regulate immune responses are likely to be crucially important. They include a subset of αβ T cells bearing CD4 and CD25 molecules, NKT cells, and γδ T cells. There are no studies so far of CD4/CD25-expressing cells in nerve biopsies in CIDP. NKT cells that secrete IL-4 and regulate the immune response toward the Th2 profile are present in the endoneurium of nerves in CIDP but not other types of neuropathy (Illes et al., 2000). T cells bearing the γδ TCR do occur in the endoneurium of inflamed nerves and may represent up to 30% of the endoneurial T-cell population (Khalili-Shirazi et al., 1998;Winer et al., 2002). γδ T cells are T cells with regulatory properties which respond to low-molecular non-peptidic ligands in response to a wide range of infections and secrete mainly Th1-like cytokines (Hayday and Tigelaar, 2003). Their presence in the peripheral nerve raises the possibility that they play a role in modulating

inflammatory responses in CIDP which deserves further investigation. Investigation of suppressor cell function in CIDP patients has been confined to a single study which suggested decreased concanavalin A-induced suppressor cell function compared with healthy controls (De Luca et al., 1999). The cell population that demonstrated the suppressor function was not identified.

Antibodies

The results of investigations seeking antibodies to myelin or Schwann cell antigens have mostly been disappointing. Early studies (Table 1) sought antibodies to myelin by immunofluorescence and identified antibodies in either no or at the most 22% of patients (Latov et al., 1981; Hughes et al., 1984; McCombe et al., 1988).

In the search for a specific protein antigenic target, recent studies have focused on P0 because it is the most abundant peripheral nerve myelin protein and induces EAN. Most studies have found antibodies in about 20% of patients and not or rarely in controls. Most striking was the study of Yan et al. (2001) already mentioned in which the Western immunoblot technique identified antibodies to P0 protein in six of 21 CIDP patients who were responsive to PE and only in one of 15 controls. We also found antibodies to P0-like and other bands by immunoblot in eight of 32 patients with CIDP and in none of 30 normal control subjects (Allen et al., 2005). Antibodies to P0 and other myelin proteins, including PMP22 and P2, have been found in small numbers of CIDP patients in some studies but not consistently, as already reviewed by us (Allen et al., 2005). With the possible exception of P0, there is no convincing evidence of antibodies to myelin proteins being formed in CIDP. By contrast, antibodies to proteins such as P0 which are expressed at the myelin–Schwann cell surface do cause demyelination upon intraneural injection (Hughes et al., 1985) and remain possible agents of demyelination in CIDP. The detection of antibodies to P0 and other myelin protein antigens is likely to be critically dependent on the conformation of the antigen which may only be realized on the intact protein in its usual membrane environment.

In the search for glycolipid antibodies, suspicion first fell on galactocerebroside because it is a major peripheral nerve glycolipid and is the major target of complement-fixing anti-myelin antibodies in animals that have been immunized with myelin. It induces a chronic EAN-like illness when injected into rabbits (Saida et al., 1979b;1981). Furthermore, injection of anti-galactocerebroside serum into nerves produces florid demyelination (Saida et al., 1979a). Nevertheless, the search for anti-galactocerebroside antibodies in CIDP has been uniformly negative (Table 2).

The search for antibodies to gangliosides has been more rewarding in other forms of inflammatory neuropathy than for any other class of molecules. The associations between antibodies to ganglioside GM1 and axonal forms of GBS and between antibodies to ganglioside GQ1b and Miller Fisher syndrome are well known (Willison and Yuki, 2002). Although antibodies to ganglioside GM1 are found in 30–80% of patients with multifocal motor neuropathy, they are not or only rarely present in symmetrical sensory and motor CIDP (Table 2). Ganglioside GM1 is a relatively minor myelin ganglioside (Gong et al., 2002) which happens to have attracted particular attention. However, searches for antibodies to other myelin gangliosides including LM1, the most abundant ganglioside in peripheral nerve myelin, have, with rare exceptions in individual cases (Usuki et al., 2005), been equally unrewarding. Despite one initial positive report, antibodies to sulfatides have not been consistently found more often in CIDP than in control subjects (Table 2). Antibodies to the glycosphingolipid SGPG are characteristic of the IgM paraprotein-associated demyelinating neuropathy and may in some instances cross-react with MAG. Despite negative initial studies, Yuki found IgM antibodies to SGPG in 12 of 30 CIDP patients and only five of 50 controls (Yuki et al., 1996), an observation which would be worth following up. Because the brunt of the pathological attack falls on the myelin and pathological studies have suggested that myelin and not the Schwann cell is the primary focus of the autoimmune attack, little attention has been paid to the Schwann cell itself. Recently, Kwa and colleagues showed that about a quarter of sera from both CIDP and GBS patients reacted with non-myelinating Schwann cells in tissue culture (Kwa et al., 2003). The antigenic target appeared to be in the leading

lamella of the Schwann cell processes. They propose that antigenic targets involved with Schwann cell–axon signaling are involved.Because axonal degeneration is common in CIDP, it is reasonable to seek antibodies to axonal antigens. Initial reports of antibodies to tubulin could not be confirmed (Table 3), and no one has published a broader search for axonal antigens. Even if they were present, they would not be expected to play a primary pathogenetic role.

Treatment Response

At least two-thirds of CIDP patients show short-term improvement with corticosteroids, intravenous Ig, or PE (Mehndiratta and Hughes, 2002; Van Schaik et al., 2002; Mehndiratta et al., 2004). Improvement begins within 2–8 days of starting treatment and wears off after a variable period of as little as 1–3 weeks but sometimes longer. All three treatments have a broad spectrum of effects on many aspects of the immune system. Thus, while benefit from these treatments supports an underlying autoimmune, or at least immune, process, the response tells us little about whether antibodies, complement, Th cells, or cytotoxic T cells are responsible. The efficacy of PE might appear at first blush to implicate antibodies, but complement and cytokines are likely to be removed by the process as well. There is a regrettable lack of information from controlled trials about the efficacy of immunosuppressive drugs. Responses to several drugs, particularly cyclosporine and cyclophosphamide, have been reported in observational studies (Mahattanakul et al., 1996; Good et al., 1998; Hughes, 2003). We have seen improvements in strength with methotrexate in seven of 10 patients (Fialho et al., 2006). The possibility of using novel immunosuppressive agents is less appealing because of reports of the development of CIDP in two patients being treated for arthritis with TNF-blocking agents (Richez et al., 2005). Despite the lack of clear evidence from immunosuppressive drugs, the responses to steroids, intravenous Ig, and PE confirm that the inflammatory process in CIDP is injurious but do not give insight into the cause or mechanism of the inflammation.

Conclusions

CIDP is an inflammatory, almost certainly auto-immune, disease in which both T cells and antibodies are involved. The confinement of the inflammation to the peripheral nerves, and sometimes to individual nerves or even segments of nerves, could be explained by endoneurial expression of chemokines and chemokine receptors for which there is preliminary evidence. Whereas related neuropathies are probably caused by antibodies to myelin glycolipids, especially gangliosides, or to MAG, the immunodominant antigens in CIDP remain undiscovered. The myelin protein antigens P2, P0, and PMP22 each induce EAN in rodent models and are candidate autoantigens in CIDP. The strongest evidence incriminates P0, to which antibodies have been found in 20% of cases. In the AIDP form of GBS, similar auto-immunity is self-terminating. Spontaneous T-mediated autoimmune neuropathy occurs due to a deficiency of regulatory T cells in mouse models. Possible failure of immunoregulation would permit the development and persistence of the inflammatory response and would repay further investigation.

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Original Articles

P-selectin glycoprotein ligand-1 variable number of tandem repeats (VNTR) polymorphism in patients with multiple sclerosis

Scalabrini D, Galimberti D, Fenoglio C, **Comi C**, De Riz M, Venturelli E, Castelli L, Piccio L, Ronzoni M, Lovati C, Mariani C, Monaco F, Bresolin N, Scarpini E. Neurosci Lett. 2005 Nov 18;388(3):149-52.

The initial step of multiple sclerosis (MS) pathogenesis is the aberrant activation of specific populations of autoreactive T lymphocytes in the periphery, followed by T cell recruitment into the brain (for review see [7]). The process leading to lymphocyte extravasation is a finely regulated sequence of steps controlled by adhesion molecules [16], including P-selectin glycoprotein ligand-1 (PSGL-1) on lymphocytes. PSGL-1 gene is located on chromosome 12q24 [23] and several polymorphisms have been described within its sequence. Among them, a variable number of tandem repeat (VNTR) polymorphism in the mucin-like region originates three possible allelic variant, named alleles A, B and C, from the largest containing 16 decameric repeats, to the smallest, consisting of 14 tandem repeats, respectively [1]. The allelic frequency of this polymorphism in Caucasians is 85% A, 14% B and 1% C, and an association of the smaller B and C alleles with a lower risk of developing acute cerebrovascular (CVD) events has been demonstrated [12]. From a functional point of view, recent findings demonstrate that activated platelets bind less efficiently to neutrophils carrying the shortest C allele. Therefore, the protective association of B and C alleles with CVD make it conceivable that binding of leukocytes to activated platelets or endothelium may vary among alleles differing in size [12], as also previously suggested by transfection studies pointing out that the distance from plasma membrane might be an important determinant of P-selectin binding activity [18]. Further association analyses excluded any association of the shorter alleles with coronary heart disease [4] and [22], although the VNTR and M62I polymoprhisms in PSGL-1 independently influence the related plasma levels [22].

On the basis of these studies, the distribution of the VNTR polymorphism was analyzed in a population of MS patients as well as in a same-size population of age-matched healthy subjects, in order to determine whether the presence of different alleles could influence either the susceptibility or the course of the disease.

Three hundred twenty-one patients with MS (114 males and 207 females; mean age at onset: 31.4 ± 0.7 years; mean disease duration: 8.8 ± 0.5 years) were consecutively recruited at MS Centers of Ospedale Maggiore Policlinico, Milan and Ospedale Maggiore, Novara. All patients underwent a standard battery of examinations, including medical history, physical and neurological examination, screening laboratory test, brain magnetic resonance imaging (MRI). Diagnosis was made in accordance to McDonald's criteria [15]. The course of MS was described as relapsing remitting (RR, n = 241, 85 males and 156 females; mean age at onset: 29.8 ± 0.6 years; mean disease duration: 9.4 ± 0.7 years), secondary progressive (SP, n = 37, 11 males and 26 females; mean age at onset: 33.8 ± 2.1 years; mean disease duration: 23.6 ± 2.5 years) or primary progressive (PP, n = 21, 11 males and 10 females; mean age at onset: 39.9 ± 2.9 years; mean disease duration: 11.6 ± 1.8 years) [14]. The remaining 22 patients had a clinical isolated syndrome (CIS, n = 22, 7 males and 15 females; mean age at onset: 34.8 ± 1.9 years; mean disease duration: 2.2 ± 0.3 years) suggestive of MS, and, at MRI, dissemination of lesions in time and space [15]. The overall clinical data collected included age at onset of MS, disease duration, time between first and second attack, number of relapses, disease severity according to the expanded disability status scale (EDSS) [10], annual relapse rate and disease progression before treatment. The control group consisted of 342 subjects matched for ethnic background, gender and age. An informed consent to participate in this study was given by all individuals.

High-molecular weight DNA was isolated from whole blood using a Flexigene Kit (Qiagen, Hildren, Gemany) as described by the manufacturer.

VNTR distribution was determined by polymerase chain reaction (PCR) assay, as previously described [1].

Allelic and genotypic frequencies were obtained by direct counting. Hardy Weinberg equilibrium was tested by using a χ^2 goodness of fit test. Fisher's exact test was used for differences in allele distribution between groups, and the odds ratio (OR) was calculated along with its 95% CI. Clinical data of subjects carrying different genotypes were compared using the Mann–Whitney U-test. Statistical significance was set at $P < 0.05$.

The VNTR allele and genotype frequencies for the study groups of MS patients and controls are reported in Table 1. The distribution of the three possible alleles A, B and C in controls was similar to the one previously reported in Caucasians [4]. No significant differences among allelic frequencies of these three alleles in MS as compared with controls were shown (A: 81.4% versus 84.2%; B: 16.7% versus 14.9%; C: 1.9% versus 0.9%, $P > 0.05$, Table 1). However, stratifying patients according to the course of the disease [14], a significantly increased frequency of the shortest C allele in PP-MS was observed, either comparing with controls (7.1% versus 0.9%, $P = 0.011$, OR: 9.3, 95% CI: 2.2–40.4) or with all remaining MS patients, who had acute inflammatory attacks at onset and an initial RR form (7.1% versus 1.5%, $P = 0.036$, OR: 5.4, 95% CI: 1.3–21.7). Besides, none of SP-MS patients was a carrier of the C allele, and B carriers converted later from RR to SP course as compared with A/A subjects (15.8 years versus 8.8 years, $P = 0.01$).

Table 1.

VNTR allele and genotype frequencies (%) in MS patients and healthy controls

VNTR allele and genotype frequencies (%) in MS patients and healthy controls

VNTR frequencies	Controls (*n* = 342)	All MS (*n* = 321)	MS subtypes			
			RR-MS (*n* = 241)	SP-MS (*n* = 37)	PP-MS (*n* = 21)	CIS (*n* = 22)
Allele						
A	576 (84.2)	523 (81.4)	392 (81.3)	61 (82.4)	34 (81)	36 (81.8)
B	102 (14.9)	107 (16.7)	81 (16.8)	13 (17.6)	5 (11.9)	8 (18.2)
C	6 (0.9)	12 (1.9)	9 (1.9)	0 (0)	3 (7.1)[*]	0 (0)
Genotype						
A/A	240 (70.2)	209 (65.1)	158 (65.6)	24 (64.9)	13 (61.9)	14 (63.6)
A/B	90 (26.3)	94 (29.3)	68 (28.2)	13 (35.1)	5 (23.8)	8 (36.4)
A/C	5 (1.5)	11 (3.4)	8 (3.3)	0 (0)	3 (14.3)	0 (0)
B/B	6 (1.7)	6 (1.9)	6 (2.5)	0 (0)	0 (0)	0 (0)
B/C	1 (0.3)	1 (0.3)	1 (0.4)	0 (0)	0 (0)	0 (0)

Values are expressed as n (%). [*]P = 0.011, PP-MS vs. controls. OR (95% CI): 9.3 (2.2–40.4). [*]P = 0.036, PP-MS vs. bout onset MS. OR (95% CI): 5.4 (1.3–21.7).

Stratifying patients by gender, no statistically significant differences were observed. No further correlation between genetic findings and available clinical variables was found as well.

According to these results, the presence of the C allele of the VNTR polymorphism might increase the susceptibility to develop PP-MS, an MS type which is progressive from the onset, without any relapse or remission, rather than the most common form of MS in which attacks of symptomatic demyelination and subsequent recovery occur.

In addition, this allelic variant is absent in SP-MS patients and the presence of the shorter B allele is likely to delay the transition from RR to SP course.

The polymorphism studied is located within a gene which exerts a relevant role in recruiting activated lymphocytes into the brain [11]. As regards PSGL-1 biological function, the presence of the C allele has been demonstrated to exert a profound effect on ligand binding, as cell transfectants expressing the shortest C allele support less efficiently interactions with endothelium [12]. Thus, the distance from plasma membrane is likely to be a crucial factor influencing P-selectin binding activity. Basing on these considerations, it could be conceivable that the A allele confers to lymphocytes the highest efficiency to bind P-selectin during acute attacks. Therefore, carriers of the shorter B or C alleles could have a less efficient recruitment of lymphocytes into the brain, implying a less severe demyelination during exacerbations. This mechanism could result over time in a slower progression of the disease to the SP form, a condition in which axonal damage is the prevalent cause of the accumulating disability. In this regard, previous data already demonstrated that acute exacerbations have a measurable and sustained effect on disability in patients with RR-MS [13]. The absence of the C allele in SP-MS patients analyzed, together with the observed longer time before SP conversion in B carriers, is consistent with the previous hypothesis. Nevertheless, difficulties to draw reliable conclusions on the function of the mutated C allele derive from the very low frequency of samples from C/C carriers (about 1 out of 1000 subjects), which would be a better model to study effects of this mutated allele. On the other hand, as P-selectin, one of PSGL-1 counterligand, has been demonstrated to mediate normal T lymphocyte surveillance through the blood brain barrier [5] and [9], an alternative hypothesis could be that patients with the C allele have reduced immune surveillance, which results in an increased susceptibility to virally induced CNS demyelination.

The high frequency of C carriers found in PP-MS patients suggests that an efficient recruitment of lymphocytes is not a crucial step during the pathogenesis of this form, which is supposed to have a lower degree of inflammation and a consistent axonal

damage [3] and [20]. Although PP and SP-MS have been considered subtypes of a chronic progressive form of MS, controversy remains with regard to the nature of PP-MS as to whether it is a distinct disease. In fact, several features are different in PP-MS as compared with bout-onset MS, including brain MRI, which could be normal [21], absent oligoclonal bands in cerebrospinal fluid [17], less perivascular cuffing and parenchymal cellular infiltration [19] and different laboratory parameters [2]. Different patterns of adhesion molecules expression have been observed in PP-MS as compared with relapsing MS patients. In particular, significant decreases in leukocyte surface expression of most of the adhesion molecules and increases in soluble ICAM-1 and L-selectin levels were found in SP- and RR-MS compared with PP-MS patients, whereas results in PP-MS patients were similar to those in controls [6]. Therefore, trafficking of peripheral leukocytes into the CNS has been supposed to play a major role in the pathogenesis of SP- and RR-MS but not in PP-MS [8].

In conclusion, the C allele of the VNTR polymorphism in PSGL-1 gene is likely to be associated with PP-MS. Besides, data in SP-MS suggest that the longer the allele, the better the efficiency in binding P-selectin, possibly influencing the course of bout-onset MS. However, confirmation studies on larger groups are needed, in order to perform a functional analysis, possibly in carriers of the rare C/C genotype. Besides, possible interactions among this and other allelic variants all over the genome, mainly in different adhesion molecules implicated in the migration of lymphocytes should be considered.

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E-selectin A561C and G98T polymorphisms influence susceptibility and course of multiple sclerosis

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1. Introduction

The initial step of multiple sclerosis (MS) pathogenesis is the aberrant activation of specific populations of autoreactive T lymphocytes in the periphery, followed by T cell recruitment into the brain (for review, see Galimberti et al., 2004). The process leading to lymphocyte extravasation is a finely regulated sequence of steps controlled by adhesion molecules (Piccio et al., 2002), including E- and P-selectins. These selectins are expressed by inflamed brain endothelium and bind T lymphocytes bearing the cutaneous lymphocyte antigen (CLA, Lenter et al., 1994 and Li et al., 1996). E-selectin recruits lymphocytes of the Th1 subtype, which produce proinflammatory cytokines and possess the CLA antigen, while does not support binding of Th2 lymphocytes, which do not express the CLA-epitope (Austrup et al., 1997 and Borges et al., 1997).

Several polymorphisms have been described within the E-selectin gene. A Single Nucleotide Polymorphism (SNP) in the coding region of the gene (A561C) causes a conservative change of a serine with an arginine at codon 128 (S128R, Wenzel et al., 1994). This mutation confers an alteration in selectin ligand binding specificity (Revelle et al., 1996), leading to a gain of function under flow conditions, possibly amplifying the number of leukocytes that roll and subsequently arrest on endothelium (Rao et al., 2002a and Yoshida et al., 2003). Rao et al. (2002b) showed that the presence of the SNP confers not only an increased capability of binding lymphocytes, but also a different specificity in their recruitment, as S128R E-selectin molecules are able to bind CLA-negative lymphocytes, suggesting that also non Th-1 subsets, mainly anti-inflammatory Th2 cells, may be recruited.

A further SNP in the E-selectin, consisting in a G to T mutation (G98T), is located in the 5'-untranslated region of the gene. Wenzel et al. (1996) found a correlation between the S128R and G98T mutations, but more recent findings did not confirm this association (Zheng et al., 2001).

On the basis of these studies, the distribution of A561C and G98T SNPs was analyzed in a population of MS patients as well as in a same-size population of age-matched healthy subjects, in order to determine whether their presence could influence the susceptibility or the course of the disease.

2. Materials and methods

2.1. Subjects

Three hundred seven patients with MS were consecutively recruited at the following MS Centers: Ospedale Maggiore Policlinico, Milan; Ospedale Maggiore, Novara and Ospedali Riuniti, Bergamo. All patients underwent a standard battery of examinations, including medical history, physical and neurological examination, screening laboratory test, brain Magnetic Resonance Imaging (MRI). Diagnosis was made in accordance with McDonald's criteria (2001). The course of MS was described as Relapsing Remitting (RR, n = 229), Secondary Progressive (SP, n = 34) or Primary Progressive (PP, n = 20; Lublin and Reingold, 1996). Thirty-two out of 229 patients with RR-MS had a benign form, defined as disease in which patients remained fully functional in all neurologic systems after 15 years from MS onset (Lublin and Reingold, 1996), and having an Expanded Disability Status Scale (EDSS; Kurtzke, 1983) lower than 4. The remaining 24 patients had a Clinical Isolated Syndrome (CIS) suggestive of MS, and, at MRI, dissemination of lesions in time and in space (McDonald et al., 2001). The clinical data collected were: age at onset of MS, disease duration, time between first and second attack, number of relapses, disease severity according to the EDSS, annual relapse rate and disease progression before treatment (Table 1). The control group consisted of 300 subjects matched for ethnic background, gender and age. An informed consent to participate in this study was given by all individuals.

Table 1. Demographic and clinical variables of subjects

	Controls	Total group	MS patients			
			RR	SP	PP	CIS[a]
Number of subjects	300	307	229	34	20	24
Gender (M:F)	115 : 185	109 : 198	80 : 149	10 : 24	11 : 9	8 : 16
Age, y	38.6 ± 0.9	48.6 ± 0.9	38.7 ± 0.8	59.6 ± 1.2	47.5 ± 2.8	34.0 ± 1.7
Age at onset, y		31.7 ± 0.6	30.7 ± 0.7	32.1 ± 1.8	39.4 ± 3.1	33.2 ± 1.9
Disease duration, y		8.3 ± 0.5	7.1 ± 0.5	19.7 ± 1.9	11.7 ± 2.0	1.3 ± 0.2
Time to SP conversion, y				10.9 ± 1.3		
Time between 1st and 2nd attack, y			3.2 ± 0.4 (n = 135)[b]			
Annual relapse rate[c]			1.0 ± 0.1 (n = 91)[b]			
EDSS		2.4 ± 0.1 (n = 186)[b]	1.8 ± 0.1 (n = 142)[b]	5.8 ± 0.2 (n = 26)[b]	5.9 ± 0.4 (n = 18)[b]	
Progression Index[c]		0.42 ± 0.04 (n = 186)[b]	0.32 ± 0.03 (n = 142)[b]	0.52 ± 0.09 (n = 26)[b]	0.89 ± 0.16 (n = 18)[b]	

Data are given as means ± S.E.M.; EDSS=Expanded Disability Status Scale; Progression Index = EDSS /disease duration (years); annual relapse rate = total amount of relapses/duration of disease. [a] Clinical Isolated Syndrome suggestive of MS with dissemination of lesions in time and in space at MRI, according to McDonald[22]. [b] Patient numbers available for respective analysis. [c] Calculated before any immunomodulatory treatment.

2.2. Genomic DNA isolation

High-molecular-weight DNA was isolated from whole blood using a Flexigene Kit (Qiagen, Hildren, Gemany) as described by the manufacturer.

2.3. G98T and A561C polymorphism determination

G98T and A561C SNPs were determined by Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLP) assay, as previously described (Zheng et al., 2001 and Ellsworth et al., 2001).

2.4. Statistical analysis

Allelic and genotypic frequencies were obtained by direct counting. Hardy Weinberg equilibrium was tested by using a χ^2 goodness of fit test. Chi-square was used to test for differences in allele distribution between the groups. The odds ratio (OR) was calculated along with its 95% CI.

3. Results

A trend towards an increase in the allelic frequency of the G98T SNP in MS patients compared with controls (17.3 versus 13.8%; P > 0.05, Table 2) was observed. Indeed, these results derive from a significantly increased number of individuals with the T/T genotype in MS population compared with healthy subjects (4.7 versus 1.0%; P < 0.01; OR: 4.7, 95% CI: 1.3–16.6; Table 2). Stratifying MS patients according to the course of the disease (Lublin and Reingold, 1996), a markedly significant increase of T/T genotype carriers in RR-MS patients versus controls was present (5.2 versus 1.0%; P < 0.01; OR: 5.5, 95%CI: 1.5–19.6; Table 2). This genotypic variant was observed also in other groups of patients with an acute onset, while it was completely absent in PP-MS patients. No significant differences in either allelic or genotypic distribution of the A561C SNP between MS patients and controls were shown (Table 3). However, considering MS patients with acute inflammatory attacks at onset and an initial RR form, a decreased frequency of the C mutated allele was observed in the

subgroup that progressed to SP-MS (8.8% versus 14.4%, P > 0.05, Table 3). To better confirm this evidence, SP-MS patients were compared with a subgroup of RR-MS patients having a benign form of the disease (Lublin and Reingold, 1996) who have a lower probability to develop SP-MS over time (Pittock et al., 2004), demonstrating a significantly decreased frequency of the C allele in SP-MS as compared with benign MS patients (23.9% versus 8.8%; P < 0.025; OR: 4.0, 95%CI: 1.2–13.0; Table 4).

Stratifying patients by gender, no statistically significant differences were observed for both SNPs. None of the possible haplotypes deriving from the combination of the two alleles was associated with MS (data not shown).

Table 2. G98T allele and genotype frequencies (%) in MS patients and healthy controls

G98T frequencies	Controls n = 300	All MS n = 307	MS subtypes			
			RR-MS n = 229	SP-MS n = 34	PP-MS n = 20	CIS n = 24
Allele						
G	517 (86.2)	508 (82.7)	373 (81.4)	60 (88.2)	35 (87.5)	40 (83.3)
T	83 (13.8)	106 (17.3)	85 (18.6)	8 (11.8)	5 (12.5)	8 (16.7)
Genotype						
G/G	220 (73.3)	215 (70.0)	156 (68.2)	27 (79.4)	15 (75)	17 (70.8)
G/T	77 (25.7)	78 (25.3)	61 (26.6)	6 (17.7)	5 (25)	6 (25)
T/T	3 (1.0)	14 (4.7)*	12 (5.2)**	1 (2.9)	0 (0)	1 (4.2)

Values are expressed as n (%).
* P < 0.01 for all MS patients vs. healthy controls; OR (95%CI) : 4.7 (1.3–16.6).
** P < 0.01 for RR-MS patients vs. healthy controls; OR (95%CI) : 5.5 (1.5–19.6).

Table 3. A516C allele and genotype frequencies (%) in MS patients and healthy controls

A561C frequencies	Controls n = 300	All MS n = 307	MS subtypes			
			RR-MS n = 229	SP-MS n = 34	PP-MS n = 20	CIS n = 24
Allele						
A	522 (87.0)	528 (86.0)	392 (85.6)	62 (91.2)	34 (85.0)	40 (83.3)
C	78 (13.0)	86 (14.0)	66 (14.4)	6 (8.8)	6 (15.0)	8 (16.7)
Genotype						
A/A	225 (75.0)	227 (74.0)	167 (73.0)	29 (85.3)	15 (75.0)	16 (66.7)
A/C	72 (24.0)	74 (24.0)	58 (25.3)	4 (11.8)	4 (20.0)	8 (33.3)
C/C	3 (1.0)	6 (2.0)	4 (1.7)	1 (2.9)	1 (5.0)	0 (0)

Values are expressed as n (%).

Table 4. A516C allele and genotype frequencies (%) in SP-MS compared with benign RR-MS patients

A561C frequencies	SP-MS n = 34	Benign RR-MS n = 32
Allele		
A	62 (91.2)	50 (76.1)
C	6 (8.8)	14 (23.9) [*]
Genotype		
A/A	29 (85.3)	19 (59.3)
A/C	4 (11.8)	12 (37.5)
C/C	1 (2.9)	1 (3.2)

Values are expressed as n (%).
[*] P < 0.025 for benign vs. SP-MS. OR (95%CI) : 4.0 (1.2–13.0).

4. Discussion

According to the present results, the presence of the T/T genotype of the G98T SNP in the E-selectin gene seems to confer an increased risk of developing MS. Moreover, it might be a risk factor towards the development of a type of MS characterized by acute attacks at onset, as this genotype is absent in PP-MS. On the contrary, the A561C polymorphism does not increase the risk of MS, but is likely to influence the course of the disease, acting as protective factor towards SP-MS in subjects affected by RR-MS.

The polymorphisms studied are located within a gene which is crucial for the recruitment of activated lymphocytes into the brain (Ley, 2003), thus an association of the T/T genotype of the G98T SNP with subtypes of MS having had a bout-onset

disease, characterized by attacks of symptomatic demyelination, could be reasonable. The complete absence of the T/T genotype in PP-MS, which is supposed to have a lower degree of inflammation and a consistent axonal damage (Bitsch et al., 2000 and Scarpini et al., 2002), is in accordance with this hypothesis.

Analysis on our MS population suggests a protective effect of the C allele towards the development of SP-MS. From a biological point of view, the A561C SNP has been demonstrated to exert a profound effect on ligand recognition and binding, as cell transfectants expressing the A561C E-selectin better support interactions with leukocytes under flow conditions (Rao et al., 2002a). This tethering mechanism could theoretically amplify the number of lymphocytes interacting with mutated cerebral endothelial cells during demyelinating attacks occurring in RR-MS. A very important point to consider is that while the wild type E-selectin recruits specifically activated Th-1 lymphocytes (Austrup et al., 1997), which produce proinflammatory cytokines and chemokines (Galimberti et al., 2004), the presence of the A561C polymorphism extends the range of lymphocytes recruited by E-selectin, including Th2 lymphocytes (Rao et al., 2002b). This subset of T cells produces anti-inflammatory cytokines, which could help to counterbalance the negative effects of proinflammatory Th1 cells. Consequently, in RR-MS patients, the presence of the C allele could be beneficial, allowing the recruitment of anti-inflammatory Th2 cells during demyelinating attacks, possibly counterbalancing the inflammatory Th1 lymphocytes, and resulting eventually in a less severe myelin damage. This mechanism could result over time in a slower progression of the disease to the SP form, a condition in which axonal damage is the prevalent cause of the accumulating disability.

In conclusion, the T/T genotype of the G98T SNP may be proposed as risk factor for bout-onset, but not PP-MS, development, while the A561C is likely to influence the course of the disease, exerting a protective effect towards progression to SP. However, longitudinal analysis on RR-MS patients will better clarify the role of the A561C with regard to the course of the disease.

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CXCL10 haplotypes and multiple sclerosis: association and correlation with clinical course

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Introduction

Multiple sclerosis (MS) is supposed to be an autoimmune disease, in which myelin proteins act as autoantigens. The initial step in the pathogenesis of MS seems to be the aberrant activation, caused by an unknown antigen, of specific populations of immune cells in the periphery, which start to proliferate and express a wide range of cell-surface adhesion molecules, leading to the interaction with the brain endothelium followed by the transmigration through the brain blood barrier into the central nervous system (CNS), where they are responsible for demyelination followed by axonal damage [1]. Several molecules are involved in this pathogenetic process, including adhesion molecules, cytokines and chemokines. Amongst chemokines, CXCL10 (interferon-γ-inducible protein-10), together with other chemokines of both α and β subfamilies, was found to be upregulated during relapses in experimental autoimmune encephalomyelitis mice [2]. CXCL10 levels were shown to be increased in cerebrospinal fluid (CSF) of patients with symptomatic attacks of inflammatory demyelination [3], but no differences were found with controls during the stable phase of the disease [4]. Moreover, considering different MS subtypes, whilst CXCL10 levels were significantly elevated in CSF in relapsing remitting (RR) and secondary progressive (SP)–MS patients, its levels in primary progressive (PP)–MS patients were similar to controls [5].

Multiple Sclerosis is a multifactorial disease, and genetic factors play an important role in orchestrating these pathological events and in changing the disease phenotype from patient to patient. At present, many efforts have been made to identify genetic

variation having a potential role in human diseases [6]. The strongest and most consistent evidence for a susceptibility gene in MS is within the major histocompatibility complex (MHC) on chromosome 6p21.3. Association with the HLA-DR2 haplotype (DRB1*1501–DQB1*0602) has been repeatedly demonstrated in several populations, primarily those of Northern European descent but with a minor extent also in continental Italian population [7]. Other HLA associations have also been reported with particular regard to Sardinia where the effect of isolated population growth on allele frequency is highlighted by the characteristic HLA genotype [8]. Although the MHC region contributes significantly to MS risk, much of the genetic effect in MS remains to be explained as the clinical heterogeneity and complex aetiology of MS represent confounding factors when studying the disease. Besides HLA, several allelic variants have been proposed as candidate for MS, such as the G98T single nucleotide polymorphism (SNP) in the E-selectin gene, which confers an increased risk to develop MS [9]. Other polymorphisms have been demonstrated to be associated with PP–MS, as the C allele of the variable number of tandem repeats polymorphism in P-selectin glycoprotein ligand-1 [10]. Lastly, some allelic variants are probably to influence the course of the disease [9].

CXCL10 gene is composed by four exons interrupted by three introns [11], and to date two SNPs have been described in exon 4, consisting in a G □ C and in a T □ C substitution in the untranslated 3' region of the gene (140 and 783 bp after exon 4 stop codon; http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?type=rsrs=rs3921 and http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?type=rsrs=rs8878, respectively). Neither association nor functional studies have been extensively carried out yet, except for a limited analysis of a 92-Caucasian individual population, resulting in a frequency of 46% for the rs3921 and of 39% for the rs8878 (http://www.ensembl.org/Homo_sapiens/snpview?snp=rs3921 and http://www.ensembl.org/Homo_sapiens/snpview?snp=rs8878).

On the basis of these studies, underlying the possible importance of allelic variants in CXCL10 in the pathogenesis of MS, a mutation scanning of CXCL10 exons was carried

out in a relatively small population of patients and controls, and allelic variants with a high (>5%) frequency were next evaluated in a larger population of MS patients as well as in a same-size population of age-matched healthy subjects through allelic discrimination, to determine whether their presence could influence the susceptibility or exert a protective effect towards the development of the disease. Besides, a possible influence of polymorphisms on clinical variables was analysed, correlating the presence of SNPs with age at onset, disease duration, time between onset and second episode, annual relapse rate and progression index (PI).

Patients and Methods
Subjects

Two hundred and twenty-six patients with MS were consecutively recruited at the following MS Centres: Ospedale Maggiore Policlinico and Ospedale Sacco, Milan and Ospedale Maggiore, Novara. All patients underwent a standard battery of examinations, including medical history, physical and neurological examination, screening laboratory tests, CSF analysis, brain magnetic resonance imaging (MRI). Diagnosis was made in accordance to McDonald's criteria and further revision [12,13]. The course of MS was described as RR (n = 174), SP (n = 36) or PP (n = 16) [14]. The clinical data collected were: age at onset of MS, disease duration, time between first and second attack, number of relapses and disease severity according to the Expanded Disability Status Scale (EDSS) [15]. An attack refers to an episode of neurological disturbance, reported either by subjective report or by objective observation, with duration of more than 24 h, preceded by a relatively stable or improving neurological state of at least 30 days [12]. The annual relapse rate prior to any immunomodulatory therapy was calculated in 83 patients with RR–MS, diagnosed at least 2 years before the recruitment, to exclude possible bias because of a higher number of relapses in the first year from disease onset [16]. Disease progression was defined by calculating the PI as a measure of accumulated disability over time (PI = EDSS/disease duration in years) [17]. Thirty-five out of 174

patients with RR–MS had a benign form; defined as patients with more than 10 years of disease duration and an EDSS lower than four [18].

The control group consisted of 235 subjects matched for ethnic background, gender and age. Both patients and controls were Caucasians originating from a limited area in Northern Italy. All patients and controls were ascertained to have parents and grandparents born in Northern Italy to ensure ethnicity. An informed consent to participate in this study was given by all individuals. All the demographic and clinical variables of subjects are summarized in Table 1.

Genomic DNA isolation

High-molecular weight DNA was isolated from whole blood using a Flexigene Kit (Qiagen, Hildren, Gemany) as described by the manufacturer. The amount of DNA for each sample was determined by measuring the optical density at 260 nm wavelengths using a spectrophotometer (Eppendorf, Hamburg, Germany). DNA samples were stored at −20°C.

Sequencing

Exons 1 through 4 were amplified using specific primers. Fragments were purified using ExoSAP-IT® Kit (United States Biochemical, Cleveland, OH, USA) and then direct sequences were performed with an ABI PRISM 3100 gene analyser (ABI, Foster City, CA, USA).

Allelic discrimination

Allelic discrimination was carried out by using the Taqman methodology. Each Taqman 5'-nuclease assay employed 25 ng of genomic DNA as template. Specific assay-on-demand products were used for genotyping G□ C and T □ C CXCL10 variants (ABI assay ID: c_482889_10 and c_482889_10, respectively). Assays were performed in 15 μl reactions in 96-well plates using an ABI PRISM ®7000 instrument

(ABI). Thermocycling consisted of 10 min at 95°C for AmpliTaqGold activation, and 40 cycles of 95°C for 15 s and 60°C for 1 min, as previously described [19].

Statistical analysis

Allelic and genotypic frequencies were obtained by direct counting. Haploview 3.2 software (Broad Institute of Harvard and MIT, Cambridge, MA, USA) was used to test for Hardy–Weinberg equilibrium and for differences in haplotype distribution between cases and controls. The odds ratio (OR) was calculated along with its 95% confidence interval (CI). Demographic and clinical variables are expressed as median (range). Non-parametric Kruskal–Wallis test, including Dunn's method for multiple comparisons, was used for differences amongst groups.

Results

Association analysis

Mutation scanning of CXCL10 exons carried out in 20 subjects (10 patients and 10 controls) confirmed the presence of two previously reported polymorphisms in exon 4, whereas no novel allelic variants were detected. Therefore, a further association analysis on the whole population was carried out for the G□ C and T □ C SNPs. Haplotype frequencies, deriving from different SNP genotype combinations, for MS patients and controls are reported in Table 2. Both MS and control populations were in Hardy–Weinberg equilibrium, and the two SNPs were in complete linkage disequilibrium (D' = 1). Despite the GGTT combination was the least prevalent in our population, this haplotype has been defined as wild type in accordance with the sequence reported in NCBI database. A slight increase of the GGTT haplotype frequency was observed in MS patients as compared with controls (20.3 vs. 16.2%, Table 2), but not to a significant extent (P > 0.05). Stratifying patients according to gender or subtype of the disease, no significant differences were observed (Table 2).

In a subgroup of 35 RR–MS patients with a benign form of the disease, an increased frequency of this haplotype was observed as compared with the remaining RR–MS patients (34.3 vs. 16.5%, P = 0.032, OR: 2.63, 95% CI: 1.15–6.03).

Correlations with clinical data

The PI was significantly lower in patients carrying the GGTT wild-type haplotype as compared either with GCTCor CCCC carriers (median values: 0.17 vs. 0.25 or 0.28, respectively, P = 0.016, Table 3). In patients who had a disease onset characterized by the attacks of demyelination, the time between disease onset and the second episode, which represents a favourable prognostic factor [20], was significantly longer in GGTT than in GCTC or CCCC carriers (median values: 3 vs. 1 or 2 years, respectively, P = 0.021, Table 3). Considering SP–MS patients, the time between the first event and the subsequent worsening to SP was longer in GGTT group as compared with GTCC or CCCC (median values: 16 vs. 10 or 9 years, P = 0.08, Table 3). No differences between GGTT carriers and non-carriers were found regarding the age at onset and relapse rate.

Discussion

According to these results, the presence of the wild-type GGTT haplotype in CXCL10 gene does not confer an increased risk of developing MS, but is probably to modulate the progression of the disease, influencing the worsening of the disability, as demonstrated by a significantly lower PI in carriers compared with non-carriers, and acting as protective factor towards the progression to SP–MS in subjects affected by RR–MS. The evidence that the frequency of the GGTT haplotype is significantly increased in benign RR–MS, as well as a longer interval between onset and first episode in wild-type haplotype carriers, further supports this hypothesis.

This is the first attempt to screen an MS population for the presence of new polymorphisms in CXCL10 gene and to correlate haplotypes in this gene with clinical parameters. CXCL10 specifically targets activated lymphocytes [21] and thus is

probably to be a crucial molecule for the recruitment of lymphocytes into the brain, occurring during attacks of symptomatic demyelination. In the past few years several studies demonstrated the presence of CXCL10 together with other inflammatory molecules in CSF from patients during exacerbations [3–5]. However, recent findings suggest a possible role for CXCL10 in myelin repair, as CXCL10 related receptor CXCR3, together with other chemokine receptors, is constitutively expressed on oligodendrocytes in normal adult human CNS tissue [22]. Therefore, the simultaneous expression of different CXC chemokine receptors on oligodendrocytes, and their respective ligands on astrocytes around MS lesions may support the hypothesis of novel functional roles for these immune system molecules, including CXCL10, in oligodendrocyte recruitment and remyelination [22].

Our results suggest that the GGTT wild-type haplotype in CXCL10 gene may be associated with a slower progression of the disability during the course of the disease, and with a longer time between the development of RR and SP–MS. Therefore, its presence seems to exert a beneficial role against the clinical progression, although further confirmatory studies are certainly required. To date, several association studies have been carried out, but only few of them analysed the possible effect of polymorphisms on the progression of the disease. To this aim, the most investigated gene has been the apolipoprotein E (ApoE), which is linked to the prevention of neurotoxicity and repair processes in a variety of neurological disorders [23]. The ApoE ε2 allele is associated with lesser disease severity in familial MS [24], whereas the ε4 allele is associated with progressive disease in women and cognitive impairment in men with MS [17,25,26].

At present, the molecular mechanism underlying the described results is still unknown. The G□ C and T □ C SNPs are located in the 3'-untranslated region of CXCL10 gene, thus an effect on the stability of the related mRNA could be conceivable [27]. However, another possibility is that CXCL10 SNPs may simply be 'markers' inherited with other unknown causative genes.

Another point to be considered is that the outcome measures described in this study, although widely used, are not probably to reflect the degree of inflammation into the brain, which, on the contrary, could be better evaluated by MRI parameters such as T2 lesion load and gadolinium-enhancing lesions [28].

Despite the mechanism of action of the studied SNPs is still poorly understood, CXCL10 genotyping could be of some help as prognostic factor, together with other clinical prognostic measures currently considered, including the development of a progressive disease course, a high number of relapses in the first 2 years from onset, a short relapse-free interval between the first two attacks, and the ApoE genotype [16].

In conclusion, the GGTT haplotype of the CXCL10 gene may be proposed as a disease modifying gene [29], slowing down the worsening of clinical disability and exerting a possible protective effect towards the progression to SP. However, as MS is probably to be a disease with both genetic and environmental components, interactions between these and other allelic variants all over the genome should be considered, together with the possible effects of non-genetic factors in modulating the course of the disease.

Tables

Table 1 Demographic and clinical variables of subjects analysed

	Controls	MS			
		Total group	RR	SP	PP
Number of subjects	235	226	174	36	16
Gender (M:F)	88:147	71:155	51:123	12:24	8:8
Age (years)	31.5 (15–74)	40 (13–78)	37 (13–64)	54 (26–73)	51 (32–78)
Age at onset (years)		29 (11–69)	28 (11–58)	33 (12–52)	40 (24–69)
Disease duration (years)		8 (1–43)	7 (1–42)	18 (3–43)	10 (2–31)
Time to SP conversion (years)				10 (1–27)	
Annual relapse rate[a]			1 (0.1–4.3) ($n = 83$)[b]		
EDSS		2 (0–8.5) ($n = 166$)[b]	1.5 (0–6.5) ($n = 130$)[b]	6 (3–8) ($n = 24$)[b]	6.2 (3–8.5) ($n = 12$)[b]
Progression index		0.25 (0–3.5) ($n = 166$)[b]	0.21 (0–3.5) ($n = 130$)[b]	0.29 (0.13–1.83) ($n = 24$)[b]	0.61 (0.23–2.5) ($n = 12$)[b]
Data are given as median (range); progression index = EDSS/disease duration (years); annual relapse rate = total amount of relapses/duration of disease; [a]calculated for RR–MS patients with a disease duration ≥2 years; [b]patient numbers available for respective analysis; EDSS, Expanded Disability Status Scale; MS, multiple sclerosis; PP, primary progressive; RR, relapsing remitting; SP, secondary progressive.					

Table 2 CXCL10 haplotype frequencies (%) in MS patients and healthy controls

Haplotypes	Controls (*n* = 235) SNP (*G*/*C*)	All MS (*n* = 226) SNP (*T*/*C*)	MS subtypes		
			RR–MS (*n* = 174)	SP–MS (*n* = 36)	PP–MS (*n* = 16)
GG/*TT*	38 (16.2)	46 (20.3)	35 (20.1)	8 (22.2)	3 (18.8)
GC/*TC*	123 (52.3)	104 (46.0)	77 (44.2)	19 (52.8)	8 (50.0)
CC/*CC*	74 (31.5)	76 (33.7)	62 (35.7)	9 (25.0)	5 (31.2)
MS, multiple sclerosis; PP, primary progressive; RR, relapsing remitting; SNP, single nucleotide polymorphism; SP, secondary progressive.					

Table 3 Demographic and clinical variables of MS patients stratified according to CXCL10 haplotypes

	GGTT (*n* = 42)	*GCTC* (*n* = 99)	*CCCC* (*n* = 68)
Disease duration (years)	11 (0.8–38)	7 (0.5–42)	8 (0.5–43)
Age at onset (years)	28.5 (14–69)	31 (11–55)	29 (14–54)
Time to SP conversion (years)	16 (6–27) (*n* = 7)[b]	10 (1–25) (*n* = 18)[b]	9 (2–19) (*n* = 9)[b]
Annual relapse rate[a]	0.6 (0.1–4.3) (*n* = 20)[b]	1 (0.2–3.3) (*n* = 37)[b]	1 (0.1–3.5) (*n* = 26)[b]
Progression index	0.17 (0–1.25)* (*n* = 31)[b]	0.25 (0–3.5) (*n* = 80)[b]	0.28 (0–1.33) (*n* = 55)[b]
Time between first and second relapse (years)[a]	3 (0.17–22)** (*n* = 27)[b]	1 (0.17–16) (*n* = 65)[b]	2 (0.17–30) (*n* = 45)[b]
Data are given as median (range); progression index = Expanded Disability Status Scale/disease duration (years); annual relapse rate = total amount of relapses/duration of disease; [a]calculated for RR–MS patients with a disease duration ≥2 years; [b]patient numbers available for respective analysis; *P = 0.016, *GGTT* vs. either *GCTC* or *CCCC*; **P = 0.021, *GGTT* vs. *GCTC*; MS, multiple sclerosis; RR, relapsing remitting; SP, secondary progressive.			

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Fas-mediated T-cell apoptosis is impaired in patients with chronic inflammatory demyelinating polyneuropathy

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Introduction

Chronic inflammatory demyelinating polyneuropathy (CIDP) is a disease of the peripheral nervous system (PNS) that is clinically characterized by symmetrical proximal and distal weakness with altered sensation and hyporeflexia/areflexia (Maisonobe et al., 1996;Berger et al., 2003). Its clinical course can be either relapsing–remitting (RR) or chronic progressive (CP) (Gorson et al., 1997;Rotta et al., 2000). Several findings, such as inflammation at the site of lesion, response to immunomodulatory treatment, and the presence of autoantibodies against myelin antigens, indicate that it is an auto-immune disease involving both humoral and cell-mediated mechanisms (Toyka and Gold, 2003;Rezania et al., 2004). Activated T cells are central in this process because they produce cytokines modulating activation of macrophages and help antibody production by B cells (Rezania et al., 2004). Whether the immune response stops or persists may depend on the immune response shutting-off system, which involves the Fas death receptor triggering apoptosis of Fas[+] effector T cells (Rezania et al., 2004). This hypothesis is supported by data from animal models and more recently from patients with auto-immune demyelinating diseases (Gold et al., 1997;Sabelko-Downes et al., 1999;Bonetti et al., 2003). Indeed, in both experimental auto-immune encephalomyelitis and experimental auto-immune neuritis, T-cell-dependent inflammation is shut down by a programmed cell death mechanism (Gold et al., 1997). Furthermore, it has been recently reported that

Schwann cells from CIDP patients can induce T-cell apoptosis, thus regulating the immune response in the PNS (Bonetti et al., 2003).

Fas induces cell apoptosis by triggering a cascade of caspases through two partly interconnected pathways; the extrinsic pathway involves caspase-8-mediated direct activation of the cascade, whereas the intrinsic pathway proceeds through mitochondrial release of cytochrome C and activation of caspase-9. Both pathways converge in the activation of effector caspases, such as caspase-3, -6, and -7. The system is under the control of several inhibitors belonging to the family of FLICE-inhibitory protein (FLIP), bcl-2, and inhibitor of apoptosis protein (IAP) (Dianzani et al., 2003). The Fas system may play a role in maintaining immune tolerance by decreasing the risk of cross-reactions with self-antigens by 'molecular mimicry' when the non-self-antigens have been cleared. Accordingly, inherited defects of Fas function cause auto-immune lymphoproliferative syndrome (ALPS), a condition that is characterized by haematological auto-immunities and non-neoplastic accumulation of lymphocytes in the spleen and lymph nodes. ALPS patients usually display expansion of atypical T cells expressing the T-cell receptor (TCR)α/β, but lacking CD4 and CD8, and markers of helper and cytotoxic lymphocytes, respectively, and therefore named double-negative T cells (DN T cells) (Straus et al., 1999;Dianzani et al., 2003). Most frequently, mutations affect the Fas gene (ALPS-Ia), but mutations of the Fas ligand (ALPS-Ib) or caspase-10 genes (ALPS-II) have been reported as well; in other patients (ALPS-III), the mutated gene is not known. In previous studies, we showed that inherited defects of the Fas function may also predispose to several common auto-immune diseases including multiple sclerosis (MS) (Comi et al., 2000;Ramenghi et al., 2000;Dianzani et al., 2003). The inherited component of the defect was suggested by the finding that defective Fas function was displayed not only by a substantial proportion of MS patients but also by their healthy parents (Comi et al., 2000). Intriguingly, the defect was significantly more frequent in patients with progressive course than in those with RR course, which suggests that Fas function may influence not only MS development but also its evolution (Comi et al., 2000).

The notion that MS shares several mechanisms with CIDP (Toyka and Gold, 2003), and the observation that inflammatory neuropathy can be a clinical presentation in patients with ALPS (Sneller et al., 1997;Vaishnaw et al., 1999;Rieux-Laucat et al., 2003), prompted us to search for Fas function defects in CIDP and evaluate its correlation with the disease course and prognostic factors.

Materials and Methods

Patients

We studied 27 patients with CIDP, diagnosed according to the American Academy of Neurology subcommittee criteria (Ad Hoc Subcommittee of the American Academy of Neurology AIDS Task Force, 1991), and 12 patients with acute inflammatory demyelinating polyneuropathy (AIDP) (Ho et al., 1995) (Table 1). Patients underwent routine analyses to rule out other causes of neuropathy, cerebrospinal fluid examination, and electrodiagnostic tests. Neurophysiologic evaluation was performed within 2 weeks from symptoms' onset in AIDP patients and between 8 and 10 weeks from symptoms' onset in CIDP patients. The testing included motor and sensory conduction velocities, nerve and muscle action potentials, distal latencies, F-wave latencies, and needle electromyogram (EMG) study. Presence of fibrillation potentials at EMG was deemed as a sign of secondary axonal damage. None of the patients had received immunomodulatory/immunosuppressive treatment in the 3 months prior to blood collection. In particular, no patient was receiving chronic immunosuppression. Previous treatments included either oral prednisolone or intravenous immunoglobulins (Igs). No patient displayed drug resistance (Table 1). All AIDP patients displayed full recovery from the acute episode. All patients gave their informed consent according to the Declaration of Helsinki (International Committee of Medical Journal Editors, 1995). The research was approved by the local ethical committee.

Immunophenotype analysis

Peripheral blood mononuclear cells (PBMCs) from 27 CIDP patients, 12 AIDP patients, and 110 healthy donors were isolated by gradient centrifugation. Expression of surface molecules was evaluated by direct immunofluorescence and cytofluorimetric analysis (FACScan, Becton Dickinson) using monoclonal antibodies (MAbs) to CD3, CD4, CD8, TCRα/β (Becton Dickinson), and Fas (Immunotech). DN T cells were detected using fluorescein isothiocyanate (FITC)-conjugated anti-TCRα/β MAbs plus phycoerythrin (PE)-conjugated anti-CD4 and CD8 MAbs. Fas expression was detected using FITC-conjugated anti-Fas MAbs plus PE-conjugated anti-CD3 MAbs. Antigenic density was expressed as the median fluorescence intensity ratio (MFI-R) of total lymphocytes according to the following formula:

$$MFI\text{-}R = \frac{\text{MFI of sample histogram (arbitrary units)}}{\text{MFI of control histogram (arbitrary units)}}$$

Analysis of Fas- or methyl prednisolone-induced apoptosis

Fas-induced cell death was evaluated, as previously reported (Ramenghi et al., 2000), on T-cell lines obtained by activating PBMCs with phytohemagglutinin (PHA) at days 0 (1 mg/mL) and 13 (0.2 mg/mL) and cultured in RPMI 1640 medium + 10% fetal bovine serum (FBS) + recombinant interleukin-2 (rIL-2) (2 U/mL) (Biogen). Fas function was assessed at day 19. Cells were incubated with control medium or anti-Fas MAb (1 µg/mL) (CH 11 clone, UBI) in the presence of rIL-2 (2.5 U/mL) to minimize spontaneous cell death. Cell survival was evaluated after 18 h by counting live cells in each well by trypan blue exclusion test. The same conditions were used to measure cell death induced by methyl prednisolone (PDN) 100 µM (Upjohn). Results were expressed as percentage of specific cell survival (SCS), calculated as follows:

$$\frac{\text{total live cell count in the assay well}}{\text{total live cell count in the control well}} \times 100$$

As normal range of SCS to Fas-mediated apoptosis, we referred to data previously obtained in our laboratory from 150 healthy donors (Dianzani et al., 2003). The data were then replicated in the 110 controls, whose test was performed in parallel with

patients. Subjects whose SCS was ≥82% (95th percentile of the normal range) were defined as Fas-resistant (Fas-r), whereas those with SCS below that value were defined as Fas-sensitive (Fas-s). PDN-mediated apoptosis was tested in 14 patients with CIDP, 12 with AIDP, and 20 controls run in parallel with patients. Moreover, patients' cell survival was compared with data previously obtained in our laboratory from 75 healthy donors (Comi et al., 2000). Hybrid cell lines were produced by polyethylene glicol (PEG)-fusing PHA-activated T cells with the Fas-s continuous cell line HUT78 and culturing fused cells in RPMI 1640 + 10% FBS + anti-Fas MAb (1 µg/mL).

Caspase activity

Fas-induced activation of caspase-8 and etoposide-induced activation of caspase-9 were evaluated on T-cell lines obtained by activating PBMCs from 11 Fas-r patients with PHA at days 0 (1 µg/mL) and 8 (0.1 µg/mL) and culturing cells with 10 U/mL IL-2. These culture conditions were found to allow maximal activation of both caspases in preliminary kinetic experiments in 10 normal donors. Four days after the second stimulation (day 12), T cells were treated or not treated with either anti-Fas MAb (CH 11, 2 µg/mL) or etoposide (10 µg/mL) (Sigma) on ice for 30 min Thereafter, cells were moved to 37°C for 3 h when treated with anti-Fas MAb, or 6 h when treated with etoposide, and then centrifuged and lysed (MBL). Protein concentration was measured using the Biorad Protein Assay (Bio-Rad), and the same amounts were used to evaluate caspase activity by fluorimetric assays (MBL). Two or more control lysates, obtained from Fas-s normal donors, were always run in parallel. The results of caspase activity were expressed as activity of either Fas-stimulated or etoposide-stimulated cells/caspase activity of unstimulated cells (relative caspase activity).

Western blotting of FLIP

The samples (150 µg of protein/lane) were loaded on a 12% polyacrylamide gel, separated under reducing conditions, and subsequently blotted on a Hybond nitrocellulose membrane (Amersham Biosciences, Inc.). The blots were blocked for at

least 1 h with 5% non-fat dry milk in phosphate-buffered saline (PBS)/Tween (PBS + 0.1% Tween 20), washed with PBS/Tween, and then incubated with a polyclonal α-FLIP antibody (1:500 in PBS/Tween, Chemicon, Inc.) for 16 h at 4°C. After washing in PBS/Tween and incubating with polyclonal secondary antibodies (horseradish peroxidase-conjugated anti-rabbit Ig 1:5000 in PBS/Tween), we developed the blots by the chemiluminescence method (ECL, Amersham Biosciences, Inc.).

Statistical analysis

Comparisons of cell survival to Fas-mediated apoptosis and relative caspase activity in the different groups were performed with the non-parametric Mann–Whitney U-test. Comparisons of single-patient data were performed with either chi-square test or Fisher's exact test. All p values are two-tailed.

Results

Fas expression in T cells

Fas expression was evaluated in T-cell lines derived from each patient on the same day in which the cell death assay was performed. No difference was found between patients and controls. MFI-R, expressed as median value (interquartile range), was 13 (7–26) in CIDP patients, 12 (7–18) in AIDP patients, and 12 (5–22) in controls. Search for DN T cells in fresh PBMCs did not reveal expansion of these cells in any patient (i.e., DN cell count was always <1%).

Fas- or PDN-mediated T-cell apoptosis in CIDP and AIDP patients

Patients with CIDP displayed significantly higher cell survival to Fas-mediated apoptosis than both normal controls and AIDP patients (median SCS: 83, 48, and 59%, respectively, p < 0.0001 and p = 0.0035) (Fig. 1), whereas no difference was found between controls and AIDP patients. The single-patient data showed that 14 of 27 (52%) CIDP patients were Fas-r compared with two of 110 (2%) normal controls

(p < 0.0001) and zero of 12 AIDP patients (p = 0.0026). To assess specificity of the defect, we evaluated PDN-induced apoptosis in 14 CIDP patients, 12 AIDP patients, and 20 controls. Results showed no difference between the three groups. Fas function stability during disease course was then evaluated by performing a second cell death assay in five Fas-r and four Fas-s CIDP patients. The first evaluation was performed at diagnosis and the second after a median time of 20 months of disease (range 18–22 months). All five Fas-r patients had a CP course, whereas two Fas-s patients had an RR course and two a CP course. In RR patients, the second evaluation was performed in the remission phase. Results showed that all patients displayed a similar SCS in both samples (i.e., variability was always <5%) (Fig. 1).

Analysis of Fas-mediated cell death in CIDP patients with different clinical courses showed that both CP and RR patients had a significantly higher cell survival than controls (median SCS: 89 vs. 48%, p < 0.0001 and 70 vs. 48%, p = 0.0008, respectively). Comparison of patients with CP vs. RR course showed that cell survival was significantly higher in CP than in RR patients (p < 0.05) (Fig. 2). Indeed, 70% (12/17) of CP patients were Fas-r compared with 20% (2/10) of RR patients (p < 0.05). Thereafter, we divided CIDP patients into two groups according to neurophysiologic features at diagnosis and found that patients with secondary axonal pattern had significantly higher cell survival than those with a pure demyelinating form (median SCS: 90 vs. 72%, p < 0.01) (Fig. 2). All patients from the former group (10/10) were Fas-r compared with only 24% (four of 17) of those from the latter group (p < 0.005). Analysis of Fas-induced cell death in CIDP patients receiving different treatments (oral prednisone vs. intravenous Igs) showed that cell survival was similar in the two groups (median SCS: 82 vs. 83%, p > 0.05).

Fas-mediated T-cell apoptosis in patients' parents and hybrid cell lines

To test whether the Fas pathway impairment had an inherited component, we evaluated Fas-mediated T-cell death in six families of Fas-r CIDP patients: mother and father from families 1–5 and father from family 6; all of them were healthy. We

found that SCS was significantly higher in these family members than in controls (p < 0.0001) and in each family there was at least one Fas-r parent (Fig. 1). In previous studies, we showed that Fas-r patients with ALPS or MS produce molecules exerting a dominant negative effect on Fas function (Comi et al., 2000;Ramenghi et al., 2000;Dianzani et al., 2003). To evaluate whether these molecules are also detectable in CIDP, we fused activated CD4$^+$ T cells derived from two Fas-r patients with the Fas-s HUT78 T-cell lines and cultured hybrid cells under the selective pressure of anti-Fas MAbs. Both fusions from the Fas-r patients gave rise to Fas-r hybrid cell lines (Fig. 1), whereas fusion from Fas-s control subjects (n = 15) did not (data not shown).

Caspase-8 and -9 activities and Western blotting of FLIP in Fas-r CIDP patients

To better characterize the apoptosis defect, we evaluated function of the extrinsic and intrinsic pathways of apoptosis by assessing activation of caspase-8 induced by Fas triggering and caspase-9 induced by etoposide, selectively acting on mitochondria, in T-cell lines from 11 Fas-r CIDP patients and 10–19 Fas-s controls. Results showed that caspase-8 activity was significantly lower in CIDP patients than in controls (p < 0.05), whereas no difference was found in caspase-9 activity (Fig. 3). To assess whether defective caspase-8 activation was ascribable to increased expression of the FLIP inhibitor, we evaluated FLIP expression by Western blotting in the T-cell lines obtained from the same group of patients. Results showed no difference between patients and controls (data not shown).

Discussion

This work shows that patients with CIDP display a significantly lower Fas-induced T-cell death than healthy controls and that a substantial proportion of them is Fas-r. These data are in line with those reported in other auto-immune disorders, such as ALPS, type 1 diabetes mellitus (T1DM), thyroid auto-immunities (TAs), and MS (Comi et al., 2000;Ramenghi et al., 2000;Dianzani et al., 2003). In particular, the proportion

of Fas-r subjects in MS is very close to that found in CIDP (Comi et al., 2000). Therefore, impaired Fas function may result in defective elimination of autoreactive T cells and confer a predisposition to CIDP.

This cross-sectional study cannot rule out the possibility that Fas resistance may have been acquired during the course of disease by selection of constitutively Fas-r T-cell subsets, driven by chronic immune activation or therapy. A longitudinal study is needed to clarify this issue. However, a preliminary analysis of nine patients, in whom Fas function was evaluated at diagnosis and 20 months later, showed that the defect is stable over time.

One possibility is that the defect is due to inherited alterations similar to those causing ALPS. This hypothesis is supported by the finding that Fas-r patients have at least one Fas-r parent. Because these parents were healthy, the defect is not a consequence of the disease. Furthermore, fusion of Fas-r T cells from patients with a Fas-s continuous T-cell line produced Fas-r hybrid cells. This suggests a role for molecules exerting a dominant negative effect on Fas function, as previously reported in other auto-immune diseases (Dianzani et al., 2003). Presumably, the defect does not involve the Fas gene because CIDP patients did not display an expansion of DN T cells in the peripheral blood, a feature that is typically associated with Fas gene mutations. Moreover, the Fas death receptor was normally expressed on patients' T cells. An involvement of Fas ligand is ruled out by the test methodology, in which the Fas receptor was triggered by an anti-Fas MAb and therefore independent from Fas ligand activity. Taken altogether, these findings point to a defect involving the Fas-signaling pathway, similar to that detected in MS, T1DM, and TAs (Dianzani et al., 2003).

Assessment of caspase activity showed that the defect mainly involved the extrinsic pathway of apoptosis, as shown by the defective activation of caspase-8. By contrast, the intrinsic pathway, acting through mitochondria and caspase-9, was not affected. Activation of caspase-8 requires aggregation of the death-inducing signaling complex

(DISC), formed by Fas, the adapter molecule FAS·associated death domain (FADD), and caspase-8. DISC formation is inhibited by FLIP, whose downregulation is crucial for the Fas sensitivity of activated lymphocytes. Increased expression of FLIP has been detected in T cells from MS patients by Semra et al. (2001), who suggested that it may be involved in the Fas function defect. By contrast, FLIP seems not to be involved in the defect displayed by our CIDP patients, who expressed levels similar to the controls.

Defective Fas function may not only predispose to CIDP development but also favour a progressive course, possibly due to a failure of the mechanism of remission which would require shutting-off of the auto-immune response. Accordingly, cell survival to Fas-mediated apoptosis was significantly higher in CP than in RR patients. This finding is in line with what we found in MS, where defective Fas function was more frequent in progressive than in RR patients (Comi et al., 2000). Moreover, the finding of an efficient T-cell apoptosis in AIDP patients with full recovery supports the hypothesis that defective T-cell Fas-function is a key factor for persistence of the auto-immune response. In this regard, it would be of interest to test Fas-mediated T-cell apoptosis in patients with acute inflammatory neuropathy who then develop a chronic disease (Odaka et al., 2003).

Another consequence of impaired T-cell apoptosis may be the tendency to develop early secondary axonal damage, probably due to persistent inflammation. As previously reported, CIDP patients frequently display axonal loss, possibly due to the influence that longstanding demyelination exerts on axonal properties (Nagamatsu et al., 1999). It is noteworthy that in our work all patients with secondary axonal damage at diagnosis, indicated by fibrillation potentials at EMG, displayed defective Fas function, whereas only 24% of patients with an electrodiagnostic evaluation consistent with a pure demyelinating form displayed the defect.

Because CP course and axonal damage are negative prognostic factors in CIDP (Bouchard et al., 1999), it can be suggested that Fas defects may favour an

aggressive disease evolution. From a clinical standpoint, testing T-cell survival to Fas-mediated apoptosis may provide information on both clinical course and prognosis, thus leading towards the most appropriate treatment. According to Kieseier et al. (2002), the occurrence of secondary axonal damage in CIDP patients may require a different treatment strategy in which immunomodulation, neuroprotection, and nerve repair promotion are combined.

In conclusion, this study provides the first evidence that defective Fas function may be a predisposing factor for CIDP development and possibly a negative prognostic marker, similar to other auto-immune diseases. To clarify this issue, an analysis of a larger group of patients followed-up since disease onset is already in progress.
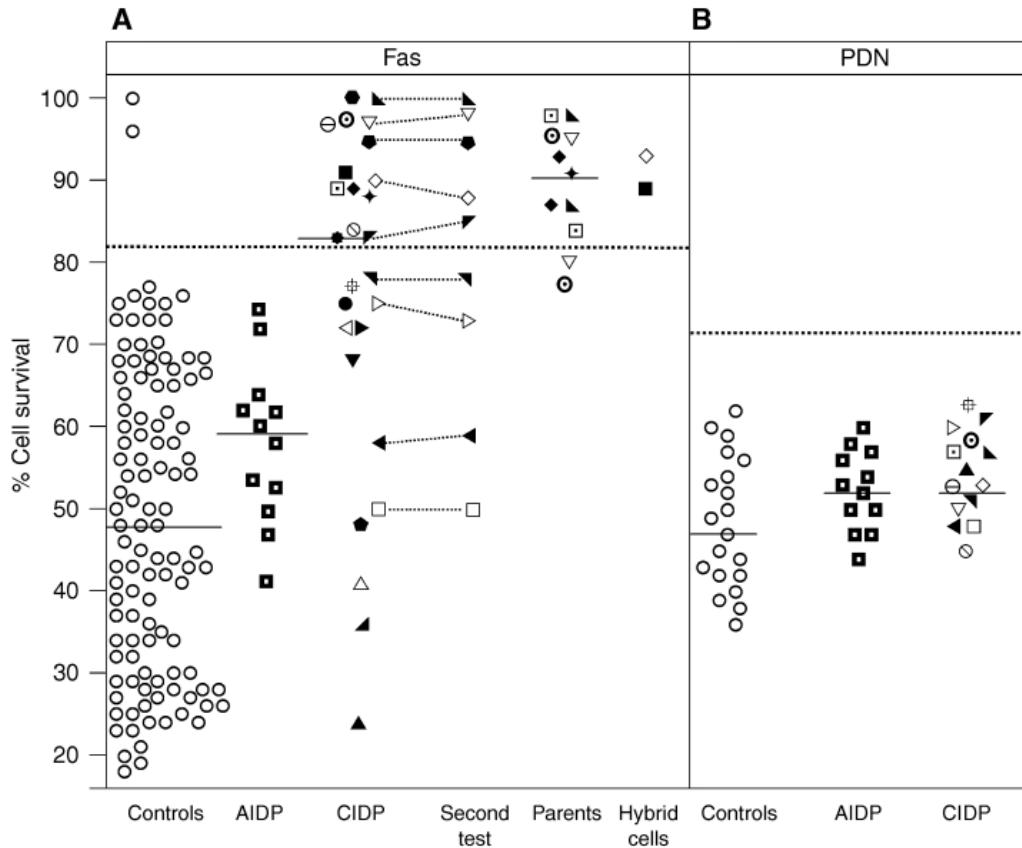
Tables

Table 1. Clinical features of chronic inflammatory demyelinating polyneuropathy (CIDP) and acute inflammatory demyelinating polyneuropathy (AIDP) patients.

|	CIDP	AIDP
Patients (*n*)	27*	12†
Gender (male:female)	21:6	6:6
Age (mean ± SD) (years)	61 ± 13	45 ± 12
Time to diagnosis (mean ± SD) (days)	65 ± 5	9 ± 4
Response to treatment (*n*)	27	12
RR course (*n*)	10	0
CP course (*n*)	17	0
Demyelinating pattern (*n*)	17‡	12
Secondary axonal pattern (*n*)	10§	0
RR, relapsing–remitting; CP, chronic progressive.		
*All cases fulfilled the criteria of the American Academy of Neurology (AAN) subcommittee *(Ad Hoc Subcommittee of the AAN AIDS Task Force, 1991)*. Patients were enrolled in the following Italian hospitals: Ospedale Maggiore at Novara (eight patients), Ospedale Sacco at Milano (six patients), Ospedale San Gerardo at Monza (five patients), Ospedale Regionale at Aosta (five patients), and Ospedale S. Maria at Castellanza (three patients).		
†All cases fulfilled the criteria for the diagnosis of AIDP *(Ho et al., 1995)*. Patients were enrolled in the Ospedale Maggiore at Novara, Italy.		
‡Patients who met at least three of the four mandatory physiological criteria proposed by the AAN *(Ad Hoc Subcommittee of the AAN AIDS Task Force, 1991)*.		
§Patients who met at least three of the four AAN physiological criteria *(Ad Hoc Subcommittee of the AAN AIDS Task Force, 1991)* and also displayed fibrillation potentials at needle electromyogram.		

Figures

Figure 1. Fas- and methyl prednisolone (PDN)-induced T-cell death in patients and controls.

(A) Fas-mediated apoptosis in healthy controls whose test was performed in parallel with patients, acute inflammatory demyelinating polyneuropathy (AIDP) patients, chronic inflammatory demyelinating polyneuropathy (CIDP) patients, second evaluation of nine CIDP patients (a dotted line joins the results of the two evaluations), parents of six patients, and hybrid cell lines. (B) PDN-mediated apoptosis in healthy controls whose test was performed in parallel with patients, AIDP patients, and CIDP patients. Each patient is marked with the same symbol in all panels. Parents have the same symbol as their son/daughter. Activated T cells were treated with anti-Fas MAb or PDN, and cell survival was assessed after 18 h. Results are expressed as percentage of specific cell survival (SCS). The dotted lines indicate the upper limit of the normal range of SCS to Fas- or PDN-mediated apoptosis,

calculated as the 95th percentile of data obtained from 150 and 75 normal donors, respectively *(Comi et al., 2000; Dianzani et al., 2003)*. The full lines indicate the median value for each group.

Figure 2. Fas-induced T-cell death in chronic inflammatory demyelinating polyneuropathy (CIDP) patients with different clinical courses and electrophysiologic patterns.
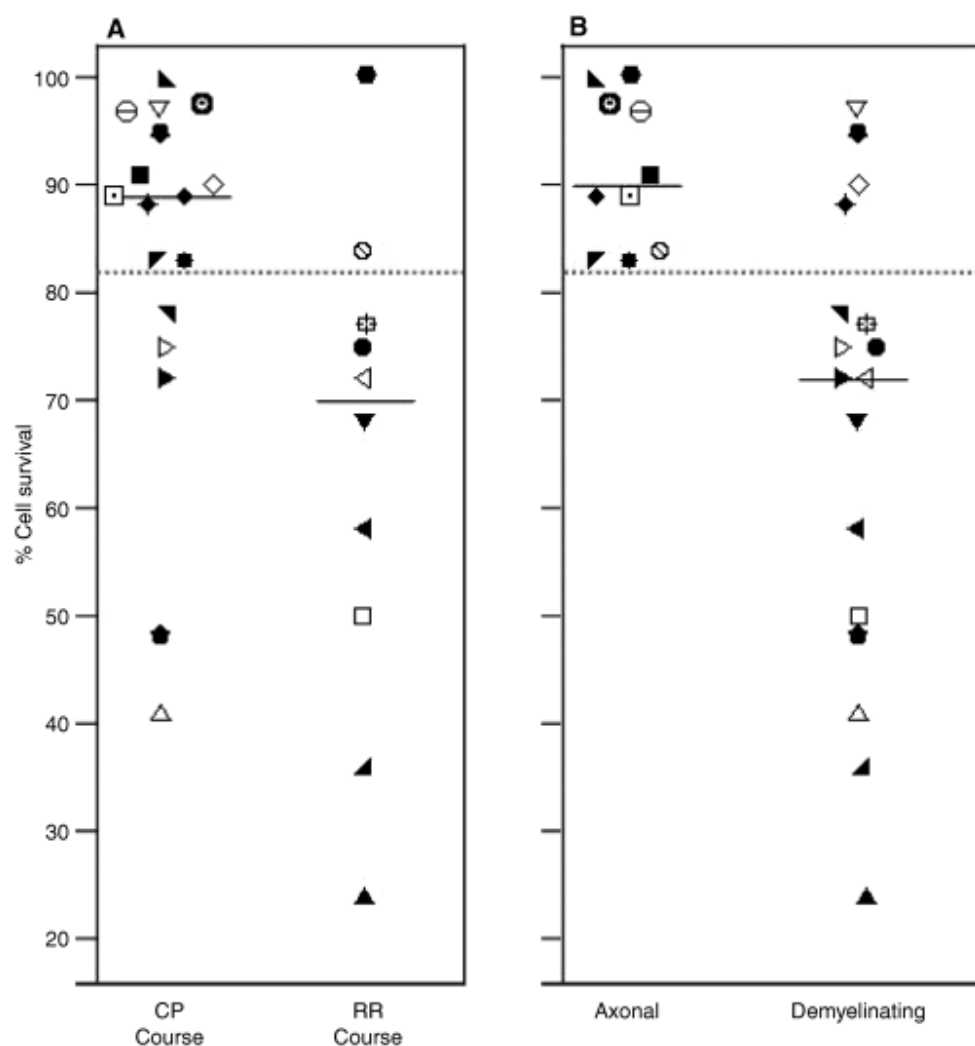
(A) Comparison of data from chronic progressive (CP) and relapsing–remitting (RR) patients. (B) Comparison of data from patients with secondary axonal pattern and pure demyelinating form. Each patient is marked with the same symbol in all panels. Results are expressed as percentage of specific cell survival to Fas-mediated

apoptosis. The dotted line indicates the upper limit of the normal range calculated as the 95th percentile of data obtained from 150 normal donors *(Dianzani et al., 2003)*. The full lines indicate the median value for each group.

Figure 3. Caspase activity in Fas-resistant chronic inflammatory demyelinating polyneuropathy (CIDP) patients.
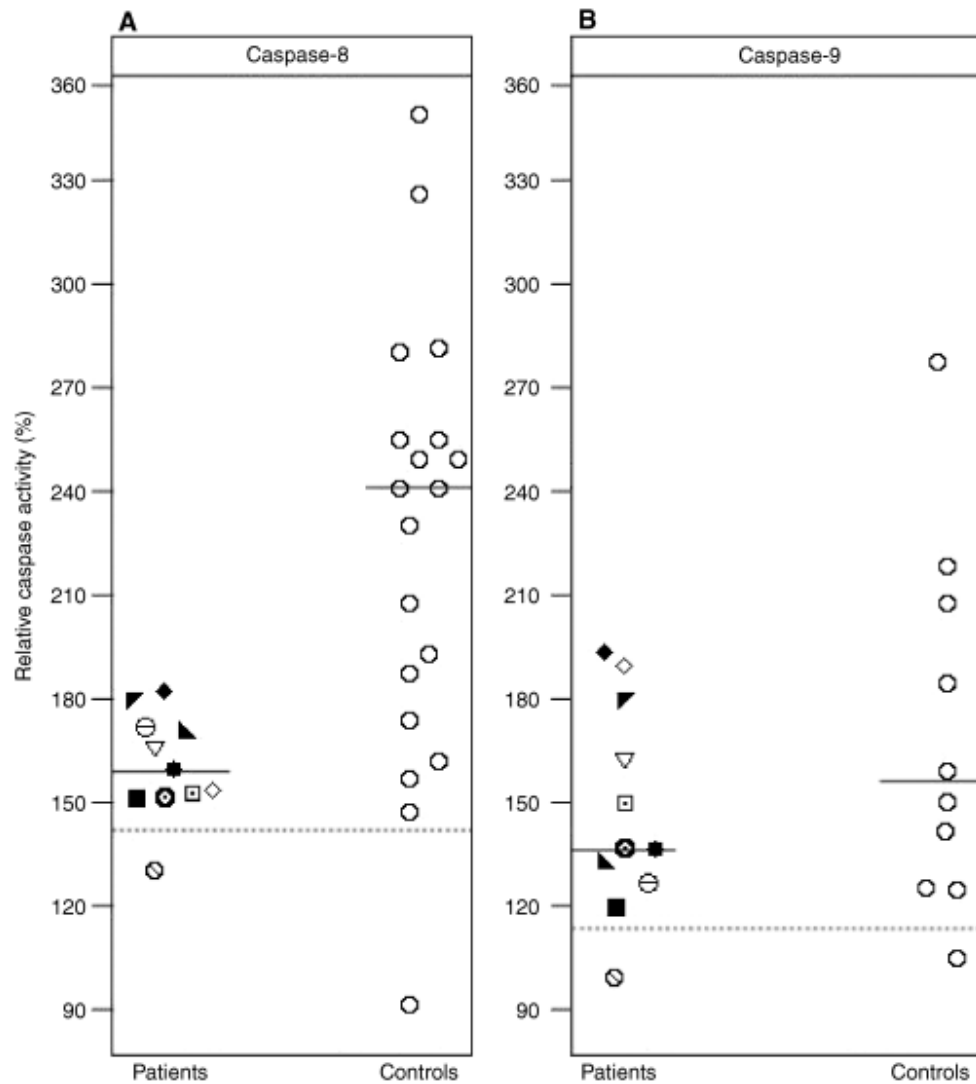
(A) Fas-induced caspase-8 activity in CIDP patients and controls. (B) Etoposide-induced caspase-9 activity in CIDP patients and controls. Each patient is marked with the same symbol in all panels. Results are expressed as percentage of relative caspase activity. The dotted line indicates the lower limit of the normal range calculated as the 5th percentile of data obtained from healthy donors. The full lines indicate the median value for each group.

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