The Role of T Lymphocytes in The Pathogenesis of Hu Antibody Associated Paraneoplastic Neurological Syndromes

Cover: This is a photograph of a model posing as a patient with Hu-PNS. Hu-PNS usually runs a severe and subacute course, leaving the patient with severe disability within weeks to months from onset of symptoms. At the time of neurological presentation, approximately 70% of patients are not known with cancer. In more than 80% of the patients, an underlying tumor is ultimately detected, most often a small cell lung cancer.

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The Role of T Lymphocytes in The Pathogenesis of Hu Antibody Associated Paraneoplastic Neurological Syndromes

De rol van T lymfocyten bij de pathogenese van paraneoplastische neurologische syndromen die met Hu antistoffen geassocieerd zijn

Proefschrift

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Aan mijn vader (1946-1999)
Aan mijn moeder

Aan Jelmer

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Chapter | 1

General Introduction

PARANEOPLASTIC SYNDROMES

Paraneoplastic syndromes or 'remote effects of cancer' result from damage or dysfunction of organs that are not invaded by a neoplasm or its metastases.[1] By definition, these disorders are not caused by tumor cell infiltration, infection, ischemia, metabolic and nutritional deficits, surgery or other forms of tumor treatment. Many paraneoplastic syndromes result from products secreted by the tumor that mimic hormones causing hypercalcemia, Cushing's syndrome, cancer cachexia, etc.[1] Although some paraneoplastic neurological syndromes (PNS) are caused by a similar mechanism (e.g. carcinoid myopathy), most PNS are immune-mediated. The finding that patients with lung cancer and paraneoplastic sensory neuropathy had antineuronal antibodies led Wilkinson and Zeromski[2] to hypothesize in 1965 that patients with the "encephalomyelitic form of carcinomatous neuropathy" should be investigated "for the presence of circulating anti-brain antibodies, particularly as lymphocytic infiltration is a prominent feature in these patients". Approximately 30 years later, the characterization of the Hu antibodies[3] has shown that these patients have developed an immune response against the Hu antigens, a family of neuronal RNA-binding proteins.[4] Since then, an ever growing number of more or less characterized paraneoplastic antibodies has been defined. Detection of paraneoplastic antibodies is clinically important because it diagnoses an often serious neurological syndrome as paraneoplastic and directs the search for an underlying neoplasm. The diagnosis and clinical management of PNS are reviewed in Chapter 2. This thesis will focus on PNS that are associated with Hu antibodies (Hu-PNS).

INCIDENCE OF HU-PNS

High titer Hu antibodies are invariably associated with a paraneoplastic neurological syndrome and the most common underlying tumor is small cell lung cancer (SCLC).[5-8] In The Netherlands, the department of Immunology, in collaboration with the department of Neurology, at Erasmus MC is the national reference laboratory for the detection of paraneoplastic antibodies.[9] Each year, approximately 15 new patients with high-titer Hu antibodies and an associated SCLC are diagnosed. With an incidence of 1,520 SCLC patients per year in the Netherlands (Netherlands Cancer Registry, Utrecht, the Netherlands), the frequency of Hu-PNS in SCLC patients is at least 0.9%.

CLINICAL ASPECTS OF HU-PNS

Symptoms and signs

The most frequent presenting symptoms and signs of Hu-PNS are sensory neuropathy (55%), subacute cerebellar ataxia (10-20%), limbic encephalitis (10-15%), brainstem encephalitis (10%) and multifocal involvement (10-30%), including autonomic dysfunction in a subset of patients.[6,8] During the course of the disease, multiple areas of the nervous system become involved in another 30% of patients with unifocal symptoms at presentation.[8] The disease usually runs a severe and subacute course, leaving the patient with severe disability within weeks to months from onset of symptoms.[6,8] After stabilization, approximately two-thirds of patients are non-ambulatory.[8] However, a more protracted and benign course has also been described in several patients, usually with sensory neuropathy.[10]

Diagnosis:

The detection of Hu antibodies in serum or CSF definitively diagnoses the neurological syndrome as paraneoplastic.[11] In most patients the CSF is abnormal showing signs of inflammation, including mononuclear pleiocytosis, oligoclonal bands and intrathecal IgG synthesis.[5] In patients with limbic encephalitis, MRI and CT may show abnormalities in the medial temporal lobes and in patients with prominent cerebellar signs and symptoms radiological evidence of cerebellar atrophy may become apparent.[12,13] Otherwise, MRI and CT changes in patients with Hu-PNS are aspecific.[5]

Underlying tumor

At the time of neurological presentation, approximately 70% of patients are not known with cancer.[5-8] In more than 80% of the patients, an underlying tumor is ultimately detected, most often a SCLC.[5-8] Other tumors include neuroblastomas, small cell prostate cancers and, at much lower frequencies, many other tumors.[5-8] After detection of Hu antibodies, a careful search for an underlying SCLC should be performed. When the thoracic CT-scan is negative, a total body FGD-PET or PET/CT scan is recommended.[14,15] When a tumor other than SCLC is detected, it may be an unrelated secondary neoplasm.[7] However, when the diagnosed extrathoracic tumor expresses Hu antigens, further tests to rule out a coexisting SCLC are probably unnecessary.[6]

In approximately 15% of patients with Hu antibodies, no tumor is found by chest radiography and CT. In some of these patients a tumor may be detected during follow-up or at post-mortem examination.[16] In other patients, spontaneous regression of an underlying SCLC may have occurred.[17]

Treatment

Several retrospective series have demonstrated that Hu-PNS patients who receive antitumor treatment have a better functional outcome.[5,8,18,19] Because of the retrospective nature of these studies, the better neurological prognosis in patients receiving treatment of the tumor could also be caused by selection. On the other hand, the results of immunotherapy in Hu-PNS are generally disappointing. Several retrospective studies reported no clear beneficial effect of steroids, cyclophosphamide, IVIg or plasma exchange.[5,8,18,19] In individual cases neurological improvement following immunotherapy has been reported.[20] Therefore, most patients with Hu-PNS not receiving antitumor treatment, will be treated with some sort of immunotherapy in an attempt to halt neurological deterioration.

Prognosis

The neurological prognosis is poor in most patients. In one series, 38% of patients were unable to walk at the time of diagnosis while 63% had become non-ambulatory after stabilization of the neurological syndrome.[8] The overall 3 months, one-year and three-year survival rates from the time of diagnosis are approximately 60%, 40% and 20%.[8]

HU ANTIBODIES

The routine laboratory detection of Hu antibodies is generally performed by an immunohistochemical technique (e.g. indirect immunofluorescence) followed by confirmation on Western blot using purified recombinant HuD protein as substrate.[9,21] Hu antibodies react selectively with the nuclei of all neurons while sparing the nucleoli.[22-25] Western blotting with neuronal extracts as substrate show reactivity of all anti-Hu sera with a triplet of antigens of 32-42 kDa.[22,23] Subsequent expression cloning experiments have demonstrated that Hu antibodies react with the neuronal HuD antigen.[4] Less than 1% of SCLC patients will develop PNS and high titer Hu antibodies. Interestingly, 16% of SCLC patients have lower titer Hu antibodies without neurological symptoms.[26] These low titer Hu antibodies were associated with limited disease stage, complete response to therapy, and longer survival.[26] These associations were not confirmed in another study that used a more sensitive and possibly less specific assay for the detection of Hu antibodies.[27]

HU ANTIGENS

HuD was the first cloned paraneoplastic antigen using anti-Hu patient serum.[4] HuD belongs to a family of neuronal RNA-binding proteins that contain two N-terminal RNA binding domains linked by a stringer to a third RNA binding domain. Other family mem-

bers include HuC[28], Hel-N1 and Hel-N2.[29,30] The Hu-proteins are highly homologous to a drosophila protein (Elav), that is critical for nervous system development of the fly.[4,31] The exact function of the Hu proteins is unknown, but their homology to Elav and their early expression during embryogenesis of the mammalian nervous system[32] suggest that they are likewise crucial for development and maintenance of the neuronal phenotype. HuD is believed to bind to AU-rich elements and regulate mRNA export and stability of several cell cycle regulatory proteins.[33,34] The Hu proteins have been implicated in multiple aspects of neuronal function, including the commitment and differentiation of neuronal precursors, as well as synaptic remodeling in mature neurons.[35]

Immunohistochemical and Western blot studies show expression of Hu proteins in all SCLC. Because sera from all patients with Hu antibodies react with recombinant HuD, HuC and Hel-N1 proteins[30,36], RT-PCR was used to identify which family member was preferentially expressed in SCLC. A study of SCLC samples from 3 patients with and 3 patients without Hu-PNS detected HuD mRNA in all samples while no HuC or Hel-N1 mRNA could be detected.[36] Using a different primer set on 9 SCLC samples from patients without Hu antibodies, King and Dropcho detected Hel-N1 in 7 and Hel-N2 message in 6 tumors.[37] The discrepancy between these studies may be explained by primer design and/or by tumor selection.

Further studies showed that the HuD gene was not mutated in SCLC, including tumors from patients with Hu-PNS.[38,39]

Immunopathological findings in Hu-PNS

Pathological examination of the nervous system generally shows loss of neurons in affected areas of the nervous system with inflammatory infiltration by $CD4^+$ T cells, $CD20^+$ B cells and plasma cells in the perivascular spaces and $CD4^+$ T cells and cytotoxic (TIA^+) $CD8^+$ T-cells in the interstitial spaces.[40-43] Affected areas reveal an increase in reactive microglia that usually surround the neurons.[40,42] The location and severity of the neuronal loss correlates with the clinical syndrome but frequently the pathological abnormalities are much more widespread than one would predict based on the neurological signs and symptoms of the patient.[40,42] Tumors from Hu-PNS patients may be more heavily infiltrated with inflammatory cells and may more often express MHC class I. [44]

Pathogenesis of Hu-PNS

All patients with high titer Hu antibodies develop neurological signs and symptoms.[5-8] The expression of the onconeural antigen HuD in both the nervous system and in the

underlying SCLC favors the hypothesis that aberrant expression of Hu proteins in the SCLC triggers an immune response that subsequently reacts with the same antigens in the nervous system.[1] An immune etiology is further supported by the inflammatory changes in the CSF, including anti-HuD specific oligoclonal bands[45], and the inflammatory changes in the nervous system found at autopsy (see above).

Remarkably, all or almost all SCLC express Hu-proteins while only 16% of patients develop low-titer Hu antibodies and less than 1% have high-titer antibodies and Hu-PNS. The production of high titer Hu antibodies in a small proportion of SCLC patients could be explained by mutations in the HuD gene that could hypothetically trigger an HuD directed immune response. However, no mutations have been detected in the HuD gene in SCLC from Hu-PNS patients.[38,39] On the other hand, there is no evidence for an association between an apparent autoimmune constitution or HLA phenotype in SCLC patients who develop Hu-PNS versus those who do not develop Hu-PNS.[46] A similar lack of association between HLA phenotype and PNS was observed in SCLC patients with Lambert-Eaton myasthenic syndrome.[47,48] These findings suggest that SCLC may provide an as yet unidentified autoantigenic ('danger') stimulus that induces PNS.[49]

Pathogenesis of Hu-PNS – Hu antibodies

Findings supporting a pathogenic role for Hu antibodies include the strong correlation between high titers of Hu antibodies in serum with development of PNS, the intrathecal synthesis and deposits of Hu antibodies in the CNS, and the highly restricted expression of Hu antigens by the nervous system and the tumor. Hu antibodies may cause neuronal dysfunction by direct binding to the Hu proteins, by antibody dependent cellular cytotoxicity (ADCC)[50] or by complement activation[51] resulting in complement-mediated destruction via the membrane attack complex and by promoting phagocytosis of opsonized cells. However, a role for Hu antibodies in the pathogenesis of Hu-PNS has never been proven. In vitro Hu antibody-mediated lysis of neurons or SCLC tumor cells could never be demonstrated.[52-54] Furthermore, passive transfer of Hu antibodies in animals did not model neurological disease, despite high Hu titers.[55] Pathologic examination of CNS tissues and tumors of Hu-PNS patients revealed Hu-IgG bound to nuclei of neurons and tumor cells,[5] but the correlation between the localization of the Hu-IgG and clinical symptoms and regions of major tissue injury was limited.[22,24] In addition, these studies are hampered by possible postmortem perivascular diffusion of Hu-IgG and possible artifacts resulting from tissue processing methods.[55] If and how Hu antibodies can enter neurons is unknown.[55] Only some evidence of antibody entry into cells has been provided for antibodies to double-stranded DNA in patients with systemic lupus erythematosus (i.e., by endocytosis of antibodies via the brush border myosin 1 receptor).[56,57] IgG1 and IgG3

are the predominant anti-Hu IgG isotypes, which can activate complement.[42] Nevertheless, only weak complement reactivity was found in brain and tumor tissues from Hu-PNS patients.[40,42] In addition, the presence of cells involved in ADCC, such as NK cells, could not be demonstrated in the involved areas of post-mortem Hu-PNS neuronal tissues.[40,42] Finally, animal immunization with HuD protein or cDNA resulted in synthesis of high titers of Hu antibodies but not in neurological disease.[55,58] The intracellular localization of Hu antigens and the inability to proof a pathogenic role for Hu antibodies despite extensive study indicate that a pathogenic role for Hu antibodies is unlikely.

Pathogenesis of Hu-PNS – cellular immunity

The detection of $CD8^+$ T cells surrounding tumor cells and neurons in affected tissues of Hu-PNS patients,[40,42,59,60] the intracellular localization of Hu antigens and the inability to demonstrate a pathogenic role for Hu antibodies drew the attention towards a role for cytotoxic T cells in the immune pathogenesis of Hu-PNS. The detection of oligoclonal $CD8^+$ T cells in infiltrated areas of nervous tissues and tumors of Hu-PNS patients, supported the involvement of these cells in neuronal loss.[59,60] In addition, the expression of HLA class I molecules in tumors[44] and in affected nervous tissues from Hu-PNS patients[59] is compatible with a pathogenic role of Hu-specific $CD8^+$ T cells. The high titers of predominantly IgG_1 Hu antibodies suggest help from Hu-specific $CD4^+$ T cells to B cells.[42,61,62] Based on these findings, the current hypothesis is that the ectopic expression of HuD in tumor cells elicits a HuD specific T cell response that subsequently attacks both the tumor and neurons. In accordance with this hypothesis previous studies have detected HuD specific T cells in the blood of patients with Hu-PNS (Fig. 1).[63-65] However, HuD specific $CD8^+$ T cells were also found in the blood of healthy controls.[64,66] These latter studies did not use control peptides or proteins and included only low patient numbers hampering the interpretation of the results. Therefore, the role of HuD specific T cells in Hu-PNS requires further study and is the focus of this thesis.

Scope of this thesis

The objective of this thesis was to further investigate the role of T cells in the pathogenesis of Hu antibody associated PNS. To reach this goal, we prospectively investigated the cellular immune status and the presence of HuD specific T cells in a large cohort of patients with Hu antibody associated PNS. Chapter 2 provides a review on the clinical aspects of PNS. To determine whether any major immune disturbance was related to Hu-PNS, we studied the immune phenotype of circulating lymphocytes in patients with Hu-PNS and

controls in Chapter 3. Chapter 4 describes a multifaceted approach to detect circulating HuD specific T cells in a large group of patients with Hu-PNS and controls. Chapter 5 illustrates the successful usage of HLA class I multimer technology to detect antigen-specific T cells in CSF. The extension of this technique to detect HuD specific T cells in the cerebrospinal fluid of Hu-PNS patients is described in Chapter 6. False positive findings encountered during our search for HuD specific T cells are analyzed in Chapter 7. Chapter 8 reports on the treatment of patients with Hu-PNS with anti-CD20 (rituximab). In the last chapter we reflect our main findings, discuss methodological issues, speculate on the implications of our results and propose future studies.

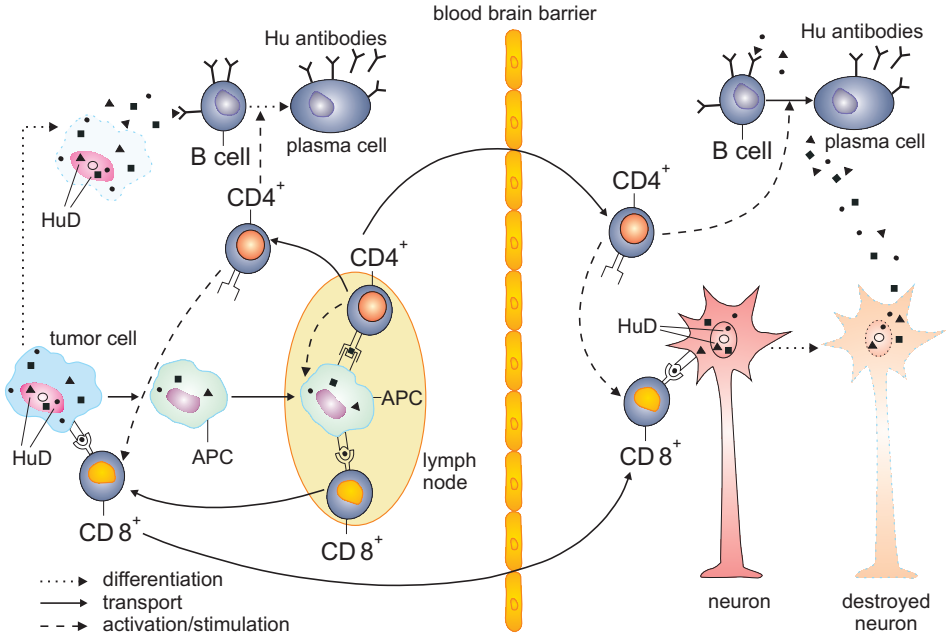

Figure 1. The hypothesized role for T cells in the pathogenesis of Hu antibody associated PNS.

HuD expressing tumor cells are phagocytozed by antigen presenting cells (APC) that migrate to lymph nodes, where they present HuD antigenic peptides to HuD specific $CD8^+$ and $CD4^+$ T cells via HLA-Class I and HLA-class II molecules, respectively. Tumor cells themselves present HuD antigens to $CD8^+$ T cells via HLA class I molecules.[42,44] The $CD4^+$ T cells support $CD8^+$ T cell activation and proliferation by cytokines such as IFN-γ and IL-2. $CD4^+$ T cells stimulate B cells through cytokines including interleukin (IL)-4 and IL-5. B cells recognize soluble Hu antigens through their B-cell receptor for Hu antigen. After activation, B cells differentiate into plasma cells, which secrete antibodies specific for Hu. Upon engagement of their TCR and accessory molecules, $CD8^+$ T cells can destroy tumor cells by secreting granzymes, perforins and cytokines such as TNF-α, or by upregulation of CD95 (Fas ligand) on tumor cells. The remnants of destroyed tumor cells can be taken up by APC, processed and presented to T cells. In addition, they can be specifically recognized by antibodies and eliminated via Fc receptor-expressing phagocytes. Cytotoxic HuD specific $CD8^+$ T cells not only slow the tumor growth, but they also cross the blood brain barrier and similarly attack neurons expressing the HuD antigen, causing severe neurological damage in these patients.

Chapter | 2

Managing paraneoplastic neurological syndromes

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ABSTRACT

Paraneoplastic neurological syndromes are remote effects of cancer that are not caused by invasion of the tumor or its metastases. Immunologic factors appear important in the pathogenesis of PNS because antineuronal autoantibodies against nervous system antigens have been defined for many of these disorders. The immunologic response is elicited by the ectopic expression of neuronal antigens by the tumor. Expression of these so-called 'onconeural' antigens is limited to the tumor and the nervous system and sometimes also the testis. At the time of presentation of the neurological symptoms, most patients have not yet been diagnosed with cancer. Detection of paraneoplastic antibodies is extremely helpful in diagnosing an otherwise unexplained, and often rapidly progressive neurological syndrome as paraneoplastic. In addition, the paraneoplastic antibodies may also direct the search for an underlying neoplasm. On the other hand, in patients known with cancer, the presentation of a paraneoplastic neurological syndrome may herald recurrence of the tumor or a second tumor. The number of paraneoplastic antibodies is still growing and at least 7 of these can now be considered 'well characterized'. Based on the clinical syndrome, the type of antibody and the presence or absence of cancer, patients are classified as having a 'definite' or 'possible' paraneoplastic syndrome. Despite the presumed autoimmune etiology of PNS, the results of various forms of immunotherapy have been disappointing with some exceptions. Rapid detection and immediate treatment of the underlying tumor appears to offer the best chance of stabilizing the patient and preventing further neurological deterioration.

INTRODUCTION

Paraneoplastic neurological syndromes (PNS) are remote effects of cancer that are by definition caused neither by invasion of the tumor or its metastases, nor by infection, ischemia, metabolic and nutritional deficits, surgery or other forms of tumor treatment.[1] Immunologic factors are believed to be important in the pathogenesis of PNS because antibodies against nervous system antigens have been defined for many of these disorders.[1] Presumably, the immunologic response is elicited by the ectopic expression of neuronal antigens by the tumor. Expression of these 'onconeural' antigens is limited to the tumor and the nervous system and sometimes also the testis. At the time of presentation of the neurological symptoms, most patients have not yet been diagnosed with cancer.[6,8,67,68] Detection of paraneoplastic antibodies can help diagnose the neurological syndrome as paraneoplastic and may direct the search for an underlying neoplasm. Often, the oncologist or hematologist will be involved in the tumor work-up. On the other hand, in patients known with cancer, the presentation of a paraneoplastic neurological syndrome may herald recurrence of the tumor or a second tumor. In these patients, however, metastatic complications of the known cancer must be ruled out first. Despite the presumed autoimmune etiology of PNS, the results of various forms of immunotherapy have been disappointing with some exceptions.[6,8,67,68] Rapid detection and immediate treatment of the underlying tumor appears to offer the best chance of stabilizing the patient and preventing further neurological deterioration.[6,8,67,68]

Pathogenesis

Pathological examination of the nervous system generally shows loss of neurons in affected areas of the nervous system with inflammatory infiltration by $CD4^+$ T-helper cells and B-cells in the perivascular spaces and cytotoxic $CD8^+$ T-cells in the interstitial spaces.[40,42,69] Examination of the cerebrospinal fluid (CSF) frequently demonstrates pleocytosis, intrathecal synthesis of IgG and oligoclonal bands, supporting an inflammatory or immune-mediated etiology.

The discovery of paraneoplastic antineuronal autoantibodies resulted in the general belief that these are immune-mediated disorders triggered by aberrant expression of 'onconeural' antigens in the tumor. Support for this hypothesis comes from the fact that the target paraneoplastic antigens are expressed both in the tumor and in the affected parts of the nervous system. Furthermore, the tumors are usually small, heavily infiltrated with inflammatory cells and spontaneous remissions at the time of neurological presentation have been described.[17,70] These findings suggest that some PNS without identifiable tumor may result from immune-mediated eradication of the tumor.[17,70] In keeping with this hypothesis, one study found more limited disease distribution and better oncologic outcome in small cell lung cancer (SCLC) patients with paraneoplastic autoantibodies.[26]

Although the paraneoplastic antibodies are synthesized intrathecally, a pathogenic role could only be proven for those paraneoplastic autoantibodies that are directed against easily accessible antigens located at the cell surface. Examples of such antigens are the acetylcholine receptor (anti-AChR muscle type in myasthenia gravis and neuronal ganglionic type in autonomic neuropathy), P/Q type voltage-gated calcium channels (anti-VGCC in Lambert-Eaton myasthenic syndrome), voltage-gated potassium channels (anti-VGPC in neuromyotonia) and the metabotropic glutamate receptor mGluR1 (anti-mGluR1 in paraneoplastic cerebellar degeneration). Most paraneoplastic antigens are located in the cytoplasm (e.g. the Yo antigen) or nucleus (e.g. the Hu and Ri antigens) and a pathogenic role for the respective antibodies could not be demonstrated.[55] In these disorders, indirect lines of evidence support the view that the cellular immune response against these antigens is responsible for the neurological damage.[63,71,72] The relative contribution of the cellular and humoral immunity to the clinical and pathological manifestations has not been resolved.[63,71,72] The paraneoplastic antibodies may, in these cases, be surrogate markers for T lymphocyte activation.[73]

A totally different mechanism seems at work in paraneoplastic cerebellar degeneration (PCD) in Hodgkin's lymphoma because the target antigens of the associated anti-Tr and anti-mGluR1 autoantibodies are not expressed in the Hodgkin tumor tissue.[74] Dysregulation of the immune system in Hodgkin's lymphoma and an etiologic role for (viral?) infections have been postulated in this disorder.

Incidence

The incidence of PNS varies with the neurological syndrome and with the tumor. Approximately 10% of patients with plasma cell disorders accompanied by malignant monoclonal gammopathies are affected by a paraneoplastic peripheral neuropathy. Over half of the patients with the rare osteosclerotic form of myeloma develop a severe predominantly motor paraneoplastic peripheral neuropathy. In other hematological malignancies, the incidence of PNS is very low with the exception of Hodgkin's disease. However, the incidence of PNS is even in Hodgkin's disease well below 1%. In solid tumors, the more common neurological syndromes are myasthenia gravis which occurs in 15% of patients with a thymoma and LEMS which affects 3% of patients with SCLC. For other solid tumors, the incidence of PNS is less than 1%.

Diagnosis

Clinical syndromes are never pathognomonic for a paraneoplastic etiology and a high index of clinical suspicion is important. Symptoms can be atypical, psychiatric or even fluctuating and PNS should often be in the differential diagnosis of otherwise unexplained neurological syndromes. Some neurological syndromes such as limbic encephalitis and subacute cerebellar degeneration associate relatively often with cancer. These are called

'classical' paraneoplastic syndromes and are underlined in Table 1.[11] Other syndromes such as sensorimotor polyneuropathy are much more prevalent and the association with cancer may be by chance. Detection of a 'well characterized' paraneoplastic antibody is extremely helpful because it proves the paraneoplastic etiology of the neurological syndrome. The paraneoplastic antibodies are generally divided into 3 categories (Table 2).[11] The 'well-characterized' antibodies are reactive with molecularly defined onconeural antigens. These antibodies are strongly associated with cancer and have been detected unambiguously by several laboratories in a reasonable number of patients with well defined neurological syndromes.[11] The partially characterized antibodies are those with an unidentified target antigen and those that have either been described by a single group

Table 1. Paraneoplastic Neurological Syndromes

Central Nervous System
Encephalomyelitis
Limbic encephalitis
Brainstem encephalitis
Subacute cerebellar degeneration
Opsoclonus - myoclonus
Stiff-person syndrome
Paraneoplastic visual syndromes
Cancer-associated retinopathy
Melanoma-associated retinopathy
Paraneoplastic optic neuropathy
Motor neuron syndromes
Subacute motor neuronopathy
Other motor neuron syndromes
Peripheral Nervous System
Subacute sensory neuronopathy
Acute sensorimotor neuropathy
Chronic sensorimotor neuropathy
Association with M-proteins
Subacute autonomic neuropathy
Paraneoplastic peripheral nerve vasculitis
Neuromuscular Junction and Muscle
Lambert-Eaton myasthenic syndrome
Myasthenia gravis
Neuromyotonia
Dermatomyositis
Acute necrotizing myopathy
Cachectic myopathy

Classical paraneoplastic syndromes are in italics.

of investigators or have been reported in only a few patients. The third group consists of antibodies that are associated with specific disorders but do not differentiate between paraneoplastic and non-paraneoplastic cases.

Because different antibodies can be associated with the same clinical findings[68] and the same antibody can be associated with different clinical syndromes[6,8], paraneoplastic antibodies should be searched for by screening rather than by focusing on a specific antibody. Recently, Pittock et al.[75] demonstrated in a large prospective series that approximately 30% of patients have more than one paraneoplastic antibody. The combination of paraneoplastic antibodies provides important additional information to narrow the search for an underlying malignancy.[75]

In the absence of paraneoplastic antibodies, additional diagnostic tests may be helpful in some paraneoplastic syndromes, although these are never specific for a paraneoplastic etiology. MRI can help diagnose limbic encephalitis and may demonstrate cerebellar atrophy several months after onset of paraneoplastic cerebellar degeneration. Examination of the CSF is generally not required for detection of paraneoplastic antibodies because these can almost always be detected in serum as well. CSF examination may show, however, signs of inflammation such as increased white cell counts, oligoclonal bands and intrathecal synthesis of IgG, indicating an immune-mediated or inflammatory etiology. In patients known to have cancer, MRI and CSF cytology are important in ruling out leptomeningeal metastases. Some paraneoplastic syndromes of the peripheral nervous system, such as Lambert-Eaton myasthenic syndrome (LEMS), myasthenia gravis and neuromyotonia are accompanied by characteristic electrophysiological changes. These findings, however, are also present in the absence of an underlying tumor. Determining the precise type of neurological syndrome may assist in the search for an underlying tumor such as SCLC in LEMS and a thymoma in myasthenia gravis.

Once a paraneoplastic diagnosis has been established or is suspected, rapid identification of the tumor becomes essential but may be difficult because most paraneoplastic syndromes develop in the early stages of cancer. The work-up generally starts with a detailed history including smoking habits, weight loss, night sweats and fever. A thorough physical examination should include palpation for pathological lymph nodes, rectal and pelvic examination and palpation of breasts and testis. Often, the tumor is detected by high resolution computed tomography (CT) of chest, abdomen and pelvis. If the CT-scan remains negative, whole body fluorodeoxyglucose positron emission tomography (FDG-PET) or PET/CT is recommended to detect an occult tumor or its metastases.[14,15,76] In addition, the type of antibody and paraneoplastic syndrome may suggest a specific underlying tumor and indicate further diagnostic tests such as mammography (may be replaced by MRI) or ultrasound of the testes or pelvis (Table 2). When all tests remain negative, repeat evaluation at 3-6 months intervals for 2-3 years is recommended.

Table 2. Antibodies, Paraneoplastic Neurological Syndromes and Associated Tumors

Antibody	Clinical syndromes	Associated tumors
Well characterized paraneoplastic antibodies		
Anti-Hu (ANNA-1)	Encephalomyelitis, limbic encephalitis, sensory neuronopathy, subacute cerebellar degeneration, autonomic neuropathy	SCLC, neuroblastoma, prostate
Anti-Yo (PCA-1)	Subacute cerebellar degeneration	Ovary, breast
Anti-CV2 (CRMP5)	Encephalomyelitis, chorea, limbic encephalitis, sensory neuronopathy, sensorimotor neuropathy, optic neuritis, subacute cerebellar degeneration, autonomic neuropathy	SCLC Thymoma
Anti-Ri (ANNA-2)	Opsoclonus-myoclonus, brainstem encephalitis	Breast, SCLC
Anti-Ma2 (Ta)[1]	Limbic/diencephalic/brainstem encephalitis, subacute cerebellar degeneration	Testicle, lung
Anti-amphiphysin	Stiff-person syndrome, encephalomyelitis, subacute sensory neuronopathy, sensorimotor neuropathy	Breast, SCLC
Anti-recoverin	Cancer associated retinopathy	SCLC
Partially characterized antibodies		
Anti-Tr (PCA-Tr)	Subacute cerebellar degeneration	Hodgkin's disease
ANNA-3	Encephalomyelitis, subacute sensory neuronopathy	SCLC
PCA-2	Encephalomyelitis, subacute cerebellar degeneration	SCLC
Anti-Zic4	Subacute cerebellar degeneration	SCLC
Anti-mGluR1	Subacute cerebellar degeneration	Hodgkin's disease
Antibodies that occur with and without cancer		
Anti-VGCC	Lambert-Eaton myasthenic syndrome, subacute cerebellar degeneration	SCLC
Anti-AchR	Myasthenia gravis	Thymoma
Anti-nAChR	Subacute autonomic neuropathy	SCLC
Anti-VGKC	Limbic encephalitis, neuromyotonia	Thymoma, SCLC

[1]Patients with brainstem encephalitis or subacute cerebellar degeneration usually associate with tumors other than testicular cancer and their sera also react with Ma1 protein.

ANNA = antineuronal nuclear antibody; SCLC =Small Cell Lung Carcinoma; VGCC = voltage gated calcium channels; PCA = Purkinje cytoplasmic antibody; mGluR1 = metabotropic glutamate receptor type 1; nAChR = nicotinic acetylcholine receptor; VGKC = voltage gated potassium channel

Because of the difficulties in diagnosing a neurological syndrome as paraneoplastic, an international panel of neurologists has established diagnostic criteria that divide patients with a suspected paraneoplastic syndrome into 'definite' and 'probable' categories. These criteria are based on the presence or absence of cancer, the presence of 'well characterized' antibodies and on the type of clinical syndrome. Patients with a definite paraneoplastic syndrome include[11]:

1. A classical syndrome (i.e. encephalomyelitis, limbic encephalitis, subacute cerebellar degeneration, opsoclonus-myoclonus, subacute sensory neuronopathy, chronic gastrointestinal pseudo-obstruction, LEMS or dermatomyositis) and cancer that develops within 5 years of the diagnosis of the neurological disorder, regardless of the presence of paraneoplastic antibodies.

2. A non-classical syndrome that objectively improves or resolves after cancer treatment, provided that the syndrome is not susceptible to spontaneous remission.
3. A non-classical syndrome with paraneoplastic antibodies (well characterized or not) and cancer that develops within 5 years of the diagnosis of the neurological disorder.
4. A neurological syndrome (classical or not) with well characterized paraneoplastic antibodies (i.e. Hu, Yo, Ri, amphiphysin, CV2 or Ma2).

Patients with a possible paraneoplastic syndrome include [11]:

1. A classical syndrome without paraneoplastic antibodies and no cancer but at high risk to have an underlying tumor (e.g. smoking habit).
2. A neurological syndrome (classical or not) without cancer but with partially characterized paraneoplastic antibodies.
3. A non-classical neurological syndrome, no paraneoplastic antibodies and cancer that presents within two years of the neurological syndrome.

Treatment and Prognosis

Despite the immunological etiology of most of the paraneoplastic neurological syndromes, the results of immunotherapy have been disappointing.[19] Exceptions are the neurological syndromes associated with paraneoplastic antibodies that are directed against antigens that are located at the surface of the cell (i.e. antigens that are accessible to circulating antibodies). These include not only disorders of the peripheral nervous system (LEMS, myasthenia gravis and neuromyotonia) but also anti-mGluR1 associated paraneoplastic cerebellar degeneration and anti-amphiphysin associated stiff-person syndrome.[77,78] Immunotherapy modalities that are recommended for these disorders include plasma exchange, immunoadsorption (extraction of patient IgG over a protein A column), steroids and i.v. Ig.

For most paraneoplastic syndromes, where the antigen is cytoplasmic or nuclear, the nervous dysfunction is probably not caused by functional interference of antibodies with the target antigen. In disorders with intracellular target antigens and a strong cellular immune reaction, plasma exchange and immunoadsorption are not expected to give much benefit. In these cases a trial of a treatment that modulates the activation and function of effector T cells makes more sense, but to date there is only limited evidence that steroids, cyclophosphamide, i.v. Ig, or other immunosuppressive therapies are effective.[79]

Hence the first goal of treatment for paraneoplastic neurological disorders is control of the tumor. In addition, anti-tumor therapy has been demonstrated to stop the paraneoplastic neurological deterioration and leave the patients, on average, in a better condition.[18] In severely debilitated patients, e.g. the elderly and bedridden, treatment of an underlying tumor is often withheld because of the very small chance of clinically relevant neurological improvement.

Table 3 provides a summary of treatment of paraneoplastic syndromes and the effect on neurological outcome.

CLINICAL SYNDROMES

The classical paraneoplastic neurological syndromes are described below. Descriptions of the non-classical syndromes that usually are not paraneoplastic but may occur in association with cancer can be found elsewhere.[80]

Encephalomyelitis

Paraneoplastic encephalomyelitis is characterized by involvement of several areas of the nervous system, including the temporal lobes and limbic system (limbic encephalitis), brainstem (brainstem encephalitis), cerebellum (subacute cerebellar degeneration), spinal cord (myelitis) dorsal root ganglia (subacute sensory neuronopathy) and autonomous nervous system (autonomic neuropathy).[5,41] Patients with predominant involvement of one area but clinical evidence of only mild involvement of other areas are usually classified according to the predominant clinical syndrome. Symptoms of limbic encephalitis, subacute cerebellar degeneration, subacute sensory neuronopathy and autonomic neuropathy are described below. Symptoms of brainstem encephalitis can include diplopia, dysarthria, dysphagia, gaze abnormalities (nuclear, internuclear or supranuclear), facial numbness and subacute hearing loss.

Underlying tumor

Although virtually all cancer types have been associated with paraneoplastic encephalomyelitis, the majority of patients have an underlying SLCL.[5-8,41] Most patients are not known with cancer when the neurological symptoms present and the SCLC may be difficult to demonstrate due to its small size. When Hu antibodies are detected or when the patient is at risk for lung cancer (smoking, age > 50 years) a careful and repeated search for an underlying SCLC is warranted. When CT-scan is negative, a total body FGD-PET scan or FDG-PET/CT may detect the neoplasm.[5-8,41] When a tumor other than SCLC is detected in a patient with Hu antibodies, it may unexpectedly express the Hu-antigen[6] or may be an unrelated secondary neoplasm.[7] When the tumor tissue is available for analysis and expresses the Hu antigen, a further work-up for a second tumor (SCLC) can probably be safely deferred.[6]

Diagnostic evaluation

MRI or CT of the brain is normal or shows aspecific changes in most paraneoplastic encephalomyelitis patients with two exceptions.[5] In 65-80% of patients with predominant limbic encephalitis, MRI and CT-scan show temporal lobe abnormalities.[12,13] Patients with a predominant cerebellar syndrome will develop cerebellar atrophy in the chronic stage. CSF is abnormal in most patients showing elevated protein, mild mononuclear pleocytosis, elevated IgG index, or oligoclonal bands.[5]

Table 3. Paraneoplastic Neurological Syndromes and their Response to Treatment

Clinical syndrome	Autoantibody	Response to immunotherapy	Response to tumor therapy	Comments
Encephalomyelitis	Hu (ANNA-1)	No established effect	Stabilizes the patient in better condition	Spontaneous improvement very rarely described
Limbic encephalitis	Hu (ANNA-1), Ma2	Some patients respond	May improve	Partial improvement may occur spontaneously
Subacute cerebellar degeneration	Yo (PCA-1)	No established effect	No effect on neurological outcome	
	Tr (PCA-Tr), mGluR1	May improve	May improve	Subacute cerebellar degeneration associated with Hodgkin's disease may also improve spontaneously
Opsoclonus - myoclonus (adults)	Ri (ANNA-2)	May improve	Partial neurological recovery	Thiamin, baclofen and clonazepam may be effective
Opsoclonus - myoclonus (pediatric)	No antibody	Two-thirds improve	Partial neurological recovery	
Stiff-person syndrome	Amphiphysin	May improve	May improve	Responds to baclofen, diazepam, valproate, vigabatrine and carbamazepine; Painful spasms may require opioids
Cancer-associated retinopathy	Recoverin	Vision may slightly improve	No established effect	
Melanoma-associated retinopathy	Anti-bipolar cells	Anecdotal vision improvement	Anecdotal vision improvement	
Paraneoplastic optic neuropathy	CV2/CRMP5	Anecdotal vision improvement	Anecdotal vision improvement	
Subacute sensory neuronopathy	Hu (ANNA-1)	No established effect; rare partial responses	Stabilizes the patient in better condition	Treatment of neuropathic pain with tricyclic AD and AED's
Chronic sensorimotor neuropathy with M-protein	MAG (IgM)	May improve	May improve	
Chronic sensorimotor neuropathy with osteosclerotic myeloma	No antibody	No established effect	Often responds	Radiotherapy, chemotherapy and surgery effective
Subacute autonomic neuropathy	Hu	No established effect	No established effect	Symptomatic treatment of orthostatic hypotension; neostigmine in pseudo-obstruction
Paraneoplastic peripheral nerve vasculitis	Hu	May improve	May improve	
Lambert-Eaton myasthenic syndrome	P/Q-type VGCC	Often responds	Often responds	2,3 diaminopyridine; cholinesterase inhibitors may be tried (efficacy unclear)
Myasthenia gravis	AChR	Often responds	Often responds	Cholinesterase inhibitors
Neuromyotonia	VGKC	May respond	Not known	Anti-epileptic drugs (carbamazepine, phenytoin)
Dermatomyositis	Mi-2	Usually responds	May respond	

ANNA = antineuronal nuclear antibody; MAG = myelin-associated glycoprotein; PCA = Purkinje cytoplasmic antibody; mGluR1 = metabotropic glutamate receptor type 1; VGCC = voltage gated calcium channels; nAChR = nicotinic acetylcholine receptor; VGKC = voltage gated potassium channel.

Antineuronal antibodies

Patients with paraneoplastic encephalomyelitis and SCLC often have Hu antibodies (also called anti-neuronal nuclear autoantibodies or ANNA-1) in their serum and CSF.[5-8] Other antibodies associated with paraneoplastic encephalomyelitis include anti-CRMP5/CV2,[73] anti-amphiphysin[81] and the less well characterized ANNA-3[82] and PCA-2 antibody.[83]

Treatment and prognosis

Tumor treatment offers the best chance of stabilizing the patient's neurological condition while immunotherapy does not appear to modify the outcome of paraneoplastic encephalomyelitis.[6,8,19] Therefore, all efforts should be directed at early diagnosis of paraneoplastic encephalomyelitis and rapid identification and treatment of the tumor. Because of incidental reports of neurological improvement following various forms of immunosuppressive treatment, a trial of one or two immunosuppressive modalities may be warranted in a single patient. However, spontaneous neurological improvement has rarely been described.[70] The overall functional outcome is bad and more than 50% of patients are confined to bed or chair in the chronic phase of the disease.[6,8,19] The median survival of patients is approximately one year from diagnosis.[6,8] Mortality is predicted by worse functional status at diagnosis, age > 60 years, involvement of more areas of the nervous system and absence of treatment.[6]

Because of the limited efficacy of plasma exchange, i.v. Ig, and corticosteroids[6,8,19] and the presumed role of cellular immunity, more aggressive immunosuppression with cyclophosphamide, tacrolimus or cyclosporine may be considered. To limit toxicity, these more aggressive immunosuppressive approaches should probably be reserved for patients who are not receiving chemotherapy.

Limbic Encephalitis

Paraneoplastic limbic encephalitis is a rare disorder characterized by the subacute onset (in days to a few months) of short-term memory loss, seizures, confusion and psychiatric symptoms suggesting involvement of the limbic system.[41,84] Hypothalamic dysfunction may occur with somnolence, hyperthermia and endocrine abnormalities. Selective impairment of recent memory is a hallmark of the disease but may not be evident in patients presenting with severe confusion or multiple seizures.[12] More than half of the patients presenting with limbic encephalitis may have an underlying neoplasm.[12] Clinically three groups of patients with PLE can be identified.[12] The first group consists of patients with Hu antibodies and lung cancer (usually SCLC). The limbic encephalitis is part of paraneoplastic encephalomyelitis and the patients have involvement of other areas outside the limbic system and brainstem. These patients are older (median age 62 years), usually smoke and are more often female.[12,85] The second group consists of young males with testicular cancer and anti-Ma2 antibodies.[86] The median age is 34 years. Symptoms are usually confined

to the limbic system, hypothalamus and brainstem. The third group has no antineuronal antibodies (approximately 40% of patients with paraneoplastic limbic encephalitis).[12,13] In these patients, the symptoms are more often confined to the limbic system, the median age is around 57 years and the associated tumor is often located in the lung.[12,85]

Underlying tumor

The associated tumor is a lung tumor in 50 to 60% of patients, usually SCLC (40 to 55%) and testicular germ cell tumors in 20%.[12,85] Other tumors include breast cancer, thymoma, Hodgkin's disease and immature teratomas.[12,13]

Diagnostic Evaluation

The diagnosis is often difficult because there are no specific clinical markers and symptoms usually precede the diagnosis of cancer.[12] MRI and CT-scan are abnormal in 65 – 80%[12,13] of patients. Abnormalities consist of increased signal on T2-weighted and FLAIR images of one or both medial temporal lobes, hypothalamus and brainstem. Early in the course of the disease, the MRI may be normal and repeat imaging may be indicated. Co-registration of FDG-PET may further improve the sensitivity of imaging.[87] CSF examination is abnormal in 80% showing transient mild lymphocytic pleocytosis with increased protein, IgG or oligoclonal bands.[12,13] Detection of paraneoplastic antibodies will help establish the diagnosis and direct a tumor search that should include the lung, breasts and testicles in the absence of paraneoplastic antibodies.

Antineuronal antibodies

Antineuronal antibodies are found in about 60% of patients with paraneoplastic limbic encephalitis. The most frequent related paraneoplastic antibodies are: anti-Hu, anti-Ma2 (with or without anti-Ma1), anti-CV2/CRMP5, and anti-amphiphysin.[12,73,88] The majority of patients with Hu antibodies have symptoms that suggest dysfunction of areas of the nervous system outside the limbic system. The related tumor in these patients is usually SCLC. Patients with only anti-Ma2 antibodies (also called anti-Ta) are young males with testicular cancer. Patients with anti-Ma2 and anti-Ma1 antibodies are significantly older and are more often female.[88] Anti-Ma1 patients are more likely to develop cerebellar dysfunction and usually harbor other tumors than testicular cancer. CV2/CRMP5 antibodies are detected in patients with SCLC or thymoma.[73] Antibodies reactive with voltage gated potassium channels (anti-VGKC) can be associated with paraneoplastic limbic encephalitis and thymoma or with non-paraneoplastic limbic encephalitis.[89,90]

Treatment and prognosis

Spontaneous complete recovery has been described, although very rarely.[85,91] Immunotherapy is largely ineffective[12], but multiple cases benefiting from anti-tumor treatment

have been reported.[12,85,92] Therefore, all efforts should be directed at identifying and treating the underlying tumor. If no tumor is found, the search should be repeated every 3-6 months for a total of 2-3 years. Irrespective of treatment, partial neurological recovery was seen in 38% of anti-Hu, 30% of anti-Ta (Ma2) and 64% of patients without antibodies.[12]

Subacute cerebellar degeneration

Paraneoplastic cerebellar degeneration is one of the most common and characteristic paraneoplastic syndromes.[41,68] In a study of 137 consecutive patients with antibody-associated paraneoplastic syndromes, 50 (37%) presented with subacute cerebellar degeneration.[68] Paraneoplastic cerebellar degeneration usually starts acutely with nausea, vomiting, dizziness and slight incoordination of walking, evolving rapidly over weeks to a few months with progressive ataxia of gait, limbs and trunk, dysarthria and often nystagmus associated with oscillopsia. The disease reaches its peak within months and then stabilizes. By this time, most patients are severely debilitated. They are generally unable to walk without support, may be unable to sit unsupported while handwriting is often impossible and feeding oneself has become difficult. The neurological signs are always bilateral but may be asymmetrical. Diplopia is common at presentation although the investigator usually cannot detect abnormalities of ocular movement. The symptoms and signs are limited to the cerebellum and cerebellar pathways, but other mild neurological abnormalities may be found on careful examination. These include hearing loss, dysphagia, pyramidal and extrapyramidal tract signs, mental status change and peripheral neuropathy.[67,93,94]

Underlying tumor

Paraneoplastic cerebellar degeneration can be associated with any cancer but the most common tumors are lung cancer (usually SCLC), ovarian cancer and lymphomas (particularly Hodgkin's lymphoma). In 60-70% of the patients, the neurological symptoms precede the diagnosis of the cancer by a few months to 2-3 years and lead to its detection.[67,68,74]

Diagnostic Evaluation

Subacute cerebellar degeneration is a rare disorder in cancer patients. On the other hand, 50% of patients presenting with acute or subacute non-familial ataxia are estimated to have an underlying malignancy.[41] MRI and CT-scan are initially normal but often reveal cerebellar atrophy later in the course of the disease. CSF examination shows mild lymphocytic pleocytosis with elevation of protein and IgG levels in the first weeks to months. Oligoclonal bands may be present. The diagnosis of paraneoplastic cerebellar degeneration is established by demonstration of specific antineuronal antibodies. The type of antibody directs the search for an underlying neoplasm (Table 2).

Antineuronal antibodies

Paraneoplastic cerebellar degeneration can be associated with various antineuronal autoantibodies. The clinical and tumor specificities of each of the antibodies are summarized in Table 2.

Anti-Yo (also called anti-Purkinje cell antibody type 1 or PCA-1), anti-Tr (PCA-Tr) and anti-mGluR1 are associated with relatively 'pure' cerebellar syndromes. Yo antibodies are associated with breast cancer and tumors of the ovaries, endometrium and fallopian tubes.[67,68,93] These antibodies are directed against the cdr proteins that are expressed by Purkinje cells and the associated tumors.[93,95] Cdr-2 specific cytotoxic T cells have been identified in the serum from patients with paraneoplastic cerebellar degeneration suggesting a pathogenic role for the cellular immune response in this paraneoplastic syndrome.[96] Anti-Tr (PCA-Tr) antibodies are directed against an unidentified cytoplasmic Purkinje cell antigen and appear specific for Hodgkin's disease.[74] Anti-mGluR1 antibodies have been found in two patients with paraneoplastic cerebellar degeneration and Hodgkin's disease. Passive transfer of patient anti-mGluR1 IgG into CSF of mice induced severe, transient ataxia.[77]

Approximately 50% of patients with cerebellar degeneration and an underlying SCLC have high titer Hu antibodies.[97] The remaining patients are likely to have anti P/Q-type VGCC antibodies. These antibodies were present in all patients who also had LEMS and in some patients with cerebellar degeneration without LEMS. In patients with anti-amphiphysin or anti-CV2/CRMP5 antibodies, the cerebellar degeneration is often part of the paraneoplastic encephalomyelitis syndrome and more widespread neurological symptoms and signs are usually found.

The more recently discovered Purkinje cell antibody (PCA-2) and the ANNA-3 antibody are associated with lung cancer and a variety of neurological syndromes including cerebellar degeneration.[83] The anti-Zic4 antibodies are strongly associated with SCLC and most patients have paraneoplastic encephalomyelitis often presenting with cerebellar dysfunction.[98] These patients often have concurrent anti-Hu or anti-CV2/CRMP5 antibodies. Patients with isolated anti-Zic4 antibodies are more likely to develop cerebellar symptoms.

Treatment and prognosis

The outcome of paraneoplastic cerebellar degeneration is generally poor and the best chance to at least stabilize the syndrome is to treat the underlying tumor.[68] Incidental improvement has been reported either spontaneously or in association with plasma-exchange, steroids, i.v. Ig, or rituximab.[99] In patients with anti-Yo-associated cerebellar degeneration, the prognosis is better for patients with breast cancer than for those with gynecologic cancer.[67] The prognosis is better in patients with paraneoplastic cerebellar degeneration associated with Hodgkin's disease and anti-Tr (PCA-Tr) or anti-mGluR1 antibodies. With successful treatment of the tumor and/or immunotherapy, symptoms may disappear and the antibodies vanish.[74,77]

Opsoclonus – Myoclonus

Opsoclonus is a disorder of ocular motility that consists of involuntary, arrhythmic, high-amplitude conjugate saccades in all directions. Opsoclonus may occur intermittently or, if more severe, constantly and it does not remit in the darkness or when the eyes are closed. Opsoclonus is often associated with diffuse or focal myoclonus, the 'dancing eyes and dancing feet syndrome', and other cerebellar and brainstem signs.[80,100,101] An excessive startle response reminiscent of hyperekplexia may also occur in opsoclonus - myoclonus.[102] In contrast to most paraneoplastic syndromes, the course of opsoclonus - myoclonus may be remitting and relapsing.[100]

Underlying tumor

Approximately 20% of adult patients with opsoclonus - myoclonus have a previously undiscovered malignancy.[101] The most commonly associated neoplasms are SCLC and breast and gynecologic cancers.[102,103] Many other tumors including thyroid and bladder cancer have also been reported.[104]

Almost 50% of children with opsoclonus - myoclonus have an underlying neuroblastoma. Conversely, approximately 2-3% of children with neuroblastoma have paraneoplastic opsoclonus – myoclonus.[105,106] Tumors in children with paraneoplastic opsoclonus - myoclonus apparently have a better prognosis than tumors in patients without this paraneoplastic syndrome.

Diagnostic Evaluation

MRI is usually normal but may show hyperintensities in the brainstem on T2 weighted images.[107] Examination of the CSF may show mild pleocytosis and protein elevation. In some patients, paraneoplastic opsoclonus - myoclonus resembles paraneoplastic cerebellar degeneration. The prominent opsoclonus and truncal rather than appendicular ataxia distinguish this syndrome from anti-Yo and anti-Hu associated paraneoplastic cerebellar degeneration.[80] Adult patients with paraneoplastic opsoclonus - myoclonus are older (median age 66 years) than patients with the idiopathic syndrome (median age 40 years). In adult patients the tumor search should be directed at the most common underlying tumors, i.e. high resolution CT of the chest and abdomen and gynecological examination and mammography (or MRI of the breasts).[103] When this is negative, FDG-PET should be considered.[76,108]

In children, non-paraneoplastic opsoclonus - myoclonus occurs as a self-limited illness and is probably the result of a viral infection of the brainstem. The search for an occult neuroblastoma should include imaging of chest and abdomen (CT scan or MRI), urine catecholamine measurements and metaiodobenzylguanidine scan.[109] When negative, the evaluation should be repeated after several months.[110]

Antineuronal antibodies

Specific antibodies are found in only a minority of patients with paraneoplastic opsoclonus – myoclonus.[103] In women, anti-Ri antibodies (or antineuronal nuclear autoantibody type 2, ANNA-2) are mostly associated with breast and gynecologic tumors. Anti-Ri has occasionally been found in bladder cancer and SCLC and may then occur in male patients.[103] Anti-Ri antibodies are directed against the Nova proteins.[111,112] Paraneoplastic opsoclonus - myoclonus can also be associated with Hu antibodies, usually as part of a more widespread paraneoplastic encephalomyelitis. Bataller et al.[113] screened a brainstem cDNA library with sera from 21 patients with (paraneoplastic) opsoclonus - myoclonus. Twenty five proteins were identified, recognized by one or two sera each, demonstrating that immunity to neuronal autoantigens in opsoclonus - myoclonus is both frequent and heterogeneous.

In children presenting with opsoclonus - myoclonus, the detection of Hu antibodies is diagnostic of an underlying neuroblastoma.[44] The frequency of Hu antibodies in neuroblastoma with paraneoplastic opsoclonus - myoclonus is approximately 10%.[44,114,115] This finding differs little from the 4-15% of anti-Hu positive sera in children with neuroblastoma who do not have opsoclonus – myoclonus.[44,114]

Treatment and prognosis

In contrast to most of the other paraneoplastic syndromes, paraneoplastic opsoclonus - myoclonus may remit either spontaneously, following treatment of the tumor, or in association with clonazepam or thiamine treatment. Most patients with idiopathic opsoclonus - myoclonus make a good recovery that seems to be accelerated by steroids or i.v. Ig. Paraneoplastic opsoclonus - myoclonus has usually a more severe clinical course and treatment with steroids or i.v. Ig appears ineffective. In a series of 14 patients with paraneoplastic opsoclonus - myoclonus, 8 patients whose tumors were treated showed complete or partial neurological recovery. In contrast, 5 of the 6 patients whose tumors were not treated died of the neurological syndrome despite steroids, i.v. Ig or plasma exchange.[103] However, improvement following the administration of steroids, cyclophosphamide, azathioprine, i.v. Ig, plasma exchange or plasma filtration with a protein A column has been described in single cases.[102,116-118]

In children, paraneoplastic opsoclonus - myoclonus may improve following treatment with ACTH, prednisone, azathioprine or i.v. Ig, but residual CNS signs are frequent.[106,110,119] Treatment of the tumor with chemotherapy is the most important predictor of good neurological recovery.[120]

Subacute Sensory Neuronopathy

Subacute sensory neuronopathy is an uncommon disorder that is probably paraneoplastic in about 20% of patients.[121,122] The symptoms begin with pain and paraesthesiae. Clumsi-

ness and unsteady gait then develop and usually become predominant. The distribution of symptoms is often asymmetrical or multifocal. The upper limbs are often affected first and are almost invariably involved with evolution. Sensory loss may also affect the face, chest or abdomen. On examination all sensory modalities are affected but the most striking abnormality is loss of deep sensation causing sensory ataxia with pseudoathetosis of the hands. Tendon reflexes are depressed or absent. In most patients, the disease progresses rapidly over weeks to months, leaving the patient severely disabled. In a few patients the neuronopathy remains stable for months with mild neurological deficits.[123] Subacute sensory neuronopathy occurs in approximately 75% of patients with paraneoplastic encephalomyelitis, is predominant in 50% and clinically pure in 25%.[6,8] Autonomic neuropathy including gastrointestinal pseudo-obstruction is common.

Underlying tumor

Subacute sensory neuronopathy is associated with lung cancer, usually SCLC, in 70-80% of patients.[6-8] Other associated tumors include breast cancer, ovarian cancer, sarcoma and Hodgkin's lymphoma.[121,122] Subacute sensory neuronopathy usually predates the diagnosis of cancer with a median delay of 3.5-4.5 months.[6,8]

Diagnostic evaluation

Electrophysiologically, the hallmark of subacute sensory neuronopathy is the absence or marked reduction of sensory nerve action potentials. Motor conduction velocities may be mildly reduced. Early in the course of the disease, CSF examination shows mild pleocytosis, with an elevated IgG and oligoclonal bands.[8,121,122] Sural nerve biopsy is rarely required for the diagnosis but may differentiate from vasculitic neuropathy.

Antineuronal antibodies

Anti-Hu is the most frequent paraneoplastic antibody in subacute sensory neuronopathy.[5-8] In this setting, Hu antibody detection has a specificity of 99% and sensitivity of 82%.[124] The absence of Hu antibodies does not rule out an underlying cancer. Anti-CRMP5/CV2 antibodies also occur with paraneoplastic peripheral neuropathies.[125] These patients usually have a sensory or sensorimotor neuropathy with less frequent involvement of the arms, but often associated with cerebellar ataxia.[73,125,126] Anti-CRMP5/CV2 antibodies are usually associated with SCLC, neuroendocrine tumors and thymoma. Amphiphysin antibodies are associated with multifocal paraneoplastic encephalomyelitis and symptoms often include sensory or sensorimotor neuropathy.[81,127,128] Associated tumors (mostly limited) are mainly SCLC, breast cancer and melanoma.

Treatment and prognosis

Immunotherapy consisting of plasma exchange, steroids and i.v. Ig is ineffective in most cases.[18,129] There maybe some exceptions to this rule.[19,20] In one study, two out of ten patients stabilized in a relatively good clinical condition following intensive treatment with a combination of steroids, cyclophosphamide and i.v. Ig.[19] Early detection and treatment of the underlying neoplasm, usually SCLC, appears to offer the best chance of stabilizing the neurological symptoms.[8,19] In patients with an identifiable tumor, anti-tumor treatment is recommended. In the absence of a tumor, anti-tumor treatment may be considered in patients with Hu antibodies, age > 50 years and a history of smoking. In patients not receiving anti-tumor therapy, a short course of immunotherapy can be considered.

Symptomatic treatment is directed at neuropathic pain and dysautonomic symptoms such as orthostatic hypotension.

Lambert-Eaton myasthenic syndrome

Lambert-Eaton myasthenic syndrome (LEMS) presents with proximal weakness of the lower extremities and fatigability. Bulbar symptoms may occur more frequently than previously reported[130] but are generally milder than in myasthenia gravis. Respiratory weakness can occur. Deep tendon reflexes, especially those in the legs, are diminished or absent but may reappear after exercise. Autonomic features ultimately develop in 95%, especially dryness of the mouth, impotence and mild/moderate ptosis.[130-132] In some patients, LEMS may develop in association with other paraneoplastic syndromes, including paraneoplastic cerebellar degeneration and encephalomyelitis.[97]

Underlying tumor

Approximately 70% of patients have cancer, almost always SCLC.[132,133] Other tumors include small cell carcinomas of the prostate and cervix, lymphomas and adenocarcinomas. The prevalence of LEMS in SCLC is estimated to be around 3%.[131,134] Clinically and serologically, the 30% without identifiable tumor are indistinguishable from the paraneoplastic LEMS patients although LEMS may have a more progressive course in patients with SCLC.[130] In patients presenting with LEMS, a smoking history and absence of the HLA-B8 genotype strongly predict an underlying SCLC.[48] Patients with SCLC and LEMS survive significantly longer than SCLC patients who do not have the paraneoplastic syndrome.[135]

Diagnostic Evaluation

The typical pattern of electromyographic abnormalities is the hallmark of LEMS. This includes a low compound muscle action potential at rest with a decreased response at low rates of repetitive stimulation (3 Hz) and an incremental response at high rates of repetitive stimulation (50 Hz) or 15-30 s of maximal voluntary contraction.[136]

Antineuronal antibodies

Most patients with LEMS have antibodies against P/Q type calcium channels that are located presynaptically in the neuromuscular junction.[136] About 20% have anti-MysB antibodies reactive with the β-subunit of neuronal calcium channels.[137]

Treatment and prognosis

Treatment of LEMS must be tailored to the individual based on severity of the symptoms, underlying disease, life expectancy and previous response to treatment. In patients with paraneoplastic LEMS, treatment of the tumor frequently leads to neurological improvement.[138] Symptomatic treatment is with drugs that facilitate the release of acetylcholine from motor nerve terminals such as 3,4-diaminopyridine (DAP).[139] In a placebo-controlled randomized trial, DAP (5-20 mg tid-qid) was effective for long-term treatment, alone or in combination with other treatments.[140] The maximum recommended daily dose of DAP is 80 mg; at higher doses seizures occur.[140] Cholinesterase inhibitors (pyridostigmine, 30-60 mg, q6h) may improve dryness of mouth but rarely relieve weakness. If these treatments are not effective enough, it must be decided if immunosuppressive therapy with steroids, azathioprine or cyclosporine is in place. Removal of the pathogenic anti-P/Q type calcium channel antibodies by plasma exchange[141] and i.v. Ig can give quick but transient relief.[132,142] LEMS responds less favorably to immunotherapy than myasthenia gravis.

Dermatomyositis

In dermatomyositis the characteristic heliotrope rash (purplish discoloration of the eyelids) often precedes the appearance of proximal muscle weakness. Other manifestations include arthralgia, myocarditis and congestive heart failure and interstitial lung disease. Clinical, electromyographical, and pathological findings of dermatomyositis are similar in patients with and without cancer.

Underlying tumor

The standardized incidence ratio for a malignant disease in dermatomyositis is 6.2 (95% confidence interval 3.9 – 10.0).[143] Dermatomyositis is associated with cancer of the ovary, lung, pancreas, stomach, colorectal and breast and with non-Hodgkin lymphoma.[144]

Diagnostic evaluation

Most patients have elevated serum creatine kinase levels and electromyographic evidence of myopathy. Muscle imaging (CT or MRI) may help in confirming the diagnosis and determining the type of an inflammatory myopathy and in selecting an appropriate biopsy site. Muscle or skin biopsy is the definitive diagnostic procedure and shows inflammatory infiltrates.[145]

Antineuronal antibodies

Antibodies to the Mi-2 protein complex are specific for dermatomyositis and are present in high titers in about 35% of cases.[146]

Treatment and prognosis

Treatment of paraneoplastic dermatomyositis is generally the same as for the patients without a tumor. Nearly all patients respond to corticosteroids.[147] Refractory patients and patients requiring a lower dose of steroids can be treated with azathioprine. Methotrexate and cyclophosphamide may also be considered.[147]

Chapter | 3

Increased proportions of circulating activated T cells in Hu antibody associated paraneoplastic neurological syndromes

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Submitted

Abstract

In paraneoplastic neurological syndromes (PNS) associated with small cell lung cancer (SCLC) and Hu antibodies, neuron-specific Hu antigens expressed by the tumor hypothetically trigger an immune response that also reacts with the same or similar antigens in the nervous system, resulting in tumor suppression and neuronal damage. To gain more insight into the hypothesized cell-mediated immune pathogenesis of these syndromes, we analyzed the circulating lymphocyte subsets in untreated patients with SCLC, PNS and Hu antibodies (n=18), SCLC without PNS (n=19) and controls (n=29) using 4- and 6-color flow cytometry. The proportions of activated $CD4^+$ and $CD8^+$ T cells were increased in PNS as compared to both control groups, suggesting a role for T cells in Hu antibody-associated PNS. Furthermore, the numbers of cytotoxic $CD8^+$ T cells were lower which may reflect activation-induced apoptosis or recompartmentalization of cytotoxic T cells. We found no evidence for immune deregulation due to reduced numbers of regulatory ($CD25^{high}$, $FoxP3^+$) $CD4^+$ T cells.

INTRODUCTION

Paraneoplastic neurological syndromes (PNS) associated with Hu antibodies are considered to have an immune-mediated aetiology. Ectopic expression of the onconeuronal Hu antigens in the tumor has been suggested to trigger an immune response also reacting with structurally related antigens expressed in the nervous system.[1] One of the most frequently involved tumors is small cell lung cancer (SCLC) and approximately 50% of patients with PNS and SCLC have high-titer Hu antibodies.[4] The tumors are usually small and spontaneous remissions at the time of neurological presentation have been described, implicating immune mediated tumor growth control.[17,70] As a pathogenic role for Hu antibodies in PNS could not be demonstrated[55] the cellular immune response against the Hu proteins has been further investigated. Pathological examination of PNS neuronal tissue revealed localized inflammatory cell infiltrates, containing B cells, $CD4^+$ T cells and $CD8^+$ T cells, in the proximity of overt neuronal cell damage.[40,42,59,60] In the peripheral blood increased numbers of circulating activated $CD4^+$ and $CD4^+$ memory T cells have been reported[63] and some studies suggested the presence of Hu-specific T cells.[63-65]

Several T cell subsets play a role in immune responses directed against auto-antigens or tumor-antigens. Activated ($HLA\text{-}DR^{+}$ [148]) $CD4^+$ T cells are known to stimulate both cellular and humoral responses against these antigens.[149,150] On the other hand, regulatory $CD4^+$ T cells, defined by their high expression of CD25 ($CD25^{high}$) and the intracellular expression of forkhead/winged helix transcription factor (FoxP3), downregulate immune responses towards auto-antigens and tumor-antigens.[150,151] In addition, there is increasing evidence for a role of cytotoxic $CD8^+$ T cells in autoimmune diseases and anti-tumor immune responses.[152,153] Most $CD8^+$ T cells are cytotoxic, express HLA-DR upon activation and differentiate from naive to memory cells and finally to terminally differentiated effector cells. Characteristic for terminally differentiated effector $CD8^+$ T cells is their history of multiple cell divisions, reduced proliferative capacity and susceptibility to activation-induced apoptosis.[154-156] Most of these cells express CD57, which is linked with chronic antigen exposure and the presence of azurophilic cytotoxic granules[157,158] and have returned to a resting state ($HLA\text{-}DR^{-}$).[156] Expression of CD56 is functionally correlated with cytotolytic activity and oligoclonal T-cell expansions often consist of $CD56^+$ and $CD57^+$ cells.[159,160]

To gain more insight into a possibly cell-mediated immune pathogenesis of Hu antibody associated PNS, we analyzed the immunophenotype of circulating lymphocyte subsets in *untreated* SCLC patients with and without Hu antibody associated PNS, and in healthy controls. In particular, we focused our studies on T cells expressing phenotypes of activated, regulatory and cytotoxic T cells.

PATIENTS AND METHODS

Patients and controls

Clinical information was obtained from patients, whose serum samples were submitted to our national reference laboratory for paraneoplastic antibodies (Erasmus MC) between January 2004 and January 2006 and were Hu antibody positive (see below). Patients designated hereafter as "PNS patients" were enrolled in this study when they had (i) high-titer serum Hu antibodies; (ii) SCLC; (iii) a "definite" diagnosis of PNS according to international guidelines[11]; and (iv) no chemotherapy or immunosuppressive therapy prior to study entry. In collaboration with pulmonologists, SCLC patients who (i) were Hu antibody negative; (ii) had no neurological signs or symptoms and (iii) had not received chemotherapy or immunosuppressive treatment prior to study entry were included in the study as SCLC controls (designated hereafter as "SCLC patients"). Apparently healthy volunteer donors were included as healthy controls (designated hereafter as "HC"). For the present study, blood was tested from 18 PNS patients, 19 SCLC patients and 29 HC. Written informed consent was obtained from all tested individuals and the local ethical review committee approved the study. Various relevant characteristics of patients and controls are shown in Table 1. In PNS, the dependence in activities of daily living was scored using the modified Rankin Scale.[8]

Flow cytometric analysis of circulating lymphocyte subsets

A single platform, whole blood, stain, lyse, no-wash method based on counting beads was used for the enumeration of B cells, T cells, NK cells, $CD4^+$ and $CD8^+$ T cells based on 4- color flow cytometry (FacsCalibur; BD Biosciences, San Diego, CA). Details of this method are described elsewhere.[161] Briefly, 100 µl of EDTA anti-coagulated whole blood was stained with cocktails of appropriately titrated monoclonal antibodies (mAb). After incubation for 15 minutes (15') at room temperature (RT), 2 ml NH_4Cl lysing buffer was added and 100 µl of Flowcount counting beads (Beckman Coulter, Marseille, France). After another 15' of incubation on RT, samples were acquired on the flow cytometer. Additional stainings were performed on the same blood sample to quantify the expression of CD56 and CD57 by the T cells. For this purpose, we used a lyse, stain and wash technique which includes an additional washing step after red cell lysis in order to reduce background fluorescence due to unbound mAb. Fluorescent minus one (FMO) controls, i.e., staining controls that employ all reagents except for the one of interest, were used to accurately identify expressing cells in the fully stained sample.[162]

Six-color flow cytometry (FacsCanto, BD Biosciences) was performed for the analysis of regulatory $CD4^+$ T cells, activated T cells and of naive, memory and terminally differentiated effector $CD8^+$ T cells. For this purpose we used thawed, living (7-amino actinomycin D-negative ($7\text{-}AAD^-$)) peripheral blood mononuclear cells, which had been cryopreserved

Table 1. Patient characteristics at the time of study entry

	PNS	SCLC*	HC
N	18	19	29
Age (median, range)	66 (51-81)	63 (41-78)	55 (42-89)
Gender			
male	4	13	14
female	14	6	15
Hu-antibodies		*Negative*	*Negative*
median titer	12,800		
range	(400-204,800)		
CMV serostatus			
positive	12	9	17
negative	6	10	12
Paraneoplastic Neurological Syndrome		*NA*	*NA*
PSN	11		
PEM	2		
PLE	3		
Pseudo-obstruction	1		
Motor neuron disease	1		
SCLC[a]	18	19	0
Limited	18	10	*NA*
Extended	0	9	*NA*
Neurological symtoms		*NA*	*NA*
Interval onset symptoms - study entry	4 months (2-15)[b]		
Interval onset symptoms - diagnosis	3 months (1-12)[b]		
Progressive at study entry[c]	15 (83%)		
Modified Rankin Score[d]		*NA*	*NA*
MRS=2	2		
MRS=3	11		
MRS=4	5		

[a]Diagnosis of SCLC was obtained by cytologic or histologic examination in all cases.

[b]Intervals are presented as medians (range).

[c]Progression of neurological symptoms was defined by the increase of at least one point on the modified Rankin scale in the two months prior to study entry.

[d]Modified Rankin score at the time of study entry.[8]

PNS = patients with SCLC and Hu antibody associated PNS; SCLC* = SCLC patients without PNS; HC = healthy controls; NA = not applicable; PSN = paraneoplastic sensory neuronopathy; PEM = paraneoplastic encephalomyelitis; PLE = paraneoplastic limbic encephalitis; MRS = modified Rankin score.

after isolation by Ficoll density gradient centrifugation.[163] In all 6-color staining panels, FMO controls were included.

The immunological definitions of the lymphocyte subsets are shown in Table 2. The following conjugated mAb were used for cell surface labelling: CD3-FITC, CD3-APC, CD3-APC-Cy7, CD4-FITC, CD4-PE-Cy7, CD8-PE, CD8-PerCP, CD8-PE-Cy7, CD16-PE,

CD19-APC, CD25-PE, CD27-FITC, CD28-FITC, CD45-PerCP, CD45RA-APC, CD56-PE, CD57-FITC and HLA-DR-APC-Cy7. All mAB were obtained from BD Biosciences with the exception of CD28 (Beckman Coulter, Miami, FL).

The intracellular staining with allophycocyanin (APC)- labeled anti-FoxP3 was performed according to its manufacturer's instructions (e-Biosciences, San Diego, CA). Briefly, $2x10^6$ PBMC were incubated with all mAb except for anti-FoxP3 for 15' in the dark at RT, washed with Phosphate Buffered Saline (PBS) and subsequently incubated with 1 ml of eBioscience fixation/permeabilization solution at 4°C for 30' in the dark. After three rounds of washing, Fc receptor binding sites were blocked by adding 2 µl (2% final) normal rat serum for 15' at 4°C. After this blocking step, 20 µl rat anti-human FoxP3 antibody was added and incubated at 4°C for 30' in the dark. Finally, the cells were washed twice with permeabilization buffer (eBiosciences), resuspended in PBS and acquired on the flow cytometer.

Analysis of list mode data processed by 4- and 6-color flow cytometry was performed using CellQuestPro and FacsDiva software, respectively (both from BD Biosciences). The analysis of the $CD4^+$ regulatory T cell fraction is detailed in Figure 1. The absolute

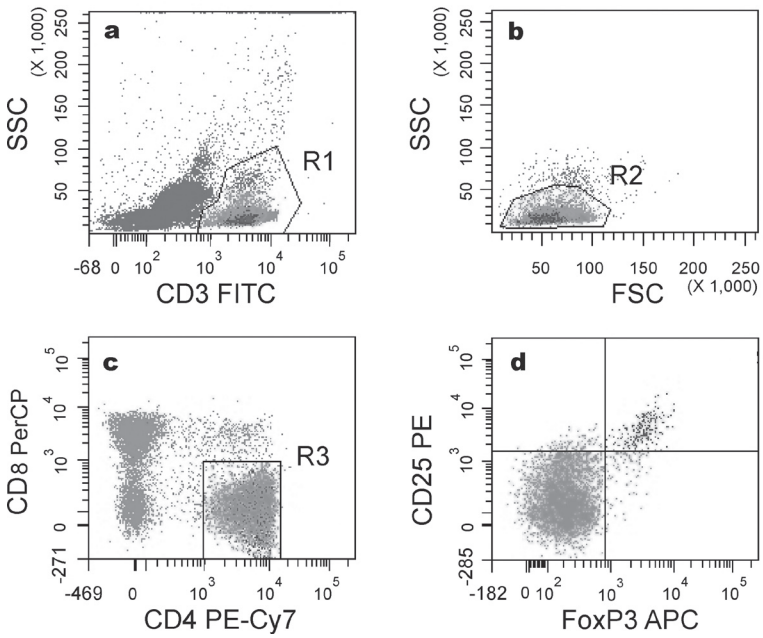

Figure 1. Flow cytometric analysis of $CD4^+$, 25^{high}, $FoxP3^+$ regulatory T cells.

The T lymphocytes ($CD3^{bright}$, low SSC signals) were selected in gate R1 (panel a). Subsequently, any remaining cellular debris and aggregates were excluded based on FSC and SSC (region R2; panel b). Next, $CD4^+$ T cells were defined by plotting events that meet criteria for both region 1 and 2 in a CD4 versus CD8 plot and selecting events positive for CD4 and negative for CD8 (region R3, panel c). Finally, within the events meeting the criteria of region R1, R2 and R3, the $CD4^+$ regulatory T cell population was defined by high expression of CD25 and intracellular expression of FoxP3 in the upper right quadrant (panel d). The units on the x- and y-axes are relative fluorescent intensity using a biexponential scale for fluorescent parameters and a linear scale for physical parameters on forward light scatter (FSC) and sideward light scatter (SSC).

numbers of T cell subsets were calculated by multiplying the percentages of T cells expressing the markers of interest, with the corresponding absolute T-cell numbers, or, in case of co-staining with CD4 or CD8, with the corresponding absolute numbers of $CD4^+$ or $CD8^+$ T cells.

Hu antibody detection

Hu IgG antibody titers were determined on rat cerebellar sections by indirect immunofluorescence (IIF)[164] and immunoreactivity was confirmed by western blotting using purified recombinant HuD in all cases.[36] Only patients with 'high-titer' serum Hu antibodies were eligible for the study (serum IIF titer ≥ 400 [164]).

Statistical analysis

Age, gender and CMV serostatus have significant impacts on the numbers and phenotypes of the lymphocyte subsets under study.[165-168] Therefore, pair wise comparisons between the various groups were done using ANCOVA with covariates age, gender and CMV serostatus (SPSS version 11.5 [SPSS, Inc., Chicago, IL]). Because the lymphocyte subset data were in general not normally distributed, the lymphocyte counts were log-transformed to approximate normal distributions and subsequently used for the comparisons between groups. Differences between groups with a *P*-value <0.05 were considered significant.

Table 2. Comparison of lymphocyte subsets in PNS patients versus SCLC patients and healthy controls

Lymphocyte subset	Immunological definition	ANCOVA		
		PNS versus SCLC	PNS versus HC	SCLC versus HC
B cells	$CD3^-,19^+,45^+$	0.71	**0.67***	0.94
NK cells	$CD3^-,16^+,45^+,56^+$	1.15	**0.59***	**0.51****
T cells	$CD3^+,45^+$	0.76	0.75	1.00
$CD4^+$ T cells	$CD3^+,4^+,45^+$	0.83	**0.70***	0.85
- regulatory	$CD3^+,4^+,25^{high},FoxP3^+$	1.03	**3.95***	**3.85***
- % activated	$CD3^+,4^+,HLADR^+$	**1.82****	**1.91****	1.05
$CD8^+$ T cells	$CD3^+,8^+,45^+$	0.74	**0.65****	0.87
- % activated	$CD3^+,8^+,HLADR^+$	**1.8***	**4.7****	1.6
- naive	$CD3^+,8^+,27^+,28^+,45RA^+$	0.91	0.74	0.82
- memory	$CD3^+,8^+,27^+,28^+,45RA^-$	0.78	0.80	1.04
- term. diff.	$CD3^+,8^+,27^-,28^-,45RA^+$	**0.56***	**0.47***	0.85
$CD57^+$ T cells	$CD3^+,45^+,57^+$	**0.50****	**0.42*****	0.84
$CD56^+$ T cells	$CD3^+,45^+,56^+$	**0.43****	**0.37****	0.85

Values are adjusted ratios of geometric means. Groups were compared by ANCOVA with adjustment for age, gender and CMV serostatus.
PNS = patients with SCLC and Hu antibody associated PNS; SCLC = SCLC patients without PNS; HC = healthy controls; term. diff. = terminally differentiated; CD3 = pan-T lymphocyte marker; CD45 = pan-leukocyte marker;
* $P<0.05$; ** $P<0.01$; *** $P<0.001$.

RESULTS

Table 2 shows the results of the comparisons between (i) PNS versus SCLC; (ii) PNS versus HC; and (iii) SCLC versus HC after adjusting for the influences of age, gender and CMV serostatus. The unadjusted data for all parameters that yielded significant differences in any of these comparisons are shown in Figures 2-4.

In the adjusted analyses (Table 2), PNS patients had a general lymphopenia, i.e., significantly lower numbers of B cells, NK cells, $CD4^+$ T cells and $CD8^+$ T cells, in comparison with HC. NK cells were also lower in SCLC than in HC (Fig. 2). In contrast, both PNS and SCLC stood out by increased numbers of regulatory $CD4^+$ T cells ($CD25^{high}$, Fox-$P3^+$) as compared to HC (Fig. 2). The proportions of activated (HLA-DR^+) cells within the $CD4^+$- and $CD8^+$ subset were higher in PNS than in SCLC or in HC (Fig. 3). In contrast, the numbers of terminally differentiated effector $CD8^+$ T cells ($CD27^-$,28^-,$45RA^+$) were lower in PNS than in SCLC or in HC (Fig. 4). These T cells generally had a resting phenotype

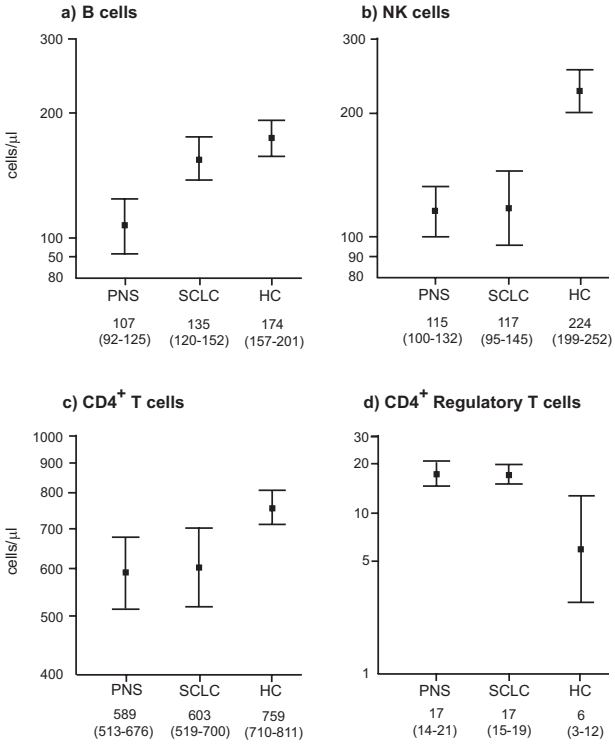

Figure 2. Numbers of B cells, NK cells, $CD4^+$ T cells and $CD4^+$ regulatory T cells in PNS and controls

Absolute numbers (i.e., cells per microliter) are given uncorrected for age, gender and CMV serostatus in PNS, SCLC and HC groups. The subsets shown are (a) B cells, (b) NK cells, (c) $CD4^+$ T cells and (d) Regulatory $CD4^+$ T cells [$CD25^{high}$, $FoxP3^+$]. Squares indicate geometric mean values, and error bars ±1 standard error for each parameter. These results are also shown at the bottom of each panel. Logarithmic scales were used for the y-axes to compress the figures. PNS = patients with SCLC and Hu antibody associated PNS; SCLC = SCLC patients without PNS; HC = healthy controls.

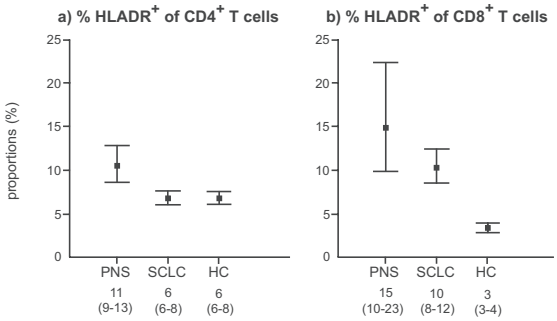

Figure 3. Proportions of activated (HLA-DR$^+$) CD4$^+$ and CD8$^+$ T cells in PNS and controls

For PNS patients, SCLC patients and healthy controls the proportions of CD4$^+$ (Fig. 3A) and CD8$^+$ T cells (Fig. 3B) expressing the activation marker HLA-DR are shown uncorrected for age, gender and CMV serostatus. Linear scales were used for the y-axes. See further the legend to Figure 2.

(i.e., HLA-DR$^-$; not shown). Finally, the numbers of T cells with a cytotoxic phenotype (i.e., those expressing CD56 or CD57) were reduced in PNS as compared to SCLC and HC (Fig. 4).

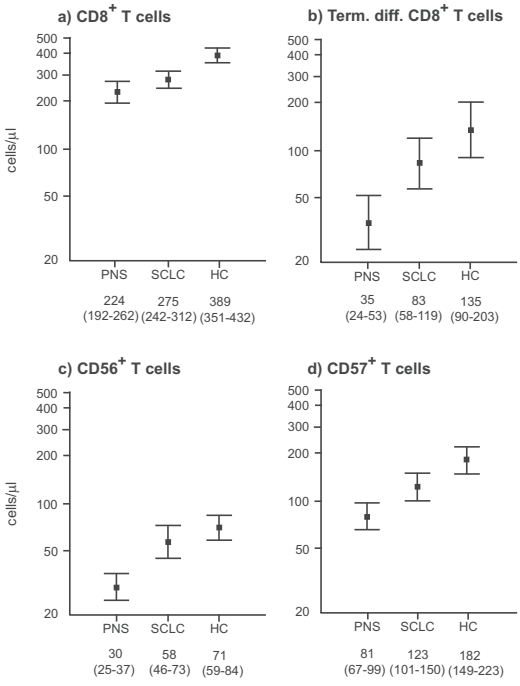

Figure 4. Numbers of CD8$^+$ T cells and T cells with a cytotoxic phenotype in PNS and controls

Absolute numbers (i.e., cells per microliter) are given uncorrected for age, gender and CMV serostatus in PNS, SCLC and HC groups. The subsets shown are (a) CD8$^+$ T cells; (b) terminally differentiated effector CD8$^+$ T cells (CD27$^-$,28$^-$,45RA$^+$) and T cells with a cytotoxic phenotype, i.e., expressing CD56 (c) or expressing CD57 (d). See further the legend to Figure 2.

When PNS patients with central- versus peripheral neurological disease were compared, no significant differences in circulating lymphocyte subset numbers were observed (data not shown). Likewise, no differences were detected between SCLC patients with limited versus extended stage of their tumors.

Discussion

Although several lines of evidence suggest a cell-mediated immune etiology in Hu antibody associated PNS[40,42,59,60,63-65], the pathogenesis of this disorder remains by and large unclear. Here, we analyzed the circulating lymphocyte subsets in SCLC patients with Hu antibody associated PNS and controls to obtain insight whether any major disturbances in immune homeostasis might be related to PNS. To differentiate between PNS and SCLC-associated lymphocyte subset alterations, SCLC patients without PNS were included as controls.

Several lymphocyte subset alterations were related to PNS, but not to SCLC. First, PNS patients had a general lymphopenia, i.e., lower numbers of B cells, NK cells, $CD4^+$ T cells and $CD8^+$ T cells, in comparison with HC (Table 2). Reduced numbers of lymphocyte subsets have been reported in untreated cancer patients[169,170] and may be caused by immune suppressive factors secreted by the tumor, malnutrition and psychological stress.[170] However, although the lymphocyte numbers showed a similar trend in PNS and SCLC, the lymphopenia was more prominent in PNS (Table 2). The general lymphopenia observed in PNS, may be interpreted as a general autoimmune phenomenon as various autoimmune disorders are associated with lymphopenia.[171-174] Also, the lymphopenia in PNS may reflect recompartmentalization of circulating lymphocytes to the sites of inflammation, i.e., neuronal and tumor tissue. This mechanism has been suggested as an explanation for the peripheral lymphopenia in sarcoidosis, where an influx of peripheral $CD4^+,103^-$ T cells has been observed in fluid harvested by broncho-alveolar lavages.[175,176]

Second, in PNS we found a significantly increased proportion of activated ($HLA\text{-}DR^+$) cells within the $CD4^+$ T-cell subset compared to SCLC and HC, confirming previous observations.[63] In addition, we observed that the activated ($HLA\text{-}DR^+$) circulating $CD8^+$ T-cells were also significantly increased in PNS. Activated T cells are well-known features in T cell-mediated autoimmune disorders such as multiple sclerosis[177,178] and polymyositis.[173,179] Therefore, observed increased proportions of activated T cells in PNS are consistent with the hypothesized role of T lymphocytes in the pathogenesis of these syndromes.

Third, the numbers of circulating, cytotoxic and terminally differentiated $CD8^+$ T cells were significantly lower in PNS, as compared to SCLC and HC. Similar observations have been made by Benyahia et al.[63], who also found reduced numbers of $CD8^+$ T cells in

patients with PNS. These findings may be explained by migration of cytotoxic T cells to the sites of inflammation (see above). Considering the increased proportion of activated T cells in PNS, our observations may also result from replicative exhaustion of cytotoxic T cells due to chronic antigenic stimulation and activation-induced apoptosis.[154-156] Replicative exhaustion of T cells is seen in the peripheral blood of patients encountering chronic antigen exposure, such as chronic viral infections and after allogeneic hematopoietic stem cell transplantation.[180,181] Moreover, in systemic lupus erythematosis (SLE), decreased resistance of activated T cells to apoptosis may cause a reduction of T cell numbers in this disease.[182,183] In PNS patients, replicative exhaustion of T cells may be due to an ongoing immune response directed to both neuronal and tumor tissue.

Other lymphocyte subset alterations observed in PNS are related to the underlying SCLC. The increased numbers of regulatory, $CD4^+$ T cells in SCLC patients with and without PNS, are in agreement with observations by others demonstrating increased numbers of regulatory T cells in cancer patients[184] and in a PNS patient with probable lung cancer.[185] The increased number of regulatory T cells in PNS contrasts with the reduction of these cells that is generally seen in autoimmunity.[151]

In SCLC patients with and without PNS, the NK cell numbers were significantly lower as compared to HC. There is some evidence that NK cell functions are decreased in lung cancer patients.[167,186,187] Decreased NK cell numbers may be related to the increased numbers of $CD4^+$ regulatory T cells[188] and indicate down-regulation of immune function in patients with SCLC.

In conclusion, SCLC patients with Hu antibody associated PNS have increased proportions of activated $CD4^+$ and $CD8^+$ T cells, as compared to SCLC patients without PNS and healthy controls. These observations indicate a role for T lymphocytes in the pathogenesis of PNS.

Acknowledgements

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Chapter | 4

No evidence for circulating HuD-specific CD8+ T cells in patients with paraneoplastic neurological syndromes and Hu antibodies

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ABSTRACT

Aim: In paraneoplastic neurological syndromes (PNS) associated with small cell lung cancer (SCLC) and Hu antibodies (Hu-PNS), Hu antigens expressed by the tumor hypothetically trigger an immune response that also reacts with Hu antigens in the nervous system, resulting in tumor suppression and neuronal damage. To gain more insight into the hypothesized $CD8^+$ T cell-mediated immune pathogenesis of these syndromes, we searched for circulating HuD-specific $CD8^+$ T cells in a large cohort of Hu-PNS patients and controls.

Patients and Methods: Blood was tested from 43 Hu-PNS patients, 31 Hu antibody negative SCLC patients without PNS and 54 healthy controls. Peripheral blood mononuclear cells (PBMC) were stimulated with HuD protein-spanning peptide pools (15-mers) and individual HuD-derived peptides (9-mers) and analyzed by cytokine flow cytometry and interferon-γ ELISPOT-assays. Additionally, HuD-based Class I HLA multimers were used to visualize HuD-specific $CD8^+$ T cells.

Results: No HuD-specific $CD8^+$ T cells could be detected in the blood of Hu-PNS patients or controls.

Conclusions: Our results do not support a role for HuD-specific $CD8^+$ T cells in Hu-PNS. Further studies should focus on the detection of circulating HuD-specific $CD4^+$ T cells and examine the antigen specificity of T cells in affected tissues.

INTRODUCTION

Paraneoplastic Neurological Syndromes (PNS) are considered as naturally occurring, successful anti-tumor immune responses in humans.[189] However, this tumor immunity goes along with autoaggression against the nervous system, resulting in severe neurological dysfunction.[190] The mechanisms responsible for the anti-tumor response and neuronal damage are poorly understood. Antigens expressed by the tumor that are normally restricted to neurons (so-called onconeuronal antigens) hypothetically trigger an immune response that cross-reacts with the same antigens in the nervous system.[1] One of the most frequently involved tumors is small cell lung cancer (SCLC) and approximately 50% of patients with PNS and SCLC have high-titer Hu antibodies (Hu-PNS). Hu antibodies are directed against a family of neuron specific, mRNA binding proteins, of which HuD is the best documented member. Consistent expression of HuD in all SCLC suggests that HuD plays a central role in triggering the immune response.[36,191]

High titers of Hu antibodies in serum- and cerebrospinal fluid suggested a pathogenic role for these antibodies, that could, however, never be proven in animal models.[55,58] Furthermore, expression of Hu antigens is exclusively intracellular, and it is therefore difficult to understand how such antibodies could target tumors or neurons.[192] In addition, pathological examination of PNS neuronal tissue demonstrated localized inflammatory cell infiltrates, containing B cells, $CD4^+$ and $CD8^+$ T cells, in the proximity of overt neuronal cell damage.[40,42,59,60] The presence of an oligoclonal $CD8^+$ T cell infiltrate in nervous tissues and tumors of Hu-PNS patients further suggests an immunopathogenic role for such cells.[59,60]

Some authors report the presence of HuD peptide-specific $CD8^+$ T cells in the blood of these patients, but also in that of apparently healthy controls (HC).[64-66] In addition, the presence of circulating HuD-specific $CD4^+$ T cells has been suggested.[63]

Here, we have investigated the presence of circulating HuD-specific T cells in a large cohort of Hu-PNS patients and controls. Despite a multifaceted approach mainly geared towards the detection of HuD-reactive $CD8^+$ T cells and, to a somewhat lesser extent, $CD4^+$ T cells, no such cells were detected in the blood of Hu-PNS patients and controls.

MATERIALS AND METHODS

Patients

Forty-three patients with high-titered Hu antibodies and a definite clinical diagnosis of PNS[11], 31 Hu antibody negative SCLC patients without neurological symptoms or signs (SCLC) and 54 apparently HC were tested. The Erasmus MC Institutional Review Board approved the study and all individuals provided written informed consent. The individuals'

Table 1. Patient characteristics at the time of study entry.

	Hu-PNS	SCLC
N	**43**	**31**
Age (median, range)	64 (4-81)[a]	61 (40-83)
Gender (M/F)	15/28	21/10
Hu-Ab titre (median, range)	12,800 (400-204,800)	Negative
CMV serostatus (pos/neg)	29/14	17/14
Paraneoplastic Neurological Syndrome		NA
PSN	27	
PEM	5	
PCD	4	
PLE/ BE	3	
Pseudo-obstruction	2	
Motor neuron disease	2	
Tumor		
No tumor	8[b]	0
SCLC	31	31
- Limited	28	17
- Extended	3	14
NSCLC	2	0
prostate	1	0
neuroblastoma	1	0
Prior treatment		
none	27	27
chemotherapy ± immunosuppression	16	4
Neurological symptoms		NA
Interval onset symptoms - study entry	5 months (2-15) [c]	
Interval onset symptoms - diagnosis	4 months (1-12) [c]	
Progressive at study entry[d]	34 (79%)	
Modified Rankin Score		NA
MRS=2	7	
MRS=3	22	
MRS=4	10	
MRS=5	4	

[a]One Hu-PNS patient was a four-year old boy with an underlying neuroblastoma. The remaining Hu-PNS patients were aged between 49 and 81 years.

[b]No tumor mass visible on CT-scan or FDG-PET scan.

[c]Median (ranges) of intervals are shown.

[d]Progression of neurological symptoms was defined by the increase of at least 1 point on the modified Rankin scale during 2 months prior to study entry.

Abbreviations: Hu-Ab = Hu antibody; CMV = cytomegalovirus; pos = positive; neg = negative; NA = not applicable; PSN = paraneoplastic sensory neuronopathy; PEM = paraneoplastic encephalomyelitis; PCD = paraneoplastic cerebellar degeneration; PLE = paraneoplastic limbic encephalitis; BE = brainstem encephalitis; SCLC = small cell lung cancer; NSCLC = non-small cell lung cancer; MRS = Modified Rankin Score.

class I HLA alleles were typed by standard diagnostic PCR at the 2-digit resolution level. Patient characteristics are shown in Table 1. Anti-Hu IgG titers were determined as described previously[8] and in Hu-PNS the dependence in activities of daily living was scored using the modified Rankin Scale.[8] Twenty-five HC were male and 29 female, their median age was 46 years (range 17 to 89) and all were Hu antibody negative. No HC had received previous chemotherapy or immunosuppressive treatment and 30 HC (56%) were CMV-seropositive.

Reagents

Ninety-three HuD protein-spanning synthetic peptides, 15-mers with 11 amino acids overlap, were pooled to constitute the HuD peptide mix (HuDmix) and smaller peptide pools (Jerini Peptide Technologies [JPT], Berlin, Germany). For interferon-γ enzyme-linked immunosorbent spot-forming (IFN-γ ELISPOT) assays and the construction of HLA class-I multimers, HuD-derived 9- and 10-mers were selected based on previous studies.[64,66] The phycoerythrin (PE)-labeled multimers and corresponding peptides used were: HLA-A*0101-^{147}ELEQLFSQY^{155}, HLA-A*0101-^{245}RLDNLLNMAY^{254}, HLA-A*0201-^{86}SLGYGFVNYI^{95}, HLA-A*0201-^{248}NLLNMAYGV^{256}, HLA-A*0201-^{315}QLFGPFGAV^{323}, HLA-A*0201-^{362}RLGDRVLQV^{370}, and HLA-A*2402-^{154}QYGRIITSRI^{163} (ProImmune, Oxford, UK). As positive and negative controls, HLA-A*0201-^{495}NLVPMVATV^{503} (CMV phosphoprotein-65 [CMV-pp65]; Beckman Coulter, San Diego, CA) and HLA-A*0201 presenting an irrelevant peptide (ProImmune), respectively, were included.

To measure general T-cell responsiveness, we used phorbol myristate acetate (PMA) plus ionomycin, or phytohemagglutinin (PHA). A peptide pool containing 15-mers spanning CMV-pp65 (JPT) was used as positive- and negative antigen-specific control in CMV seropositive and seronegative individuals, respectively.

Cytokine flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated within 12 hours after venipuncture and stimulated in duplicate as described elsewhere.[193] Briefly, $2x10^6$ PBMC were incubated at 37°C in a CO_2 incubator for 18 hours with 1 μg/ml HuDmix, 1 μg/ml CMV-pp65, 1 μg/ml ionomycin plus 25 ng/ml PMA, or without antigen. After 2 hours of stimulation, brefeldin A was added to one of the duplicate tubes allowing for intracellular accumulation of cytokines in activated T cells. Brefeldin A was not added to the second tube to allow detection of secreted cytokines in the supernatant. In some individuals, additional stimulation was performed in the presence of co-stimulatory monoclonal antibodies (mAb) directed against CD28 and CD49d (BD Biosciences, San Jose, CA). Stimulated PBMC were stained and analyzed using anti-CD3 conjugated with peridinyl chlorophyllin (PerCP), anti-CD8 conjugated with allophycocyanin (APC), anti-interferon (IFN)-γ conjugated with fluorescein isothiocyanate (FITC), anti-tumor necrosis factor (TNF)-α conjugated with PE,

or appropriate isotype control mAb (all from BD Biosciences).[193] $CD4^+$ T cells were defined as $CD3^+,8^-$. Positive responses were defined by (i) percentage of cytokine-positive $CD4^+$ or $CD8^+$ T cells >2 times the negative control (i.e., no antigen) *and* (ii) ≥ 0.1% of the total number of $CD4^+$ or $CD8^+$ T cells, each after subtraction of isotype control results.

Detection of secreted cytokines

The secretion levels of IFN-γ, TNF-α, interleukin (IL)-2, IL-4, IL-5 and IL-10 were measured in supernatants using a cytometric bead array (BD Biosciences). Based on CMV data (not shown), a positive result was defined as cytokine concentration >2 times background (no antigen) and a minimum level of 50 pg/ml.

IFN-γ ELISPOT

PBMC ($2x10^5$/well) were pre-stimulated in duplicate in 96-well plates with culture medium containing 3 µg/ml HuD 9-mers, HuDmix, CMV-pp65, PHA, or no antigen for 1.5 hours at 37°C and 5% CO_2.[194] The PBMC were subsequently transferred to anti-IFN-γ-coated ELISPOT plates (Nalge Nunc, Rochester, NY) for a further 18-hour incubation. The ELISPOT assay was performed using standard protocols and automated reading (AELVIS GmbH, Hanover, Germany).[194] The mean number of spot-forming cells (SFC) in duplicate wells was used as assay outcome. Positive results were defined by numbers of SFC/well >3 times background (no antigen) and a minimum of 15 SFC/well.

Detection of HuD-specific $CD8^+$ T cells using Class-I HLA multimers

Thawed PBMC were stained as described previously.[195] Following acquisition of $1x10^5$ viable (i.e., 7-amino-actinomycin-D [7AAD] negative) $CD8^+$ T cells, a positive result required a percentage of ≥ 0.1% of viable $CD8^+$ T cells binding the HuD multimer *and* a brightly staining HuD multimer-binding $CD8^+$ T-cell population that did not overlap with the dimly staining irrelevant multimer-binding T-cell population.

RESULTS

Cytokine flow cytometry of PBMC after stimulation with HuD-derived peptides

Stimulation with PMA and ionomycin induced IFN-γ production in both $CD4^+$ and $CD8^+$ T cells of all individuals (not shown). Whilst all CMV-seropositive individuals specifically responded to CMV-pp65, no HuD-specific T-cell reactivity was observed in any of the Hu-PNS patients, SCLC patients or HC (Figs. 1a, b). Similar results were obtained when intracellular TNF-α (not shown) or secreted cytokines (Fig. 1c) were measured. The use of co-stimulatory antibodies in combination with HuDmix did not result in the detection of HuD-specific T-cell reactivity (not shown).

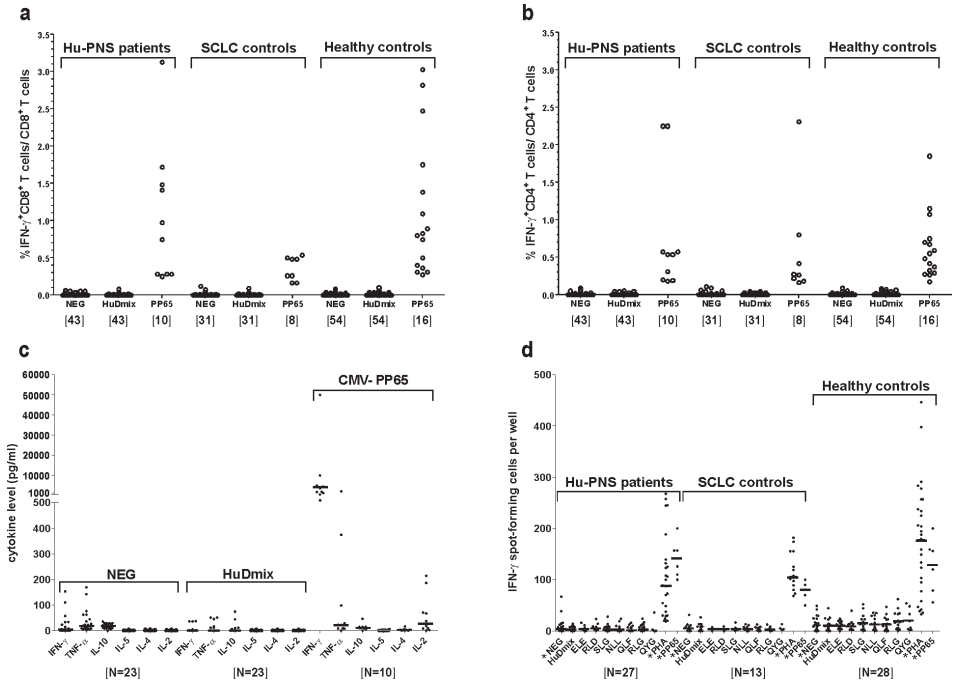

Fig. 1. Cytokine production in response to HuDmix and HLA-matched HuD 9-mers.

Proportions of $CD8^+$ (panel a) and $CD4^+$ (panel b) T cells expressing intracellular IFN-γ after stimulation with HuDmix in Hu-PNS patients, SCLC and healthy controls. Each dot represents the result observed in a single individual. (panel c) After stimulation of PBMC with HuDmix and control antigens, the indicated cytokines were measured in assay supernatants. The results are shown for Hu-PNS patients only. Horizontal lines indicate median values of each group. (panel d) ELISPOT assay showing the number of IFN-γ SFC after stimulation of $2x10^5$ PBMC with HuDmix or HuD 9-mers. Each dot represents the mean result of duplicates for each stimulus in each individual. (panels a-d) Responses to CMV antigens are shown for CMV seropositive individuals only, as they were consistently negative in CMV seronegative individuals (not shown). The numbers of individuals tested are given in between brackets.

Abbreviations: NEG = negative control (incubation without antigen); HuDmix = HuD protein-spanning peptide pool; PP65 = CMV pp-65 protein-spanning peptide pool; IL = interleukin; TNF = tumor necrosis factor; IFN = interferon; SFC = spot-forming cell; PHA = phytohemagglutinin; ELE, RLD, SLG, NLL, QLF, RLG and QYG designate individual HuD-based peptides.

IFNγ-ELISPOT assay on PBMC stimulated with HuD-derived 9-mers

All individuals responded to PHA as determined by IFNγ-ELISPOT assay. In addition, all CMV-seropositive individuals responded to CMV-pp65. However, no T cell reactivity towards the previously described class-I HLA-binding HuD peptides[64] was detected in individuals with the appropriate HLA types in any of the study groups (Fig. 1d).

Analysis of HuD-specific $CD8^+$ T cells using Class-I HLA multimers

Finally, we investigated the presence of HuD-specific $CD8^+$ T cells in PBMC using Class-I HLA multimers containing HuD-derived peptides. Whilst CMV-seropositive individuals with the appropriate HLA types showed distinct populations of pp65 multimer-binding

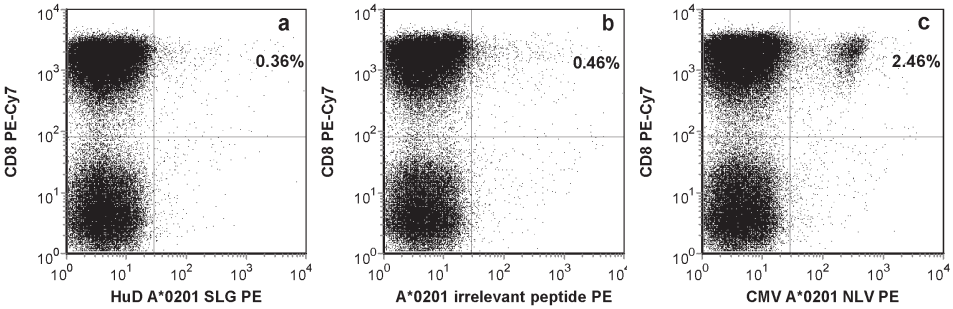

Fig. 2. Analysis of HuD peptide-loaded, Class-I HLA multimer-binding CD8⁺ T cells.

PBMC from a CMV-seropositive Hu-PNS patient were stained with HLA-A*0201 multimers loaded with the HuD peptide SLGYGFVNYI (panel a), an irrelevant peptide (panel b) or with the CMV-pp65 peptide NLVPMVATV (panel c). All data shown are obtained after selection of T cells (i.e., CD3⁺, low sideward scatter signals).[195] Binding of Class-I HLA multimers (panels a-c, horizontal axes) was analyzed in relation to CD8 expression. In this example, the proportion of SLGYGFVNYI multimer-binding CD8⁺ T cells (0.36%) was similar to that of irrelevant multimer-binding CD8⁺ T cells (0.46%); binding resulted in low-intensity fluorescence signals only. In contrast, 2.46% of CD8⁺ T cells bound the NLVPMVATV multimer resulting in high-intensity fluorescence signals from most CD8⁺ T cells.

CD8⁺ T cells, no HuD-specific CD8⁺ T cells were observed in individuals with the appropriate HLA types in any of the study groups (Fig. 2).

Discussion

We set out to detect HuD-specific T cells in the blood of Hu-PNS patients as they are postulated to play a pivotal role in the immunopathology of this disease. Although we applied three different approaches we could not detect circulating HuD-specific T cells.

First, to induce cytokine responses in T cells, we used 15-mer protein-spanning peptide pools that have the advantage of covering the full protein sequence and of eliciting both CD8⁺ and CD4⁺ T-cell responses[193], as demonstrated by the CD8⁺ and CD4⁺ CMV-pp65-specific T-cell responses. However, no CD8⁺ or CD4⁺ HuD-specific T-cell responses were observed. These results are at variance with the detection of HuD-specific CD4⁺ T-cell proliferative responses in PBMC of Hu-PNS patients by Benyahia et al.[63] This discrepancy may be explained by differences in read-out (i.e., 3-day lymphocyte proliferation in Benyahia's study[63] vs. overnight cytokine production in ours) and the use of recombinant HuD protein[63] vs. a protein-spanning 15-mer peptide pool (this manuscript).

We then studied responses to HLA class-I binding HuD peptides that were previously selected.[64] Using the same experimental setup and HuD 9-mer peptides, Rousseau et al.[64] reported HuD-specific T-cell reactivity in 7/10 Hu-PNS patients and in 3/10 HC.[64] In that small study a positive response was defined as an experimental value ≥ 2 times above background. With that criterion, 3 PNS, 2 SCLC and 4 HC would have been classified as HuD T-cell responders in our study. However, using that cut-off we would also have

detected T-cell reactivity in individuals whose Class-I HLA molecules did not have the appropriate binding motifs (data not shown). Therefore, we used more stringent cut-off levels resulting in a negative outcome. As most patients in both studies had progressive neurological disease and were tested shortly after start of symptoms and prior to therapy, differences between the study populations do not explain this discrepancy.

Finally, we could not detect HuD-specific circulating $CD8^+$ T cells using Class-I HLA multimers with the same fine specificities as defined by Rousseau et al.[64] The absence of detectable circulating HuD-specific $CD8^+$ T cells may not be surprising. In a PCR-based study, Plonquet et al.[59] detected the same T-cell clone in neoplastic and nervous tissues, but not in blood. This finding suggests that T cells involved in the pathogenesis of Hu-PNS circulate in concentrations below detection level. An immune response taking place in the central nervous system parenchyma may deplete the circulating pool of $CD8^+$ T cells with that specificity.[196] Furthermore, vaccination studies in melanoma patients demonstrate that clinically effective anti-tumor immune responses may occur despite low levels of melanoma-specific cytotoxic T cells, i.e., below the detection limit of multimer-based assays.[197,198]

In conclusion, we were unable to detect HuD-specific T cells in a large cohort of Hu-PNS patients and controls. However, two of our three assays were designed for the detection of $CD8^+$ T cells only. The IgG_1 isotype predominance of serum Hu antibodies in Hu-PNS indicates a T-helper response to the Hu antigen.[42] Therefore, further studies are warranted that focus on the detection of circulating HuD-specific $CD4^+$ T cells. In this context, regulatory $CD4^+$ T cells – which down regulate immune responses towards auto-antigens and tumor-antigens – are of interest. Although the numbers of regulatory T cells are increased in cancer patients[184] and in PNS patients[185,199]; their (possibly impaired) function in PNS remains to be studied. Finally, examination of the antigen-specificity of T cells in affected tissues may shed further light on the role of HuD-specific T cells in the pathogenesis of Hu-PNS.

Acknowledgement

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Chapter | 5

Resolution of EBV+ CNS lymphoma with appearance of CSF EBV-specific T cells

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ABSTRACT

Current knowledge of EBV-specific T cell responses in the CSF of patients with EBV-related lymphoproliferative disease (EBV-LPD) in the CNS is very limited. Here, we present two recipients of hematopoietic stem cell transplants with EBV-LPD in the CNS. EBV-specific $CD8^+$ T lymphocytes were detected in CSF and peripheral blood using class I HLA multimers loaded with EBV-derived peptides. The appearance of EBV-specific $CD8^+$ T cells in CSF and blood correlated with neurological improvement and disappearance of EBV-LPD. These observations suggest a role for EBV specific $CD8^+$ T cells in the control of EBV-LPD in the CNS.

INTRODUCTION

Epstein-Barr virus (EBV)-related lymphomas of the central nervous system (CNS) are mostly associated with AIDS but are also seen after allogeneic hematopoietic stem cell transplantation (SCT), and usually have a fatal outcome.[200,201] Reactivating EBV infections are normally controlled by EBV-specific T lymphocytes. However, patients with severe T-cell deficiencies, such as after SCT, are at risk for EBV reactivation progressing to B-cell lymphoproliferative disease (EBV-LPD). The protective and therapeutic role of peripheral blood EBV-specific T cells against EBV-LPD has been subject of various studies.[202-207] However, little is known about EBV-specific T-cell responses in the cerebrospinal fluid (CSF) of patients with EBV-LPD in the CNS.

For the identification of virus-specific T lymphocytes, fluorochrome-conjugated, peptide–loaded class I major histocompatibility complex (MHC) multimers have been developed and studied in animal but not in human CSF.[208-210] Here, we present two patients with EBV-LPD in the CNS. Using MHC class I multimers loaded with EBV-derived peptides, EBV-specific $CD8^+$ T cells in CSF and peripheral blood were monitored and related to EBV load and clinical course.

CASE REPORTS

Patient 1

A 65-year old man presented with progressive paraparesis 61 days after autologous SCT given as treatment for extramedullary plasmacytomas. On examination the leg weakness was MRC grade 4+/4- proximally and 4+/3 distally. Knee and ankle reflexes were absent and both plantar responses were indifferent. All sensory modalities were diminished in both legs and the patient was unable to walk. MRI of the spine showed subtle linear enhancement at the ventral side of the spinal cord. CSF examination revealed an elevated protein level (1.0 g/l) and mononuclear pleocytosis (118 x 10^6 cells/l). High EBV DNA concentrations were detected in CSF and plasma between days 65 and 78 (Fig. 1A). Ninety percent of the B lymphocytes in the CSF were monoclonal (s-IgG^+, s$Ig\lambda^+$), consistent with the presence of EBV-LPD. No monoclonal B cells were detected in blood and bone marrow. During the first week in hospital, the patient's sensory level ascended to T9 while the leg weakness progressed to MRC grade 4/3 proximally and 4/2 distally. On day 75, the patient started to recover spontaneously; he regained the ability to walk and was discharged at day 93. By that time, the EBV DNA in the blood had become undetectable and remained so for the entire duration of follow-up (Fig. 1A). The patient died from recurrent plasmacytomas on day 319 post SCT.

Patient 2

A 53-year old woman was treated with an allogeneic SCT for chronic B-lymphocytic leukemia (B-CLL). On day 69 post SCT, she developed generalized lymphadenopathy with elevated plasma EBV load (Fig. 1C). Two monoclonal B-cell populations were detected in peripheral blood and bone marrow: one with the residual B-CLL phenotype ($CD19^{+}20^{dim}5^{+}$ $sIgG^{dim}$ $sIg\kappa^{dim}$) and one with a novel phenotype ($CD19^{+}20^{+}5^{-}$ $sIgG^{dim}$ $sIg\kappa^{dim}$). She was treated with rituximab (anti-CD20, 375 mg/m^2 i.v., day 70) and cyclosporine was tapered. Two days later, aphasia and MRC 4 paresis of the left hand developed. CT scan and MRI of the brain were normal. Mild mononuclear pleocytosis (23 x 10^6 cells/l) and high EBV load were detected in the CSF on days 72 and 73 (Fig. 1C). Immunophenotyping of the CSF lymphocytes revealed monoclonal B cells with the novel $CD19^{+}20^{+}5^{-}$ $sIgG^{dim}$ $sIg\kappa^{dim}$ phenotype, confirming CNS localization of EBV-LPD. Over the next days, the paresis of the left hand deteriorated (MRC 2) and weakness of the proximal muscles of the left arm developed (MRC 4). In addition, she became increasingly confused. EBV DNA and monoclonal B cells with the EBV-LPD phenotype were no longer detectable in the CSF day 76. Because EBV-LPD was still detectable in the blood at that time, she received a 2nd dose of rituximab. Upon this intervention, blood EBV DNA transiently increased to 158,000 geq/ml on day 82 to decline rapidly afterwards (Fig. 1C). On day 79, her orientation and aphasia started to improve considerably. EBV DNA and monoclonal B cells with the EBV-LPD phenotype became undetectable in the blood on day 91, whilst EBV DNA and monoclonal B cells remained undetectable in the CSF during further follow-up (Fig. 1C). However, the patient became comatose on day 100 and the MRI was now consistent with progressive multifocal leukoencephalopathy. JC virus was detected in the CSF and the patient died on day 130 post SCT.

MATERIALS AND METHODS:

Quantitative EBV-specific polymerase chain reaction (PCR) assay

Early diagnosis of EBV reactivation in the CNS has become possible by the use of quantitative PCR to detect EBV DNA in CSF.[211] EBV DNA levels were measured in plasma and CSF by quantitative PCR as described previously.[212] Results >50 geq/ml were considered positive.

Flow cytometric detection of monoclonal B lymphocytes

Leukocytes from CSF, peripheral blood and bone marrow were isolated, resuspended in phosphate-buffered saline (PBS) and immunostained as previously described.[213] Four-color flow cytometry was performed using a FACSCalibur (BD Biosciences, San Jose, CA) and list mode data analysis was performed as described elsewhere.[214]

Quantification of CD8+ T lymphocytes specific for class I HLA-restricted, EBV-encoded epitopes

Depending on cell yields from CSF (≥10,000 leukocytes per staining), 3 phycoerythrin (PE)-labeled class I HLA multimers (ProImmune, Oxford [UK]; see Table 1) were used, individually or after pooling, for staining in combination with monoclonal antibodies (mAb) CD3 conjugated with peridinyl chlorophyllin (PerCP) and CD8 conjugated with allophycocyanin (APC) (both from BD Biosciences). Leukocytes were incubated with cocktails of multimers and mAb for 30 minutes at room temperature, washed once and analyzed by flow cytometry. Within the $CD8^+$ T cells the proportions of multimer-binding T cells was assessed (Fig. 2).

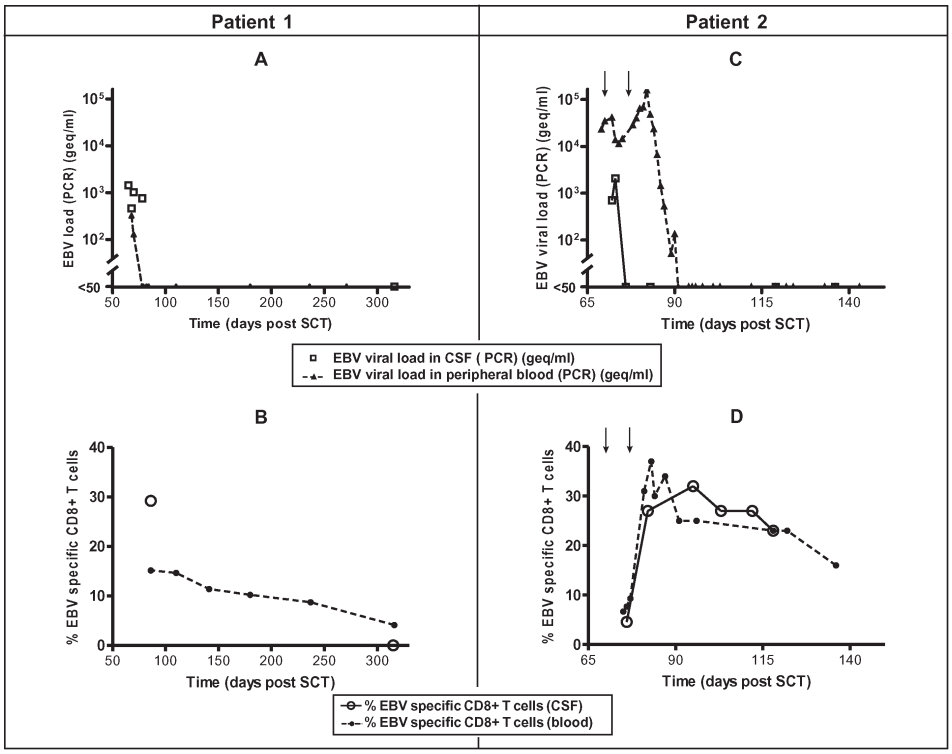

Fig. 1. EBV viral load and EBV specific CD8+ T cells in CSF and blood.

EBV viral load was monitored in CSF and peripheral blood from patients 1 (panel A) and 2 (panel C) using quantitative PCR. EBV specific $CD8^+$ T lymphocytes were detected in CSF and peripheral blood from patients 1 (panel B) and 2 (panel D) using Class I HLA multimers loaded with EBV-derived peptides. The % multimer-binding cells are expressed as fractions of total $CD8^+$ T cells. Arrows (panels C and D) indicate Rituximab infusions on days 70 and 76.

RESULTS

EBV specific CD8+ T cells in CSF and blood

Patient 1

Upon neurological improvement (day 86), a major proportion, i.e., 29%, of the CD8+ T cells in the CSF was EBV-specific (Figs. 1B and 2). At day 315, i.e., well after neurological recovery, EBV-specific CD8+ T cells were no longer detectable in the CSF (Fig. 1B). On day 86, 15% of the CD8+ T cells in the blood were EBV specific to decrease gradually afterwards (Fig. 1B).

Patient 2

On day 76, 5% of CD8+ T cells in the CSF were EBV specific. This proportion increased to 32% on day 95, when neurological symptoms had improved, and decreased gradually afterwards (Fig. 1D). On day 82, EBV-specific T-cells in the blood had increased to 37% of CD8+ T cells to decline gradually afterwards (Fig. 1D).

Fine specificities of EBV specific T cells

The cell yields from 3 CSF specimens allowed us to compare the fine specificity of EBV-specific T-cell responses in CSF and blood (Table 1). In patient 1 (day 86), CD8+ T cells specific for the lytic EBV epitope BMLF1 constituted the largest detectable subset in CSF (28%) and blood (14%). Similarly, in patient 2, BMLF1-specific CD8+ T cells were the largest detectable subset on day 76 in CSF (4%) and blood (7%). However, by day 95 the subsets specific for the latent epitopes LMP2 and EBNA3A had become the largest subsets in CSF (25% and 7%, respectively), whilst BMLF1-specific T cells were only 1% of CD8+ T

Table 1. CD8+ T cells specific for individual EBV peptides in CSF and blood.

			% of CD8+ T cells binding EBV multimer					
			Patient 1		Patient 2			
EBV Antigen (coordinates)	**Presenting HLA allele**	**Amino acid sequence**	CSF	Blood	CSF	Blood	CSF	Blood
			Day 86		Day 76		Day 95	
BMLF1 (280-288)	A* 0201	GLCTLVAML	28.3	13.9	3.8	6.6	1.3	1.0
LMP2 (426-434)	A* 0201	CLGGLLTMV	0.3	0.1	0.6	0.8	24.5	12.5
EBNA 3A (379-387)	B* 0702	RPPIFIRRL	0.6	1.1	0.2	0.3	7.3	12.9

HLA typing of Patient 1: HLA-A*0201, A3, B*0702, B62.
HLA typing of Patient 2: HLA-A*0201,--, B*0702, B62.

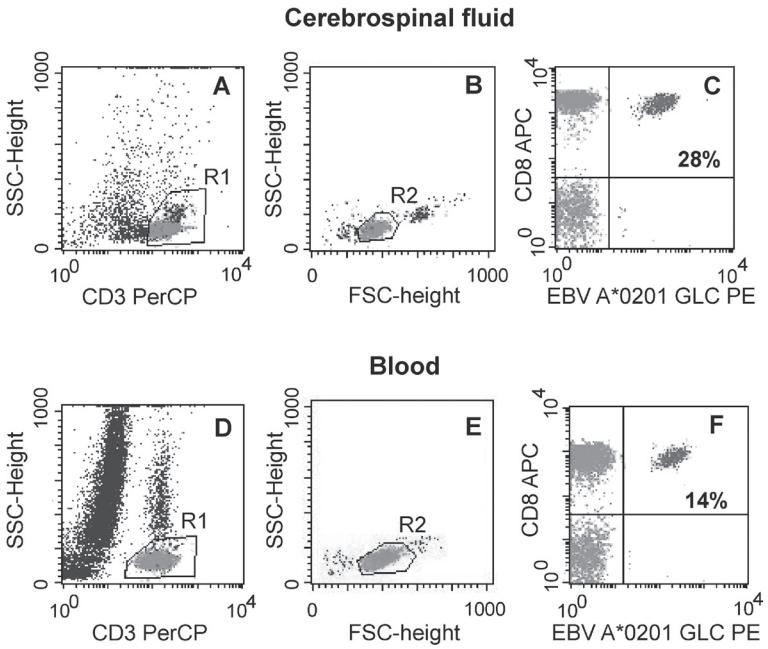

Fig. 2. Flow cytometric detection of class I HLA-restricted, EBV peptide specific CD8+ T lymphocytes (patient 1).
The T lymphocytes ($CD3^{bright}$, low SSC) were selected in gate R1. Subsequently, any remaining cellular debris and aggregates were excluded based on FSC (gate R2). Within the T cells, the reactivity with class I HLA multimers was then plotted against CD8 fluorescence. The % multimer-binding cells (expressed as fraction of total $CD8^+$ T cells) are shown in the upper right quadrants of histograms C (CSF) and F (blood).
Abbreviations: SSC: side scatter, FSC: forward scatter, CD3: T lymphocyte marker, EBV A* 0201 GLC PE: phycoerythrin-labeled HLA class I multimer presenting the peptide GLCTLVAML (derived from the EBV protein BMLF1).

cells. At that time, LMP2 and EBNA-3A-specific subsets also constituted the largest detectable fractions EBV-specific $CD8^+$ T cells in the blood.

Discussion

Regression of systemic EBV-LPD is associated with a rapid increase of EBV-specific T-cell counts in the peripheral blood.[206] This report is, to our knowledge, the first to demonstrate the presence of significant proportions of EBV specific $CD8^+$ T cells in the CSF of patients with EBV-LPD in the CNS in correlation with neurological improvement and disappearance of EBV-LPD. Patient 1 recovered without any specific treatment. Patient 2 improved neurologically after rituximab administration and tapering of cyclosporin. Although rituximab may have contributed to systemic clearance of EBV-LPD, the impact of this treatment on CNS lymphoma is unlikely.[215,216] Instead, we suggest that EBV-specific T-cell recovery may have contributed to the control of EBV-LPD in the CNS of this patient.

In both patients, the frequency of EBV-specific cells among the $CD8^+$ T cells was higher in CSF than in blood, indicating selective recruitment and/or proliferation of EBV-specific T cells within the CNS. The relative proportions of $CD8^+$ T cells directed towards individual EBV epitopes were similar in CSF and peripheral blood, indicating that the kinetics of the EBV-specific $CD8^+$ T-cell responses were similar in both compartments. In patient 2, most of the EBV-specific CD8+ T cells in the CSF were initially reactive with the lytic epitope BMLF1. After improvement of the neurological symptoms, the antigen-specificity of these EBV-specific CD8+ T cells shifted towards the latent epitopes LMP2 and EBNA-3A. This observation is similar to the shift in recognition from lytic to latent epitopes occurring in the transition from primary to the persistent phase of EBV infection.[217,218] A similar course of T cell epitope specificity has been observed in a heart transplant patient with EBV-LPD upon treatment with autologous EBV specific cytotoxic T lymphocytes (CTL).[205] Here, the CTL precursor frequency toward the lytic antigen BMLF1 was high in the initial tumor regression phase and tended to decrease during the long term tumor regression phase, whereas the CTL precursor frequency towards three latent epitopes was low in the initial tumor regression phase, but increased during the long term regression phase. Because only a single observation was made in patient 1, we have no information about a potential shift in epitope dominance.

The small number of patients and limited number of observations restrict the pathogenetic conclusion that can be drawn from our observations. In combination with the recently reported effectiveness of allogeneic EBV specific T-cell immunotherapy in a patient with EBV-LPD in the CNS[219], our data support the view that EBV-specific $CD8^+$ T cells play a role in the control of EBV-LPD in the CNS.

Chapter | 6

The cerebrospinal fluid of patients with Hu-associated paraneoplastic neurological syndromes does not contain detectable numbers of HuD-specific CD8+ T cells

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Submitted

ABSTRACT

To investigate the putative role of HuD-specific cellular immunity in the pathogenesis of Hu antibody-associated paraneoplastic neurological syndromes (Hu-PNS), we determined the presence of HuD-specific T cells in the cerebrospinal fluid (CSF) of 13 Hu-PNS and 4 control patients. CSF-derived T cells were expanded and assayed for HuD reactivity using HuD protein-spanning overlapping 15-mer peptides and individual HuD 9-mers in interferon-γ ELISPOT-assays. Additionally, fresh and expanded CSF-derived T cells were stained with HLA class I multimers presenting HuD 9-mer peptides. Despite this multifaceted approach, no HuD-specific $CD8^+$ T cells were detected.

INTRODUCTION

The mechanisms responsible for tumor suppression[26] and neuronal damage in paraneoplastic neurological syndromes (PNS) are poorly understood.[1] In PNS associated with small cell lung cancer (SCLC) and Hu antibodies (Hu-PNS), Hu antigens expressed by the tumor hypothetically trigger an immune response that recognizes the same or similar antigens in the nervous system.[1,36] Hu antibodies are directed against a family of neuron specific proteins including HuD, HuC and Hel-N1. HuD expression in all SCLC tumor cells suggests that the HuD antigen may play an important role in initiating the immune response.[36,191]

Despite high serum- and cerebrospinal fluid (CSF) titers, a pathogenic role of Hu antibodies could never be proven in animal models.[55,58] The occurrence of oligoclonal $CD8^+$ T cells in nervous tissues and tumors of Hu-PNS patients suggests their involvement in neuronal degradation.[59,60] Some studies have described the presence of HuD peptide-specific $CD8^+$ T cells in blood of Hu-PNS patients, but also in healthy controls.[64-66] We were unable to confirm the presence of such cells in the blood of 43 Hu-PNS patients or 85 controls.[220] PNS patients often have CSF pleocytosis in the early stages of disease, consistent with a cellular immune response in this compartment.[12,221] In addition, patients with paraneoplastic cerebellar degeneration (PCD) associated with a specific antibody response to the tumor and brain antigen cdr2, have expanded populations of MHC class I-restricted cdr2-specific CTLs in their blood[222] and feature CSF pleocytosis with increased percentages of activated T cells.[71]

Antigen-specific T cells, potentially involved in CNS viral infections and multiple sclerosis (MS), have been identified in relatively high concentrations in CSF as compared with simultaneously obtained peripheral blood (PB) samples.[223-227] These studies suggest that the T cells located within the CSF may reflect a T cell response in the CNS parenchyma. Because fresh brain biopsies of Hu-PNS patients are generally unavailable for functional T cell studies, we investigated the presence of HuD-reactive T cells in CSF samples of 13 Hu-PNS and 4 control patients.

MATERIALS AND METHODS

Patients and controls

Thirteen patients were included in the study featuring (i) high titer serum Hu antibodies; (ii) a "definite" diagnosis of PNS[11]; and (iii) progressive neurological disease as defined by the increase of at least one point on the modified Rankin scale during the 4 weeks prior to study entry.[8] These patients are referred to as Hu-PNS patients; their characteristics are shown in Table 1. In addition, 4 patients with non-inflammatory neurological diseases

(i.e., normal pressure hydrocephalus and herniated cervical disc) were included as controls. Fresh samples of CSF and peripheral blood (PB) were prospectively collected. The Erasmus MC Institutional Review Board approved the study and all patients provided written informed consent. Hu antibodies were detected by indirect immunofluorescence (IIF) on rat cerebellar sections[164] and confirmed by Western blotting using purified recombinant HuD.[36] Hu antibody (Ab) titers of paired serum and CSF samples were determined by endpoint titration on Western blot.[36]

Intrathecal IgG synthesis was calculated using the formula of Schuller et al[228], as follows: Intrathecal IgG synthesis (mg/l) = CSF IgG (mg/l) – 30 + [CSF albumin (mg/l) – 210] x serum IgG (g/l) / 60. In this formula, 30 mg/l is the upper limit of normal CSF IgG concentration, 210 mg/l is the mean normal concentration of CSF albumin, and 60 is the equivalent albumin transudation of 0.1% of serum IgG. Using the formula, the percentage of CSF IgG derived from serum and intrathecal production respectively can be calculated. Subsequently, the Hu Ab-specific activity (ASA) in serum or CSF was calculated by the following formula[229]: 1 unit of Hu-ASA$_{\text{serum or CSF}}$ = Hu Ab titers$_{\text{erum or CSF}}$ / [IgG]$_{\text{serum or CSF}}$ (mg/ml) x 10^{-3}. Intrathecal Hu Ab synthesis was then calculated by the formula: Intrathecal Hu-ASA = (CSF ASA - [serum ASA x % of IgG from serum])/% of intrathecal [IgG]. A ratio of intrathecal ASA/serum ASA of Hu antibody >2 was considered a positive intrathecal synthesis.[229]

Human leukocyte antigen (HLA) typing was performed on peripheral blood mononuclear cells (PBMC) by standard serological methods and molecular typing at 4-digit resolution (Sanquin Blood Bank, Rotterdam, The Netherlands).

The 4 control patients with non-inflammatory neurological diseases were male, aged between 52 and 79 years, Hu antibody seronegative, and had not received prior chemotherapy or immunosuppression. The control patients expressed either the HLA-A*0101 (n=2), HLA-A*0201 (n=1) alleles, or both (n=1).

Reagents

Ninety-three synthetic peptides, each 15 amino acids (aa) long with 11 aa overlaps and together spanning the entire HuD protein, were pooled to constitute (i) the HuD protein-spanning peptide pool (referred to as "HuDmix" hereafter) and (ii) 20 smaller so-called matrix peptide pools, which were designed so that each peptide was present in two of these pools.[230] The peptides were obtained from Jerini Peptide Technologies (Berlin, Germany). For interferon-γ enzyme-linked immunosorbent spot-forming (IFN-γ ELISPOT) assays and the construction of HLA class-I multimers, HuD-derived 9- and 10-mer peptides were selected based on previous studies.[64,66] The phycoerythrin (PE)-labeled multimers and corresponding peptides used were: HLA-A*0101-^{147}ELEQLFSQY^{155}, HLA-A*0101-^{245}RLDNLLNMAY^{254}, HLA-A*0201-^{86}SLGYGFVNYI^{95}, HLA-A*0201-^{248}NLLNMAYGV^{256}, HLA-A*0201-^{315}QLFGPFGAV^{323}, HLA-A*0201-^{362}RLGDRVLQV^{370}, and HLA-A*2402-^{154}QYGRIITSRI^{163} (ProImmune, Oxford, UK). As negative control, a HLA-A*0201 multimer

presenting an irrelevant peptide (ProImmune) was included. Phytohemagglutinin (PHA; Roche Diagnostics GmbH, Mannheim, Germany) served as positive control for general T-cell responsiveness in the ELISPOT assay.

Enumeration of $CD4^+$ and $CD8^+$ T cells in fresh and expanded CSF cells

Erythrocytes and leukocytes were enumerated within one hour after obtaining the CSF sample. For immune phenotyping, cells were concentrated from 3 ml CSF by centrifugation (8 min at 450 ×*g*) and resuspended in 100 µl phosphate-buffered saline (PBS). The cells were incubated for 15 min at room temperature (RT) in the dark with 10 µl of each of the following monoclonal antibodies (mAb): CD45 conjugated with peridinyl chlorophyllin (CD45-PerCP), CD3 conjugated with fluorescein isothiocyanate (CD3-FITC), CD4 conjugated with PE-Cy7 (CD4-PE-Cy7) and CD8 conjugated with allophycocyanin (CD8-APC). All mAb were purchased from BD Biosciences (San Jose, CA). Subsequently, the cells were washed, resuspended in PBS containing 1% paraformaldehyde, and acquired on a FACScanto flow cytometer. Analysis was performed using FACSdiva software (both from BD Biosciences).

Generation of CSF-derived T cell lines and antigen presenting cells

In order to obtain sufficient T cell numbers for in vitro assays, CSF-derived T cells were expanded nonspecifically as described previously.[231,232] Briefly, cells were concentrated from 10 ml fresh CSF by centrifugation (8 min at 450 ×*g*), washed once with PBS containing 3% fetal bovine serum (FBS), and resuspended in T cell medium (TCM) consisting of RPMI 1640 supplemented with antibiotics, 10% heat-inactivated human pooled serum and 50 U/ml human recombinant interleukin-2 (rIL-2). Thereafter, the cells were divided over 10 wells of a 96-well round-bottomed microtiter plate and stimulated with a "feeder-mix" in a total volume of 150 µl/well. This feeder-mix consisted of TCM, 1µg/ml PHA and 1 x 10^5 gamma-irradiated (3000 rad) allogeneic PBMC pool derived from 8 healthy donors. The T cell lines thus generated *in vitro,* hereafter referred to as CSF-TCL, were assayed after one or two rounds of mitogenic stimulation. Autologous B lymphoblastic cell lines (BLCL) were used as antigen-presenting cells (APC), and were established by infecting patient PBMC with Epstein-Barr virus (EBV) as described previously.[194]

IFN-γ ELISPOT assay

Autologous BLCL (5 x 10^3 cells/well) were pulsed in quadruplicate with 3 µg/ml HuDmix, 20 HuD matrix pools, individual HuD 9-mers, and controls as described previously.[233] After 1 hour, CSF-TCL were added in duplo in two different concentrations (1 x 10^4 or 3 x 10^4 T cells/well) in a total volume of 150 µl/well and incubated for another 1.5 hour. The T cell/APC cell suspensions were subsequently transferred for overnight incubation to anti-IFN-γ coated ELISPOT plates (Silent Screen; Nalge Nunc, Rochester, NY). The

ELISPOT assay was performed using standard protocols[194] and an automated ELISPOT reader (AELVIS GmbH, Hanover, Germany). The mean number of spot-forming cells (SFC) in duplicate wells was used as assay outcome. Based on results obtained using peptides that did not fit the binding groove of the class I HLA allele under study (not shown), positive results were defined by a number of SFC > 2–fold above background (i.e., in the absence of antigenic stimulation) *and* a minimum number of 15 SFC/well. In this way, the detection level of the assay was defined as 0.05% and 0.15% of T cells when testing 3 x 10^4 cells and 1 x 10^4 cells, respectively.

HuD peptide-loaded class I HLA multimer assay

HuD peptide-loaded class I HLA multimers were used for the detection of peptide-specific T cells in fresh CSF samples (10 ml) and CSF-TCL of patients and controls expressing the appropriate HLA class I alleles. Immunostaining was performed as described previously.[195,223] Depending on cell yields in CSF samples, HLA-matched HuD multimers were stained individually or after pooling. For each staining at least 10^4 leukocytes were used. On each occasion, a multimer presenting an irrelevant peptide was incubated separately. Cell suspensions were incubated with multimers for 30 min at RT and subsequently with the mAbs CD45-PerCP, CD3-FITC and CD8-APC for 15 min. After one wash, cells were resuspended in PBS followed by flow cytometric data acquisition using all cells in each sample.

For CSF-TCL, 2 x 10^6 T cells were incubated separately with each HLA-matched multimer for 30 min at RT and subsequently with CD3-FITC and CD8-APC mAb. Cells were resuspended in PBS containing 1 µg/ml 7-aminoactinomycin D (7-AAD; Sigma, St. Louis, MO, USA), followed by flow cytometric data acquisition from 1 x 10^6 viable (i.e. 7-AAD negative) cells.

For fresh CSF samples, a positive result required (i) a percentage of ≥1.0% of $CD8^+$ T cells binding HuD multimer following acquisition of at least 2,500 $CD8^+$ T cells; *and* (ii) a brightly staining HuD multimer-binding $CD8^+$ T cell population whose fluorescence intensity exceeded that of the dimly staining irrelevant multimer-binding T cell population. These requirements were essentially the same for the CSF-TCL, except for the minimum required percentage of T cells binding HuD multimer, i.e. ≥ 0.01% viable $CD8^+$ T cells following acquisition of at least 500,000 viable $CD8^+$ T cells.

RESULTS

Patient and CSF characteristics

At the time of CSF sampling all Hu-PNS patients had progressive neurological signs or symptoms and their MRS had deteriorated by at least one point over the four weeks prior

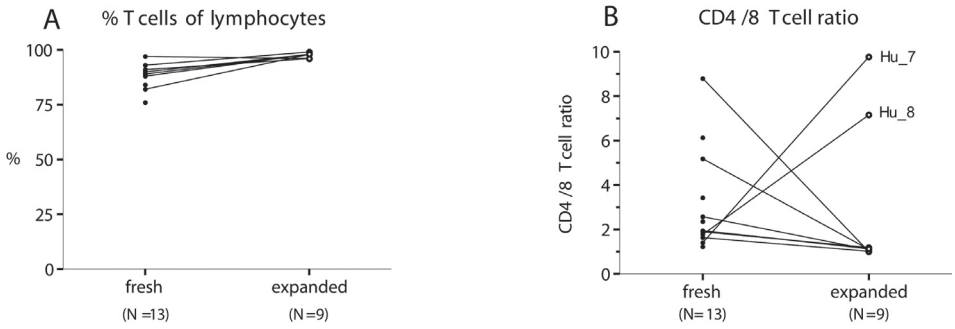

Figure 1. Generation of CSF-TCL of Hu-PNS patients

The CSF cells from nine of the thirteen Hu-PNS patients were expanded as described in Materials & Methods. Prior to and after expansion of the CSF-derived cells, the percentages of $CD3^+$ T cells (expressed as fraction of lymphocytes; panel A) and the ratio between $CD4^+$ and $CD8^+$ cells within the $CD3^+$ T cells were determined (panel B). CSF-TCL = CSF derived T cell line.

to study entry. CSF abnormalities were detected in all but one patient (Hu-12). The protein concentration was increased (>0.60 g/l) in 8 patients, the IgG index increased (>0.60) in 11 patients (data not shown) and mononuclear pleiocytosis (> 4.0 cells/µl) was observed in 6 patients (Table 1). Six out of 8 patients with paraneoplastic sensory neuronopathy (PSN) had mononuclear pleocytosis, indicating that they also suffered from inflammation of the central nervous system that was clinically not apparent.[234] Six patients with PSN and three with limbic encephalitis had intrathecal Hu antibody synthesis, indicated by an $ASA_{intrathecal}$/ ASA_{serum} ratio >2. The leukocytes in the CSF of the Hu-PNS patients were predominantly lymphocytes (median 78%, range 52% to 98%), and consisted mainly of $CD3^+$ T cells (Fig. 1A). Within the $CD3^+$ T cells, the $CD4^+$ T cells predominated over $CD8^+$ T cells (i.e. $CD4^+/CD8^+$ T cell ratio >1 in all 13 patients (Fig. 1B).

Expansion of CSF T cells

Because of the generally low concentrations of lymphocytes in CSF and the limited amount of CSF that can reasonably be sampled, we generated CSF-TCL from 9 Hu-PNS patients and 4 non-HuPNS control patients in order to obtain sufficient cell numbers for further studies. CSF cells were cultured during a median of 17 days (range 10 to 26 days), and expanded a median of 1,040 fold (range 300-13,300). The expansion cultures yielded a median of 31 x 10^6 cells (range 6-69 x 10^6 cells), and consisted of mainly (>98%) $CD3^+$ T cells. In 7 out of 9 CSF-TCL from Hu-PNS patients there was a predominant outgrowth of $CD3^+,8^+$ T cells, whereas in the remaining 2 CSF-TCL (patients Hu-7 and 8) the $CD3^+,4^+$ T cells predominated (Fig. 1B).

IFNγ-ELISPOT assays using HuD protein spanning peptide pools

To study whether or not HuD-specific T cells could be detected among CSF-TCL from Hu-PNS patients, CSF-TCL were assayed for HuD reactivity. HuD-specific T cells were

Table 1. Patient and CSF characteristics at study entry

PNS Patient	Age/ gender	Hu-Ab titer serum	CSF					PNS	Tumor	Therapy	symptoms-diagnosis (months after onset)	symptoms-assay (months after onset)	MRS	HLA
			Protein (g/L)	Hu-Ab titer	ASA_{IT}/ ASA_{serum} [1]	MNC (cells/μL)	PNC (cells/μL)							
Hu_1	64/F	102,400	0.38	2,048	3.2	2.3	<0.3	PLE	Lung[2]	None	1	1.5	3	A*0201
Hu_2	61/F	204,800	1.70	32,768	2.3	<0.3	<0.3	PLE	SCLC	Chemo	1	1.5	5	A*0101
Hu_3	66/M	102,400	0.33	256	0	1.0	<0.3	PCD	SCLC	None	2.5	3.5	3	A11,A31
Hu_4	69/F	51,200	0.65	512	0.4	3.3	<0.3	PLE + PSN	SCLC	None	6	6.5	3	A*0101
Hu_5	51/F	102,400	0.44	2,048	6.5	2.3	<0.3	PLE + PSN	No[3]	None	8	8.5	3	A*0101, A*0201
Hu_6	71/F	102,400	1.18	2,048	5.6	14.0	0.3	PSN	SCLC	None	1.3	1.5	3	A*2402
Hu_7	75/M	3,200	0.48	128	12.2	5.3	<0.3	PSN	SCLC	None	2	4	4	A*0201
Hu_8	66/M	25,600	0.76	4,096	10.7	10.0	<0.3	PSN	NSCLC	None	2.5	3	3	A*0101
Hu_9	53/F	51,200	0.66	512	2.6	6.7	<0.3	PSN	SCLC	None	2.5	3.0	2	A*0201
Hu_10	67/F	51,200	0.76	256	0	11.0	<0.3	PSN	No[3]	None	2.5	3	3	A*0201
Hu_11	56/F	6,400	1.89	256	6.3	3.0	1.0	PSN	SCLC	IVIG	6	6.5	3	A*0201
Hu_12	64/F	102,400	0.15	256	0	1.7	0.7	PSN	NSCLC	Chemo	12	15	2	A*0201
Hu_13	61/M	51,200	1.38	8,192	6.1	16.0	<0.3	PSN	Lung[2]	None	2.5	3	3	A*0101

[1]The ratio of intrathecal Hu antibody-specific activity (ASA_{IT}) / serum ASA.[229] A ratio > 2 indicates intrathecal Hu antibody synthesis.

[2]Tumor mass visible on CT-scan.

[3]No tumor mass visible on CT-scan or FDG-PET scan.

Hu-PNS patient = patient with Hu antibody associated PNS; Hu-Ab = Hu antibody; CSF= cerebrospinal fluid; ASA = antibody specific activity; IT = intrathecal; MNC = mononuclear cells; PNC = polynuclear cells; PNS = paraneoplastic neurological syndrome; MRS = modified Rankin score; HLA = HLA-class I phenotype; F = female; M = male; PLE = paraneoplastic limbic encephalitis; PCD = paraneoplastic cerebellar degeneration; PSN = paraneoplastic sensory neuronopathy; SCLC = small cell lung cancer; NSCLC = non-small cell lung cancer; IVIG = intravenous immunoglobulins.

identified by IFNγ-ELISPOT assays using HuD protein overlapping 15-mer peptides and using autologous BLCL as APC. Background signals, but not HuD-specific signals, increased when using CSF-TCL at a concentration of 3 x 10^4 T cells per well instead of 1 x 10^4 T cells per well (not shown). Irrespective of the diagnosis or treatment, the CSF-TCL of both Hu-PNS and control patients showed similar positive T cell responses upon stimulation with the T cell mitogen PHA (Fig. 2A). In contrast, no HuD 15-mer specific responses were seen in either Hu-PNS or control patients (Fig. 2A).

IFNγ-ELISPOT assays using HuD derived 9-mer peptides

Next, the reactivity of the CSF-TCL towards a selected set of HLA-A*0101, A*0201 and A*2402 binding 9-mer HuD peptides was determined. These peptides have previously been identified and employed to detect HuD-specific $CD8^+$ T cells in PB of Hu-PNS patients.[64] In none of our Hu-PNS and control patients, T-cell reactivity against these peptides was observed (Fig. 2B).

Analysis of HuD-specific T cells in CSF and CSF-TCL using HLA class I HuD multimers

HLA class I multimers based on the HLA-A*0101**,** HLA-A*0201 and HLA-A*2402 alleles were loaded with the appropriate 9-mer HuD peptides. These reagents were tested on fresh CSF samples from 6 Hu-PNS patients and on CSF-TCL from 8 Hu-PNS patients and 4 controls, all expressing the appropriate HLA-A alleles. No positive results were observed in any of the samples tested with these multimers. In addition, no differences in specific staining with HLA class I HuD multimers were observed between CSF-TCL from Hu-PNS patients and those from the control patients (data not shown). Figure 3 shows a representative example of CSF-TCL from patient Hu-10 (for explanation, see legend).

DISCUSSION

To address the possible role of HuD-specific T cells in Hu-PNS[1], we determined the presence of these cells in the CSF from 13 Hu-PNS patients and 4 non-HuPNS control patients. To obtain sufficient numbers of T cells from CSF for these analyses, T-cell lines (TCL) were generated by non-specific expansion and subsequently assayed for reactivity in an IFN-γ ELISPOT assay against overlapping 15-mer peptides spanning the entire HuD protein, and against HuD-derived 9-mer peptides that had been studied by others.[64,66] Given a detection limit of 0.05-0.15% of expanded T cells depending on the input number of cells in the assays, no HuD reactive T cells were detected among CSF-TCL derived from Hu-PNS patients or controls. In this context it is noteworthy that with a similar IFN-γ ELISPOT assay, myelin-specific T cells have been detected in the CSF of MS patients in frequencies ranging from 0.01% of expanded $CD4^+$ T cells[235] to 0.2% of freshly isolated CSF T cells.[225,226]

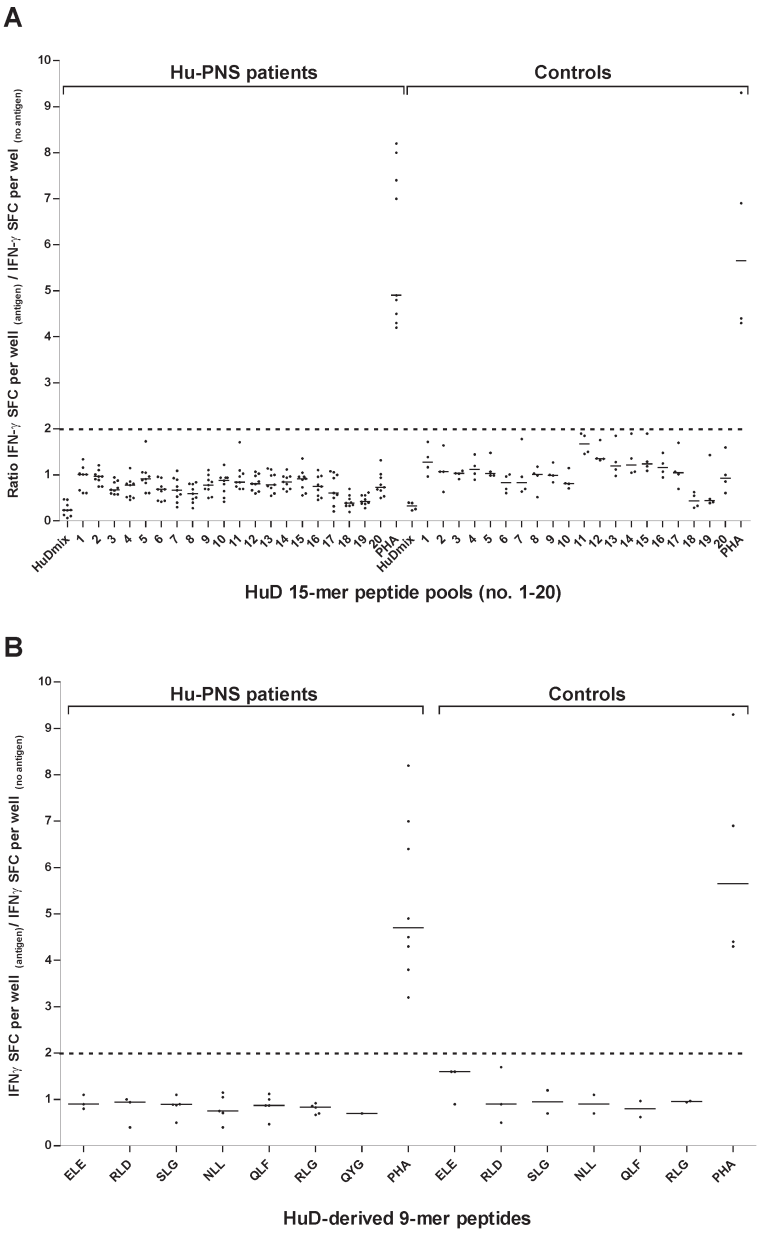

Figure 2. HuD peptide specific T cell responses

Expanded CSF-derived T cell cultures were stimulated with pools of HuD-derived 15-mer peptides or PHA (Panel A) or with individual 9-mer HuD-derived peptides (Panel B), and assayed by IFN-γ ELISPOT as described in Materials and Methods. Results of assays based on 1×10^4 T cells are shown. The ratios between the number of IFN-γ spot-forming cells (SFC) following stimulation in the presence or absence of antigen or mitogen are shown for individual Hu-PNS and control patients; horizontal bars indicate median values for each peptide, peptide pool or PHA. The horizontal line at ratio = 2, indicating the threshold for assay positivity, is shown as a reference.

Abbreviations: CSF-TCL = CSF derived T cell line; HuDmix = HuD protein spanning 15-mer peptide pool; PHA = phytohemagglutinin; IFN = interferon; ELE, RLD, SLG, NLL, QLF, RLG and QYG designate individual 9-mer peptides.

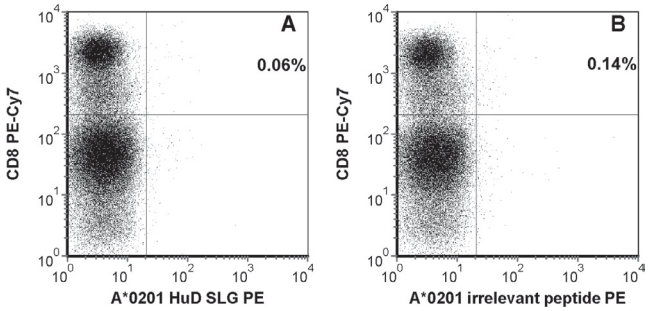

Figure 3. Staining of CSF cells with HLA Class I multimers loaded with HuD peptides

Expanded CSF-derived T cells obtained from patient Hu-10 (expressing HLA-A*0201) were stained separately with four different HuD-HLA-A*0201 multimers and the HLA-A*0201 multimer loaded with the irrelevant peptide as negative control. Here, a representative example is shown of CSF-TCL stained with HLA-A*0201 multimers loaded with the HuD peptide SLGYGFVNYI (Panel A), or with the irrelevant peptide (Panel B). Data shown are obtained after selection of T cells (i.e., $CD3^+$, low sideward scatter signals).[195] Binding of Class I HLA multimers (horizontal axes) was analyzed in relation to CD8 expression (vertical axes). The markers were set to discriminate between $CD8^{bright}$ and other T cells, and between detectable and nondetectable binding of multimers. In this example, the proportion of HuD multimer binding cells (0.06% of $CD8^+$ T cells) was below that of irrelevant multimer-binding cells (0.14% of $CD8^+$ T cells); any binding resulted in low-intensity fluorescence signals only.

We also used HuD peptide-loaded HLA-class I multimers to directly visualize HuD-specific $CD8^+$ T cells. Using this technology, the detection limit of our assay was 1% for freshly isolated CSF T cells, and 0.01% for CSF-TCL. Again, no HuD-specific T cells were detected, either in freshly isolated CSF T cells or CSF-TCL. The same approach has been successful to detect virus-specific $CD8^+$ T cells in CSF, albeit in frequencies well above 1% of $CD8^+$ T cells.[223,224] HLA Class I multimers have been successfully applied to detect transaldolase (TAL)-specific $CD8^+$ T cells in the blood from MS patients after cloning.[236] In the latter study, PBMC were first depleted of $CD4^+$ T cells, stimulated with TAL peptides for 7 days and subsequently assayed for multimer binding, revealing frequencies of approximately 1% of TAL-specific $CD8^+$ T cells after culture.[236]

Autoantigen-specific $CD4^+$ T cells have been detected in the CSF from patients with MS using a combination of different approaches such as pre-depletion of CD4-negative cells[235], pre-stimulation of CSF cells with antigen and IL-2 such as in limiting dilution assays[227,237], and alternative read outs such as lymphocyte proliferation[227,237,238] in addition to ELISPOT.[225,226] Using these approaches, myelin-reactive T cells could be detected in frequencies ranging from 0.005 to 0.2 % of CSF $CD4^+$ T cells.[225-227,235,237]

Possible explanations of our negative results are the preferential expansion of non-HuD reactive T cells and failure of HuD-specific T cells to produce IFN-γ (i.e., anergic T cells). The majority of T cells in the CSF were $CD4^+$, but $CD8^+$ T cells were preferentially expanded in our culture system. Although 15-mer protein-spanning peptide pools elicit both $CD8^+$ and $CD4^+$ T-cell responses[193,239], the preferential expansion of $CD8^+$ T cells may have hampered the detection of $CD4^+$ HuD-specific T cells. Although the detection level of our multimer assay on fresh CSF was only 1.0 % of $CD8^+$ T cells, the negative outcome

of HuD multimer staining on fresh CSF renders the presence of $CD8^+$ HuD-specific T cells in the CSF less likely.

In conclusion, we were unable to detect HuD-specific T cells in the CSF of Hu-PNS patients and non-Hu-PNS control patients, questioning the postulated role of HuD-specific T cells in the immunopathology of Hu-PNS. The IgG_1 isotype predominance and high titers of Hu antibodies in Hu-PNS indicate a T-helper response to the Hu antigen.[42] Furthermore, the presence of intrathecal Hu antibody production in the majority of Hu-PNS patients is also indicative for the involvement of local Hu-specific $CD4^+$ T cells.[232] Whilst our approaches have been mainly geared towards the detection of $CD8^+$ T-cell responses against HuD, future studies of HuD-specific T-cell responses should focus on those mediated by $CD4^+$ T cells.

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Chapter | 7

The impact of impurities in synthetic peptides on the outcome of T-cell stimulation assays

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ABSTRACT

Protein-spanning peptide pools have proven valuable as a screening tool for detecting T-lymphocyte responses against a wide range of proteins. We have used this approach in our search for T cells reactive to the onconeural protein HuD. We found positive responses in only 3 of 127 individuals; however, these were highly unusual in that the same class I HLA alleles and peptides were involved. These T-cell responses were not confirmed when peptides re-synthesized by the same manufacturer with similar and with higher purity levels were used. Our observations indicated that these T cell responses were not directed against the designed HuD peptides. Here, we report on (i) MALDI-FT Mass Spectrometry based comparisons of the peptide batches that did -and did not- elicit T cell responses and (ii) a detailed analysis of the various by-products of peptides, irrespective of T-cell assay outcome. We found numerous differences between the peptide batches, such as omissions of amino acids in the primary structure of the peptides. Furthermore, some batches revealed strong interactions with calcium ions or contained sulfated peptides. Our data reveal that different batches from the same peptide may contain artefacts that influence the outcome of HLA-restricted T-cell response assays.

INTRODUCTION

The analysis of peptide-specific T lymphocytes is an important tool for studies analyzing immune responses against a wide range of microbes (e.g., bacteria, viruses and parasites), against autoantigens and against malignant tumors. The acquired immune response involves the activation of T cells upon their encounter with specific antigen. T lymphocytes express T-cell receptors (TCR) for the recognition of antigens. Interaction of the TCR with its specific antigen leads to the activation of T cells often resulting in the secretion of cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α. In order to be recognized by a TCR antigens have to be presented by antigen presenting cells (APC) via the human MHC complex (human leukocyte antigen, HLA). MHC molecules have specific binding grooves for linear antigens (peptides). The two major T-cell subsets, i.e., $CD4^+$ and $CD8^+$, serve specific and different immune functions but a detailed description of these functions is beyond the scope of this article. $CD4^+$ and $CD8^+$ T cells specific for defined antigens can be identified through detection of intracellular cytokines after *ex vivo* stimulation on peripheral blood mononuclear cells (PBMC; i.e., T cells and APC) with the relevant peptides.[240] Protein-spanning peptide pools, i.e., mixtures of overlapping 15-mer peptides, have been shown capable of efficiently stimulating both $CD4^+$ and $CD8^+$ T-cell responses.[193,241] Such protein-spanning peptide pools have been shown to be valuable tools for the detection of T-cell responses against virally encoded proteins if the fine specificity of these responses is unknown.[193,241] However, the preparation of such a pool for a 100 kD protein using peptides of ~95% purity, for example, would require a significant investment. Therefore, peptides with purities of approximately 70% are often used in screening studies for antigen-specific T cells.[193,242,243] However, the impact of the contents of the 30% impure part of these peptides on the outcome of T-cell assays is unknown.

To detect circulating T cells directed against the onconeural HuD antigen[4], we stimulated peripheral blood cells from individuals with HuD protein-spanning pool of 15-mer peptides with purities of at least 70%. In this study of 127 individuals, we found $CD8^+$ T cell responses in only 3 cases. All 3 individuals shared HLA-A*2401$^+$ and HLA-B*1801$^+$, and the $CD8^+$ T cell responses were directed against the same three 15-mers, i.e., peptide #38, #40 and #86 (batch 1, 71% purity; Fig. 1). This highly unusual result could not be reproduced when using a new batch of peptides with 82% purity from the same manufacturer (batch 2; Fig. 1), or with peptides with a higher level of purity (93%; batch 3; Fig. 1). These findings indicated that the observed T-cell responses were not directed against the HuD antigen.

Here, we report on MALDI-FT mass spectrometry based comparisons of the peptide batches that did - and did not - elicit T-cell responses. During these analyses we encountered numerous differences in the peptide batches such as omissions of one or more amino acids, but also strong calcium ion interactions irrespective of T cell assay outcomes; in addition we found various sulfated peptides.

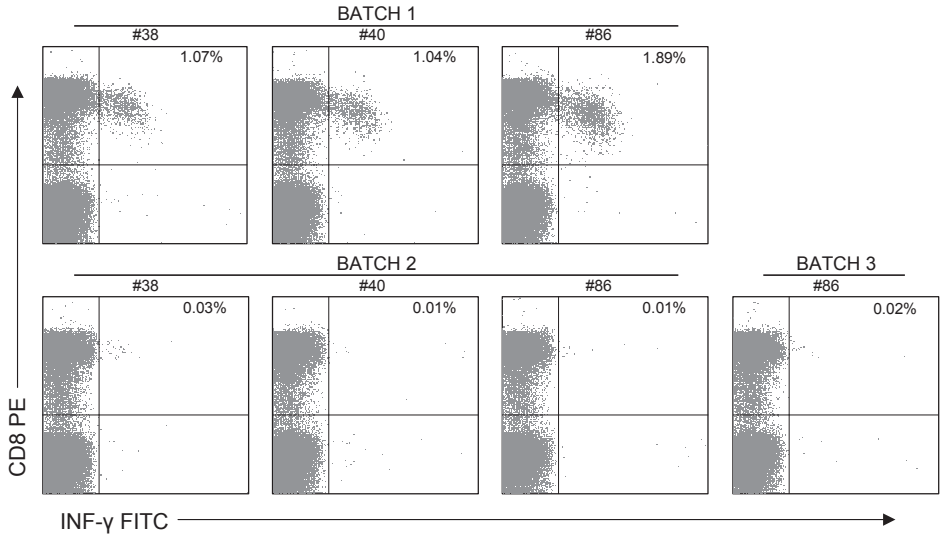

Figure 1.

Assessment of $CD8^+$ T cell-responses to peptides, i.e., batch 1 (71% purity peptides #38, #40 and #86); batch 2 (82% purity peptides #38, #40 and #86), and batch 3 (94% purity peptide #86). All data shown are obtained after selection of T cells (i.e., $CD3^+$, low side scatter). To calculate the percentages of IFN-γ producing $CD8^+$ T cells in response to peptide, thresholds were set on the unstimulated cells (not shown) and the percentage of IFN-γ producing $CD8^+$ T cells in the unstimulated sample were subtracted from the corresponding percentages in the peptide-stimulated samples. According to these criteria, the T cell responses towards batches 2 and 3 were regarded negative.
CD3 = T-cell marker; IFN = interferon.

Experimental

The final purity of the synthesized product is a function of the coupling efficiency of amino acids, the number of amino acids incorporated in the synthesized peptide and the chromatography methods used.[244] Impurities such as salts, deletion peptides (formed due to incomplete coupling), protecting groups and modified peptides can range from 10-50% by weight. To obtain purities of >70%, purification of the synthetic peptides is generally carried out on a semi-preparative HPLC system. Additional purification can lead to purities of >90%. Fractions containing the peptide are collected and lyophilized. The actual purity depends on how conservatively the fractions are pooled. Users need to specify what level of purity is required. According to our requirements, all peptides had >70% purity and some were prepared as to have >90% purity. Characterization of by-products can be achieved by liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS).[245] Here we use MALDI-FTMS to characterize such by-products. The very high mass accuracy and high sensitivity of this technique eliminates the need for time consuming pre-fractionation.

Synthetic peptides

As mentioned above the peptides of batch 1 elicited $CD8^+$ T-cell responses whereas those of batches 2 and 3 did not.

For batch 1, custom 15-mer peptide synthesis was performed on an APEX 396 synthesizer (Advanced ChemTech, Giessen, Germany) at JPT Peptide Technologies GmbH ([JPT]; Berlin, Germany) using a standard Fmoc-based solid phase synthesis protocol on an TCP-resin (Trichlorophenyl-resin) support. Double couplings (2 x 45min) were performed using 4 equiv. Fmoc-amino acid/ *N*-[(1H-benzotriazol-1-yl)-(dimethylamino)methylene]-N-methyl-methanaminium hexafluorophosphate *N*-oxide (HBTU) (4 eq.)/ diisopropylethylamine (DIEA) (8 eq.) in dimethylformamide (DMF) (coupling concentration: 0.25 M). For Fmoc-deblocking a solution of 20% piperidine in dimethylformamide was used. Finally, peptide-resin cleavage was performed with a solution consisting of 2% triisopropylsilane, 5% water, and 5% phenol in trifluoroacetic acid (TFA) for 2 h. After precipitation in tert-butyl methyl ether peptides were purified when not fulfilling the target specification of 70% by preparative HPLC using a Merck Hitachi D-7000 preparative HPLC-system on a Merck Lichrosorb RP-18 (250 mm x 25 mm ID, 7 µm) column. Peptides were eluted using a linear gradient, eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in 80% acetonitrile/20% water (v/v). After lyophilisation all peptides were shown to have a purity >70% according to HPLC (HP1100, Agilent, Waldbronn, Germny) and consistent MALDI-TOF-MS data (Voyager-DE, Biospectrometry Workstation, Perseptive Biosystems Inc., Framingham, MA, USA).[246] All peptides were accompanied by Analytical Data Sheets including the raw data of HPLC and MS. Batch 2 was prepared by JPT two years after batch 1. The synthesis method of batch 2 differed from that of batch 1 in that a SyroII peptide synthesizer (MultiSynTech, Witten Germany), a Tentagel©polyhydroxybutyrate (TG-PHB)-resin and a Benzotriazole-1-yl-oxy-tri-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) coupling reagent were used. Batch 3 was prepared as batch 2 and was subsequently purified by HPLC to a target specification of >90%.

The individual 15-mer peptides were dissolved in DMSO (Sigma, St. Louise, USA) at 100 mg/ml and a small portion of the dissolved peptides was diluted to 0.2 mg/ml and aliquoted for use in the T cell assays. The peptides were stored at –80°C.

Detection of antigen-specific T cells in a cytokine flow cytometry assay

Peripheral blood mononuclear cells (PBMC) were isolated within 12 hours after venipuncture and stimulated as described elsewhere.[193,241] Briefly, 2 x 10^6 PBMC were incubated at 37°C in a CO_2 incubator for 18 hours with 1 µg/ml synthetic peptide, 1 µg/ml ionomycin plus 25 ng/ml phorbol myristate acetate (PMA) (positive control) or without antigen (negative control). After 2 hours of stimulation, brefeldin A was added to one of the duplicate tubes allowing for intracellular accumulation of cytokines in activated T cells. Stimulated PBMC were stained and analyzed using CD3 monoclonal antibody (mAb) conjugated

with peridinyl chlorophyllin (PerCP), CD8 mAb conjugated with allophycocyanin (APC), anti-interferon (IFN)-γ mAb conjugated with fluorescein isothiocyanate (FITC), anti-tumor necrosis factor mAb (TNF)-α conjugated with PE, or the appropriate isotype control mAb (all from BD Biosciences, San Jose [CA]). $CD4^+$ T cells were defined as $CD3^+,8^-$. Responses were regarded as positive if the percentage of cytokine-positive $CD4^+$ or $CD8^+$ T cells was >2 times the negative control (i.e., no antigen) *and* ≥ 0.1% of the total number of $CD4^+$ or $CD8^+$ T cells, each after subtraction of isotype control results.

MALDI-FT Mass Spectrometry

Sample preparation

The concentration of our peptide stock solutions was 0.2 mg/mL in DMSO, which corresponds to 100 pmol/µL. Prior to mass spectral analysis, the peptides were diluted 10 fold in water containing 0.1% TFA and 0.5 µL of this dilution was mixed with 0.5 µL of the matrix solution (10 mg/mL DHB in 0.1% TFA) on a target plate and allowed to dry at ambient temperatures. Thus, about 5 pmol of the peptide is deposited on the target plate. This relatively large amount of material (in comparison to peptide calibration mixture where usually 1 pmol is deposited on the plate) enables the detection of contaminants down to 0.1 % (intensity relative to the peptide signal intensity, base peak = 100 %) at a signal to noise ratio (S/N) > 10. A contaminant is considered present when the monoisotopic and the first isotope peak are present at a S/N > 10.

Instrumentation

The measurements were performed by MALDI-FT-MS (Apex Q 9.4 Tesla equipped with a combi-source, Bruker Daltonics).[247] For each measurement 50 scans were added and for each scan ions generated by 10 laser shots were accumulated in a mass range of 800–4000 *m/z*. Total accumulation time was 3 minutes. FTMS spectra were processed with a Gaussian filter and 2 zero fillings. A standard peptide calibration mix (Bruker Daltonics) which contains angiotensin I and II, substance P, Bombesin, Renin Substrate, ACTH clip 1-17, ACTH clip 18-39 and Somatostatin 28 was used for external calibration. MS/MS experiments were also performed on a Bruker Ultraflex MALDI-TOF mass spectrometer as described previously.[248]

Results and Discussion

Low mass and high mass impurities

General aspects

The FTMS mass spectra of the synthetic peptides revealed that several peaks at masses lower than that of the synthetic peptide were present. It is important to note that a particular minor low mass component, as detected by FTMS, may not represent a peptide impurity but results instead from dissociation of the synthetic peptide itself.[249] Such dissociations may occur within the ion source or during transit to the ICR cell. These metastable fragmentations are the result of the relatively large ion lifetime (1 s) of the ions prior to detection in FT, compared to the much shorter lifetimes associated with more conventional MS techniques, such as time-of-flight (10^{-4} s), and thus they are a direct consequence of RRKM theory.[250] To minimize the occurrence of such fragmentations, our FTMS apparatus uses an in-source pulsed gas and accumulates ions in a hexapole ion guide at peak pressures prior to pump down and transmission to the ICR cell.[247] To further reduce these fragmentations the laser power is set at 5% above the threshold value.

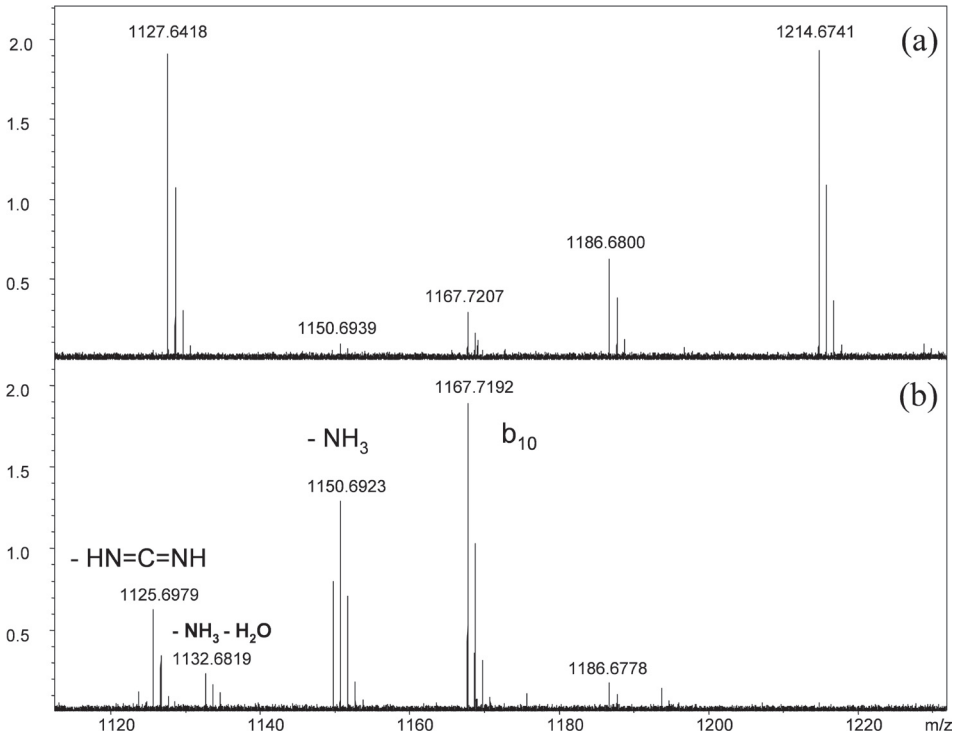

Figure 2.

(a) Partial FTMS mass spectrum of the synthetic peptide #40 RIITSRILVDQVTGV ; (b) the same mass spectrum run under conditions of induced fragmentation (quadrupole voltage = - 60 V. (Y-axis: intensity relative to base peak = protonated peptide.)

Nevertheless, even under such controlled conditions, minor peaks might appear which may represent fragment ions.

In Figure 2a a small part of the mass spectrum of the synthetic peptide 40 (RIITSRIL-VDQVTGV, protonated form, m/z = 1669.9959) is given. In this small mass range at least five monoisotopic peaks are present. Of particular interest are the weak signals at m/z 1167.7207 (0.3% of base peak) and m/z 1150.6939 (0.08%). It was observed that the MS/MS mass spectrum of the protonated peptide at m/z 1669.9959 was dominated by the b_{10} fragment at 1167.7207. Naturally then, the concern arises that this peak in Figure 2a might not be a peptide impurity, but instead may correspond to the b_{10} fragment of peptide 40. To differentiate between fragmentation and a real peptide impurity a potential of -60 V was applied to the quadrupole collision cell (without any mass isolation), thereby inducing fragmentation of all peptides, and a second mass spectrum was recorded. Peaks that result from dissociation will increase in intensity, whereas those that correspond to impurities will disappear because these peptides will dissociate. The partial FT mass spectrum of peptide 40, recorded with induced dissociation is given in Figure 2b. It can be seen that certain peaks disappear or are considerably reduced in intensity. These are the true peptide impurities. Other peaks, namely fragment peaks, become much more prominent, such as the b_{10} peak at m/z 1167.71920 and its fragments at m/z 1150.6923 (loss of NH_3) and m/z 1125.6980 (loss of [C, H_2, N_2], likely HN=C=NH being loss of carbodiimide from an R group). The purpose of our study was to detect any differences in identity or relative amounts of contaminants in different batches of the same peptide.

Comparison of peptide batches 1 and 2

It may be argued that for comparative purposes, running spectra under induced fragmentation conditions is not necessary. Nevertheless, all spectra were run without and with induced dissociation and the differences between batches 1 and 2 were identified in terms of peptide impurities. For peptides 38 and 40 such experiments revealed a similar difference between batches 1 and 2. Exact mass measurements show that for both peptides batch 1 (which elicited the positive reaction in our T cell analyses, see Fig. 1) contained a peptide lacking the amino acids I or L and T. The MS/MS spectrum of peptide 38 (EQLFSQYGRIITSRI) is dominated by a b_{14} peak, corresponding to the loss of the C-terminal amino acid isoleucine, i.e. – I – H_2O. The MS/MS spectrum of the –I (L) –T peak contained a very intense signal for the b_{12} peak, again corresponding to loss of the C-terminus isoleucine. From these data we conclude that in the peptide impurity, the I amino acid is still at the C-terminus. Similar experiments were performed on the – I (L) –T peak for peptide 40 and a comparative analysis of the results for peptides 38 and 40 strongly indicated that peptides 38 and 40 both lack the adjacent amino acids IT. Thus, for peptides 38 and 40 we could identify one major, common difference between batches 1 and 2. The situation for peptide 86, GFVTMTNYDEAAMAI, appeared more complicated as revealed

by comparison of the spectra of batches 1 and 2. In Figure 3 are given the partial FT mass spectra of batches 1 and 2. Panel A shows the partial mass spectrum of batch 1 and panel B the partial mass spectrum of batch 2. Batch 1 contains additional intense signals corresponding to peptides lacking V or A (but not both). In addition, the protonated sodium salt (or the sodiated peptide) of the –V peak ($-H^+ + Na^+$) is clearly present as indicated. The peak at 1572.6029 is not the protonated potassium salt ($-H^+ + K^+$) as might otherwise have reasonably been expected, but rather, according to the exact mass, the protonated calcium salt ($-2H^+ + Ca^{2+}$): the calculated mass for the $-H^+ + K^+$ component is 1572.6111, that for - $2H^+ + Ca^{2+}$ is 1572.6035, compare to the measured value of 1572.6029 ± 0.0012 (external calibration). The intensity of the –V peak is ca. 11 % of that of m/z 1633.72348, whereas the S/N ratio is 12 % of m/z 1633.72348. Thus these –V and –A components are major contaminant contributors. The MS/MS spectra of GFVTMTNYDEAAMAI and the –V and –A components are given in Figure 4. The major ions are b type product ions ($b_2 - b_{14}$, $b_2 - b_{13}$). The MS/MS spectrum of the –V component is precisely as predicted for the sequence GFTMTNYDEAAMAI. The MS/MS spectrum of the –A peak reveals that this impurity consists of two components, namely GFVTMTNYDEAAMI and GFVTMTNYDEAMAI, although for the latter we cannot say which A of the original AA sequence is missing. Analysis of the FT mass spectral data revealed other impurities in the two batches and these are listed in Table 1. Batch 1 contains more impurities than batch 2 and most of these correspond to peptides lacking one or two amino acids. Again, true impurities, as opposed to fragments, were identified by their disappearance under fragmentation conditions; thus the – G component listed in Table 1 is a true impurity and not a y_{14} fragment. Such experiments, as well as exact mass measurements, show that the – M peak listed in Table 1 (at m/z 1502.6831) is not a b_{14} fragment corresponding to loss of the C-terminus isoleucine (m/z = 1502.6291. The differences between batches 1 and 2 may be explained by the use of different peptide synthesizers and differences in synthesis protocols, i.e. use of different resins and different coupling reagents, see Experimental.

Earlier work has shown that peptides that do not contain basic amino residues (R, K, H) show nearly complete delocalization of charge in the $[M + H^+]$ ions.[251] This means that an ensemble of $[M + H^+]$ ions is formed with the proton residing at various sites of the peptide molecule in a more or less random way. Such protonated peptides fragment mainly to b-type ions. As can be seen from Figure 4a this is indeed the case: the peptide GFVTMTNYDEAAMAI shows an almost complete b series of ions. That the b-ions become more intense at higher masses can be explained in terms of charge delocalization in the product ions, which makes large ions more stable than small ones. Thus the MS/MS mass spectrum of peptide 86 can be rationalized in terms of charge delocalization both in the precursor and in the product ions.

It is well known that R has the most significant influence on the fragmentation behavior of protonated peptides.[251] This is because R has the largest proton affinity of all amino

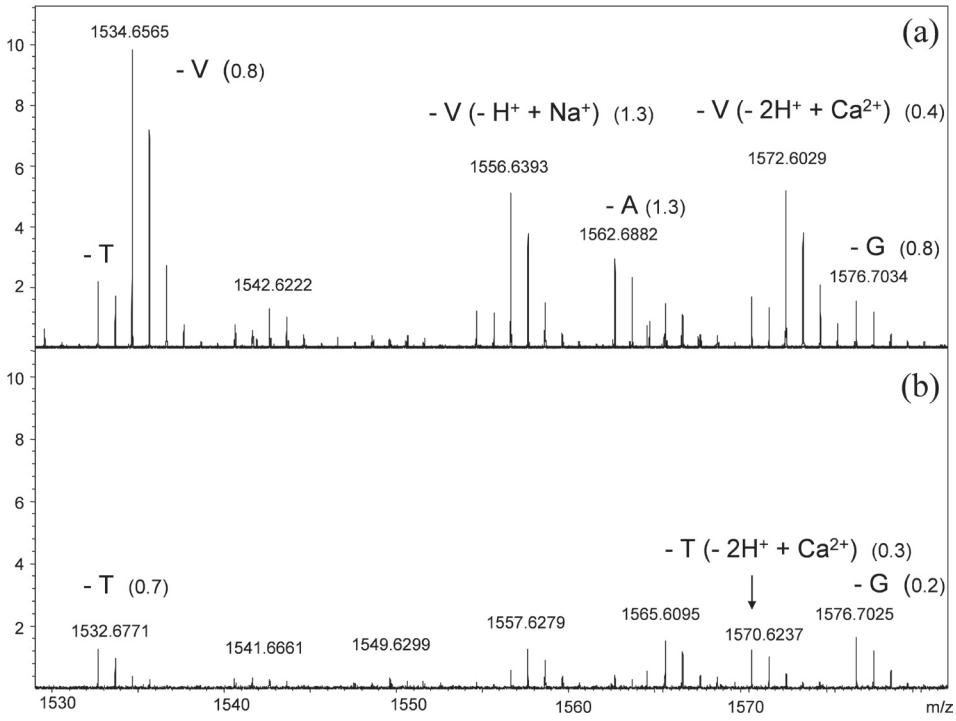

Figure 3.

Partial FTMS mass spectra of the synthetic peptide #86 GFVTMTNYDEAAMAI; (a) batch 1; (b) batch 2. Values in parentheses refer to the mass deviation (in ppm) of the calculated and measured masses (external calibration). (Y-axis: intensity relative to base peak = protonated peptide.)

acids[252] leading to charge localization in the protonated peptide. This is well illustrated by the dissociation behavior of peptides 38 (EQLFSQYGRIITSRI) and 40 (RIITSRILVDQVTGV) compared to that of peptide 86 (GFVTMTNYDEAAMAI). While peptide 86 shows an almost complete b-series of ions, the MS/MS spectra of peptides 38 and 40 show only one major fragment at b_{10} and b_{13} respectively.

The synthetic peptides studied contained signals of various intensities at masses higher than that of the protonated peptides. Thus, it was observed that the mass spectra of the peptides 86, GFVTMTNYDEAAMI, and 87, MTNYDEAAMAIASLN, contained very intense signals for peaks nominally 38 Daltons higher. These signals were as intense as those for the protonated species. Exact mass measurements indicate that these peaks appear at 37.9468 Daltons higher. Surprisingly, this corresponds to exchange of two protons by one Ca^{2+} (calc. + 37.9470) and not to the exchange of one proton by one K^+ (calc. + 37.9558 Dalton). Presumably the presence of the two (adjacent) acidic amino acids D and E in the peptides 86 and 87 makes exchange with calcium possible leading to a bidentate structure $COO^-\bullet\bullet\bullet\bullet Ca^{2+}\bullet\bullet\bullet\bullet{}^-OOC$.

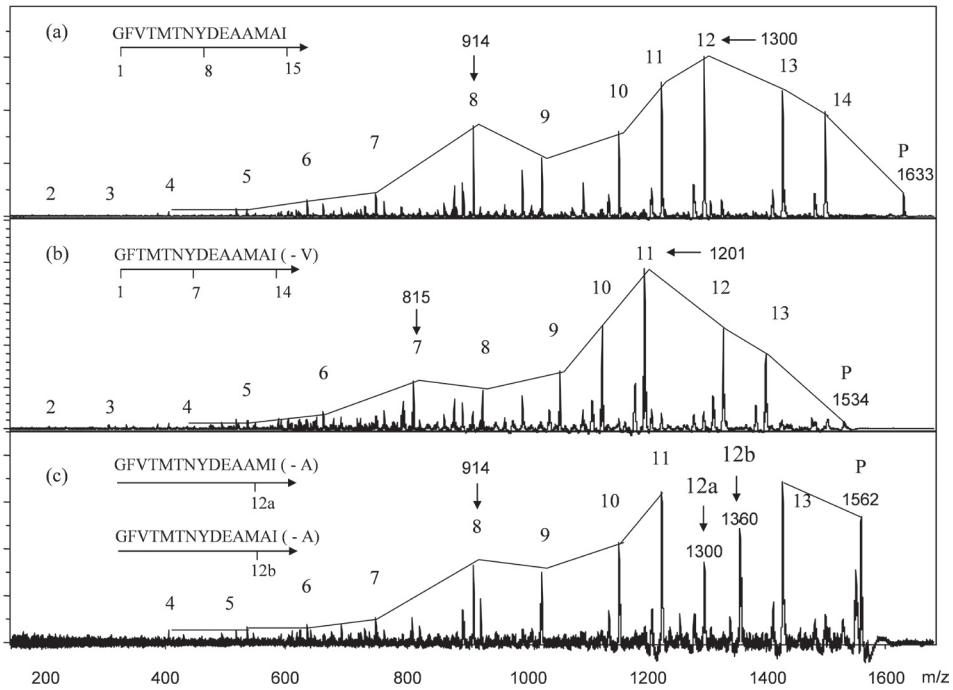

Figure 4.

MS/MS spectra of (a) GFVTMTNYDEAAMAI, (b) the –V deletion peptide and (c) the –A deletion peptide. The MS/MS mass spectrum of panel b is consistent with the sequence GFVTMTNYDEAAMAI, whereas the spectrum in panel c shows that two –A deletion peptides are present as indicated.

Several peptides, e.g. peptide 38, contained intense signals at nominally 80 Daltons higher than that for the protonated peptide. Exact mass measurements show that these peaks appear at 79.9572 ± 0.0006 Daltons higher, corresponding to a sulfated (calc. + 79.9568) rather than to a phosphorylated (calc.+ 79.9663) peptide. The MS/MS spectra of these sulfated peptides show a loss of HSO_3, i.e. the sulfated peptides fragment back to the protonated peptide, precluding identification of the sulfation site. We observed that when the peptide has a serine (S) amino acid, these sulfated peptides were invariably present. For example, the intensity of the sulfated peptide for peptide 38 is 11 % of the protonated signal, whereas for peptide 86, which does not contain S, no sulfated peptide could be detected.

Comparison of batches having different purities

Synthetic peptides can be purchased with different degrees of purities, e.g. 70% and 90% purity with an approximate 2.5-fold corresponding price difference. For the peptide 86 we compared the MALDI-FTMS spectra for batches having a purity of 82% (batch 2) and 93% (batch 3) respectively according to the manufacturer's analysis. These batches result

Table 1. Impurity peptides present in batches 1, 2 and 3 of peptide #86, GFVT1MT2NYDEA1A1MA2I (m/z 1633.7236)

Exact mass	Amino acid missing	Batch 1 (70.6%)		Batch 2 (81.9%)		Batch 3 (93.4%)		Sequence confirmed by MS/MS
		Present[a]/ absent	Relative intensity (%)	Present[a]/ absent	Relative intensity (%)	Present[a]/ absent	Relative intensity (%)	
1576.7022	- G	+	1.9	+	2.0	+	1.3	yes
1486.6552	- F	+	6.3	-		-		yes
1534.6552	- V	+	11.7	-		-		yes
1502.6831	- M	+	1.2	-		-		no[b]
1532.6759	- T_2	+	2.7	+	1.5	+	0.9	yes[c]
1519.6807	- N	-		+	1.6	+	3.9	yes
1518.6967	- D	+	1.0	-		-		no[b]
1562.6865	- A_1	+	3.5	-		-		yes
1562.6865	- A_2	+		-		-		yes
1520.6396	- I	+	0.5	-		-		no[b]
1429.6338	- GF	+	1.0	-		-		no[b]
1387.5868	- FV	+	1.3	-		-		yes
1477.6338	- VG	+	0.5	-		-		no[b]

[a] Impurity is considered present if S/N for monoisotope and 1st isotope peak is > 10

[b] Signal too weak or interference due to neighboring peaks.

[c] Confirmed for batch 2 only; interference of – V for batch 1.

S/N = signal to noise ratio.

from the same peptide synthesis method. We focused on the presence and abundance of truncated and deletion peptide impurities in both batches, but we only found differences with respect to the abundance of impurities, see Table 1. It also appeared that the 90% purity batch contained much less calcium, as the adduct at nominal 38 Daltons higher was significantly less abundant in the 90% purity batch. However, it is entirely possible that the synthetic peptides contain material that does not appear in our MALDI-FTMS spectra, such as very low molecular weight material.

If we compare batches corresponding to different synthesis methods (batches 1 versus 2 and 3) and different purities (batches 1 versus 2 versus 3) we find some interesting features. All batches contain the truncated –G peptide in similar amounts and therefore the presence of this peptide is not related to the synthesis method or to the level of purity. Also, it can be seen, that although batches 2 and 3 clearly contain less deletion peptides, batches 2 and 3 contain a different deletion peptide, namely –N.

Implications for T-cell studies

In studies where numerous T cell responses are detected, e.g., when T-cell responses directed against cytomegalovirus (CMV) are analyzed in CMV seropositive individuals,

erroneous T-cell responses may easily go unnoticed. However, unexpected T-cell responses, such as the ones we found in our search for HuD-specific T cells, alert to the potential presence of erroneous elements in the peptides used. Our data indicate that it is important to further investigate such findings using re-synthesized peptides and to perform a thorough quality assessment of the synthesized peptides.

Conclusions

In our comparative MALDI-FTMS analysis of batches of the same peptides that did, and did not, elicit T-cell responses we observed numerous differences, such as omissions of one or more amino acids in batches that elicited T cell responses. Surprisingly, we also encountered strong calcium ion interactions and sulfated peptides in peptide batches irrespective of T-cell assay outcome. We recommend that (i) relevant T-cell responses be confirmed using re-synthesized peptides preferably with higher purity; and (ii) if possible, a thorough mass spectrometry quality assessment be performed in peptide spanning methods for those peptides that give positive responses.

Acknowledgements

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Chapter | 8

An uncontrolled trial of rituximab for antibody associated paraneoplastic neurological syndromes

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ABSTRACT

Anti-CD20 monoclonal antibody (rituximab) is effectively used in the treatment of B-cell lymphomas. Recent reports in the literature suggest that antibody associated autoimmune disorders may respond to rituximab. We therefore treated nine patients with anti-Hu or anti-Yo associated paraneoplastic neurological syndromes (PNS) with a maximum of four monthly IV infusions of rituximab (375 mg/m^2). In this uncontrolled, unblinded trial of rituximab, three patients improved ≥1 point on the Rankin Scale (RS). One patient with limbic encephalitis improved dramatically (RS from 5 to 1). Further studies of rituximab in autoantibody associated PNS are warranted.

INTRODUCTION

Paraneoplastic neurological syndromes (PNS) are autoimmune disorders that are in approximately 50% associated with antineuronal autoantibodies reactive with onconeural antigens.[36] Well characterized onconeural antibodies include anti-Hu (ANNA-1)[6,8] and anti-Yo (PCA-1).[67,68,253] Despite aggressive anti-tumor and immunosuppressive treatment, the neurological outcome in antibody associated PNS is grim and only 5-7% of patients with Hu antibodies improve ≥1 point on the Rankin Scale.[6,8] The prognosis of Yo patients is even worse: in two series of 55 and 34 patients, none of the patients improved neurologically.[67,253] Although the precise pathogenesis of anti-Hu and anti-Yo associated PNS is unknown, it has been postulated that the antibodies or antigen-specific cytotoxic T cells[63,71], or both, play a role.[1] A pathogenic role of the Hu and Yo antibodies has been suggested by high CSF titers and intrathecal synthesis of these antibodies[221] but could never be proven in animal models.[254,255]

Rituximab (Roche Ltd, Basel, Switzerland) is a chimeric anti-CD20 monoclonal antibody that is approved for the treatment of CD20+ B-cell non-Hodgkin's lymphoma. CD20 is a surface membrane antigen expressed mainly by B-cell precursors and mature B-cells and appears to play an important functional role in B-cell activation, proliferation and differentiation. In a recent randomized controlled trial in autoimmune rheumatoid arthritis, rituximab provided significant improvement in disease symptoms with concomitant lowering of rheumatoid factor.[256] Some efficacy of rituximab has been reported in open-label studies in a variety of other antibody associated autoimmune disorders including polyneuropathy associated with IgM antibodies.[257,258]

We hypothesized that rituximab induced elimination of circulating B lymphocytes in patients with newly diagnosed anti-Hu and anti-Yo associated PNS would result in the prevention of the development of antibody secreting cells, in a reduction of autoantibody titers and in clinical improvement or stabilization.

MATERIALS AND METHODS

Patients

From February 2001, we identified nine patients with newly diagnosed antibody-associated PNS (Table 1). Eight patients had Hu and one patient had Yo antibodies, all at titers ≥3200. Five Hu patients suffered widespread PEM/SN while the syndrome was more restricted in three (limbic encephalitis, cerebellar ataxia and sensory neuronopathy) (Table 1). The Yo patient suffered from typical paraneoplastic cerebellar degeneration (PCD). Six patients had a histologically confirmed neoplasm and in the other three patients a lung

tumor was radiologically suspected (bronchoscopic biopsy negative). In seven patients the tumor was diagnosed only after the onset of the neurological symptoms.

Treatment

After obtaining informed consent, patients were treated with a maximum of four monthly IV infusions of rituximab (375 mg/m^2). The first dose was given as inpatient; subsequent doses were administered in day care. Patients were pretreated with acetaminophen 1,000 mg PO and clemastine 2 mg IV to reduce the frequency of side effects. The only side effect that occurred during the infusions was transient lowering of blood pressure, which required a slower infusion rate for one of the nine patients. There were no infections despite elimination of all detectable circulating B cells in eight patients. Patients 2, 6 and 8 received concomitant standard chemotherapy for SCLC without infectious complications.

Outcome

The neurological disability was assessed using a modified Rankin scale (RS).[18] On the modified RS a score of 0 represents an asymptomatic patient; 1, symptoms that do not interfere with lifestyle; 2, symptoms that lead to some restriction of lifestyle but do not prevent totally independent existence; 3, symptoms significantly interfere with lifestyle or prevent totally independent existence; 4, symptoms clearly prevent independent existence, although the patient does not need constant attention; 5, severe disability with total dependence requiring constant attention; 6, death from neurological cause. A patient was considered functionally improved if there was a decrease of at least 1 point in RS measured after completing 16 weeks of treatment compared to the RS just prior to the first rituximab dose. The treatment was stopped when a patient deteriorated neurologically or when the RS increased by 1 point or more. The functional outcome was considered 'successful' when a patient with RS ≤ 3 improved or stabilized (i.e. remained ambulatory) and when a patient with RS ≥ 4 (bedridden patient) improved to ≤ 3 (ambulatory), as defined by Keime-Guibert et al.[19]

Laboratory evaluations

IgG and IgM titers of the paraneoplastic antibodies were determined on rat cerebellar sections by peroxidase immunohistochemistry and by Western blotting using purified recombinant HuD and CDR62 antigens, as described.[36,95] Serum and CSF were sampled prior to each cycle of rituximab. Multiple samples from the same patients were titrated in a single experiment by serial endpoint dilutions on rat cerebellar sections. A difference of 2 or more dilution steps was considered significant. Absolute numbers of circulating B lymphocytes were determined prior to each cycle of rituximab by a single-platform flow cytometric assay as detailed elsewhere.[259] In that assay, B lymphocytes were identified by the CD19 marker, whose expression was not affected by rituximab therapy.

To find further evidence for a pathogenic role of antibodies or cytotoxic T lymphocytes, we measured cytokines that are involved in the Th1 or Th2 immune response. The cytokines IFN-γ, TNF-α, IL-10, IL-2, IL-4 and IL-5 were measured in serum and CSF with the cytokine bead array (Beckton Dickinson, San Diego, CA). Normal serum values for all cytokines tested were <15 pg/mL.

RESULTS

In all patients the neurological symptoms progressed in the two weeks prior to study entry. Eight of the patients had not received any immunosuppressive therapy while patient 8 had been treated unsuccessfully with prednisone (60 mg per day) for two weeks prior to start of rituximab. Three patients received standard chemotherapy for SCLC at the same time that they were treated with rituximab. All three achieved a complete tumor remission (Table 1) and no adverse effects of the combination treatment were observed. Patient 5 was still in CR when she developed PNS and was treated with rituximab.

Following rituximab, three patients (# 2, 4 and 8) improved functionally as indicated by a decrease in RS of one point or more (Table 2). Patient 2 presented with seizures. After control of the seizures with phenytoin, he continued to deteriorate and finally suffered complete loss of short-term memory, was severely disoriented and required constant at-

Table 1. Patient characteristics

Patient No	Age/ Sex	Antibody	Syndrome	Sympt.- Diagnosis (months)	Tumor	Tumor – Sympt. (months)	Tumor – Diagnosis interval (months)	Tumor treatment	Response tumor
1	68/M	Hu	PCD	8	Prostate	-107	-115	Hormonal	PD
2	59/M	Hu	PLE	2	SCLC	2	0	Chemo and RT	CR
3	55/F	Hu	PEM	9	Lung (CT, PET)	9	0	No	-
4	48/F	Yo	PCD	1	Ovarian	0	-1	Surgery	PR
5	58/F	Hu	BE/PSN	1	SCLC	-6	-7	Chemo and RT	CR
6	56/F	Hu	PSN	4	SCLC	5	1	Chemo	CR
7	69/M	Hu	PEM/SN	3	Lung (PET)	5	2	No	
8	52/F	Hu	PEM/SN	2	SCLC	3	1	Chemo and RT	CR
9	80/M	Hu	PEM/SN	8	Lung (CT)	8	0	No	-

PCD, paraneoplastic cerebellar degeneration; PLE, paraneoplastic limbic encephalitis; PEM, paraneoplastic encephalomyelitis; BE, brainstem encephalitis; PSN, paraneoplastic sensory neuronopathy; SCLC, small cell lung cancer; RT, radiotherapy; PD, progressive disease; CR, complete remission; PR, partial remission.

tention (RS = 5). After diagnosis of paraneoplastic limbic encephalitis, a SCLC was found. One week after the first cycle of chemotherapy, he received rituximab. Four weeks later, his neurological condition had greatly improved (RS=1). He was discharged home and returned to a completely independent life. He completed 4 monthly cycles of rituximab and phenytoin was stopped without recurrence of seizures. Patient 4 developed PCD shortly after diagnosis of a lymph node metastasis in the groin, probably from an undetected ovarian cancer. She was bedridden at the start of treatment (RS=4). Following rituximab, she regained the ability to walk a block around the house and she finished 4 cycles (RS = 3). Patient 8 developed sensory and motor neuronopathy and cerebellar ataxia. PEM/SN was diagnosed and a SCLC was detected. She was treated with chemotherapy and rituximab and regained the ability to walk with support (RS from 4 to 3). Because of the severity of the remaining symptoms (painful sensory neuronopathy), she declined the fourth cycle of rituximab. Three patients deteriorated, two of whom died from the neurological syndrome, and three remained stable. The functional outcome was considered 'successful' in 5 of 9 patients, as defined by Keime-Guibert et al.[19]

Following rituximab, circulating CD19+ B cell levels became undetectable (i.e., <1 cell/μl) in 8 of 9 patients during the entire study period and were severely reduced in the other patient. Despite the successful elimination of circulating B cells, a significant decrease in serum IgG titer was detected in only 2 patients while the titer increased in one patient and remained stable in 6 patients (Table 2). In 6 patients, CSF titers after at least one cycle of rituximab were obtained. Only the CSF from patient 8 demonstrated a significant decrease in titer (from 64 to 8) with persisting high serum titer (Table 2). The CSF titer increased in patient 5 and remained stable in the other 4 patients.

Because of the lack of correlation between clinical response and IgG antibody titers, we further studied the IgM anti-Hu and anti-Yo titers. In 7/8 patients, we could detect anti-Hu IgM whereas no anti-Yo IgM reactivity was observed in patient 4. In patient 2, the serum

Table 2. Neurological outcome and IgG autoantibody titers in serum and CSF following rituximab treatment

Patient No.	Antibody	No. cycles rituximab	Outcome	RS start	Change RS	Serum IgG titer start	Serum IgG titer end	CSF IgG titer start	CSF IgG titer end
1	Hu	1	Stable	3	0	3200	3200	8	16
2	Hu	4	Improved	5	-4	6400	1600	200	NA
3	Hu	2	Died	4	2	3200	3200	64	128
4	Yo	4	Improved	4	-1	6400	12800	128	64
5	Hu	2	Worse	4	1	12800	12800	32	128
6	Hu	1	Died	3	3	3200	1600	32	NA
7	Hu	3	Stable	3	0	3200	25600	2048	1024
8	Hu	3	Improved	4	-1	12800	12800	64	8
9	Hu	1	Stable	4	0	51200	3200	32	NA

RS, Rankin score; NA, not available

IgM titer dropped from 12800 to 1600. In all other patients, the IgM titers remained stable at relatively low levels or decreased non-significantly.

In patient 2, the serum cytokines IFN-γ (261 pg/ml), IL-2 (3820 pg/ml) and IL-5 (180 pg/ml) were elevated while TNF-α, IL-10 and IL-4 were normal (<15 pg/ml). In all other patients serum cytokine levels were in the normal range as were all CSF cytokines measured.

Discussion

Three out of 9 PNS patients treated with rituximab had functional improvement as indicated by a decrease of at least one point on the Rankin scale. In 5 out of 9 patients the functional outcome was considered 'successful' according to the Keime-Guibert et al. criteria.[19] In previous studies that use the same outcome criteria, 0-7% of patients with anti-Hu associated PNS improved one point or more on the RS while 22-31% had a successful outcome.[8,19] In anti-Yo associated PNS, 0-5% of patients improved on the RS while 10-14% had a successful outcome.[67,68] These results suggest that rituximab may have some effect in antibody associated PNS although the numbers are too small for a definite conclusion. However, alternative explanations for the favorable outcome in our patients are likely and include concomitant anti-tumor treatment, early start of treatment and spontaneous improvement. Several studies have demonstrated that effective treatment of the tumor is important to at least stabilize anti-Hu associated PNS.[6,8,18] The improvement in two of our anti-Hu patients may have been confounded by the complete tumor remission achieved during the study period. Spontaneous remission of anti-Hu associated limbic encephalitis has been described[8] and could also have occurred in patient 2, who improved dramatically following rituximab. In all three patients who improved, the PNS diagnosis was established within 2 months of the onset of symptoms and was followed shortly by start of treatment. Because most PNS ultimately result in the destruction of neurons[1], early diagnosis and treatment are crucial.

The pathogenesis of PNS is heterogenic.[1] While some PNS such as Lambert-Eaton myasthenic syndrome and a rare form of PCD[77] are clearly caused by pathogenic autoantibodies directed at cell surface epitopes, such a role could never be proven for Hu and Yo antibodies that are directed at intracellular antigens.[55] Rituximab administration resulted in successful elimination of circulating CD19+ B cells. However, no consistent effect on serum or CSF antibody titers was observed and we did not find any correlation between clinical response and antibody titers. These observations raise the question whether rituximab did contribute to the functional improvement observed in three patients and, if so, by which mechanism. The detection of antigen-specific cytotoxic T cells in anti-Hu and anti-Yo associated PNS[63,71] suggests that the cellular immunity may play a pathogenic role.

Rituximab induced elimination of circulating B-cell results in significant clinical improvement in rheumatoid arthritis.[256] In this disorder B-cells may function as antigen-presenting cells and are important for T-cell activation. However, such roles for B-cells have not been demonstrated in PNS.

The improvement in 3 of 9 PNS patients following rituximab treatment warrants further studies. Because rituximab does not easily cross the blood brain barrier[260], concomitant intrathecal administration may enhance its efficacy in the treatment of PNS of the central nervous system.

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Chapter | 9

General Discussion

The primary aim of this thesis was to study the presence of HuD-specific T cells in peripheral blood and CSF of patients with Hu-PNS. However, despite a multifaceted approach, no such cells were detected. Several aspects that may explain the lack of detection are discussed below.

Study design and technical issues

Based on the observed infiltrating oligoclonal $CD8^+$ T cells in tumor and nervous tissues of Hu-PNS patients,[59,60] we focused primarily on $CD8^+$ T cells and to a lesser extent on $CD4^+$ T cells. The use of 15-mer protein-spanning peptide pools with 11 aa overlaps has the advantage that both $CD8^+$ and $CD4^+$ T-cell responses may be detected,[193] as was the case for $CD8^+$ and $CD4^+$ CMV-specific T cell-responses (Chapter 5). The lack of detection of HuD specific $CD4^+$ T cells in our studies contrasts with the previously observed HuD-specific $CD4^+$ T cell proliferative responses in PBMC of PNS patients.[63] This discrepancy may be explained by differences in read-out (i.e., 3-day lymphocyte proliferation[63] vs. overnight cytokine production in ours) or by the use of recombinant HuD protein[63] vs. protein-spanning 15-mer peptide pool by us. In addition, our subsequent studies using individual HuD derived 9-mers and Class-I HLA multimers were designed to detect HuD-specific $CD8^+$ T cells. Because the IgG_1 isotype predominance and high titers of Hu antibodies indicate a T-helper response to the Hu antigen[42], further study of HuD specific $CD4^+$ T cells is warranted.

For the analysis of HuD specific T cells we used various well-validated techniques,[193-195,198,223,231,232,261] known for their ability to detect even low-frequency antigen-specific T cells.[198] However, the absence of detectable circulating HuD specific $CD8^+$ T cells may not be surprising. Plonquet et al.[59] detected in a PCR-based study an identical T-cell clone in both neoplastic and nervous tissues but not in blood. This finding indicates that T cells likely involved in the pathophysiology of Hu-PNS may circulate in the blood below detection level. A cellular immune response that takes place in the central nervous system parenchyma can deplete the circulating pool of antigen-specific $CD8^+$ T cells.[196] In addition, vaccination studies in melanoma patients demonstrate that a clinically effective anti-tumor immune response can occur despite very low levels of antigen-specific cytotoxic T cells, well below the detection threshold (<1:5,000-10,000 $CD8^+$ T cells) of multimer technology.[197,198]

Several other limitations in our assays may explain our negative results. First, we used a limited number of HuD 9-mers. However, these HuD 9-mers were carefully selected based on both HLA binding capacity and previous results demonstrating circulating T cells specific for these HuD peptides in both Hu-PNS patients and controls.[64,66] Second, we employed - for logistical reasons - B cells and monocytes as antigen presenting cells (APC) in the stimulation assays and not the more potent dendritic cells. On the other hand, using B cells and monocytes as APC, antigen-specific T cells have been detected in various

disorders using ELISPOT and cytokine flow cytometry assays.[193,198] Finally, we lacked a true positive control, i.e., HuD specific T cell lines. We compensated for this lack by using both mitogenic T-cell responses as general measures of T-cell responsiveness and antigen (CMV)-specific T cell responses as positive controls in our studies.

Overall, we used several well-validated and sensitive techniques to analyze the presence of HuD specific $CD8^+$ T cells. However, for the analysis of HuD specific $CD4^+$ T cells we used only one, potentially suboptimal, technique. Importantly, our inability to detect HuD-specific T cells in blood and CSF using currently available methods does not rule out a pathogenic role of these cells in patients with Hu-PNS.

Wrong compartment?

The presence of activated T cells in the peripheral blood of Hu-PNS patients (Chapter 3) and the signs of inflammation in the CSF of the majority of Hu-PNS patients (Chapter 5) suggest that pathogenic, antigen-specific T cells must be present in the peripheral blood and CSF. Because the frequency of antigen-specific T cells in patients with CNS viral infections[223,224] and multiple sclerosis[226,227] is higher in CSF (i.e., >1% of $CD8^+$ T cells and >0.05% of T cells respectively) than in the blood, further research focused on the detection of antigen-specific T cells in the CSF of Hu-PNS patients seems worthwhile. The observed inflammatory T cell infiltrates in PNS neuronal[40,42,59,60] and tumor tissues[59,60], suggest that an important compartment to search for antigen-specific T cells would be the infiltrated neuronal and tumor tissue. However, it is difficult to obtain considerable amounts of tumor tissue because most SCLC patients are treated with chemotherapy following a diagnostic bronchoscopic biopsy.[262] Brain biopsies are never routinely obtained in Hu-PNS patients and a major drawback of studying post-mortem tissue is that the inflammatory processes may be burned-out and that the remnant T cells may be irrelevant bystanders.

Wrong antigen?

Hu antibodies are directed against a family of Hu antigens (HuD, HuC, HelN1 and HelN2). We focused our studies on T cells specific for HuD because HuD is expressed by both neurons and tumor cells of Hu-PNS patients.[6,36,44,191] However, as SCLC is a neuro(endocrine) tumor, neurons and tumor cells may very well share the expression of antigens other than HuD. These antigens expressed in the tumor, may elicit the primary cellular immune response resulting in tumor cell death and release of tumor antigens such as HuD and causing a secondary humoral immune response. In celiac disease, recent observations suggest that a key event in disease development is the activation of T cells that are directed against gluten proteins that have been converted by the enzyme tissue transglutaminase (TG2). However, IgA autoantibodies directed against the TG2 enzyme itself are found in all celiac patients and support the diagnosis of celiac disease.[263-265] Similarly, the T cell antigens important in the pathogenesis of Hu-PNS may differ from the B cell antigens

(i.e., Hu antigens) in these syndromes. Although HuD remains the antigen of interest, T cell antigens other than HuD may need further attention when future studies focusing on HuD specific T cells remain negative.

Future studies

First, the sensitivity of stimulation assays for the detection of HuD specific T cells can be further improved. In MS research HLA Class I multimers have been successfully applied to identify PB-derived MBP-specific $CD8^+$ TCL[266] and to detect PB transaldolase (TAL)-specific $CD8^+$ T cells.[236] In the latter study, PBMC were first depleted of $CD4^+$ T cells, stimulated with TAL peptides for 7 days and subsequently assayed for multimer binding, revealing frequencies of approximately 1% of TAL-specific $CD8^+$ T cells.[236] CSF autoantigen-specific T cells have been detected in patients with MS using a combination of different approaches such as pre-depletion of CD4-negative cells[235], pre-stimulation of CSF cells with antigen and IL-2 (i.e., in limiting dilution assays[227,237]), and a different read out technique, i.e., the proliferation assay[227,237,238] in addition to the ELISPOT assay.[225,226] Using these approaches, myelin-reactive T cells could be detected in frequencies ranging from 0.005 to 0.2 % of CSF $CD4^+$ T cells.[225-227,235,237] These data suggest that future research, including additional approaches such as pre-stimulation of T cells with the HuD antigen, is worthwhile in Hu-PNS patients. Also, the sensitivity of stimulation assays can be improved by using dendritic cells as APC.[96] These cells can be transduced with the HuD gene or externally loaded with HuD derived peptides.[96] Activated $HLADR^+$ T cells that were found in increased proportions in Hu-PNS (Chapter 3), are probably the T cells of interest. Sorting and analyzing these cells with the assays proposed may increase the chances of detecting antigen-specific T cells in Hu-PNS.

Second, further analysis of HuD specific $CD4^+$ T cells may provide more insight in Hu-PNS. Attempts to reproduce the HuD protein induced specific proliferation of $CD4^+$ T cells[63], combined with further functional assays on proliferating cells (i.e., delineation of the minimal epitope, analysis of cytotoxic function), can be performed on PBMC and CSF-T cell lines. In these proliferation assays it is important to use HuD protein and control proteins that are synthesized and highly purified in the same manner, to avoid T-cell responses against contaminant bacterial proteins.

Third, the analysis of viable T cells, derived from fresh nervous and tumor tissue of Hu-PNS patients, may give more insight into the pathogenesis of Hu-PNS. A detailed analysis of TCR characteristics (i.e., clonality)[59,60] would include: (i) screening of cDNA derived from biopsy tissue to clonally expand TCR-V-beta sequences by complementarity-determining region 3 (CDR3) spectratyping (a screening technique for TCR repertoire analysis)[267]; (ii) staining tissue sections with appropriate anti-TCR V-beta mAbs and identification of autoaggressive T cells in situ according to morphological criteria[59,60]; (iii) cellular cloning of T cells by limiting dilution of expanded, tissue derived T cells; (iv) molecular cloning and

characterization of TCR alpha and beta genes following gene transfer into T cells; and finally (v) the use of such TCR-transduced T cells to screen peptide or cDNA expression libraries to identify potential target antigens.[268,269]

Fourth, the contribution of Hu antibodies in the pathogenesis of Hu-PNS may be reconsidered. Recently IgG, purified from a patient with SPS and amphiphysin antibodies, was intraperitoneally injected into rats that had received encephalitogenic T-helper lymphocytes specific for myelin basic protein, to induce immune-mediated leaky blood-brain barrier.[78] The rats developed clinical signs resembling human SPS in a dose dependent manner suggesting a pathogenic role for the amphiphysin antibodies. Similar in vivo experiments using Hu antibodies in animals with leaky blood-brain barriers may shed new light on the role for these antibodies in Hu-PNS. However, several caveats must be kept in mind. The anti-amphiphysin IgG was purified from a patient who had responded very well to plasma exchange. Hu-PNS patients rarely improve after plasma exchange and the findings of Sommer and co-workers do not exclude that the neurological disorder was caused by unidentified IgG antibodies directed against cell surface antigens.[270]

Fifth, studies addressing a possible link between HLA phenotype and the development of Hu-PNS are scarce[46] reporting only a limited number of patients. From our considerably larger cohort of SCLC patients with and without Hu-PNS, detailed HLA phenotypes are available, enabling further research on this issue.

Finally, studies focusing on the relation between the fine-specificity of serum- and CSF Hu antibodies and clinical parameters such as the underlying tumor type and the PNS- and tumor-related prognosis, are currently ongoing. The outcome of these studies may, for example, facilitate tumor diagnosis in Hu-PNS patients.

Conclusion

In conclusion, some of our findings support the hypothesized T cell-mediated pathogenesis of Hu-PNS, but we were unable to confirm the presence of HuD specific $CD8^+$ T cells in blood and CSF.

References

1. Darnell RB, Posner JB. Paraneoplastic syndromes involving the nervous system. N Engl J Med. 2003; 349: 1543-1554
2. Wikinson PC, Zeromski J. Immunofluorescent detection of antibodies against neurons in sensory carcinomatous neuropathy. Brain 1965; 88: 529-538
3. Graus F, Cordon-Cardo C, Posner JB. Neuronal antinuclear antibody in sensory neuronopathy from lung cancer. Neurology 1985; 35: 538-543
4. Szabo A, Dalmau J, Manley G et al. HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. Cell 1991; 67: 325-333
5. Dalmau J, Graus F, Rosenblum MK, Posner JB. Anti-Hu--associated paraneoplastic encephalomyelitis/sensory neuronopathy. A clinical study of 71 patients. Medicine (Baltimore). 1992; 71: 59-72
6. Graus F, Keime-Guibert F, Rene R et al. Anti-Hu-associated paraneoplastic encephalomyelitis: analysis of 200 patients. Brain 2001; 124: 1138-1148
7. Lucchinetti CF, Kimmel DW, Lennon VA. Paraneoplastic and oncologic profiles of patients seropositive for type 1 antineuronal nuclear autoantibodies. Neurology 1998; 50: 652-657
8. Sillevis Smitt P, Grefkens J, de Leeuw B et al. Survival and outcome in 73 anti-Hu positive patients with paraneoplastic encephalomyelitis/sensory neuronopathy. J Neurol. 2002; 249: 745-753
9. Sillevis Smitt PA, Leeuw BD, Vecht CJ. Immunologie in de medische praktijk. XVI. Paraneoplastische syndromen van het zenuwstelsel: pathogenese en diagnostiek. Ned Tijdschr Geneeskd. 1998; 142: 1652-1658
10. Graus F, Bonaventura I, Uchuya M et al. Indolent anti-Hu-associated paraneoplastic sensory neuropathy. Neurology 1994; 44: 2258-2261
11. Graus F, Delattre JY, Antoine JC et al. Recommended diagnostic criteria for paraneoplastic neurological syndromes. J Neurol Neurosurg Psychiatry 2004; 75: 1135-1140
12. Gultekin SH, Rosenfeld MR, Voltz R et al. Paraneoplastic limbic encephalitis: neurological symptoms, immunological findings and tumour association in 50 patients. Brain 2000; 123: 1481-1494
13. Lawn ND, Westmoreland BF, Kiely MJ et al. Clinical, magnetic resonance imaging, and electroencephalographic findings in paraneoplastic limbic encephalitis. Mayo Clin Proc. 2003; 78: 1363-1368
14. Antoine JC, Cinotti L, Tilikete C et al. [18F]fluorodeoxyglucose positron emission tomography in the diagnosis of cancer in patients with paraneoplastic neurological syndrome and anti-Hu antibodies. Ann Neurol. 2000; 48: 105-108
15. Rees JH, Hain SF, Johnson MR et al. The role of [18F]fluoro-2-deoxyglucose-PET scanning in the diagnosis of paraneoplastic neurological disorders. Brain 2001; 124: 2223-2231
16. Llado A, Carpentier AF, Honnorat J et al. Hu-antibody-positive patients with or without cancer have similar clinical profiles. J Neurol Neurosurg Psychiatry 2006; 77: 996-997
17. Darnell RB, DeAngelis LM. Regression of small-cell lung carcinoma in patients with paraneoplastic neuronal antibodies. Lancet 1993; 341: 21-22
18. Keime-Guibert F, Graus F, Broet P et al. Clinical outcome of patients with anti-Hu-associated encephalomyelitis after treatment of the tumor. Neurology 1999; 53: 1719-1723
19. Keime-Guibert F, Graus F, Fleury A et al. Treatment of paraneoplastic neurological syndromes with antineuronal antibodies (Anti-Hu, anti-Yo) with a combination of immunoglobulins, cyclophosphamide, and methylprednisolone. J Neurol Neurosurg Psychiatry 2000; 68: 479-482

20. Oh SJ, Dropcho EJ, Claussen GC. Anti-Hu-associated paraneoplastic sensory neuropathy responding to early aggressive immunotherapy: report of two cases and review of literature. Muscle Nerve 1997; 20: 1576-1582
21. Moll JWB, Vecht CJ. Immune diagnosis of paraneoplastic neurological disease. Clin Neurol Neurosurg. 1995; 97: 71-81
22. Dalmau J, Furneaux HM, Cordon-Cardo C, Posner JB. The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues. Am J Pathol. 1992; 141: 881-886
23. Dalmau J, Furneaux HM, Gralla RJ et al. Detection of the anti-Hu antibody in the serum of patients with small cell lung cancer--a quantitative western blot analysis. Ann Neurol. 1990; 27: 544-552
24. Dalmau J, Furneaux HM, Rosenblum MK et al. Detection of the anti-Hu antibody in specific regions of the nervous system and tumor from patients with paraneoplastic encephalomyelitis/sensory neuronopathy. Neurology 1991; 41: 1757-1764
25. Graus F, Ribalta T, Campo E et al. Immunohistochemical analysis of the immune reaction in the nervous system in paraneoplastic encephalomyelitis. Neurology 1990; 40: 219-222
26. Graus F, Dalmou J, Rene R et al. Anti-Hu antibodies in patients with small-cell lung cancer: association with complete response to therapy and improved survival. J Clin Oncol. 1997; 15: 2866-2872
27. Monstad SE, Drivsholm L, Storstein A et al. Hu and voltage-gated calcium channel (VGCC) antibodies related to the prognosis of small-cell lung cancer. J Clin Oncol. 2004; 22: 795-800
28. Sakai K, Gofuku M, Kitagawa Y et al. A hippocampal protein associated with paraneoplastic neurologic syndrome and small cell lung carcinoma. Biochem Biophys Res Commun. 1994; 199: 1200-1208
29. King P. Hel-N2: a novel isoform of Hel-N1 which is conserved in rat neural tissue and produced in early embryogenesis. Gene 1994; 151: 261-265
30. Dropcho EJ, King PH. Autoantibodies against the Hel-N1 RNA-binding protein among patients with lung carcinoma: an association with type I anti-neuronal nuclear antibodies. Ann Neurol. 1994; 36: 200-205
31. Campos AR, Grossman D, White K. Mutant alleles at the locus elav in Drosophila melanogaster lead to nervous system defects. A developmental-genetic analysis. J Neurogenet. 1985; 2: 197-218
32. Marusisch MF, Furneaux HM, Henion P, Weston JA. Hu neuronal proteins are expressed in proliferation neurogenic cells. J Neurobiol. 1994; 25: 143-155
33. Keene JD. Why is Hu where? Shuttling of early-response-gene messenger RNA subsets. Proc Natl Acad Sci U S A. 1999; 96: 5-7
34. Brennan CM, Steitz JA. HuR and mRNA stability. Cell Mol Life Sci. 2001; 58: 266-277
35. Deschenes-Furry J, Perrone-Bizzozero N, Jasmin BJ. The RNA-binding protein HuD: a regulator of neuronal differentiation, maintenance and plasticity. Bioessays 2006; 28: 822-833
36. Manley GT, Smitt PS, Dalmau J, Posner JB. Hu antigens: reactivity with Hu antibodies, tumor expression, and major immunogenic sites. Ann Neurol. 1995; 38: 102-110
37. King PH, Dropcho EJ. Expression of Hel-N1 and Hel-N2 in small cell lung carcinoma. Ann Neurol. 1996; 39: 679-681
38. Carpentier AF, Voltz R, DesChamps T et al. Absence of HuD gene mutations in paraneoplastic small cell lung cancer tissue. Neurology 1998; 50: 1919
39. Sekido Y, Bader SA, Carbone DP et al. Molecular analysis of the HuD gene encoding a paraneoplastic encephalomyelitis antigen in human lung cancer cell lines. Cancer Res. 1994; 54: 4988-4992
40. Bernal F, Graus F, Pifarre A et al. Immunohistochemical analysis of anti-Hu-associated paraneoplastic encephalomyelitis. Acta Neuropathol (Berl). 2002; 103: 509-515

41. Henson R. Cancer and the nervous system: The neurologic complications of systemic malignant disease. Oxford: Blackwell Scientific. 1982
42. Jean WC, Dalmau J, Ho A, Posner JB. Analysis of the IgG subclass distribution and inflammatory infiltrates in patients with anti-Hu-associated paraneoplastic encephalomyelitis. Neurology 1994; 44: 140-147
43. Wanschitz J, Hainfellner JA, Kristoferitsch W et al. Ganglionitis in paraneoplastic subacute sensory neuronopathy: a morphologic study. Neurology 1997; 49: 1156-1159
44. Dalmau J, Graus F, Cheung NK et al. Major histocompatibility proteins, anti-Hu antibodies, and paraneoplastic encephalomyelitis in neuroblastoma and small cell lung cancer. Cancer 1995; 75: 99-109
45. Rauer S, Kaiser R. Demonstration of anti-HuD specific oligoclonal bands in the cerebrospinal fluid from patients with paraneoplastic neurological syndromes. Qualitative evidence of anti-HuD specific IgG-synthesis in the central nervous system. J Neuroimmunol. 2000; 111: 241-244
46. Uchuya M, Fleury A, Graus F et al. Lack of association between human leukocyte antigens and the anti-Hu syndrome in patients with small-cell lung cancer. Neurology 1998; 50: 565-566
47. Wirtz PW, Willcox N, Roep BO et al. HLA-B8 in patients with the Lambert-Eaton myasthenic syndrome reduces likelihood of associated small cell lung carcinoma. Ann N Y Acad Sci. 2003; 998: 200-201.
48. Wirtz PW, Willcox N, van der Slik AR et al. HLA and smoking in prediction and prognosis of small cell lung cancer in autoimmune Lambert-Eaton myasthenic syndrome. J Neuroimmunol. 2005; 159: 230-237
49. Darnell RB. Onconeural antigens and the paraneoplastic neurologic disorders: at the intersection of cancer, immunity, and the brain. Proc Natl Acad Sci U S A. 1996; 93: 4529-4536
50. Archelos JJ, Hartung HP. Pathogenetic role of autoantibodies in neurological diseases. Trends Neurosci. 2000; 23: 317-327
51. Storch MK, Piddlesden S, Haltia M et al. Multiple sclerosis: in situ evidence for antibody- and complement-mediated demyelination. Ann Neurol. 1998; 43: 465-471
52. Hormigo A, Lieberman F. Nuclear localization of anti-Hu antibody is not associated with in vitro cytotoxicity. J Neuroimmunol. 1994; 55: 205-212
53. Tanaka K, Ding X, Tanaka M. Effects of antineuronal antibodies from patients with paraneoplastic neurological syndrome on primary-cultured neurons. J Neurol Sci. 2004; 217: 25-30
54. Verschuuren JJ, Dalmau J, Hoard R, Posner JB. Paraneoplastic anti-Hu serum: studies on human tumor cell lines. J Neuroimmunol. 1997; 79: 202-210
55. Sillevis Smitt PA, Manley GT, Posner JB. Immunization with the paraneoplastic encephalomyelitis antigen HuD does not cause neurologic disease in mice. Neurology 1995; 45: 1873-1878
56. Alarcon-Segovia D, Ruiz-Arguelles A, Llorente L. Broken dogma: penetration of autoantibodies into living cells. Immunol Today. 1996; 17: 163-164
57. Yanase K, Smith RM, Puccetti A et al. Receptor-mediated cellular entry of nuclear localizing anti-DNA antibodies via myosin 1. J Clin Invest. 1997; 100: 25-31
58. Carpentier AF, Rosenfeld MR, Delattre JY et al. DNA vaccination with HuD inhibits growth of a neuroblastoma in mice. Clin Cancer Res. 1998; 4: 2819-2824
59. Plonquet A, Gherardi RK, Creange A et al. Oligoclonal T-cells in blood and target tissues of patients with anti-Hu syndrome. J Neuroimmunol. 2002; 122: 100-105
60. Voltz R, Dalmau J, Posner JB, Rosenfeld MR. T-cell receptor analysis in anti-Hu associated paraneoplastic encephalomyelitis. Neurology 1998; 51: 1146-1150
61. Brown DM, Dilzer AM, Meents DL, Swain SL. CD4 T cell-mediated protection from lethal influenza: perforin and antibody-mediated mechanisms give a one-two punch. J Immunol. 2006; 177: 2888-2898

62. Zeng G, Wang X, Robbins PF et al. CD4(+) T cell recognition of MHC class II-restricted epitopes from NY-ESO-1 presented by a prevalent HLA DP4 allele: association with NY-ESO-1 antibody production. Proc Natl Acad Sci U S A. 2001; 98: 3964-3969
63. Benyahia B, Liblau R, Merle-Beral H et al. Cell-mediated autoimmunity in paraneoplastic neurological syndromes with anti-Hu antibodies. Ann Neurol. 1999; 45: 162-167
64. Rousseau A, Benyahia B, Dalmau J et al. T cell response to Hu-D peptides in patients with anti-Hu syndrome. J Neurooncol. 2005; 71: 231-236
65. Tanaka M, Maruyama Y, Sugie M et al. Cytotoxic T cell activity against peptides of Hu protein in anti-Hu syndrome. J Neurol Sci. 2002; 201: 9-12
66. Plonquet A, Garcia-Pons F, Fernandez E et al. Peptides derived from the onconeural HuD protein can elicit cytotoxic responses in HHD mouse and human. J Neuroimmunol. 2003; 142: 93-100
67. Rojas I, Graus F, Keime-Guibert F et al. Long-term clinical outcome of paraneoplastic cerebellar degeneration and anti-Yo antibodies. Neurology 2000; 55: 713-715
68. Shams'ili S, Grefkens J, de Leeuw B et al. Paraneoplastic cerebellar degeneration associated with antineuronal antibodies: analysis of 50 patients. Brain 2003; 126: 1409-1418
69. Denny-Brown D. Primary sensory neuropathy with muscular changes associated with carcinoma. J Neurol Neurosurg Psychiatry 1948; 11: 73-87
70. Byrne T, Mason WP, Posner JB, Dalmau J. Spontaneous neurological improvement in anti-Hu associated encephalomyelitis. J Neurol Neurosurg Psychiatry 1997; 62: 276-278
71. Albert ML, Austin LM, Darnell RB. Detection and treatment of activated T cells in the cerebrospinal fluid of patients with paraneoplastic cerebellar degeneration. Ann Neurol. 2000; 47: 9-17
72. Tanaka M, Tanaka K, Tokiguchi S et al. Cytotoxic T cells against a peptide of Yo protein in patients with paraneoplastic cerebellar degeneration and anti-Yo antibody. J Neurol Sci. 1999; 168: 28-31
73. Yu Z, Kryzer TJ, Griesmann GE et al. CRMP-5 neuronal autoantibody: marker of lung cancer and thymoma-related autoimmunity. Ann Neurol. 2001; 49: 146-154
74. Bernal F, Shams'ili S, Rojas I et al. Anti-Tr antibodies as markers of paraneoplastic cerebellar degeneration and Hodgkin's disease. Neurology 2003; 60: 230-234
75. Pittock SJ, Kryzer TJ, Lennon VA. Paraneoplastic antibodies coexist and predict cancer, not neurological syndrome. Ann Neurol. 2004; 56: 715-719
76. Younes-Mhenni S, Janier MF, Cinotti L et al. FDG-PET improves tumour detection in patients with paraneoplastic neurological syndromes. Brain 2004; 127: 2331-2338
77. Sillevis Smitt P, Kinoshita A, De Leeuw B et al. Paraneoplastic cerebellar ataxia due to autoantibodies against a glutamate receptor. N Engl J Med. 2000; 342: 21-27
78. Sommer C, Weishaupt A, Brinkhoff J et al. Paraneoplastic stiff-person syndrome: passive transfer to rats by means of IgG antibodies to amphiphysin. Lancet 2005; 365: 1406-1411
79. Vernino S, O'Neill BP, Marks RS et al. Immunomodulatory treatment trial for paraneoplastic neurological disorders. Neuro-oncol. 2004; 6: 55-62
80. Posner J. Paraneoplastic Syndromes. Neurologic complications of cancer. Philadelphia: FA Davis. 1995: 353-385
81. Dropcho EJ. Antiamphiphysin antibodies with small-cell lung carcinoma and paraneoplastic encephalomyelitis. Ann Neurol. 1996; 39: 659-667
82. Chan KH, Vernino S, Lennon VA. ANNA-3 anti-neuronal nuclear antibody: marker of lung cancer-related autoimmunity. Ann Neurol. 2001; 50: 301-311
83. Vernino S, Lennon VA. New Purkinje cell antibody (PCA-2): marker of lung cancer-related neurological autoimmunity. Ann Neurol. 2000; 47: 297-305
84. Corsellis JA, Goldberg GJ, Norton AR. "Limbic encephalitis" and its association with carcinoma. Brain 1968; 91: 481-496

85. Alamowitch S, Graus F, Uchuya M et al. Limbic encephalitis and small cell lung cancer. Clinical and immunological features. Brain 1997; 120 (Pt 6): 923-928
86. Voltz R, Gultekin SH, Rosenfeld MR et al. A serologic marker of paraneoplastic limbic and brain-stem encephalitis in patients with testicular cancer. N Engl J Med. 1999; 340: 1788-1795
87. Kassubek J, Juengling FD, Nitzsche EU, Lucking CH. Limbic encephalitis investigated by 18FDG-PET and 3D MRI. J Neuroimaging. 2001; 11: 55-59
88. Dalmau J, Graus F, Villarejo A et al. Clinical analysis of anti-Ma2-associated encephalitis. Brain 2004; 127: 1831-1844
89. Pozo-Rosich P, Clover L, Saiz A et al. Voltage-gated potassium channel antibodies in limbic encephalitis. Ann Neurol. 2003; 54: 530-533
90. Vincent A, Buckley C, Schott JM et al. Potassium channel antibody-associated encephalopathy: a potentially immunotherapy-responsive form of limbic encephalitis. Brain 2004; 127: 701-712
91. Taylor RB, Mason W, Kong K, Wennberg R. Reversible paraneoplastic encephalomyelitis associated with a benign ovarian teratoma. Can J Neurol Sci. 1999; 26: 317-320
92. Rosenfeld MR, Eichen JG, Wade DF et al. Molecular and clinical diversity in paraneoplastic immunity to Ma proteins. Ann Neurol. 2001; 50: 339-348
93. Furneaux HM, Rosenblum MK, Dalmau J et al. Selective expression of Purkinje-cell antigens in tumor tissue from patients with paraneoplastic cerebellar degeneration. N Engl J Med. 1990; 322: 1844-1851
94. Hammack J, Kotanides H, Rosenblum MK, Posner JB. Paraneoplastic cerebellar degeneration. II. Clinical and immunologic findings in 21 patients with Hodgkin's disease. Neurology 1992; 42: 1938-1943
95. Fathallah-Shaykh H, Wolf S, Wong E et al. Cloning of a leucine-zipper protein recognized by the sera of patients with antibody-associated paraneoplastic cerebellar degeneration. Proc Natl Acad Sci U S A. 1991; 88: 3451-3454
96. Darnell RB, Albert ML. cdr2-specific CTLs are detected in the blood of all patients with paraneoplastic cerebellar degeneration analyzed. Ann Neurol. 2000; 48: 270-271
97. Mason WP, Graus F, Lang B et al. Small-cell lung cancer, paraneoplastic cerebellar degeneration and the Lambert-Eaton myasthenic syndrome. Brain 1997; 120 (Pt 8): 1279-1300
98. Bataller L, Wade DF, Graus F et al. Antibodies to Zic4 in paraneoplastic neurologic disorders and small-cell lung cancer. Neurology 2004; 62: 778-782
99. Shams'ili S, de Beukelaar J, Gratama JW et al. An uncontrolled trial of rituximab for antibody associated paraneoplastic neurological syndromes. J Neurol. 2006; 253: 16-20
100. Anderson NE, Budde-Steffen C, Rosenblum MK et al. Opsoclonus, myoclonus, ataxia, and encephalopathy in adults with cancer: a distinct paraneoplastic syndrome. Medicine (Baltimore). 1988; 67: 100-109
101. Digre KB. Opsoclonus in adults. Report of three cases and review of the literature. Arch Neurol. 1986; 43: 1165-1175
102. Wirtz PW, Sillevis Smitt PA, Hoff JI et al. Anti-Ri antibody positive opsoclonus-myoclonus in a male patient with breast carcinoma. J Neurol. 2002; 249: 1710-1712
103. Bataller L, Graus F, Saiz A, Vilchez JJ. Clinical outcome in adult onset idiopathic or paraneoplastic opsoclonus-myoclonus. Brain 2001; 124: 437-443
104. Prestigiacomo CJ, Balmaceda C, Dalmau J. Anti-Ri-associated paraneoplastic opsoclonus-ataxia syndrome in a man with transitional cell carcinoma. Cancer 2001; 91: 1423-1428
105. Altman AJ, Baehner RL. Favorable prognosis for survival in children with coincident opsomyoclonus and neuroblastoma. Cancer 1976; 37: 846-852
106. Rudnick E, Khakoo Y, Antunes NL et al. Opsoclonus-myoclonus-ataxia syndrome in neuroblastoma: clinical outcome and antineuronal antibodies-a report from the Children's Cancer Group Study. Med Pediatr Oncol. 2001; 36: 612-622

107. Hormigo A, Dalmau J, Rosenblum MK et al. Immunological and pathological study of anti-Ri-associated encephalopathy. Ann Neurol. 1994; 36: 896-902
108. Linke R, Schroeder M, Helmberger T, Voltz R. Antibody-positive paraneoplastic neurologic syndromes: value of CT and PET for tumor diagnosis. Neurology 2004; 63: 282-286
109. Swart JF, de Kraker J, van der Lely N. Metaiodobenzylguanidine total-body scintigraphy required for revealing occult neuroblastoma in opsoclonus-myoclonus syndrome. Eur J Pediatr. 2002; 161: 255-258
110. Hayward K, Jeremy RJ, Jenkins S et al. Long-term neurobehavioral outcomes in children with neuroblastoma and opsoclonus-myoclonus-ataxia syndrome: relationship to MRI findings and anti-neuronal antibodies. J Pediatr. 2001; 139: 552-559
111. Buckanovich RJ, Yang YY, Darnell RB. The onconeural antigen Nova-1 is a neuron-specific RNA-binding protein, the activity of which is inhibited by paraneoplastic antibodies. J Neurosci. 1996; 16: 1114-1122
112. Yang YY, Yin GL, Darnell RB. The neuronal RNA-binding protein Nova-2 is implicated as the autoantigen targeted in POMA patients with dementia. Proc Natl Acad Sci U S A. 1998; 95: 13254-13259
113. Bataller L, Rosenfeld MR, Graus F et al. Autoantigen diversity in the opsoclonus-myoclonus syndrome. Ann Neurol. 2003; 53: 347-353
114. Antunes NL, Khakoo Y, Matthay KK et al. Antineuronal antibodies in patients with neuroblastoma and paraneoplastic opsoclonus-myoclonus. J Pediatr Hematol Oncol. 2000; 22: 315-320
115. Pranzatelli MR, Tate ED, Wheeler A et al. Screening for autoantibodies in children with opsoclonus-myoclonus-ataxia. Pediatr Neurol. 2002; 27: 384-387
116. Dropcho EJ, Kline LB, Riser J. Antineuronal (anti-Ri) antibodies in a patient with steroid-responsive opsoclonus-myoclonus. Neurology 1993; 43: 207-211
117. Jongen JL, Moll WJ, Sillevis Smitt PA et al. Anti-Ri positive opsoclonus-myoclonus-ataxia in ovarian duct cancer. J Neurol. 1998; 245: 691-692
118. Nitschke M, Hochberg F, Dropcho E. Improvement of paraneoplastic opsoclonus-myoclonus after protein A column therapy. N Engl J Med. 1995; 332: 192
119. Mitchell WG, Davalos-Gonzalez Y, Brumm VL et al. Opsoclonus-ataxia caused by childhood neuroblastoma: developmental and neurologic sequelae. Pediatrics 2002; 109: 86-98
120. Russo C, Cohn SL, Petruzzi MJ, de Alarcon PA. Long-term neurologic outcome in children with opsoclonus-myoclonus associated with neuroblastoma: a report from the Pediatric Oncology Group. Med Pediatr Oncol. 1997; 28: 284-288
121. Chalk CH, Windebank AJ, Kimmel DW, McManis PG. The distinctive clinical features of paraneoplastic sensory neuronopathy. Can J Neurol Sci. 1992; 19: 346-351
122. Horwich MS, Cho L, Porro RS, Posner JB. Subacute sensory neuropathy: a remote effect of carcinoma. Ann Neurol. 1977; 2: 7-19
123. Graus F, Bonaventura I, Uchuya M et al. Indolent anti-Hu-associated paraneoplastic sensory neuropathy. Neurology 1994; 44: 2258-2261
124. Molinuevo JL, Graus F, Serrano C et al. Utility of anti-Hu antibodies in the diagnosis of paraneoplastic sensory neuropathy. Ann Neurol. 1998; 44: 976-980
125. Antoine JC, Honnorat J, Camdessanche JP et al. Paraneoplastic anti-CV2 antibodies react with peripheral nerve and are associated with a mixed axonal and demyelinating peripheral neuropathy. Ann Neurol. 2001; 49: 214-221
126. Honnorat J, Antoine JC, Derrington E et al. Antibodies to a subpopulation of glial cells and a 66 kDa developmental protein in patients with paraneoplastic neurological syndromes. J Neurol Neurosurg Psychiatry 1996; 61: 270-278
127. Pittock SJ, Lucchinetti CF, Parisi JE et al. Amphiphysin autoimmunity: paraneoplastic accompaniments. Ann Neurol. 2005; 58: 96-107

128. Saiz A, Dalmau J, Butler MH et al. Anti-amphiphysin I antibodies in patients with paraneoplastic neurological disorders associated with small cell lung carcinoma. J Neurol Neurosurg Psychiatry 1999; 66: 214-217
129. Uchuya M, Graus F, Vega F et al. Intravenous immunoglobulin treatment in paraneoplastic neurological syndromes with antineuronal autoantibodies. J Neurol Neurosurg Psychiatry 1996; 60: 388-392
130. Wirtz PW, Wintzen AR, Verschuuren JJ. Lambert-Eaton myasthenic syndrome has a more progressive course in patients with lung cancer. Muscle Nerve 2005; 32: 226-229
131. Elrington GM, Murray NM, Spiro SG, Newsom-Davis J. Neurological paraneoplastic syndromes in patients with small cell lung cancer. A prospective survey of 150 patients. J Neurol Neurosurg Psychiatry 1991; 54: 764-767
132. O'Neill JH, Murray NM, Newsom-Davis J. The Lambert-Eaton myasthenic syndrome. A review of 50 cases. Brain 1988; 111 (Pt 3): 577-596
133. Wirtz PW, Nijnuis MG, Sotodeh M et al. The epidemiology of myasthenia gravis, Lambert-Eaton myasthenic syndrome and their associated tumours in the northern part of the province of South Holland. J Neurol. 2003; 250: 698-701
134. Hawley RJ, Cohen MH, Saini N, Armbrustmacher VW. The carcinomatous neuromyopathy of oat cell lung cancer. Ann Neurol. 1980; 7: 65-72
135. Wirtz PW, Lang B, Graus F et al. P/Q-type calcium channel antibodies, Lambert-Eaton myasthenic syndrome and survival in small cell lung cancer. J Neuroimmunol. 2005; 164: 161-165
136. Motomura M, Johnston I, Lang B et al. An improved diagnostic assay for Lambert-Eaton myasthenic syndrome. J Neurol Neurosurg Psychiatry 1995; 58: 85-87
137. Rosenfeld MR, Wong E, Dalmau J et al. Cloning and characterization of a Lambert-Eaton myasthenic syndrome antigen. Ann Neurol. 1993; 33: 113-120
138. Chalk CH, Murray NM, Newsom-Davis J et al. Response of the Lambert-Eaton myasthenic syndrome to treatment of associated small-cell lung carcinoma. Neurology 1990; 40: 1552-1556
139. McEvoy KM, Windebank AJ, Daube JR, Low PA. 3,4-Diaminopyridine in the treatment of Lambert-Eaton myasthenic syndrome. N Engl J Med. 1989; 321: 1567-1571
140. Sanders DB, Massey JM, Sanders LL, Edwards LJ. A randomized trial of 3,4-diaminopyridine in Lambert-Eaton myasthenic syndrome. Neurology 2000; 54: 603-607
141. Newsom-Davis J, Murray NM. Plasma exchange and immunosuppressive drug treatment in the Lambert-Eaton myasthenic syndrome. Neurology 1984; 34: 480-485
142. Bird SJ. Clinical and electrophysiologic improvement in Lambert-Eaton syndrome with intravenous immunoglobulin therapy. Neurology 1992; 42: 1422-1423
143. Buchbinder R, Forbes A, Hall S et al. Incidence of malignant disease in biopsy-proven inflammatory myopathy. A population-based cohort study. Ann Intern Med. 2001; 134: 1087-1095
144. Hill CL, Zhang Y, Sigurgeirsson B et al. Frequency of specific cancer types in dermatomyositis and polymyositis: a population-based study. Lancet 2001; 357: 96-100
145. Mastaglia FL, Garlepp MJ, Phillips BA, Zilko PJ. Inflammatory myopathies: clinical, diagnostic and therapeutic aspects. Muscle Nerve 2003; 27: 407-425
146. Targoff IN. Update on myositis-specific and myositis-associated autoantibodies. Curr Opin Rheumatol. 2000; 12: 475-481
147. Griggs R, Mendell, JR, Miller, RG. Evaluation and treatment of myopathies. Philadelphia: F.A. Davis Company. 1995
148. van den Berg AP, van Son WJ, Janssen RA et al. Recovery from cytomegalovirus infection is associated with activation of peripheral blood lymphocytes. J Infect Dis. 1992; 166: 1228-1235
149. Crane IJ, Forrester JV. Th1 and Th2 lymphocytes in autoimmune disease. Crit Rev Immunol. 2005; 25: 75-102
150. Knutson KL, Disis ML. Tumor antigen-specific T helper cells in cancer immunity and immunotherapy. Cancer Immunol Immunother. 2005; 54: 721-728

151. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol. 2004; 22: 531-562
152. Blanco P, Viallard JF, Pellegrin JL, Moreau JF. Cytotoxic T lymphocytes and autoimmunity. Curr Opin Rheumatol. 2005; 17: 731-734
153. Lonchay C, van der Bruggen P, Connerotte T et al. Correlation between tumor regression and T cell responses in melanoma patients vaccinated with a MAGE antigen. Proc Natl Acad Sci U S A. 2004; 101 Suppl 2: 14631-14638
154. Borthwick NJ, Lowdell M, Salmon M, Akbar AN. Loss of CD28 expression on CD8(+) T cells is induced by IL-2 receptor gamma chain signalling cytokines and type I IFN, and increases susceptibility to activation-induced apoptosis. Int Immunol. 2000; 12: 1005-1013
155. Hamann D, Kostense S, Wolthers KC et al. Evidence that human CD8+CD45RA+CD27- cells are induced by antigen and evolve through extensive rounds of division. Int Immunol. 1999; 11: 1027-1033
156. Speiser DE, Migliaccio M, Pittet MJ et al. Human CD8(+) T cells expressing HLA-DR and CD28 show telomerase activity and are distinct from cytolytic effector T cells. Eur J Immunol. 2001; 31: 459-466
157. Brenchley JM, Karandikar NJ, Betts MR et al. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. Blood 2003; 101: 2711-2720
158. Gratama JW, Kardol M, Naipal AM et al. The influence of cytomegalovirus carrier status on lymphocyte subsets and natural immunity. Clin Exp Immunol. 1987; 69: 16-24
159. Morley JK, Batliwalla FM, Hingorani R, Gregersen PK. Oligoclonal CD8+ T cells are preferentially expanded in the CD57+ subset. J Immunol. 1995; 154: 6182-6190
160. Pittet MJ, Speiser DE, Valmori D et al. Cutting edge: cytolytic effector function in human circulating CD8+ T cells closely correlates with CD56 surface expression. J Immunol. 2000; 164: 1148-1152
161. Mandy FF, Nicholson JK, McDougal JS. Guidelines for performing single-platform absolute CD4+ T-cell determinations with CD45 gating for persons infected with human immunodeficiency virus. Centers for Disease Control and Prevention. MMWR Recomm Rep. 2003; 52: 1-13
162. Roederer M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. Cytometry 2001; 45: 194-205
163. Gratama JW, Lipovich-Oosterveer MA, Ronteltap C et al. Natural immunity and graft-vs-host disease. Transplantation 1985; 40: 256-260
164. Moll JW, Antoine JC, Brashear HR et al. Guidelines on the detection of paraneoplastic antineuronal-specific antibodies: report from the Workshop to the Fourth Meeting of the International Society of Neuro-Immunology on paraneoplastic neurological disease, held October 22-23, 1994, in Rotterdam, The Netherlands. Neurology 1995; 45: 1937-1941
165. Gratama JW, Naipal AM, Oosterveer MA et al. Effects of herpes virus carrier status on peripheral T lymphocyte subsets. Blood 1987; 70: 516-523
166. Jentsch-Ullrich K, Koenigsmann M, Mohren M, Franke A. Lymphocyte subsets' reference ranges in an age- and gender-balanced population of 100 healthy adults--a monocentric German study. Clin Immunol. 2005; 116: 192-197
167. Nakamura H, Kawasaki N, Hagiwara M et al. Cellular immunologic parameters related to age, gender, and stage in lung cancer patients. Lung Cancer 2000; 28: 139-145
168. Olsson J, Wikby A, Johansson B et al. Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study. Mech Ageing Dev. 2000; 121: 187-201
169. Caras I, Grigorescu A, Stavaru C et al. Evidence for immune defects in breast and lung cancer patients. Cancer Immunol Immunother. 2004; 53: 1146-1152

170. Hadden JW. Immunodeficiency and cancer: prospects for correction. Int Immunopharmacol. 2003; 3: 1061-1071
171. Behan WM, Behan PO, Micklem HS, Durward WF. Abnormalities of lymphocyte subsets in polymyositis. Br Med J (Clin Res Ed). 1983; 287: 181-182
172. Erkeller-Yusel F, Hulstaart F, Hannet I et al. Lymphocyte subsets in a large cohort of patients with systemic lupus erythematosus. Lupus 1993; 2: 227-231
173. Iannone F, Cauli A, Yanni G et al. T-lymphocyte immunophenotyping in polymyositis and dermatomyositis. Br J Rheumatol. 1996; 35: 839-845
174. Viguier M, Fouere S, de la Salmoniere P et al. Peripheral blood lymphocyte subset counts in patients with dermatomyositis: clinical correlations and changes following therapy. Medicine (Baltimore). 2003; 82: 82-86
175. Chailleux E, Bignon JD, Peyrat MA et al. Lymphocyte subsets, phytohaemagglutinin responsiveness of blood lymphocytes, and interleukin 2 production in sarcoidosis. Thorax 1985; 40: 768-773
176. Kolopp-Sarda MN, Kohler C, De March AK et al. Discriminative immunophenotype of bronchoalveolar lavage CD4 lymphocytes in sarcoidosis. Lab Invest. 2000; 80: 1065-1069
177. Hafler DA, Fox DA, Manning ME et al. In vivo activated T lymphocytes in the peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. N Engl J Med. 1985; 312: 1405-1411
178. Scolozzi R, Boccafogli A, Tola MR et al. T-cell phenotypic profiles in the cerebrospinal fluid and peripheral blood of multiple sclerosis patients. J Neurol Sci. 1992; 108: 93-98
179. Benveniste O, Cherin P, Maisonobe T et al. Severe perturbations of the blood T cell repertoire in polymyositis, but not dermatomyositis patients. J Immunol. 2001; 167: 3521-3529
180. Fletcher JM, Vukmanovic-Stejic M, Dunne PJ et al. Cytomegalovirus-specific CD4+ T cells in healthy carriers are continuously driven to replicative exhaustion. J Immunol. 2005; 175: 8218-8225
181. Lin MT, Tseng LH, Frangoul H et al. Increased apoptosis of peripheral blood T cells following allogeneic hematopoietic cell transplantation. Blood 2000; 95: 3832-3839
182. Wang H, Xu J, Ji X et al. The abnormal apoptosis of T cell subsets and possible involvement of IL-10 in systemic lupus erythematosus. Cell Immunol. 2005; 235: 117-121
183. Wirleitner B, Obermoser G, Bock G et al. Induction of apoptosis in human blood T cells by 7,8-dihydroneopterin: the difference between healthy controls and patients with systemic lupus erythematosus. Clin Immunol. 2003; 107: 152-159
184. Wolf AM, Wolf D, Steurer M et al. Increase of regulatory T cells in the peripheral blood of cancer patients. Clin Cancer Res. 2003; 9: 606-612
185. de Andres C, Esquivel A, de Villoria JG et al. Unusual magnetic resonance imaging and cerebrospinal fluid findings in paraneoplastic cerebellar degeneration: a sequential study. J Neurol Neurosurg Psychiatry 2006; 77: 562-563
186. LeFever AV, Funahashi A. Phenotype and function of natural killer cells in patients with bronchogenic carcinoma. Cancer Res. 1991; 51: 5596-5601
187. Nielsen LR, Kimose HH, Linnet L et al. Analysis of the decreased NK (natural killer) activity in lung cancer patients, using whole blood versus separated mononuclear cells. J Clin Lab Immunol. 1989; 29: 71-77
188. Smyth MJ, Teng MW, Swann J et al. CD4+CD25+ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. J Immunol. 2006; 176: 1582-1587
189. Darnell RB, Posner JB. Observing the invisible: successful tumor immunity in humans. Nat Immunol. 2003; 4: 201
190. de Beukelaar JW, Sillevis Smitt PA. Managing paraneoplastic neurological disorders. Oncologist 2006; 11: 292-305

191. King PH. Differential expression of the neuroendocrine genes Hel-N1 and HuD in small-cell lung carcinoma: evidence for down-regulation of HuD in the variant phenotype. Int J Cancer 1997; 74: 378-382
192. Darnell RB. Onconeural antigens and the paraneoplastic neurologic disorders: at the intersection of cancer, immunity, and the brain. Proc Natl Acad Sci U S A. 1996; 93: 4529-4536
193. Kern F, Faulhaber N, Frommel C et al. Analysis of CD8 T cell reactivity to cytomegalovirus using protein-spanning pools of overlapping pentadecapeptides. Eur J Immunol. 2000; 30: 1676-1682
194. de Waal L, Yuksel S, Brandenburg AH et al. Identification of a common HLA-DP4-restricted T-cell epitope in the conserved region of the respiratory syncytial virus G protein. J Virol. 2004; 78: 1775-1781
195. Gratama JW, van Esser JW, Lamers CH et al. Tetramer-based quantification of cytomegalovirus (CMV)-specific CD8+ T lymphocytes in T-cell-depleted stem cell grafts and after transplantation may identify patients at risk for progressive CMV infection. Blood 2001; 98: 1358-1364
196. Stevenson PG, Bangham CR, Hawke S. Recruitment, activation and proliferation of CD8+ memory T cells in an immunoprivileged site. Eur J Immunol. 1997; 27: 3259-3268
197. Coulie PG, Karanikas V, Colau D et al. A monoclonal cytolytic T-lymphocyte response observed in a melanoma patient vaccinated with a tumor-specific antigenic peptide encoded by gene MAGE-3. Proc Natl Acad Sci U S A. 2001; 98: 10290-10295
198. Knutson KL, dela Rosa C, Disis ML. Laboratory analysis of T-cell immunity. Front Biosci. 2006; 11: 1932-1944
199. de Beukelaar JW, Sillevis Smitt P, Hop WC, Kraan J, Hooijkaas H, Verjans GMGM, Gratama JW. Increased proportions of circulating activated CD4+ and CD8+ T cells in Hu antibody associated Paraneoplastic Neurological Syndromes. Submitted
200. Dubuisson A, Kaschten B, Lenelle J et al. Primary central nervous system lymphoma report of 32 cases and review of the literature. Clin Neurol Neurosurg. 2004; 107: 55-63
201. Newell ME, Hoy JF, Cooper SG et al. Human immunodeficiency virus-related primary central nervous system lymphoma: factors influencing survival in 111 patients. Cancer 2004; 100: 2627-2636
202. Comoli P, Labirio M, Basso S et al. Infusion of autologous Epstein-Barr virus (EBV)-specific cytotoxic T cells for prevention of EBV-related lymphoproliferative disorder in solid organ transplant recipients with evidence of active virus replication. Blood 2002; 99: 2592-2598
203. Haque T, Wilkie GM, Taylor C et al. Treatment of Epstein-Barr-virus-positive post-transplantation lymphoproliferative disease with partly HLA-matched allogeneic cytotoxic T cells. Lancet 2002; 360: 436-442
204. Rooney CM, Smith CA, Ng CY et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. Blood 1998; 92: 1549-1555
205. Sherritt MA, Bharadwaj M, Burrows JM et al. Reconstitution of the latent T-lymphocyte response to Epstein-Barr virus is coincident with long-term recovery from posttransplant lymphoma after adoptive immunotherapy. Transplantation 2003; 75: 1556-1560
206. Smets F, Latinne D, Bazin H et al. Ratio between Epstein-Barr viral load and anti-Epstein-Barr virus specific T-cell response as a predictive marker of posttransplant lymphoproliferative disease. Transplantation 2002; 73: 1603-1610
207. Wagner HJ, Cheng YC, Huls MH et al. Prompt versus preemptive intervention for EBV lymphoproliferative disease. Blood 2004; 103: 3979-3981
208. Altman JD, Moss PA, Goulder PJ et al. Phenotypic analysis of antigen-specific T lymphocytes. Science 1996; 274: 94-96

209. Gallimore A, Glithero A, Godkin A et al. Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. J Exp Med. 1998;187:1383-1393
210. Moniuszko M, Brown C, Pal R et al. High frequency of virus-specific CD8+ T cells in the central nervous system of macaques chronically infected with simian immunodeficiency virus SIVmac251. J Virol. 2003;77:12346-12351
211. Weinberg A, Li S, Palmer M, Tyler KL. Quantitative CSF PCR in Epstein-Barr virus infections of the central nervous system. Ann Neurol. 2002;52:543-548
212. Niesters HG, van Esser J, Fries E et al. Development of a real-time quantitative assay for detection of Epstein-Barr virus. J Clin Microbiol. 2000;38:712-715
213. Hegde U, Filie A, Little RF et al. High incidence of occult leptomeningeal disease detected by flow cytometry in newly diagnosed aggressive B-cell lymphomas at risk for central nervous system involvement: the role of flow cytometry versus cytology. Blood 2005;105:496-502
214. Fukushima PI, Nguyen PK, O'Grady P, Stetler-Stevenson M. Flow cytometric analysis of kappa and lambda light chain expression in evaluation of specimens for B-cell neoplasia. Cytometry 1996;26:243-252
215. Harjunpaa A, Wiklund T, Collan J et al. Complement activation in circulation and central nervous system after rituximab (anti-CD20) treatment of B-cell lymphoma. Leukemia & lymphoma. 2001;42:731-738
216. Wong ET, Tishler R, Barron L, Wu JK. Immunochemotherapy with rituximab and temozolomide for central nervous system lymphomas. Cancer 2004;101:139-145
217. Catalina MD, Sullivan JL, Bak KR, Luzuriaga K. Differential evolution and stability of epitope-specific CD8(+) T cell responses in EBV infection. J Immunol. 2001;167:4450-4457
218. Hislop AD, Annels NE, Gudgeon NH et al. Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection. J Exp Med. 2002;195:893-905
219. Wynn RF, Arkwright PD, Haque T et al. Treatment of Epstein-Barr-virus-associated primary CNS B cell lymphoma with allogeneic T-cell immunotherapy and stem-cell transplantation. Lancet Oncol. 2005;6:344-346
220. de Beukelaar JW, Verjans GM, van Norden Y, Milikan JC, Kraan J, Hooijkaas H, Sintnicolaas K, Gratama JW, Sillevis Smitt PA. No evidence for circulating HuD-specific CD8+ T cells in patients with paraneoplastic neurological syndromes and Hu antibodies. Cancer Immunol Immunother. 2007; in press
221. Furneaux HF, Reich L, Posner JB. Autoantibody synthesis in the central nervous system of patients with paraneoplastic syndromes. Neurology 1990;40:1085-1091
222. Albert ML, Darnell JC, Bender A et al. Tumor-specific killer cells in paraneoplastic cerebellar degeneration. Nat Med. 1998;4:1321-1324
223. de Beukelaar JW, van Arkel C, van den Bent MJ et al. Resolution of EBV(+) CNS lymphoma with appearance of CSF EBV-specific T cells. Ann Neurol. 2005;58:788-792
224. Greten TF, Slansky JE, Kubota R et al. Direct visualization of antigen-specific T cells: HTLV-1 Tax11-19- specific CD8(+) T cells are activated in peripheral blood and accumulate in cerebrospinal fluid from HAM/TSP patients. Proc Natl Acad Sci U S A. 1998;95:7568-7573
225. Olsson T, Zhi WW, Hojeberg B et al. Autoreactive T lymphocytes in multiple sclerosis determined by antigen-induced secretion of interferon-gamma. J Clin Invest. 1990;86:981-985
226. Sun JB, Olsson T, Wang WZ et al. Autoreactive T and B cells responding to myelin proteolipid protein in multiple sclerosis and controls. Eur J Immunol. 1991;21:1461-1468
227. Zhang J, Markovic-Plese S, Lacet B et al. Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. J Exp Med. 1994;179:973-984

228. Schuller EA, Benabdallah S, Sagar HJ et al. IgG synthesis within the central nervous system. Comparison of three formulas. Arch Neurol. 1987; 44: 600-604
229. Vega F, Graus F, Chen QM et al. Intrathecal synthesis of the anti-Hu antibody in patients with paraneoplastic encephalomyelitis or sensory neuronopathy: clinical-immunologic correlation. Neurology 1994; 44: 2145-2147
230. Hoffmeister B, Kiecker F, Tesfa L et al. Mapping T cell epitopes by flow cytometry. Methods 2003; 29: 270-281
231. Milikan JC, Kuijpers RW, Baarsma GS et al. Characterization of the varicella zoster virus (VZV)-specific intra-ocular T-cell response in patients with VZV-induced uveitis. Exp Eye Res. 2006; 83: 69-75
232. Verjans GM, Feron EJ, Dings ME et al. T cells specific for the triggering virus infiltrate the eye in patients with herpes simplex virus-mediated acute retinal necrosis. J Infect Dis. 1998; 178: 27-34
233. Verjans GM, Dings ME, McLauchlan J et al. Intraocular T cells of patients with herpes simplex virus (HSV)-induced acute retinal necrosis recognize HSV tegument proteins VP11/12 and VP13/14. J Infect Dis. 2000; 182: 923-927
234. Croft PB, Urich H, Wilkinson M. Peripheral neuropathy of sensorimotor type associated with malignant disease. Brain 1967; 90: 31-66
235. Van der Aa A, Hellings N, Medaer R et al. T cell vaccination in multiple sclerosis patients with autologous CSF-derived activated T cells: results from a pilot study. Clin Exp Immunol. 2003; 131: 155-168
236. Niland B, Perl A. Evaluation of autoimmunity to transaldolase in multiple sclerosis. Methods Mol Med. 2004; 102: 155-171
237. Chou YK, Bourdette DN, Offner H et al. Frequency of T cells specific for myelin basic protein and myelin proteolipid protein in blood and cerebrospinal fluid in multiple sclerosis. J Neuroimmunol. 1992; 38: 105-113
238. Lisak RP, Zweiman B. In vitro cell-mediated immunity of cerebrospinal-fluid lymphocytes to myelin basic protein in primary demyelinating diseases. N Engl J Med. 1977; 297: 850-853
239. Crawford MP, Yan SX, Ortega SB et al. High prevalence of autoreactive, neuroantigen-specific CD8+ T cells in multiple sclerosis revealed by novel flow cytometric assay. Blood 2004; 103: 4222-4231
240. Kern F, Surel IP, Brock C et al. T-cell epitope mapping by flow cytometry. Nat Med. 1998; 4: 975-978
241. Maecker HT, Dunn HS, Suni MA et al. Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. J Immunol Methods 2001; 255: 27-40
242. Coleman S, Clayton A, Mason MD et al. Recovery of CD8+ T-cell function during systemic chemotherapy in advanced ovarian cancer. Cancer Res. 2005; 65: 7000-7006
243. ter Meulen J, Badusche M, Kuhnt K et al. Characterization of human CD4(+) T-cell clones recognizing conserved and variable epitopes of the Lassa virus nucleoprotein. J Virol. 2000; 74: 2186-2192
244. Benoiton N. Chemistry of peptide synthesis. Ed., Taylor & Francis Group, Andover. 2005
245. Sanz-Nebot V, Benavente, F., Toro, I., Barbosa, J. Separation and characterization of complex crude mixtures produced in the synthesis of therapeutic peptide hormones by liquid chromatography coupled to electrospray mass spectrometry (LC-ES-MS). Analytica Chimica Acta. 2004; 521: 25-36
246. Wenschuh H., Jerini Peptide Technologies, Berlin, Germany. Personal communication.
247. Baykut G, Jertz R, Witt M. Matrix-assisted laser desorption/ionization fourier transform ion cyclotron resonance mass spectrometry with pulsed in-source collision gas and in-source ion accumulation. Rapid Commun Mass Spectrom. 2000; 14: 1238-1247
248. Suckau D, Resemann A, Schuerenberg M et al. A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. Anal Bioanal Chem. 2003; 376: 952-965

249. O'Connor PB, Costello CE. A high pressure matrix-assisted laser desorption/ionization Fourier transform mass spectrometry ion source for thermal stabilization of labile biomolecules. Rapid Commun Mass Spectrom. 2001; 15: 1862-1868
250. Rosenstock HM, Wallenstein MB, Wahrhaftig AL, Eyring H. Absolute Rate Theory for Isolated Systems and the Mass Spectra of Polyatomic Molecules. Proc Natl Acad Sci U S A. 1952; 38: 667-678
251. van Dongen WD, Ruijters HF, Luinge HJ et al. Statistical analysis of mass spectral data obtained from singly protonated peptides under high-energy collision-induced dissociation conditions. J Mass Spectrom. 1996; 31: 1156-1162
252. Gorman GS, Speir, J.P., Turner, C.A., Amster, I.J. Proton affinities of the 20 common alpha-amino acids. J. Am. Chem. Soc. 1992; 114: 3986-3988
253. Peterson K, Rosenblum MK, Kotanides H, Posner JB. Paraneoplastic cerebellar degeneration. I. A clinical analysis of 55 anti-Yo antibody-positive patients. Neurology 1992; 42: 1931-1937
254. Graus F, Illa I, Agusti M et al. Effect of intraventricular injection of an anti-Purkinje cell antibody (anti-Yo) in a guinea pig model. J Neurol Sci. 1991; 106: 82-87
255. Sillevis Smitt P, Manley GT, Posner JB. High titer antibodies but no disease in mice immunized with the paraneoplastic antigen HuD. Neurology 1994; 44: 982S
256. Edwards JC, Szczepanski L, Szechinski J et al. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. N Engl J Med. 2004; 350: 2572-2581
257. Pestronk A, Florence J, Miller T et al. Treatment of IgM antibody associated polyneuropathies using rituximab. J Neurol Neurosurg Psychiatry 2003; 74: 485-489
258. Renaud S, Gregor M, Fuhr P et al. Rituximab in the treatment of polyneuropathy associated with anti-MAG antibodies. Muscle Nerve 2003; 27: 611-615
259. Reimann KA, O'Gorman MR, Spritzler J et al. Multisite comparison of CD4 and CD8 T-lymphocyte counting by single- versus multiple-platform methodologies: evaluation of Beckman Coulter flow-count fluorospheres and the tetraONE system.The NIAID DAIDS New Technologies Evaluation Group. Clin Diagn Lab Immunol. 2000; 7: 344-351
260. Pels H, Schulz H, Manzke O et al. Intraventricular and intravenous treatment of a patient with refractory primary CNS lymphoma using rituximab. J Neurooncol. 2002; 59: 213-216
261. Tan LC, Gudgeon N, Annels NE et al. A re-evaluation of the frequency of CD8+ T cells specific for EBV in healthy virus carriers. J Immunol. 1999; 162: 1827-1835
262. Jackman DM, Johnson BE. Small-cell lung cancer. Lancet 2005; 366: 1385-1396
263. Basso D, Guariso G, Fogar P et al. Insights in the laboratory diagnosis of celiac disease. Lupus 2006; 15: 462-465
264. Sollid LM, Jabri B. Is celiac disease an autoimmune disorder? Curr Opin Immunol. 2005; 17: 595-600
265. Stepniak D, Koning F. Celiac disease--sandwiched between innate and adaptive immunity. Hum Immunol. 2006; 67: 460-468
266. Zang YC, Li S, Rivera VM et al. Increased CD8+ cytotoxic T cell responses to myelin basic protein in multiple sclerosis. J Immunol. 2004; 172: 5120-5127
267. Skulina C, Schmidt S, Dornmair K et al. Multiple sclerosis: brain-infiltrating CD8+ T cells persist as clonal expansions in the cerebrospinal fluid and blood Proc Natl Acad Sci U S A. 2004; 101: 2428-2433
268. Dornmair K, Goebels N, Weltzien HU et al. T-cell-mediated autoimmunity: novel techniques to characterize autoreactive T-cell receptors. Am J Pathol. 2003; 163: 1215-1226
269. Hohlfeld R, Wekerle H. Autoimmune concepts of multiple sclerosis as a basis for selective immunotherapy: from pipe dreams to (therapeutic) pipelines. Proc Natl Acad Sci U S A. 2004; 101 Suppl 2: 14599-14606
270. Burns TM. A step forward for stiff-person syndrome. Lancet 2005; 365: 1365-1367

Summary

Paraneoplastic neurological syndromes (PNS) result from damage or dysfunction of the nervous system that is by definition not caused by tumor cell infiltration, infection, ischemia, metabolic and nutritional deficits, surgery or other forms of tumor treatment. Immunologic factors appear important in the pathogenesis of PNS because antineuronal antibodies against nervous system antigens have been defined for many of these disorders. The immunologic response is elicited by the ectopic expression of neuronal antigens by the tumor. Expression of these so-called 'onconeural' antigens is limited to the tumor and the nervous system. At the time of presentation of the neurological symptoms, most patients have not yet been diagnosed with cancer. Detection of paraneoplastic antibodies is extremely helpful in diagnosing an otherwise unexplained, and often rapidly progressive neurological syndrome as paraneoplastic. In addition, the paraneoplastic antibodies may also direct the search for an underlying neoplasm. The diagnosis and clinical management of PNS are reviewed in **chapter 2**. One of the most frequently involved solid tumors in PNS is small cell lung cancer (SCLC) and approximately 50% of patients with PNS and SCLC have high-titer Hu antibodies. This thesis focused on PNS that are associated with Hu-antibodies (Hu-PNS).

Hu antibodies are directed against a family of neuron specific, mRNA binding proteins (HuD, HuC and Hel-N1). The expression of the onconeural antigen HuD in both the nervous system and in the underlying SCLC favors the hypothesis that aberrant expression of Hu proteins in the SCLC triggers an immune response that subsequently reacts with the same or similar antigens in the nervous system. The immune response results non-only in extensive loss of neurons but also in a better tumor prognosis in SCLC patients with Hu antibodies compared to patients without Hu antibodies. However, the mechanisms responsible for the tumor suppression and neuronal damage are poorly understood. Despite high serum- and cerebrospinal fluid (CSF) titers, a pathogenic role of Hu antibodies could never be proven in vitro or in animal models. Pathological examination of PNS neuronal tissue demonstrated localized inflammatory cell infiltrates, containing B cells, $CD4^+$ and $CD8^+$ T cells, in the proximity of overt neuronal cell damage. The detection of oligoclonal $CD8^+$ T cells in infiltrated areas of nervous tissues and tumors of Hu-PNS patients suggested the involvement of these cells in neuronal loss. In addition, the expression of HLA class I molecules in tumors and in affected nervous tissues from Hu-PNS patients is compatible with a pathogenic role of Hu-specific $CD8^+$ T cells. The high titers of predominantly IgG 1 Hu antibodies suggest help from Hu-specific $CD4^+$ T cells to B cells. Based on these findings, the current hypothesis is that the ectopic expression of HuD in tumor cells elicits a HuD specific T cell response that subsequently attacks both the tumor and neurons. In accordance with this hypothesis previous studies have detected

HuD specific T cells in the blood of patients with Hu-PNS. However, HuD specific $CD8^+$ T cells were also found in the blood of healthy controls. Interpretation of the latter studies is hampered by limited control experiments (i.e., the lack of using control peptides and control proteins) and low patient numbers.

The objective of this thesis was to further investigate the role of T cells in the pathogenesis of Hu-PNS. To gain more insight into the hypothesized cell-mediated immune pathogenesis of these syndromes, we analyzed the circulating lymphocyte subsets in untreated patients with SCLC, Hu-PNS, SCLC without PNS and healthy controls in **chapter 3**. The proportions of activated $CD4^+$ and $CD8^+$ T cells were increased in Hu-PNS as compared to both control groups, suggesting a role for T cells in Hu-PNS. **Chapter 4** describes a multifaceted approach to detect circulating HuD specific T cells in a large group of patients with Hu-PNS and controls. Blood was tested from 43 Hu-PNS patients, 31 Hu antibody negative SCLC patients without PNS and 54 healthy controls. Peripheral blood mononuclear cells (PBMC) were stimulated with HuD protein-spanning peptide pools (15-mers) and individual HuD-derived peptides (9-mers) and analyzed by cytokine flow cytometry and interferon-γ ELISPOT-assays. Additionally, HuD-based Class I HLA multimers were used to visualize HuD-specific $CD8^+$ T cells. However, no HuD-specific $CD8^+$ T cells could be detected in the blood of Hu-PNS patients or controls.

The inability to detect circulating HuD-specific T cells may be due to the low levels of HuD-specific T cells in the peripheral blood compartment, i.e., below the detection level of our assays. Because in other CNS disorders antigen-specific T cells are present in higher frequencies in the CSF than in the blood, we subsequently focussed our studies on the CSF compartment. **Chapter 5** illustrates the successful usage of HLA class I multimer technology to detect antigen-specific T cells in CSF. The extension of this technique to detect HuD specific T cells in the CSF of Hu-PNS patients is described in **chapter 6**. In this study, we analyzed the presence of HuD specific T cells in the CSF of 13 Hu-PNS patients and controls. CSF was prospectively collected and CSF-derived T cells were expanded and assayed for HuD reactivity using HuD-derived peptides in interferon-γ ELISPOT assays. Additionally, fresh and expanded CSF-derived T cells were stained with HLA class-I multimers presenting HuD peptides. However, although the CSF from 12 of 13 Hu-PNS patients showed inflammatory changes, no HuD-specific $CD8^+$ T cells were detected with either method.

During our search for HuD specific T cells we encountered false-positive responses that are analyzed in **chapter 7**. Our data reveal that different batches from the same peptide may contain artefacts that influence the outcome of HLA-restricted T-cell response assays.

Finally, **chapter 8** reports on the treatment of patients with Hu-PNS with anti-CD20 (rituximab). In this uncontrolled, unblinded trial of rituximab, two of 8 Hu-PNS patients improved neurologically after rituximab therapy but this improvement was not accom-

panied by a significant reduction in serum or CSF Hu antibody titers. The neurological improvement may be explained by the reduction of antigen-presenting B cells.

Overall, some of our findings support the hypothesized T cell-mediated pathogenesis of Hu-PNS, but we were unable to confirm the presence of HuD specific $CD8^+$ T cells in blood and CSF. Several aspects that may explain the lack of detection of such cells in our studies are described in **chapter 9**. In the same chapter, we propose future studies directed at the continued search for antigen-specific T cells in Hu-PNS, as more research is required to either confirm or reject a role for T cells in these syndromes.

Samenvatting

Paraneoplastische Neurologische Syndromen (PNS) zijn ernstige neurologische syndromen die samenhangen met kanker. PNS worden niet direct veroorzaakt door ingroei van de tumor zelf of door metastasen, noch door vasculaire, metabole, infectieuze of met de behandeling samenhangende oorzaken. Immunologische factoren blijken een belangrijke rol te spelen bij de pathogenese van PNS aangezien bij vele van deze syndromen antineuronale (paraneoplastische) antistoffen tegen het zenuwstelsel worden aangetoond. De immuunrespons wordt uitgelokt door aberrante expressie van neuronale eiwitten in de tumor. De expressie van deze zogenaamde "onconeuronale antigenen" is beperkt tot de tumor en het zenuwstelsel. Bij presentatie van de neurologische symptomen is meestal nog niet bekend dat de patiënt een onderliggende tumor heeft. Daarom vormen de paraneoplastische antistoffen een belangrijk hulpmiddel bij de diagnose van PNS en bij het bepalen van de onderliggende maligniteit. De klinische benadering van PNS wordt toegelicht in **hoofdstuk 2**.

Vrijwel alle typen tumoren kunnen aanleiding geven tot PNS. Van de solide tumoren komt het kleincellig longcarcinoom (SCLC) het meest frequent voor bij PNS en bij ongeveer de helft van patiënten met PNS en SCLC kunnen Hu antistoffen worden aangetoond. Het in dit proefschrift beschreven onderzoek richt zich op PNS geassocieerd met Hu antistoffen (Hu-PNS). Hu antistoffen reageren met een familie van neuronspecifieke RNA bindende eiwitten (HuD, HuC en HelN1). Het tot expressie komen van het onconeuronale antigeen HuD in zowel het zenuwstelsel als in het onderliggend SCLC ondersteunt de hypothese dat aberrante expressie van Hu eiwitten in SCLC een immuunrespons uitlokt die zich vervolgens richt op dezelfde of gelijksoortige eiwitten in het zenuwstelsel. Deze immuunrespons resulteert in het verlies van neuronen. Echter, de immuunrespons resulteert ook in een verbeterde tumor gerelateerde prognose bij SCLC patiënten met Hu antistoffen in vergelijking met SCLC patiënten zonder Hu antistoffen.

De mechanismen die verantwoordelijk zijn voor het onderdrukken van de tumor en de neurologische schade zijn nog niet ontrafeld. Een pathogene rol voor Hu antistoffen kon ondanks hoge titers in serum en liquor nooit aangetoond worden in *in vitro* studies of in diermodellen. Pathologisch-anatomisch (PA) onderzoek van aangedaan zenuwweefsel van Hu-PNS patiënten toont lokale ontstekings-infiltraten bestaande uit B cellen, $CD4^+$ en $CD8^+$ T cellen. De detectie van oligoclonale $CD8^+$ T cellen in geïnfiltreerde gebieden van het zenuw- en tumor weefsel van Hu-PNS patiënten suggereert de betrokkenheid van deze cellen bij het verlies van neuronen. Tevens past de expressie van HLA klasse I moleculen op tumoren en aangedaan zenuwweefsel van Hu-PNS patiënten bij een pathogene rol van Hu-specifieke $CD8^+$ T cellen. Ook suggereren de hoge titers van voornamelijk IgG1 Hu

antistoffen hulp van Hu-specifieke $CD4^+$ T cellen aan B cellen. Op basis van deze bevindingen is de huidige hypothese dat aberrante expressie van HuD in tumorcellen een HuD-specifieke T cel respons uitlokt die zich richt op zowel de tumor als het zenuwweefsel. In overeenstemming met deze hypothese zijn in eerdere studies HuD specifieke T cellen aangetoond in het bloed van patiënten met Hu-PNS. Deze werden echter ook aangetoond in het bloed van gezonde donoren. De interpretatie van deze studies wordt bemoeilijkt door het beperkt gebruik van controle experimenten en lage patiënt aantallen.

Het doel van de studies beschreven in dit proefschrift was om meer inzicht te krijgen in de veronderstelde T cel-gemedieerde immuun pathogenese van Hu-PNS. Hiertoe onderzochten we de lymfocyten subsets in het bloed van onbehandelde patiënten met SCLC en Hu-PNS, met SCLC zonder PNS en gezonde controlepersonen in **hoofdstuk 3**. De percentages van geactiveerde $CD4^+$ en $CD8^+$ T cellen waren verhoogd in Hu-PNS in vergelijking met beide controle groepen, hetgeen een rol voor T cellen suggereert in Hu-PNS. In **hoofdstuk 4** wordt beschreven hoe met diverse technieken getracht is om circulerende HuD specifieke T cellen aan te tonen in een grote groep van Hu-PNS patiënten en controlepersonen. In deze studie werd het bloed onderzocht van 43 Hu-PNS patiënten, 31 Hu seronegatieve SCLC patiënten zonder PNS en 54 gezonde controles. Mononucleaire cellen werden uit het bloed geïsoleerd en gestimuleerd met HuD-eiwit overlappende peptiden pools (15-meren) en individuele HuD peptiden (9-meren). Cytokine productie door geactiveerde T cellen werd vervolgens uitgelezen met cytokine flow cytometrie en zogenaamde interferon-gamma Enzyme-linked immunosorbent spot (ELISPOT) assays. Daarnaast werd gebruik gemaakt van HuD-specifieke HLA klasse I multimeren om HuD specifieke $CD8^+$ T cellen te detecteren. Ondanks het gebruik van deze gevalideerde en gevoelige technieken konden HuD specifieke T cellen niet worden aangetoond in het bloed van Hu-PNS patiënten of controlepersonen.

Het niet kunnen aantonen van HuD-specifieke T cellen is mogelijk te verklaren door de zeer lage aantallen HuD-specifieke T cellen in het bloed waardoor deze met de toegepaste technieken niet detecteerbaar zijn. Omdat bij andere centraal zenuwstelsel aandoeningen antigeen-specifieke T cellen in hogere frequentie in de liquor dan in het bloed aangetoond worden, hebben we ons onderzoek vervolgens gericht op de analyse van antigeen-specifieke T cellen in de liquor. In **hoofdstuk 5** wordt aangetoond dat met behulp van HLA klasse I multimeren antigeen-specifieke $CD8^+$ T cellen niet alleen in het bloed maar ook in de liquor kunnen worden gedetecteerd. Het toepassen van deze techniek om HuD specifieke T cellen aan te tonen in de liquor van Hu-PNS patiënten wordt beschreven in **hoofdstuk 6**. In dit onderzoek werd de aanwezigheid van HuD specifieke T cellen onderzocht in de liquor van 13 Hu-PNS patiënten en controlepersonen. T cellen afkomstig uit de liquor werden aspecifiek geëxpandeerd en getest op reactiviteit met HuD peptiden in interferon-gamma ELISPOT assays. Tevens werden zowel 'verse' als geëxpandeerde T

cellen uit de liquor getest op binding met HuD-specifieke HLA klasse I multimeren. Bij 12 van de 13 Hu-PNS patiënten werden in de liquor aanwijzingen gevonden voor een ontsteking. Echter, bij geen van de Hu-PNS patiënten of controlepersonen konden HuD-specifieke $CD8^+$ T cellen worden aangetoond in de liquor.

Tijdens onze zoektocht naar HuD-specifieke T cellen detecteerden we vals-positieve reacties die worden beschreven in **hoofdstuk 7**. In dit hoofdstuk wordt aangetoond dat verschillende batches van hetzelfde peptide onzuiverheden kunnen bevatten die een grote invloed hebben op de uitkomst van T cel assays.

In **hoofdstuk 8** wordt een niet-gecontroleerde, niet-geblindeerde trial beschreven van de behandeling van Hu-PNS patiënten met anti-CD20 (rituximab). Twee van de 8 Hu-PNS patiënten vertoonden neurologische verbetering na de behandeling met rituximab. Deze verbetering ging echter niet gepaard met een significante verlaging van de Hu antistof titers in serum of liquor van deze patiënten. De neurologische verbetering is mogelijk toe te schrijven aan de afname van het aantal antigeen-presenterende B cellen.

Samenvattend toont het werk beschreven in dit proefschrift enige aanwijzingen voor een rol voor T cellen bij de pathogenese van Hu-PNS, maar de aanwezigheid van HuD specifieke T cellen kon niet worden bevestigd. Mogelijke verklaringen voor dit negatieve resultaat worden beschreven in **hoofdstuk 9**. In datzelfde hoofdstuk wordt de aanzet geleverd tot toekomstig onderzoek gericht op de detectie van antigeen-specifieke T cellen in Hu-PNS, aangezien verder onderzoek noodzakelijk is om de rol van T cellen bij de pathogenese van deze syndromen op te helderen.

List of abbreviations

15-mers	peptides consisting of 15 amino acids
7-AAD	7-amino actinomycin D
9-mers	peptides consisting of 9 amino acids
aa	amino acids
APC	antigen presenting cell
APC	allophycocyanin
CD	cluster of differentiation
CFC	cytokine flow cytometry
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
CT	computerized tomography
DC	dendritic cell
DHB	2,5-Dihydroxybenzoic acid
DMF	Dimethylformamide
DNA	deoxyribonucleic acid
EBV	Epstein Barr Virus
EBV-LPD	EBV-related lymphoproliferative disease
ELISPOT	enzyme-linked immunosorbent spot-forming assay
F	female
FDG-PET	fluorodeoxyglucose positron emission tomography
FITC	fluorescein isothiocyanate
FMO	fluorescent minus one
Fmoc based	9-Fluorenylmethoxycarbonyl-based
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyl uranium hexafluorophosphate
HC	healthy control
HLA	Human Leukocyte Antigen
HPLC	High Performance Liquid Chromatography
Hu	antigen called after the first patient's initials
Hu-Ab	Hu antibody
ICR cell	Ion Cyclotron Resonance cell
IFN	interferon
Ig	immunoglobulin
IIF	indirect immunofluoresence
IL	interleukin

IVIg	intravenous immunoglobulins
LC-ESI-MS	Liquid Chromatography coupled to Electrospray Ionization Mass Spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
M	male
m/z	Ratio of mass to number of charges (regardless of sign).
mAb	monoclonal antibody
Maldi-FT MS	Matrix-assisted Laser Desorption/Ionization-Fourier Transform Mass Spectrometry
Maldi-TOF MS	Matrix-assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry
MHC	major histocompatibility complex
min	minutes
MNC	mononuclear cells
MRC	Medical Research Council
MRI	magnetic resonance imaging
mRS	modified Rankin Scale
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry
NA	not applicable
NEG	negative control
NSCLC	non-small cell lung cancer
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCD	paraneoplastic cerebellar degeneration
PCR	polymerase chain reaction
PE	phycoerythrin
PEM	paraneoplastic encephalomyelitis
Per-CP	peridinyl chlorophyllin
PET	positron emission tomography
PHA	Phytohemagglutinin
PLE/BE	paraneoplastic limbic encephalitis/ brainstem encephalitis
PMA/Ion	phorbol myristate acetate / ionomycin
PNC	polynuclear cells
PNS	paraneoplastic neurological syndromes
PP65	CMV phosphoprotein (pp)-65
PSN	paraneoplastic sensory neuronopathy
RNA	ribonucleic acid
RRKM theory	Rice-Ramsperger-Kassel-Marcus theory

RT	room temperature
SCLC	small cell lung cancer
SCT	stem cell transplantation
SFC	spot forming cell
SLE	systemic lupus erythematosus
SPS	stiff-person syndrome
TCP-resin	Trichlorophenyl resin
TCR	T cell receptor
TFA	Trifluoroacetic acid
TNF	tumor necrosis factor
Yo	antigen called after the first patient's initials

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Curriculum Vitae

Janet Willemien Karin de Beukelaar was born on July 9th 1977 in Woerden. She attended primary school in Cobham, England, and secondary school at the Marnix Gymnasium in Rotterdam, where she graduated in 1995. In the same year she started Medical School at the University of Antwerp in Belgium. She continued Medical School from 1996 onwards at the Erasmus University in Rotterdam. During her study she joined the student fraternity "S.S.R. Rotterdam" and studied several subjects in Law School (Erasmus University Rotterdam). She conducted research at the department of Medical Oncology (Prof.dr. T.A.W. Splinter), which offered her the opportunity to present her work at scientific meetings in New Orleans, San Francisco, Paris, and Crete. She obtained her medical degree in 2003 (*Cum Laude*) and in the same year she started the work described in this thesis at the departments of Neurology (Prof.dr. P.A.E. Sillevis Smitt) and Medical Oncology (Dr. J.W. Gratama) at the Erasmus MC (Centrumlocatie and Daniel den Hoed). In 2005, she carried out part of her research project at the department of Virology (Dr. G.M.G.M. Verjans and Prof.dr. A. Osterhaus) at the same medical center. During her PhD project she was active in various committees of the Molecular Medicine Research School, was a member of the European "PNS EuroNetwork", started her SMBWO Immunology training and received the young investigator award at the International Symposium of Preventive Oncology (ISPO) in 2004. In December 2006, she started her Neurology residency at the Erasmus MC (head Prof.dr. P.A.E. Sillevis Smitt).

She lives together with her fiancé Jelmer Bergsma in Rotterdam and likes to spend her time on social activities, art (painting, photography) and music.

List of publications

1. **JWK de Beukelaar**, C van Arkel, MJ van den Bent, MB van 't Veer, GJ van Doornum, JJ Cornelissen, PAE Sillevis Smitt, JW Gratama. Resolution of EBV^+ CNS lymphoma with appearance of CSF EBV-specific T cells. Ann Neurol 2005;58:788-792

2. **JWK de Beukelaar**, PAE Sillevis Smitt. Managing paraneoplastic neurological disorders. Oncologist 2006;11:292-305

3. **JWK de Beukelaar**, GMGM Verjans, Y van Norden, JC Milikan, J Kraan, H Hooijkaas, K Sintnicolaas, JW Gratama, PAE Sillevis Smitt. No evidence for circulating HuD-specific $CD8^+$ T cells in patients with paraneoplastic neurological syndromes and Hu antibodies. Cancer Immunol Immunother 2007; in press

4. **JWK de Beukelaar**, JW Gratama, PAE Sillevis Smitt, GMGM Verjans, J Kraan, TM Luider, PC Burgers. The impact of impurities in synthetic peptides on the outcome of T-cell stimulation assays. Rapid Commun Mass Spectrometry 2007;21:1282-1288

5. S Shams'ili, **JWK de Beukelaar**, JW Gratama, H Hooijkaas, MB van 't Veer, PAE Sillevis Smitt. An uncontrolled trial of rituximab for antibody associated paraneoplastic neurological syndromes. J Neurol. 2006;253:16-20

6. **JWK de Beukelaar**, PAE Sillevis Smitt, WC Hop, J Kraan, H Hooijkaas, GMGM Verjans, JW Gratama. Increased proportions of circulating activated $CD4^+$ and $CD8^+$ T cells in Hu antibody associated Paraneoplastic Neurological Syndromes. Submitted

7. **JWK de Beukelaar**, JC Milikan, GMGM Verjans, Y van Norden, C Lamers, MJ van den Bent, JEC Bromberg, E Hulsenboom, K Sintnicolaas, JW Gratama, PAE Sillevis Smitt. The cerebrospinal fluid of patients with Hu-associated paraneoplastic neurological syndromes does not contain detectable numbers of HuD-specific $CD8^+$ T cells. Submitted

8. JW Gratama, **JWK de Beukelaar**, J Bergsma, Y van Dooren, Y van Norden, M Klimek, GMGM Verjans, MJ van den Bent, A Kusuma, J Kraan, PAE Sillevis Smitt. Lymphocyte subset analysis in cerebrospinal fluid of patients with Hu antibody associated paraneoplastic syndromes and healthy controls. To be submitted

9. **JWK de Beukelaar**, PAE Sillevis Smitt. Hoofdstuk 6: Paraneoplastische Neurologische Syndromen. Handboek NeuroImmunologie 2004;183-206

10. PAE Sillevis Smitt, SKL Polman, **JWK de Beukelaar**, RH Enting, JM Kros, H Hooijkaas. Klinische les: Diagnose van Paraneoplastische Neurologische Syndromen. Ned Tijdschr Geneeskd 2007; in press

11. **JWK de Beukelaar**, JW Gratama, H Hooijkaas, PAE Sillevis Smitt. Paraneoplastic Neurologic Syndromes: a window to occult tumors under immune attack. Daniel den Hoed Cancer News 2004;2:29-33

12. **JWK de Beukelaar**, HAP Pols, MC Zillikens, P van Biezen, PLA van Daele. How stressful can it be to catch a thief? Case Report and analysis of the influence of stress on calcium metabolism. Am J Med 2004;116:279-80

13. **JWK de Beukelaar**, HAP Pols, MC Zillikens, P van Biezen, PLA van Daele. De invloed van stress op calciummetabolisme. Nederlands tijdschrift voor Calcium- en Botstofwisseling 2005;2:31-33 & 46-47

14. **JWK de Beukelaar**, TAW Splinter, CCD van der Rijt, PIM Schmitz, WJ Kirkels. Survival and prognostic value of the response to induction chemotherapy and additional treatment of patients with locally advanced inoperable urothelial cancer. Int J Mol Med 2002;10(2):65