PARANEOPLASTIC NEUROLOGICAL SYNDROMES ASSOCIATED WITH ANTI-HU ANTIBODIES:

ARE T LYMPHOCYTES INVOLVED OR NOT?

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Colofon

The studies described in this thesis were performed at the Departments of Neurology and Medical Oncology from the Erasmus University Medical Center in Rotterdam, The Netherlands.

This research project was supported by the Gratama Foundation (Teteringen, The Netherlands), Erasmus MC Grant (Rotterdam, The Netherlands) and ZonMW AGIKO Stipendium (Den Haag, The Netherlands).

Printing of this thesis was sponsored by Sysmex, Duke Scientific Corporation, Beckman Coulter, BD Biosciences and Zebra Bioscience.

ISBN: 978-90-8891-305-1

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Lay out and cover design: Jan Heijnen, Hieke Denijs
Printed by: Proefschriftmaken.nl || Printyourthesis.com
Published by: Uitgeverij BOXPress, Oisterwijk

Paraneoplastic Neurological Syndromes associated with anti-Hu antibodies: are T lymphocytes involved or not?

Paraneoplastische Neurologische Syndromen
geassocieerd met anti-Hu antistoffen:
betrokkenheid van T lymfocyten of niet?

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. H.G. Schmidt
en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 5 oktober 2011 om 15:30 uur

door

Marieke Theodora de Graaf
geboren te Alkmaar

PROMOTIECOMMISSIE

Promotor:	Prof.dr. P.A.E. Sillevis Smitt
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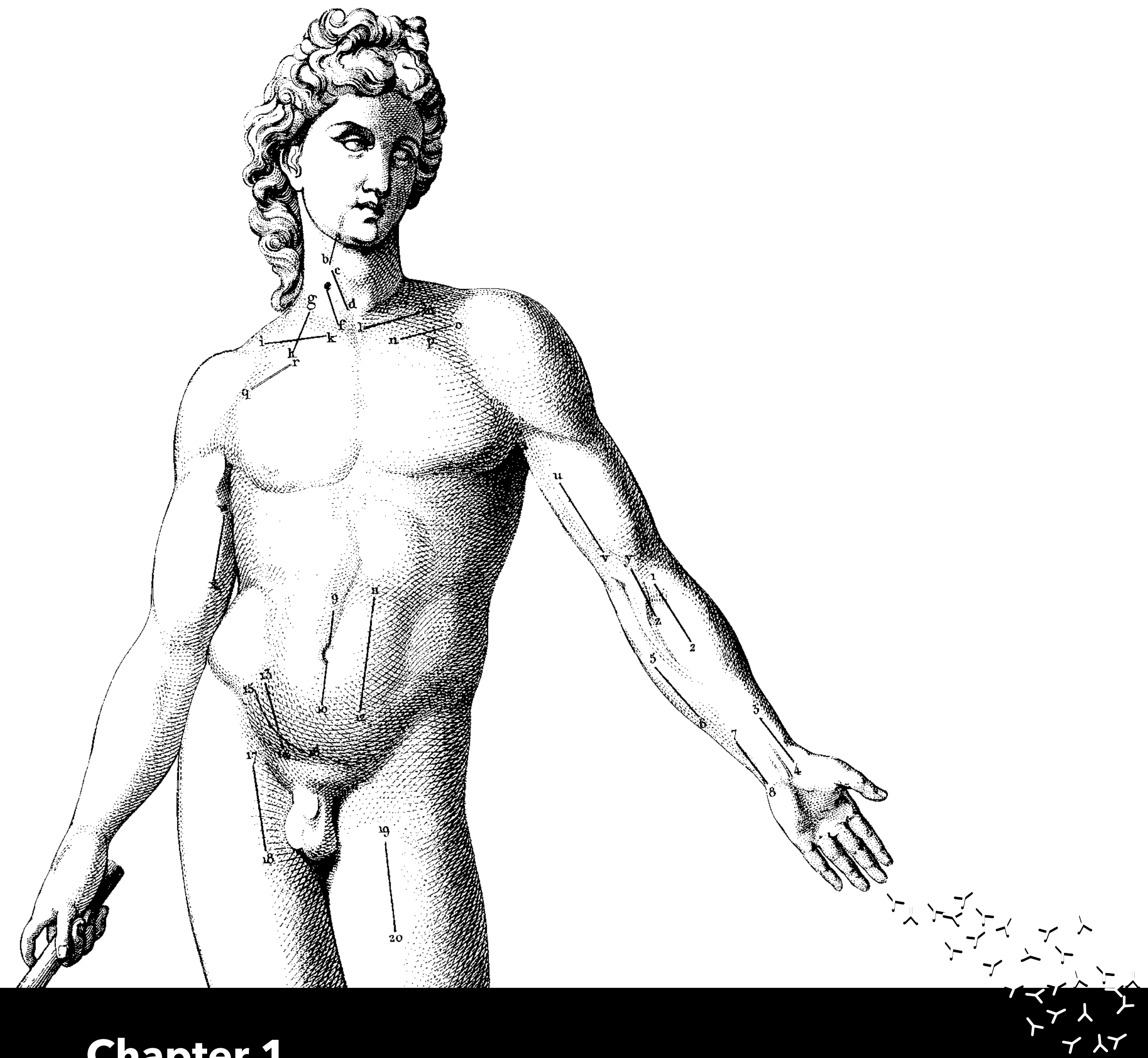
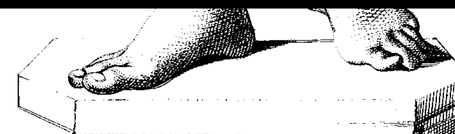


Chapter 1

General Introduction



PARANEOPLASTIC NEUROLOGICAL SYNDROMES

Clinical aspects

Paraneoplastic neurological syndromes (PNS) are remote effects of cancer that are not caused by invasion of the tumor or its metastases, nor by infection, ischemia, metabolic and nutritional deficits, surgery or other forms of tumor treatment[1]. PNS may effect any level of the nervous system (central or peripheral nervous system, including neuromuscular junction and muscle), and are devastating neurological syndromes leaving most of the patients severely disabled within a few months. In Chapter 2 the diagnosis and clinical management of PNS are reviewed. One of the most frequently involved tumors in PNS is small cell lung cancer (SCLC), and approximately 50% of patients with PNS and SCLC have high-titer antibodies against the onconeural HuD antigen (anti-Hu)[2]. Detection of anti-Hu antibodies in serum or cerebrospinal fluid (CSF) definitively diagnoses the neurological syndrome as paraneoplastic[3, 4]. The first goal of treatment for PNS is antitumor therapy, which halts neurological deterioration and improves functional outcome[5-9]. Additional immunotherapy with steroids, cyclophosphamide, rituximab, intravenous immunoglobulin (IVIg) or plasma exchange has disappointing results[6, 9-16]. This thesis will focus on PNS associated with anti-Hu antibodies (Hu-PNS).

Pathogenesis of Hu-PNS

The general model for Hu-PNS is that the normally neuron-specific expression of the HuD antigen, combined with immune privilege in the brain, accounts for the immunogenicity of the HuD antigen when it is expressed in SCLC[17]. However, the observation that all SCLC express the HuD antigen[18], while only 1% of patients develop Hu-PNS with high-titer anti-Hu antibodies[1] and low-titer anti-Hu antibodies are present in an additional 16% of SCLC patients[19, 20], does not fit into this model. Therefore, an alternative model has been proposed recently in which a natural immune response to HuD is absent - to keep the nervous system from autoimmune attack - and HuD tolerance has to be broken before Hu-PNS develops[21]. This alternative model is based on the danger theory[22], which states that the default reaction of T lymphocytes to antigens on non-hematopoietic tissues is tolerance, and it is the role of blood-derived antigen presenting cells, particularly dendritic cells, to detect and report to T lymphocytes in situations of dangerous tissue distress[23]. The finding that there is a strong tolerance to HuD in normal resting mice, while HuD-deficient mice generate HuD-specific T lymphocytes without peptide stimulation[21], supports this model. In cancer, only if tissue cells are damaged by tumor cell infiltration, or made to die necrotically due to severe ischemia or infection, danger signals will be produced to stimulate antigen presenting cells and subsequently an antitumor response will be generated[24]. If this model holds true for Hu-PNS, there has to be some kind of tissue distress, e.g., cytokine production by the tumor[25], in SCLC patients developing Hu-PNS which is absent in SCLC patients without Hu-PNS. Apparently, this tissue distress is present in a minority of SCLC patients, given that only a small population of SCLC patients succumbs to neuronal degeneration.

The immune response that is triggered in Hu-PNS, not only reacts with the tumor but is also directed against HuD expressed in the nervous system. Ninety-five percent of SCLC patients with Hu-PNS have limited disease[26, 27], which could partly be explained by lead time bias as a search is performed towards an asymptomatic SCLC in 60%-80% of patients[26]. Alternatively, the anti-tumor immune response might be responsible for the indolent course and extended survival in many Hu-PNS patients[20, 26, 27]. However, the price of tumor control is high, because PNS also cause neuronal damage resulting in severe neurological symptoms. While all patients with high anti-Hu titers suffer from PNS, an additional 16% of SCLC patients have low-titer anti-Hu antibodies without PNS[19, 20, 27]. In these patients, the presence of low-titered anti-Hu antibodies was correlated with limited stage disease, complete response to chemotherapy, and improved survival[9, 15, 19, 27]. Although, in another study, no association between the presence of anti-Hu antibodies and the extent of disease or survival was found[28]. The lack of neurological symptoms in patients with low titers of anti-Hu antibodies suggests that antitumor immunity can be dissected from neurological autoimmunity.

Cellular immunity

Previous studies have clearly demonstrated that anti-Hu antibodies do not play a pathogenic role in Hu-PNS and tumor control[29-36], but rather are a useful diagnostic marker[4]. Pathological examination of affected areas of the nervous system in Hu-PNS shows loss of neurons with localized inflammatory cell infiltrates, containing B cells, $CD4^+$ T-helper lymphocytes and cytotoxic $CD8^+$ T lymphocytes[34, 37-43]. T lymphocyte receptor analysis supported a direct effector role of cytotoxic $CD8^+$ T lymphocytes, the same clones being likely operative in neuronal damage and immune-mediated tumor growth control[39, 44]. The cerebrospinal fluid (CSF) of Hu-PNS patients generally is abnormal, showing signs of inflammation, including mononuclear pleocytosis, intrathecal synthesis of IgG and HuD-specific oligoclonal bands[8, 45-49]. In blood, Hu-PNS patients had a variety of imbalances within their lymphocyte subsets as compared with SCLC patients and healthy controls: i. a lymphopenia of B, $CD4^+$ and $CD8^+$ T lymphocytes[50]; ii. increased proportions of activated $CD4^+$ and $CD8^+$ T lymphocytes[41, 50]; iii. increased numbers of $CD4^+$ memory T lymphocytes[41]; iv. reduced numbers of terminally differentiated effector $CD8^+$ T lymphocytes and lymphocytes with a cytotoxic phenotype ($CD56^+$ and $CD57^+$)[50]; and v. down-regulation of expression levels of FOX-P3, TGF-β and CTLA-4 mRNA in regulatory T-lymphocyte subsets suggesting regulatory T-lymphocyte dysfunction[51].

Additionally, difficulty in treating these disorders with strategies directed at the humoral immune response[11, 16] support a role for T-lymphocyte mediated mechanisms in PNS, although therapies directed at the cellular immune response alone or both humoral and cellular immunity[5, 10, 14] have not been successful either. Overall, these findings suggest the occurrence of both B- and T-lymphocyte mediated immune responses in blood and CSF of Hu-PNS patients[34, 40, 47].

Cerebrospinal fluid in Hu-PNS

In CSF of Hu-PNS patients, high-titered anti-Hu antibodies can be detected. Furthermore, 93% of patients have an abnormal CSF[45]: pleocytosis (38%), elevated protein levels (80%) or presence of oligoclonal bands (59%). These oligoclonal bands are HuD-specific[47]. A study in 295 PNS patients with anti-Hu (58%), anti-Yo (20%), anti-CV2 (8%), anti-Ma (6%), anti-Ri (5%), or anti-Tr (3%) antibodies showed elevated numbers of CSF cells in 47% of patients before the 3rd month after disease onset, while after the 3rd month only 28% of patients had a CSF pleocytosis[45]. This evolution suggests a (sub)acute inflammation phase in the central nervous system (CNS) during the first months of PNS, followed by a non-inflammatory phase[45]. Moreover, this CSF pleocytosis is consistent with a cellular immune response in this compartment[49]. In patients with paraneoplastic cerebellar degeneration (PCD) and anti-Yo antibodies directed against the cdr2 antigen, this CSF pleocytosis has been shown to contain increased percentages of activated T lymphocytes[52]. In CNS viral infections and multiple sclerosis (MS), antigen-specific T lymphocytes have been identified in relatively high concentrations in CSF[53-56]. This suggest that the T lymphocytes located within the CSF may reflect a T-lymphocyte response in the CNS parenchyma. Because fresh brain biopsies of Hu-PNS patients are generally unavailable for functional T-lymphocyte studies, investigation of CSF samples of Hu-PNS patients is a good alternative.

HuD-specific T lymphocytes

The current hypothesis is that the ectopic expression of HuD in tumor cells[18] elicits a HuD-specific T-lymphocyte response that subsequently attacks both the tumor and neurons. Previous studies have studied the presence of these HuD-specific T lymphocytes, but most evidence is circumstantial. Stimulation with the HuD antigen resulted in higher proliferative responses of peripheral blood mononuclear cells (PBMC) in Hu-PNS patients compared to SCLC patients and healthy controls[41], while ELISPOT assays with 19 different HuD-peptides showed a significant IFN-γ secretion in 7/10 Hu-PNS patients[38]. The presence of HuD-specific CD8$^+$ T lymphocytes is suggested by the observation that activated CD8$^+$ T lymphocytes from a Hu-PNS patient lysed autologous fibroblasts injected with the recombinant HuD protein[57], while human leukocyte antigen (HLA) class I-restricted CD8$^+$ cytotoxic T-lymphocyte activity directed against five HuD-peptides was observed in three Hu-PNS patients[58]. Additionally, eight different HuD-peptides elicited specific cytotoxic T lymphocytes in a humanized murine model after peptide vaccination[59]. Several studies directly looking for circulating HuD-specific CD8$^+$ T lymphocytes were either negative[60] or inconclusive showing specific T-lymphocyte responses in controls[38, 59]. The failure to detect HuD-specific CD8$^+$ T lymphocytes could be related to the limited sensitivity of the assays used and the low frequency and affinity of cytotoxic T lymphocytes involved in antitumor and autoimmunity when compared to e.g., virus-specific cytotoxic T lymphocytes[53]. However, recently HuD133-specific CD8$^+$ T lymphocytes were detected in blood of one HLA-A0301 Hu-PNS patient and HuD157-specific CD8$^+$ T lymphocytes in blood and CSF of two HLA-A0201 Hu-PNS patients[61]. These HuD157-specific CD8$^+$ T lymphocytes were shown to exhibit a different phenotype, producing type 2 cytokines including IL-13 and IL-5 instead of IFN-γ[61]. This is an alternative explanation for the failure to detect HuD-specific T lymphocytes in earlier studies.

IMMUNO ASSAYS

Flow cytometry

A very useful technique to investigate leukocyte and lymphocyte populations in blood and CSF is flow cytometry. With this technique it is possible to detect a large spectrum of cellular characteristics even in samples with low-cellularity like CSF[62], which also facilitates the detection of low-frequency cells[63]. Additionally, absolute cell numbers can be counted[63]. Examination of blood and CSF cell populations in various neuro-inflammatory disorders by flow cytometry is used to further elucidate the immunopathogenesis of these diseases. For example, in multiple sclerosis various studies have examined frequencies of different lymphocyte subsets in CSF in order to study pathogenesis[64-77], determine disease activity[78-83] and evaluate therapy[84-86]. In Chapter 3 applications, possibilities and technical challenges of CSF flow cytometry are reviewed.

Antigen-specific T lymphocytes

Numerous techniques to measure antigen-specific T lymphocytes are also based on flow cytometry. For this purpose, flow cytometry is the only platform that combines the detection of antigen-specific T lymphocytes with multi-parametric analysis of these cells, including phenotypic markers, intracellular molecules, function and proliferation[87]. The following techniques are all based on flow cytometry. Secreted mediators (e.g., cytokines) in antigen-activated cells are directly visualized by intracellular cytokine staining[88], while the cytokine capture assay permits the detection, analysis and isolation of viable cytokine-secreting T lymphocytes[89]. Upon CD4$^+$ T-lymphocyte activation, CD154 expression, which

is transiently expressed on the cell surface, is measured[90] and for cytotoxicity, CD107 expression on the cell surface is determined[91]. Major histocompatibility complex (MHC)-peptide multimers bind directly to the antigen receptor of a specific T lymphocyte and are detected by flow cytometry[92]. Furthermore, antibodies specific for a unique T cell receptor (TCR) or surface activation markers (e.g., upregulation of CD25 or CD69) can be used to detect antigen-specific T lymphocytes by flow cytometry. Specific proliferation of cells can be measured with the carboxyfluoroscein diacetate succinimidyl ester (CFSE)-dilution or bromo-deoxy-uridine (BrdU)-incorporation assays[93, 94].
In addition, several techniques without using flow cytometry are used for detection of antigen-specific T lymphocytes. Proliferation of lymphocytes is studied by measuring the incorporation of ^{3}H-thymidine into the DNA of proliferating lymphocytes[95]. In cytotoxicity assays, ^{51}Chromium labelled target cells are incubated with cytolytic effector lymphocytes and release of radioactivity is measured[96]. Alternatively, non-radioactive labelling dyes can be used[97]. Another way to measure cellular activation is through the quantification of secreted mediators (e.g., cytokines) by specific ligand assays (e.g., enzyme-linked immunosorbent assay [ELISA])[98] and with the enzyme-linked immunosorbent spot-forming assay (ELISPOT) in which mediator-secreting cells can be enumerated[99].

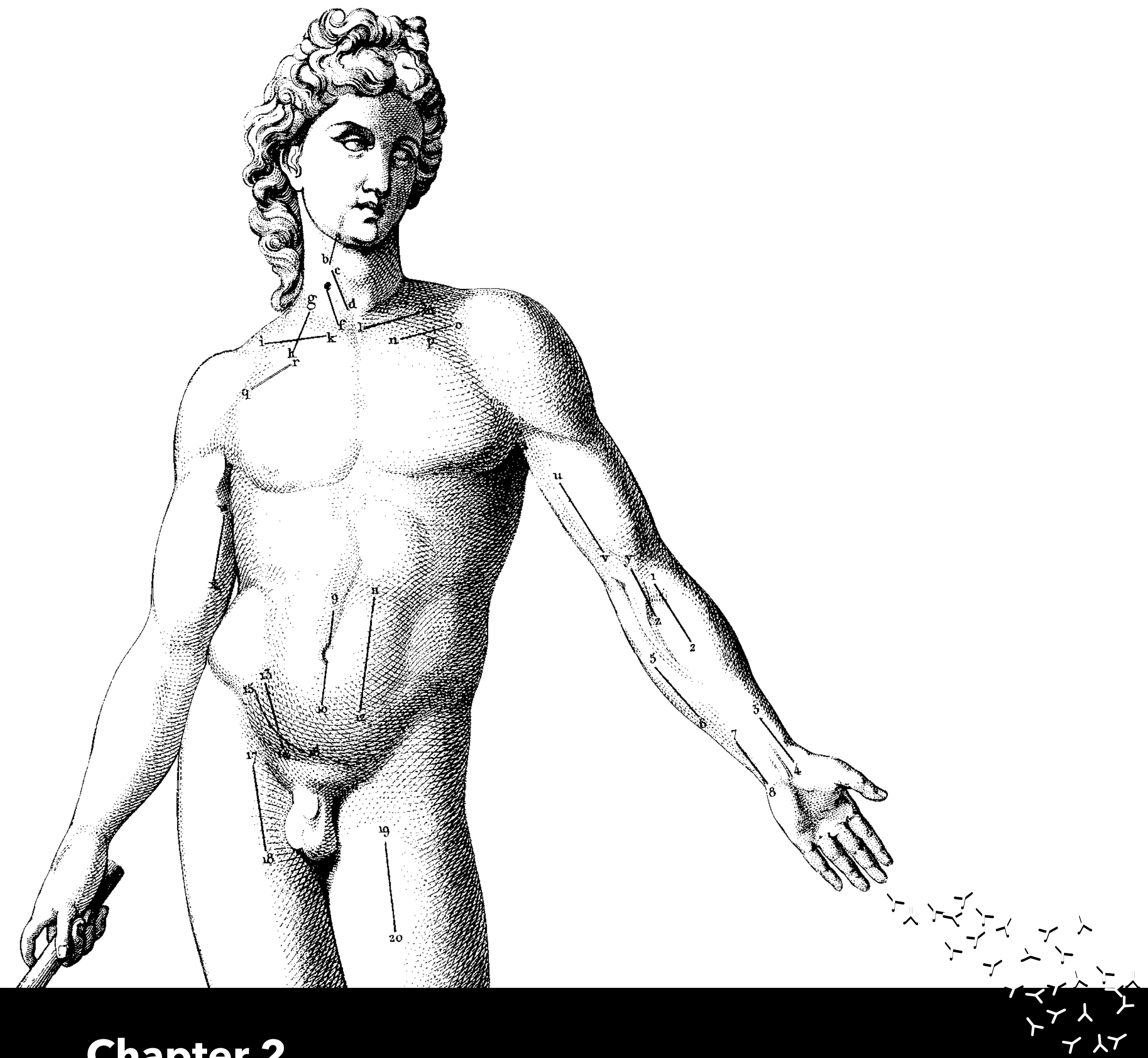
HLA association

Another way to study autoimmune diseases in which T lymphocytes are involved, is by looking at possible HLA-association. T-lymphocyte mediated immune responses are elicited by peptide fragments derived from antigens bound to HLA molecules on the cell surface of antigen-presenting lymphocytes. These HLA molecules are polygenic; every individual possesses a set of HLA molecules, determined in the HLA genes, which have different ranges of peptide-binding specificities. In other autoimmune diseases, including myasthenia gravis, type I diabetes mellitus and celiac disease[100-102], these HLA genes play an important role in an individual's susceptibility to the disease. Individuals with a specific HLA haplotype have an increased risk of mounting an autoimmune reaction against a specific antigen and thereby developing an autoimmune disease[103].

SCOPE OF THIS THESIS

In this thesis we aimed to further investigate the role of T lymphocytes in the pathogenesis of Hu-PNS. The clinical aspects of Hu-PNS are reviewed in **Chapter 2**. To reach our goal, we studied CSF as well as blood of Hu-PNS patients. The first part of this thesis describes CSF studies. To optimize the use of flow cytometry in CSF (**Chapter 3**), we investigated the effect of adding medium directly after CSF sampling on the viability of CSF cells

(**Chapter 4**) and determined reference values for absolute numbers and percentages of leukocyte and lymphocyte subsets in CSF (**Chapter 5**). In CSF of Hu-PNS patients, we studied lymphocyte subsets (**Chapter 6**) and searched for the presence of HuD-specific T lymphocytes (**Chapter 7**). The second part of this thesis is attributed to studies in blood of Hu-PNS patients. In earlier studies, detection of HuD-specific T lymphocytes by stimulation of PBMC or T lymphocytes of Hu-PNS patients has been shown to be extremely difficult (see above). The finding of a predisposition of HLA-DQ2^{+} individuals to develop Hu-PNS (**Chapter 8**) gives us the opportunity to stimulate more specifically. However, false-positive findings in T-lymphocyte stimulations due to peptide contamination (**Chapter 9**) is another challenge in these stimulation assays. Our ultimate goal is development of a treatment for Hu-PNS. Therefore, we conducted a clinical trial in which we treated Hu-PNS patients with human chorionic gonadotropin (hCG) (**Chapter 10**). Our main findings, discussion and future perspectives are presented in **Chapter 11** and **Chapter 12** summarizes this thesis.

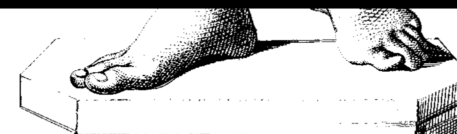


Chapter 2

Neurological Paraneoplasias

Marieke T. de Graaf and Peter A.E. Sillevis Smitt

In: Wick MR: Metastatic carcinomas of unknown origin; Chapter 2: Paraneoplastic syndromes associated with MCUOs. New York, Demos, 2008: pp 27-41



INTRODUCTION

Paraneoplastic syndromes are defined as remote effects of cancer that are not caused by invasion of the tumor or its metastases, nor by infection, ischemia, metabolic and nutritional deficits, surgery or other forms of tumor treatment[2, 104]. Paraneoplastic syndromes can present with a plethora of symptoms affecting many organ systems (Table 1). The incidence of paraneoplastic syndromes varies widely both by the type of syndrome and by the underlying cancer. Cachexia, anorexia, and weight loss are for instance extremely common in advanced cancer patients. On the other hand, most of the antibody-associated paraneoplastic neurological syndromes (PNS) are very rare, with an incidence of well below 1% of cancer patients[2].

Table 1 | Paraneoplastic syndromes associated with carcinomas

Neurological	**Systemic**
Central nervous system	Cachexia, anorexia, weight loss[b]
Encephalomyelitis[a]	Fever
Limbic encephalitis[a]	Nonbacterial thrombotic endocarditis
Brainstem encephalitis	Orthostatic hypotension
Subacute cerebellar degeneration[a]	Systemic lupus erythematosus
Opsoclonus-myoclonus	
Stiff-person syndrome	**Endocrine/metabolic**
Paraneoplastic visual syndromes	Acromegaly
Cancer-associated retinopathy	Carcinoid syndrome
Melanoma-associated retinopathy	Cushing's syndrome
Paraneoplastic optic neuropathy	Galactorrhea
Motor neuron syndromes	Gynecomastia
Subacute motor neuronopathy	Hyperamylasemia
Other motor neuron syndromes	Hypercacitonemia
	Hypercalcemia[b]
Peripheral nervous system	Hyperglycemia
Subacute sensory neuronopathy[a]	Hypertension
Acute sensorimotor neuropathy	Hyperthyroidism
Chronic sensorimotor neuropathy[b]	Hypoglycemia
Association with M-proteins	Hyponatremia[b]
Subacute autonomic neuropathy	Hypophosphatemia
Paraneoplastic peripheral nerve vasculitis	Hypouricemia
	Lactic acidosis
Neuromuscular junction and muscle	
Lambert-Eaton myasthenic syndrome[a,b]	**Cutaneous**
Myasthenia gravis	Acanthosis nigrans
Neuromyotonia	Acquired hypertrichosus lanuginose
Dermatomyositis[a]	Acquired ichthyosis
Acute necrotizing myopathy	Acquired palmoplantar keratoderma
Cachectic myopathy[b]	Acrokeratosis (Bazex's syndrome)
	Clubbing[b]
Hematologic	Dermatomyositis
Anemia[b]	Erythema annulare centrifugum
Dysproteinemia (amyloidosis)	Erythema gyratum repens
Eosinophilia	Exfoliative dermatitis
Hypercoagulability	Florid cutaneous papilllomatosis
Leukocytosis/leukoerythroblastic reaction	Hypertrophic pulmonary osteoarthropathy
Polycythemia	Pemphigus vulgaris
Thrombocytopenic purpura	Pityriasis rotunda
	Pruritus
Renal	Sign of Leser-Trélat
Glomerulopathies	Superficial thromboflebitis[b]
Tubulointerstitial disorders	Sweet's syndrome
	Tripe palms
	Vasculitis

[a]Classical paraneoplastic neurological syndromes.
[b]More common paraneoplastic syndromes.

Several mechanisms may be involved in the pathogenesis of paraneoplastic syndromes, including factors secreted by the tumors and other immunological mechanisms (Table 2). Most systemic, endocrine and metabolic, and cutaneous paraneoplastic syndromes are caused by tumor-secreted substances. Examples include the secretion of parathyroid hormone-related peptide by the tumor, resulting in hypercalcemia or the secretion of adrenocorticotropic hormone (ACTH), resulting in Cushing's syndrome. Immunological factors on the other hand appear to be important in the pathogenesis of most PNS and some cutaneous syndromes such as paraneoplastic pemphigus. The precise pathogenesis of many paraneoplastic disorders has not yet been elucidated. Some disorders, once thought to be paraneoplastic, are not; e.g., progressive multifocal leukoencephalopathy, once considered paraneoplastic, has proven to be an opportunistic infection caused by a papovavirus.

Table 2 | Pathogenetic mechanisms in paraneoplastic syndromes

Mechanism	Example of paraneoplastic syndrome
Toxic substance released by tumor	ACTH release by SCLC causes Cushing's syndrome Cytokine release by tumor or immune system (TNF-α, IL-1, IL-6, IFN-γ) causes cachectic myopathy
Competition for substrate	Carcinoid tumors compete for tryptophan causing pellagra Sarcomas competing for glucose cause hypoglycemia
Autoimmune process	LEMS caused by autoantibodies against VGCC Paraneoplastic pemphigus caused by autoantibodies against desmoglein

ACTH = adrenocorticotropic hormone; SCLC = small cell lung cancer; LEMS = Lambert-Eaton myasthenic syndrome; VGCC = voltage-gated calcium channels.

The diagnosis of paraneoplastic syndromes is straightforward when they develop in a patient known to have cancer, once metastatic complications have been ruled out. However, the majority of paraneoplastic syndromes occur in patients not yet diagnosed with cancer. In this situation, detection of paraneoplastic autoantibodies can help direct the search toward an underlying tumor. In a patient with carcinoma of unknown origin, the type of antibody can also give a clue to the most likely site of the primary tumor. When the paraneoplastic syndrome is caused by toxic factors secreted by the tumor, symptoms often respond favorably to treatment of the underlying tumor. However, in immune-mediated paraneoplastic syndromes, the response to antitumor treatment and/or immunosuppressive or immunomodulatory treatment is usually less satisfying.

NEUROLOGICAL PARANEOPLASTIC SYNDROMES

In the pathogenesis of PNS, immunologic factors are believed to be important because antibodies and T-cell responses against nervous system antigens have been defined for many of these disorders[104]. Hypothetically, 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[105-108]. Detection of paraneoplastic antibodies can help diagnose the neurological syndrome as paraneoplastic and may direct the search for an underlying neoplasm. In patients known to have cancer, the presentation of a PNS may presage the recurrence of the tumor or a second tumor. In these patients, however, metastatic complications of the known cancer must first be ruled out. Despite the presumed autoimmune etiology of PNS, the results of various forms of immunotherapy have been disappointing, with some exceptions[105-108]. Rapid detection and immediate treatment of the underlying tumor appears to offer the best chance of stabilizing the patient and preventing further neurological deterioration[105-108].

Pathogenesis

The discovery of paraneoplastic antineuronal autoantibodies resulted in the general belief that PNS 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 and heavily infiltrated with inflammatory cells, and spontaneous remissions at the time of neurological presentation have been described[109, 110]. These findings suggest that some PNS without identifiable tumor may result from immune-mediated eradication of the tumor[109, 110]. 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[111]. Although the paraneoplastic antibodies are synthesized intrathecally[47, 112, 113], 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 (VGCC in Lambert-Eaton myasthenic syndrome [LEMS]), voltage-gated potassium channels (anti-VGPC in neuromyotonia), and the metabotropic glutamate receptor mGluR1 (anti-mGluR1 in paraneoplastic cerebellar degeneration)[104, 114]. 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[115]. In these disorders, indirect lines of evidence support the view that the cellular immune response against these antigens is responsible for the neurological damage[41, 52, 116]. The relative contribution of the cellular and humoral immunity to the clinical and pathological manifestations has not been resolved[41, 52, 116, 117]. The paraneoplastic antibodies may, in these cases, be surrogate markers for T-lymphocyte activation[118].

Incidence and diagnosis

The incidence of PNS varies with the neurological syndrome and with the tumor. 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%. Neurological syndromes are never pathognomonic for a paraneoplastic etiology and a high index of clinical suspicion is important. Some syndromes such as limbic encephalitis and subacute cerebellar degeneration associate relatively often with cancer. These are called 'classical' paraneoplastic syndromes and are presented in Table 1[3]. 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 three categories (Table 3)[3]. The 'well-characterized' antibodies are reactive with molecularly defined onconeural antigens (Figure 1). 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[3]. The partially characterized antibodies are those with an unidentified target antigen and those that have either been described by a single group of investigators or 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 nonparaneoplastic cases.

Table 3 | 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)[a]	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
Anti-NMDAR	Limbic encephalitis	Teratoma
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-VGPC	Limbic encephalitis, neuromyotonia	Thymoma, SCLC

[a]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 cancer; VGCC = voltage-gated calcium channels; PCA = Purkinje cytoplasmic antibody; mGluR1 = metabotropic glutamate receptor type 1; nAChR = nicotinic acetylcholine receptor; VGPC = voltage-gated potassium channels.

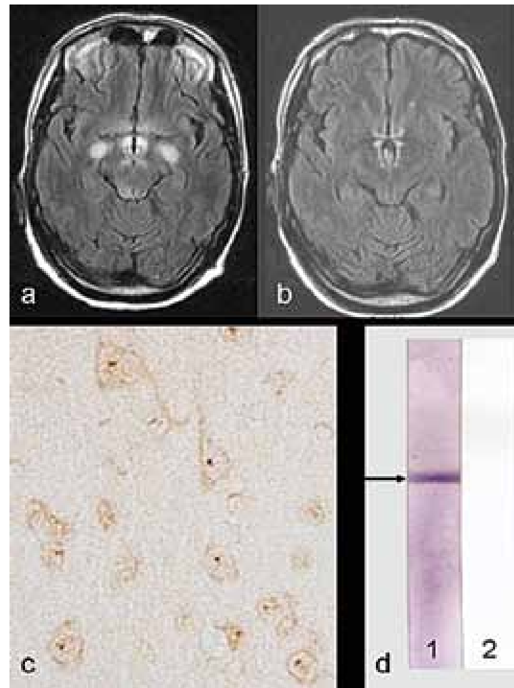


Figure 1 | Ma2-encephalitis

A 41-year old man presented with severe limbic encephalitis. Biopsy from a para-aortal mass showed undifferentiated carcinoma. (**a**) MRI of the brain showed on FLAIR images hyperintense abnormalities in the medial temporal lobes and around the third ventricle. The patient had high titer anti-Ma2 antibodies reactive with cytoplasmic and nuclear antigens in rat cortex (**c**) and purified recombinant Ma2 protein (**d**). He subsequently tested positive for AFP and β-HCG serum markers and was treated for presumed testicular cancer. During chemotherapy the limbic encephalitis improved clinically and radiologically (**b**).

Diagnosing a neurological syndrome as paraneoplastic requires the exclusion of other possible causes by a reasonably complete workup. Because of the difficulties in diagnosis, 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 the type of clinical syndrome (Table 4)[3]. Unfortunately, almost 50% of patients with a definite PNS do not have any of the 'well-characterized' paraneoplastic antibodies[3]. In these patients, early diagnosis of the tumor is often difficult resulting in delay in tumor treatment.

Table 4 | Diagnostic criteria for paraneoplastic neurological syndromes[3]

Definite paraneoplastic neurological syndrome	
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 nonclassical syndrome that objectively improves or resolves after cancer treatment, provided that the syndrome is not susceptible to spontaneous remission
3	A nonclassical 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., anti-Hu, Yo, Ri, amphiphysin, CV2 or Ma2)
Possible paraneoplastic neurological syndrome	
1	A classical syndrome without paraneoplastic antibodies and no cancer, but at high risk of having an underlying tumor (e.g., smoking habit)
2	A neurological syndrome (classical or not) without cancer but with partially characterized paraneoplastic antibodies
3	A nonclassical neurological syndrome, no paraneoplastic antibodies, and cancer that presents within 2 years of the neurological syndrome

LEMS = Lambert-Eaton myasthenic syndrome.

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 workup 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[119-121]. 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 3). When all tests remain negative, repeat evaluation at three- to six-month intervals for two to three years is recommended.

Treatment and prognosis

Despite the immunological etiology of most of the PNS, the results of immunotherapy have been disappointing[12]. 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[114, 122]. Immunotherapy modalities that are recommended for these disorders include plasma exchange, immunoadsorption (extraction of patient IgG over a protein A column), steroids, and intravenous immunoglobulins (IVIg). For most PNS, 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, IVIg, or other immunosuppressive therapies are effective[123].
Hence, the first goal of treatment for PNS is control of the tumor. In addition, antitumor therapy has been demonstrated to stop the paraneoplastic neurological deterioration and leave the patients, on average, in a better condition[9, 106, 107]. 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.

CLINICAL DESCRIPTION

PNS may affect any level of the nervous system (central or peripheral nervous system including neuromuscular junction and muscle). Most PNS are rapidly progressive, often leaving the patient severely debilitated within weeks to months[9, 105-107]. However, slow progression, relapses, or a benign course does not exclude the diagnosis. This section describes the classical PNS.

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)[26, 124]. 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, approximately 75% of patients have an underlying SCLC[26, 105, 106, 124, 125]. More than 70% of the patients are not known to have cancer when the neurological symptoms present, and the SCLC may be difficult to demonstrate due to its small size. When anti-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[119, 120].

Diagnostic evaluation

MRI or CT of the brain is normal or shows nonspecific changes in most paraneoplastic encephalomyelitis patients, with two exceptions[26]. In 65% to 80% of patients with predominant limbic encephalitis, MRI and CT scans show temporal lobe abnormalities[126, 127], and in patients with a predominant cerebellar syndrome, MRI and CT will ultimately show cerebellar atrophy. Cerebrospinal fluid (CSF) is abnormal in most patients with elevated protein, mild mononuclear pleocytosis, elevated IgG index, or oligoclonal bands[26].

Antineuronal antibodies

Patients with paraneoplastic encephalomyelitis and SCLC often have anti-Hu antibodies (also called antineuronal nuclear autoantibodies or ANNA-1) in their serum and CSF[26, 105, 106, 125]. Other less-frequent antibodies are anti-CRMP5/CV2[118], anti-amphiphysin[128] and the less well-characterized ANNA-3 [129] and PCA-2 antibody[130].

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[12, 105, 106]. 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. Because of the limited efficacy of plasma exchange, IVIg, and corticosteroids[12, 105, 106] 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. 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[12, 105, 106].

Limbic encephalitis

Limbic encephalitis is 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[124, 131]. Hypothalamic dysfunction may occur with somnolence, hyperthermia, and endocrine abnormalities. Approximately two-thirds of patients with paraneoplastic limbic encephalitis develop involvement of other areas of the nervous system during the course of the disease (i.e., encephalomyelitis). Several specific antibody-related clinical syndromes have been identified[127, 132-134].

Underlying tumor

More than half of the patients presenting with limbic encephalitis will have an underlying neoplasm[127]. The associated tumor is a lung tumor in 50% to 60% of the patients, usually SCLC (40%-55%) and testicular germ-cell tumors in 20%[126, 127, 135]. Other tumors include breast cancer, thymoma, Hodgkin's disease, and teratomas[126, 127].

Diagnostic evaluation

The diagnosis is often difficult, especially when the patient presents with psychiatric symptoms, because there are no specific clinical markers, and symptoms usually precede the diagnosis of cancer[127, 136]. An MRI scan may show increased signal on T2-weighted and fluid attenuated inversion recovery (FLAIR) images of one or both medial temporal lobes, hypothalamus, and brainstem in 65% to 80% of the patients[126, 127]. Early in the course of the disease, the MRI may be normal, and repeat imaging may be indicated. Coregistration of FDG-PET may further improve the sensitivity of imaging[137]. CSF examination is abnormal in 80% of the patients, showing transient mild lymphocytic pleocytosis with increased protein, IgG, or oligoclonal bands[126, 127].

Antineuronal antibodies

Several antibody-related clinical syndromes have been identified that vary with the associated tumors. The first group consists of patients with anti-Hu antibodies and lung cancer (usually SCLC). Patients may also have anti-amphiphysin (SCLC) or anti-CV2/CRMP5 (SCLC or thymoma) antibodies[118]. Limbic encephalitis is part of paraneoplastic encephalomyelitis, and 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[127, 135]. The second group consists of young males with testicular cancer and anti-Ma2 antibodies (Figure 1)[138]. The median age is 34 years, and symptoms are usually confined to the limbic system, hypothalamus, and brainstem. Patients with anti-Ma2 and anti-Ma1 antibodies are significantly older and are more often female[139]. Anti-Ma1 patients are more likely to develop cerebellar dysfunction and usually harbor other tumors than testicular cancer. The third group consists of young women with ovarian teratomas who harbor antibodies reactive to the *N*-methyl-D-aspartate (NMDA) receptor[132-134]. The patients develop subacute psychiatric symptoms, seizures, and hypopnea, requiring mechanical ventilation. Patients may also develop choreic or dystonic movements. The CSF invariably shows signs of inflammation including lymphocytosis, and increased protein and oligoclonal banding. The anti-NMDA receptor antibodies are sometimes only detectable in CSF (and not in serum). The fourth group has antibodies reactive to voltage-gated potassium channels (anti-VGPC) that can be associated either with paraneoplastic limbic encephalitis and thymoma or with nonparaneoplastic limbic encephalitis[140, 141]. Most patients present with or develop seizures that may progress into nonconvulsive status epilepticus. Patients may have concomitant autonomic or peripheral nerve dysfunction (neuromyotonia) and rapid eye movement sleep behaviour abnormalities. Hyponatremia is frequent while the CSF is usually acellular. The last group has no antineuronal antibodies (approximately 20%-40% of patients with paraneoplastic limbic encephalitis)[126, 127]. 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[127, 135].

Treatment and prognosis

Spontaneous complete recovery has been described, although very rarely[135, 142]. Immunotherapy is largely ineffective in patients with antibodies reactive to intracellular antigens (anti-Hu, Ma2, amphiphysin or CV2/CRMP5)[127], but multiple cases benefiting from anti-tumor treatment have been reported[127, 135, 143]. Therefore, all efforts should be directed at identifying and treating the underlying tumor. If no tumor is found, the search should be repeated every three to six months for a total of two to three years. Irrespective of treatment, partial neurological recovery was seen in 38% of anti-Hu patients, 30% of anti-Ta (Ma2) patients, and 64% of patients without antibodies[127]. In contrast, patients with antibodies reactive with easily accessible cell-surface antigens often respond dramatically to immunotherapy and antitumor treatment. Ovarian teratoma patients often clinically improve following resection of the tumor and/or treatment with corticosteroids, IVIg, or plasma exchange[133]. In patients with anti-VGPC-associated limbic encephalitis, treatment with corticosteroids, IVIg, or plasma exchange results in significant improvement in 70% to 80% of the cases[140].

Cerebellar degeneration

Paraneoplastic cerebellar degeneration is one of the most common and characteristic paraneoplastic syndromes[107, 124]. In a study of 137 consecutive patients with antibody-associated paraneoplastic syndromes, 50 (37%) patients presented with subacute cerebellar degeneration[107]. 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 or sit without support, writing is often impossible, and feeding themselves is quite difficult. 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[108, 144, 145].

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% to 70% of the patients, the neurological symptoms precede the diagnosis of the cancer by a few months to two to three years and lead to its detection[107, 108, 146].

Diagnostic evaluation

Subacute cerebellar degeneration is a rare disorder in cancer patients. On the other hand, 50% of patients presenting with acute or subacute nonfamilial ataxia are estimated to have an underlying malignancy[124]. MRI and CT scans are initially normal but often reveal cerebellar atrophy later in the course of the disease. FDG-PET and single-photon emission CT (SPECT) scans may show cerebellar hypermetabolism and increased perfusion during the acute stage of the illness[147]. 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.

Antineuronal antibodies

Paraneoplastic cerebellar degeneration can be associated with various antineuronal autoantibodies (Table 3). 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. Anti-Yo antibodies are associated with breast cancer and tumors of the ovaries, endometrium, and fallopian tubes[107, 108, 148]. These antibodies are directed against the calcium-dependent regulator (cdr) proteins that are expressed by Purkinje cells and the associated tumors[148, 149]. 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[52]. Anti-Tr (PCA-Tr) antibodies are directed against an unidentified cytoplasmic Purkinje cell antigen and appear to be specific for Hodgkin's disease[146]. 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[114].
Approximately 50% of patients with cerebellar degeneration and an underlying SCLC have high titer anti-Hu antibodies[150]. The remaining patients are likely to have anti P/Q-type VGCC antibodies. These antibodies were present in all patients who had LEMS and in some patient 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[130]. The anti-Zic4 antibodies are strongly associated with SCLC, and most patients have paraneoplastic encephalomyelitis often presenting with cerebellar dysfunction[151]. 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[107]. Incidental improvement has been reported either spontaneously or in association with plasma exchange, steroids, IVIg, or rituximab[152]. In patients with anti-Yo-associated cerebellar degeneration, the prognosis is better for patients with breast cancer than for those with gynaecologic cancer[108]. 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[114, 146].

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[153-155]. An excessive startle response reminiscent of hyperekplexia may also occur in opsoclonus-myoclonus[156]. In contrast to most paraneoplastic syndromes, the course of opsoclonus-myoclonus may be remitting and relapsing[155].

Underlying tumor

Approximately 20% of adult patients with opsoclonus-myoclonus have a previously undiscovered malignancy[154]. The most commonly associated neoplasms are SCLC and breast and gynaecologic cancers[156, 157]. Almost 50% of children with opsoclonus-myoclonus have an underlying neuroblastoma. Conversely, approximately 2% to 3% of children with neuroblastoma have paraneoplastic opsoclonus-myoclonus[158, 159]. 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[160]. Examination of the CSF may show mild pleocytosis and protein elevation. 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 gynaecological examination and mammography (or MRI of the breasts)[157]. When this is negative, FDG-PET should be considered[121, 161]. In children, nonparaneoplastic 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 or MRI scan), urine catecholamine measurements, and metaiodobenzylguanidine scan[162].

Antineuronal antibodies

Specific antibodies are found in only a minority of patients with paraneoplastic opsoclonus-myoclonus[157]. In women, anti-Ri antibodies (or antineuronal nuclear autoantibody type 2, ANNA-2) are mostly associated with breast and gynaecologic tumors. Anti-Ri has occasionally been found in bladder cancer and SCLC and may then occur in male patients[153, 163]. Paraneoplastic opsoclonus-myoclonus can also be associated with anti-Hu antibodies, usually as part of a more widespread paraneoplastic encephalomyelitis. In children presenting with opsoclonus-myoclonus, the detection of anti-Hu antibodies is diagnostic of an underlying neuroblastoma[164]. The frequency of anti-Hu antibodies in neuroblastoma with paraneoplastic opsoclonus-myoclonus is approximately 10%[164-166]. This finding differs little from the 4% to 15% of anti-Hu positive sera in children with neuroblastoma who do not have opsoclonus-myoclonus[164, 165].

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 IVIg. Paraneoplastic opsoclonus-myoclonus usually has a more severe clinical course, and treatment with steroids or IVIg appears ineffective. In a serie of 14 patients with paraneoplastic opsoclonus-myoclonus, eight patients whose tumors were treated showed complete or partial neurological recovery. In contrast, five of the six patients whose tumors were not treated died of the neurological syndrome despite steroids, IVIg, or plasma exchange[157]. However, improvement following the administration of steroids, cyclophosphamide, azathioprine, IVIg, plasma exchange, or plasma filtration with a protein A column has been described in single cases[156, 167-169]. In children, paraneoplastic opsoclonus-myoclonus may improve following treatment with ACTH, prednisone, azathioprine or IVIg, but residual central nervous system signs are frequent[159, 170, 171]. Treatment of the tumor with chemotherapy is the most important predictor of good neurological recovery[172].

Subacute sensory neuronopathy

The symptoms of subacute sensory neuronopathy begin with pain and paraesthesia[173, 174]. Clumsiness 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[175]. Subacute sensory neuronopathy occurs in approximately 75% of patients with paraneoplastic encephalomyelitis, is predominant in 50% of patients and clinically pure in 25% of patients[105, 106]. Autonomic neuropathy including gastrointestinal pseudo-obstruction is common.

Underlying tumor

Subacute sensory neuronopathy is probably paraneoplastic in about 20% of patients. In 70% to 80% of patients, subacute sensory neuronopathy is associated with SCLC[105, 106, 125]. Other associated tumors include breast cancer, ovarian cancer, sarcoma, and Hodgkin's lymphoma[173, 174]. Subacute sensory neuronopathy usually predates the diagnosis of cancer with a median delay of 3.5 to 4.5 months[105, 106].

Diagnostic evaluation

Electrophysiology shows absence or marked reduction of sensory nerve action potentials with normal or mildly reduced motor conduction velocities. Early in the course of the

disease, CSF examination shows mild pleocytosis, with an elevated IgG and oligoclonal bands[106, 173, 174]. 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[26, 105, 106, 125]. In this setting, anti-Hu antibody detection has a specificity of 99% and sensitivity of 82%[176]. The absence of anti-Hu antibodies does not rule out an underlying cancer. Anti-CRMP5/CV2 antibodies also occur with paraneoplastic peripheral neuropathies[177]. These patients usually have a sensory or sensorimotor neuropathy with less frequent involvement of the arms, but it is often associated with cerebellar ataxia[118, 177, 178]. Anti-CRMP5/CV2 antibodies are usually associated with SCLC, neuroendocrine tumors, and thymoma. Anti-amphiphysin antibodies are associated with multifocal paraneoplastic encephalomyelitis, and symptoms often include sensory or sensorimotor neuropathy[128, 179, 180]. Associated tumors (mostly limited) are mainly SCLC, breast cancer, and melanoma.

Treatment and prognosis

Immunotherapy consisting of plasma exchange, steroids, and IVIg is ineffective, with only some exceptions[9, 12, 181, 182]. 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 IVIg[12]. Early detection and treatment of the underlying neoplasm, usually SCLC, appears to offer the best chance of stabilizing the neurological symptoms[9, 106]. In patients with an identifiable tumor, antitumor treatment is recommended. In the absence of a tumor, antitumor treatment may be considered in patients with anti-Hu antibodies, age >50 years, and a history of smoking. In patients not receiving antitumor 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

LEMS presents with proximal weakness of the lower extremities and fatigability. Bulbar symptoms may occur more frequently than previously reported[183], 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% of patients, especially dryness of the mouth, impotence, and mild/moderate ptosis[183-185]. In some patients, LEMS may develop in association with other paraneoplastic syndromes, including paraneoplastic cerebellar degeneration and encephalomyelitis[150].

Underlying tumor

Approximately 70% of patients have cancer, almost always SCLC[184, 186]. 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%[185, 187]. Clinically and serologically, the 30% without an identifiable tumor are indistinguishable from the paraneoplastic LEMS patients, although LEMS may have a more progressive course in patients with SCLC[183]. In patients presenting with LEMS, smoking history and absence of the HLA-B8 genotype strongly predict an underlying SCLC [183]. Patients with SCLC and LEMS survive significantly longer than SCLC patients who do not have the paraneoplastic syndrome[183].

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 to 30 seconds of maximal voluntary contraction[188].

Antineuronal antibodies

Most patients with LEMS have antibodies against P/Q type calcium channels that are located presynaptically in the neuromuscular junction[188]. About 20% have anti-MysB antibodies reactive to the β-subunit of neuronal calcium channels[189].

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[190]. Symptomatic treatment is with drugs that facilitate the release of acetylcholine from motor nerve terminals such as 3,4-diaminopyridine (DAP)[191]. In a placebo-controlled randomized trial, DAP (5-20 mg) was effective for long-term treatment, alone or in combination with other treatments [192]. The maximum recommended daily dose of DAP is 80 mg; at higher doses, seizures occur[192]. Cholinesterase inhibitors (pyridostigmine, 30-60 mg) 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 order. Removal of the pathogenic anti-P/Q type calcium channel antibodies by plasma exchange[193] and IVIg can give quick but transient relief[184, 194]. 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)[195]. Dermatomyositis is associated with cancer of the ovary, lung, pancreas, stomach, colorectum, and breast, and with non-Hodgkin's lymphoma[196].

Diagnostic evaluation

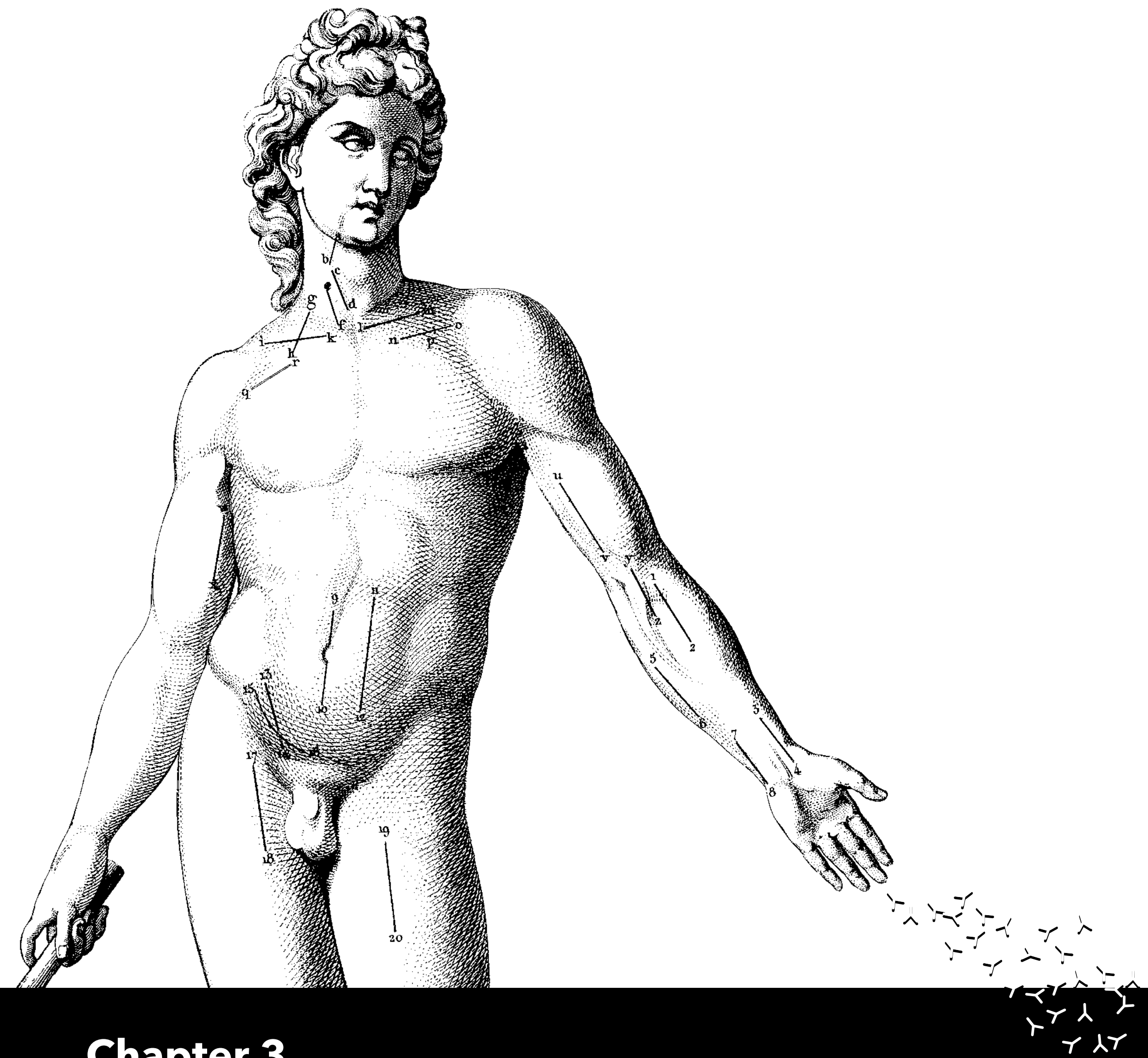
Most patients have elevated serum creatine kinase levels and electromyographic evidence of myopathy. Muscle imaging (CT or MRI) may help in confirming the diagnosis and determining the type of inflammatory myopathy and in selecting an appropriate biopsy site. Muscle or skin biopsy is the definitive diagnostic procedure and shows inflammatory infiltrates[197].

Antineuronal antibodies

Antibodies to the Mi-2 protein complex are specific for dermatomyositis and are present in high titers in about 35% of cases[198].

Treatment and prognosis

Treatment of paraneoplastic dermatomyositis is generally the same as for the patients without a tumor. Nearly all patients respond to corticosteroids [199]. Refractory patients and patients requiring a lower dose of steroids can be treated with azathioprine, methotrexate, or cyclophosphamide[199].



Chapter 3

Flow cytometric characterization of cerebrospinal fluid cells

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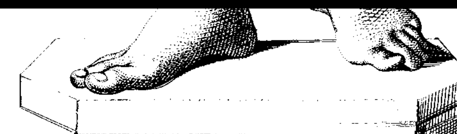
Cytometry Part B: Clinical Cytometry, 2011 May 12 (Epub)



ABSTRACT

Flow cytometry facilitates the detection of a large spectrum of cellular characteristics on a per cell basis, determination of absolute cell numbers and detection of rare events with high sensitivity and specificity. White blood cell (WBC) counts in cerebrospinal fluid (CSF) are important for the diagnosis of many neurological disorders. WBC counting and differential can be performed by microscopy, haematology analyzers or flow cytometry. Flow cytometry of CSF is increasingly being considered as the method of choice in patients suspected of leptomeningeal localization of haematological malignancies. Additionally, in several neuro-inflammatory diseases such as multiple sclerosis (MS) and paraneoplastic neurological syndromes (PNS), flow cytometry is commonly performed to obtain insight into the immunopathogenesis of these diseases. Technically, the low cellularity of CSF samples, combined with the rapidly declining WBC viability, makes CSF flow cytometry challenging. Comparison of flow cytometry with microscopic and molecular techniques shows that each technique has its own advantages and are ideally combined. We expect that increasing the number of flow cytometric parameters that can be simultaneously studied within one sample, will further refine the information on CSF cell subsets in low-cellular CSF samples and enable to define cell populations more accurately.

INTRODUCTION

White blood cell (WBC) counts and their differential into mononuclear (MNC) and polymorphonuclear cells (PMN) in cerebrospinal fluid (CSF) are critical in the diagnosis of many infectious and inflammatory neurological disorders[200]. In acute bacterial meningitis, WBC counts usually range between several hundred to more than 60,000/µl, predominantly PMN (90%-95% of WBC count), although early in the disease WBC counts can be lower than 100 WBC/µl[201]. In viral meningitis, cell counts are usually between 10 - 1,000 WBC/µl, but may exceed 1,000/µl. Here, MNC predominate, but in the very acute stages of disease PMN can account for more than 80% of leukocytes[201]. In multiple sclerosis (MS), two-thirds of patients have a normal CSF cell count and a low level of mononuclear pleocytosis is found in one-third of the cases[201]. The CSF WBC count is usually normal (<5 leukocytes/µl) in patients with the Guillain-Barré syndrome, whereas in case of an increased WBC count other diagnoses should be considered[202]. In paraneoplastic neurological syndromes (PNS), 47% of patients have a lymphocytic pleocytosis before the 3rd month after onset of the neurological symptoms, while after the 3rd month only 28% of patients have elevated cell counts[45]. CSF WBC counts are also routinely determined in patients with suspected leptomeningeal metastases of solid or haematological malignancies and half of these patients have a lymphocytic pleocytosis[203, 204].

MICROSCOPY

Generally, cell counts and differential can be obtained by evaluating cell number and morphology in microscopic slides, by automatic counting based on cellular scatter properties or by flow cytometry in which antigen expression of cells is assessed in combination with light scatter properties. Specifically, microscopic counting of WBC and red blood cells (RBC) is performed using Neubauer or Fuchs-Rosenthal counting chambers, which contain a microscopically visible counting grid and are used with a fixed sample volume. Staining with Samson or Türk reagent may be added to the procedure to facilitate WBC counting and perform differential[204]. However, in CSF samples with low cellularity (<5 leukocytes/µl), differential by microscopy is not performed. When the WBC count is higher than the upper reference value, most laboratories perform cytospin centrifugation of the sample, followed by Wright or May-Grunwald-Giemsa staining to enable morphological differential of cells in CSF. This technique permits rapid differential between monocytes, lymphocytes and granulocytes, which is of utmost importance for patients with acute neurological disease[62]. Counting of erythrocytes is important to exclude traumatic bleeding as the cause of an elevated WBC count[205]. Although microscopic counting and differential has for a long time been used in routine CSF analysis, the clinical laboratory faces several challenges in performing it. First, the analysis is time-consuming and should ideally be performed within 1 hour after lumbar puncture as CSF cell counts decrease rapidly after sampling[206]. Second, counting and differential results have relatively high intra- and interobserver variation[205], as have other manual microscopic techniques. Furthermore, skilled and continuously trained technicians are required for this assay on a 24 hours/7 day basis[207].

HAEMATOLOGY ANALYZERS

Nowadays an increasing number of clinical laboratories replace the microscopic technique by haematology analyzers (HA) for cell counting and differential in CSF[205, 207, 208]. HA may provide fast, low-cost and standardized cell counting of CSF and other body fluids such as ascites or pleural fluid. However, special attention is needed regarding background signal, carry-over and interference in view of the low cell concentrations in these fluids. Two dedicated, FDA-approved, applications for CSF counting and differential on HA are available, i.e., the ADVIA® 120/2120 CSF assay (Siemens AG, München, Germany) and the Body Fluid mode on a XE-5000™ analyzer (Sysmex, Kobe, Japan)[205, 208]. ADVIA® CSF assay uses light scatter and absorbance measurement for counting and differential, after mixing the sample with CSF reagent to sphere and fix the cells. Not only RBC, WBC and PMN/MNC are reported, but also lymphocytes, monocytes and eosinophils. XE-5000™ Body Fluid

mode uses sheath flow impedance for counting RBC, while light scatter and fluorescence intensity after DNA/RNA staining is used to analyse WBC. The application reports WBC, PMN/MNC and high-fluorescent cells. For CSF WBC counting, comparison of the Fuchs-Rosenthal counting chamber with both the XE-5000™ analyzer and the ADVIA® 120/2120 CSF assay showed linearity between 1 and 10,000 cells per µl[205, 208, 209]. Automatic MNC counts also correlated well with manual counts, but PMN counts showed poor agreement being almost two-fold higher using the XE-5000 analyzer[209]. Especially in low WBC counts (<20 cells per µl) high imprecision was observed in both techniques compared to manual counting[205, 208]. The detection limit of the XE-5000 is 10 cells per µl. When the WBC count is below that limit, differential into MNC and PMN cannot be made[209]. In these cases, manual counting or evaluation of stained cytospin slides should be performed. In automatic cell counting of low-cellular samples the same problem arises as in flow cytometry (see *Rapid decline of leukocyte counts upon lumbar puncture* section): due to high imprecision, under- or overestimation of CSF WBC counts may lead to erroneous results. In clinical practice, HA are more widely available and have lower material costs than flow cytometers.

CYTOMORPHOLOGY

Morphologic examination of CSF cells is performed on cytospin preparations stained with May-Grunwald-Giemsa[204]. Whilst highly specific (>95%), conventional cytomorphological analysis is associated with only a limited sensitivity with up to 20% to 60% false-negative results[204, 210]. Interpretation of cytological findings may be difficult because of paucity of cells in CSF and possible morphological similarities between benign and malignant cells[211]. Cytomorphological examination is used in patients with suspected leptomeningeal dissemination of solid tumours or haematological malignancies. Typically, only 50% of patients have malignant cells identified by cytomorphological examination on the first lumbar puncture[212]. This yield is increased to 80% with a second CSF examination[203], but even three lumbar punctures will still miss tumour cells in approximately 10% of patients[213]. Despite its low sensitivity, CSF cytologic examination has been the gold standard for leptomeningeal metastasis because of its 100% specificity[213]. If clinical suspicion is high, gadolinium-enhanced MRI of the brain and spine can provide definitive evidence of leptomeningeal metastasis, even without a positive CSF cytology[214, 215].

IMMUNOCYTOCHEMISTRY

Immunocytochemistry allows the detection of cell surface antigens on CSF cells by cytospins. For detection of leptomeningeal localization of haematological malignancies a sensitivity of 89% to 95% and a specificity of 89% to 100% was shown by this technique[216]. For CSF samples with low cell counts, immunocytochemistry should be used subsequent to cytomorphology and the selection of the antibodies should be determined by the cytological findings in combination with the patient's history[217, 218]. Alternatively, it is stated that this technique should only be used when CSF cytomorphology fails in patients with a strong suspicion of leptomeningeal metastases[219]. Compared to flow cytometry, immunocytochemistry gave similar results in detection of high-density surface markers, whereas for analysis of antigens that are expressed at low density immunocytochemistry may be more reliable in some applications[220]. Since flow cytometry is used in the detection of CNS involvement of haematological malignancies besides cytomorphological analysis, as discussed in the *Applications of flow cytometry to study CSF* section, immunocytochemistry has no major role anymore. In contrast, immunohistochemistry is still used in combination with cytology in the detection of leptomeningeal metastases of solid tumours.

POLYMERASE CHAIN REACTION (PCR)

PCR requires the selection of primers specific for tumour cell-derived DNA. In haematological B-cell malignancies, analysis of immunoglobulin heavy chain gene rearrangements in the third complementarity determining region (CDR3) by PCR in blood and bone marrow cells is a state-of-the-art technique for diagnosis, monitoring response to treatment and detection of minimal residual disease[221, 222]. Presence of clonally rearranged CDR3 is the molecular signature of malignant B lymphocytes and is present in 80%-95% of B-cell lymphomas and leukaemias[223]. Until now this technique has not been generally applied to CSF samples. PCR can also be used for detection of leptomeningeal metastasis in solid malignancies. Although it would be ideal to use primers for DNA sequences common to all metastatic cells, the use of sequences for specific primary cancer histopathologies might provide a more practical option, as many are known already[210, 224-226]. Additional molecular tumour markers or oncogenes can be used for other types of cancer and might eliminate the need for biopsy in selected patients[226].

FLOW CYTOMETRY

In patients with suspected leptomeningeal metastases of haematological tumours, flow cytometry of CSF samples is used in addition to cell counting and cytomorphology. In this review we will focus on the applications and recent developments of CSF flow cytometry. Although this procedure has only a narrow clinical indication, it has significant prognostic and therapeutic implications in individual patients.

Use of polychromatic flow cytometry

The advent of polychromatic flow cytometry, i.e. advanced instrumentation and reagent development[227], allows detection of a large spectrum of cellular characteristics, even in samples with small amounts of cells like CSF. Apart from differentiating between major leukocyte subsets by assessing granularity and volume of cells, a wide range of cell populations can be specified by immunophenotyping using surface membrane, cytoplasmatic and nuclear antigens[62, 63, 211]. However, intracellular staining should be limited to those cases in which it is essential to reach the immunological conclusion, because its use is associated with relatively pronounced cell loss[63]. The number of characteristics on one single cell that can be determined in a single tube, depends on the number of fluorescent colours available on the flow cytometer used and the number of monoclonal antibodies per tube. The applicability of the assay can even be further enhanced by combining two antigens expressed by non-overlapping cell subsets on a single fluorochrome (e.g., CD4 present on T lymphocytes and CD19 present on B lymphocytes)[211]. In CSF, the simultaneous assessment of 13 parameters (11 colours plus forward and sideward scatter) has been reported in this way[211]. However, problems with spectral overlap and colour compensation increase when more than 6 colours are used, but these problems can be reduced if markers and fluorochromes are combined judiciously[228].

Absolute cell counts

Frequencies of different WBC populations in CSF are most widely investigated. However, knowledge of absolute numbers of the major cell populations can be of great help to evaluate the sample[229]. Due to possible cell loss during concentration and centrifugation steps[63, 230], absolute cell counts may be an underestimation of the real CSF cell number. Addition of counting beads to the monoclonal antibody-stained CSF cell suspension allows accurate enumeration of absolute numbers of cell subsets[63]. By using this technique, we showed that PNS patients stood out by highly increased absolute counts of the major lymphocyte subsets in CSF, but above all, by B-lymphocyte counts that had increased more than 20-fold as compared to controls without neurological disease[231]. In these patients, the frequency of B lymphocytes (expressed as fraction of lymphocytes) had increased only three-fold[231]. When merely frequencies would have been studied, this enormous B-lymphocyte expansion suggesting an important role for these cells in PNS, would have remained unnoticed. This result indicates that assessment of absolute counts besides frequencies is also important in CSF.

Cellular composition of normal CSF

To use CSF flow cytometry in research of neuro-inflammatory diseases, knowledge of the composition of cells in normal CSF is needed. Because CSF of healthy controls is usually not available due to ethical considerations[232], controls with non-inflammatory neurological diseases (NIND) are often included instead[233]. However, Svenningsson et al.[234, 235] did study normal CSF by assessing the percentages of lymphocyte subsets in CSF of 34 healthy individuals, after informed consent, with 2- or 3-colour flowcytometry. In addition, we studied both absolute numbers and percentages of leukocyte, lymphocyte, T lymphocyte and dendritic cell subsets by 6-colour flow cytometry in 84 individuals without neurological disease undergoing spinal anaesthesia (Table 1)[236]. The two published studies[234, 235], as well as our data[236], showed that normal CSF is predominantly composed of $CD4^+$ T lymphocytes, mostly with a central memory phenotype, and in addition contains very low frequencies of B lymphocytes, NK cells and NKT lymphocytes (Table 1). Dendritic cells, both myeloid as well as plasmacytoid, were also present in normal CSF, although in very low frequencies (Table 1)[72, 236].

Table 1 | Reference values of WBC subsets in CSF (adapted from Chapter 5)

Subset	Absolute number[a]
Leukocytes	1.12 (0.40 – 3.17)
Granulocytes	0.08 (0.02 – 0.43)
Monocytes	0.23 (0.08 – 1.11)
Lymphocytes	0.66 (0.16 – 1.88)
T cells	0.62 (0.15 – 1.83)
$CD4^+$ T cells	0.44 (0.08 – 1.43)
$CD8^+$ T cells	0.13 (0.04 – 0.40)
NKT cells	0.01 (0.00 – 0.06)
B cells	0.00 (0.00 – 0.03)
NK cells	0.01 (0.00 – 0.05)
Dendritic cells	0.04 (0.01 – 0.18)
Myeloid	0.02 (0.00 – 0.13)
Plasmacytoid	0.01 (0.00 – 0.03)

[a]Medians (5th-95th percentiles) of absolute numbers x10^6/l are given.

Sensitivity and specificity

Flow cytometry is a sensitive method capable of detecting abnormal monoclonal B lymphocytes, which comprise as little as 0.01% of total lymphocytes[237, 238]. The detection rate of CSF involvement in hematological malignancies is up to 86% higher in flow cytometry than in conventional cytomorphological analysis[204, 211, 237-243] (Table 2). Although it was previously suggested that this method may not be suitable in the evaluation of samples with low cellularity[220], it has been shown that CSF T lymphocytes, the predominant lymphocyte subset in CSF, can be reliably detected in samples with a cell count lower than 5 leukocytes/μl[241]. Kleine et al.[244] showed that precision of lymphocyte flow cytometry is high (coefficient of variance [CV] ≤10%) provided that a sufficient number of events has been acquired[245]. However, the CV may increase to values up to 30% for the minor subsets in CSF, e.g. NK cells and NKT lymphocytes[244].

Table 2 | Comparison of flow cytometry and conventional cytomorphology in detection of CSF involvement in haematological malignancies

Malignancies Reference	N	CSF involvement[a]	FC+/CC+	FC+/CC-	FC-/CC+	Detection rate by using CC alone[b]	Detection rate by using FC alone[c]
Lymphoma							
Finn, 1998[242]	27	10	6	3	1	7/10 (70%)	9/10 (90%)
Hegde, 2005[238]	60	14	2	12	0	2/14 (14%)	14/14 (100%)
Quijano, 2009[211]	123	25	7	17	1	8/25 (32%)	24/25 (96%)
Sancho, 2010[246]	105	22	7	15	0	7/25 (28%)	22/22 (100%)
Acute leukaemia							
Subira, 2001[241]	168	21	11	7	3	14/21 (67%)	18/21 (86%)
Sayed, 2009[243]	45	23	8	13	2	10/23 (43%)	21/23 (91%)
Lymphoma, leukaemia							
French, 2000[237]	35	9	4	3	2	6/9 (67%)	7/9 (78%)
Roma, 2002[240]	53	21	12	9	0	12/21 (57%)	21/21 (100%)
Nückel, 2006[239]	45	18	12	3	3	15/18 (83%)	15/18 (83%)
Bromberg, 2007[204]	219	43	15	24	4	19/43 (44%)	39/43 (91%)

[a]CSF involvement was diagnosed when flow cytometry, conventional cytomorphology or both were positive.
[b]Detection rate of CSF involvement by using conventional cytomorphology alone: ([FC+/CC+] + [FC-/CC+])/CSF involvement.
[c]Detection rate of CSF involvement by using flow cytometry alone: ([FC+/CC+] + [FC+/CC-])/CSF involvement.
FC = flow cytometry; CC = conventional cytomorphology.

Several studies comparing flow cytometry and conventional cytomorphology to detect CSF involvement in haematological malignancies (Table 2) describe samples in which flow cytometry is positive whilst cytology is negative[204, 211, 238, 246]. Presence of neurological symptoms compatible with leptomeningeal disease is highly suggestive for CNS involvement in such patients, whereas absence of symptoms and lack of recurrence of CNS disease during clinical follow-up indicate a false-positive flow cytometric result[204, 211]. Results of clinical follow-up were documented in three studies. Sancho et al.[246] found that a flow cytometry-positive, cytology-negative result was associated with a higher probability of CNS relapse in aggressive B-cell lymphomas, as compared to samples with absence of neoplastic cells by both methods, whereas Hegde et al.[238] observed that 5/11 patients with a flow cytometry-positive, cytology-negative result relapsed in the clinical central nervous system and died despite having received active treatment. In addition, Bromberg et al.[204] described the absence of CNS recurrence in only 1/24 flow cytometry-positive, cytology-negative patients. These follow-up data indicate that flow cytometric analysis of CSF samples has a low risk of being false-positive in patients with haematological malignancies. In flow cytometry-negative samples on initial staging, Hegde et al.[238] observed that 3/40 patients at increased risk and 0/41 patients at low risk for central nervous system involvement relapsed in the central nervous system. This low risk of a false-negative result in flow cytometry, can also be deducted from Table 2 which shows a low number of flow cytometry-negative, cytology-positive samples in all studies.

Rare event detection

When leptomeningeal localization of a haematologic malignancy is suspected, the presence of a pathological (monoclonal) population and phenotypic characterization of that population can be assessed by using the proper antibody reagent panel adapted to the number of cells and previous histological and immunophenotypical diagnosis or suspected diagnosis together with appropriate gating strategies[63]. Pathological cells usually occur at very low percentages in the order of 0.01% in CSF. In addition, CSF samples contain a limited number of cells rendering pathologic cells in CSF very rare. To detect low numbers of rare cells, the background fluorescence of the reagents should be minimal, and a sufficient cell number is required to analyze lymphocyte subsets reproducibly. Therefore, the volume of the CSF sample should ideally be minimally 5 ml and cell loss during processing be prevented as discussed in the *Technical pitfalls* section.

APPLICATIONS OF FLOW CYTOMETRY TO STUDY CSF

CNS involvement with lymphoproliferative disorders

Involvement of the central nervous system (CNS) is a relatively uncommon complication of leukaemia and lymphoma[211, 241], which is suspected in patients who develop neurological symptoms or signs[211] or in patients at high risk of CNS localization[204]. It has grave prognostic significance and requires important therapeutic decisions including the administration of intrathecal chemotherapy[204, 241]. Leptomeningeal localization is diagnosed by conventional cytomorphological analysis through identification of malignant lymphocytes in CSF[63, 247]. However, this technique has a relatively high rate of false-negative results in up to 60% of cases[219, 248]. Recent reports suggest that multiparameter flow cytometric assessment of CSF samples could improve the efficiency of detection of CNS involvement, due to its high specificity and greater sensitivity[204, 247, 249]. Table 2 gives an overview of studies which investigated the value of flow cytometry and conventional cytomorphology in detection of CSF involvement in haematological malignancies. These studies showed that the use of flow cytometry alone increased the detection rate of CSF involvement up to 86% compared to the use of cytomorphology alone. Combined use of flow cytometry and cytomorphology increased the detection rate with 17%-86% compared to cytomorphology alone. Therefore, the National Comprehensive Cancer Network (USA) has recommended the routine use of flow cytometry in conjunction with cytomorphological analysis for the diagnosis of CNS lymphoma[250]. CNS involvement is diagnosed if one of these diagnostic procedures is positive. For detection of haematological malignancies, flow cytometry depends on the analysis of light chain restriction (Figure 1) and/or aberrant antigen expression, which should be interpreted within the context of the patient's histological diagnosis[251]. Still, cytomorphological examination of CSF has additional diagnostic and possibly prognostic value and should still be performed in conjunction with flow cytometry[204].

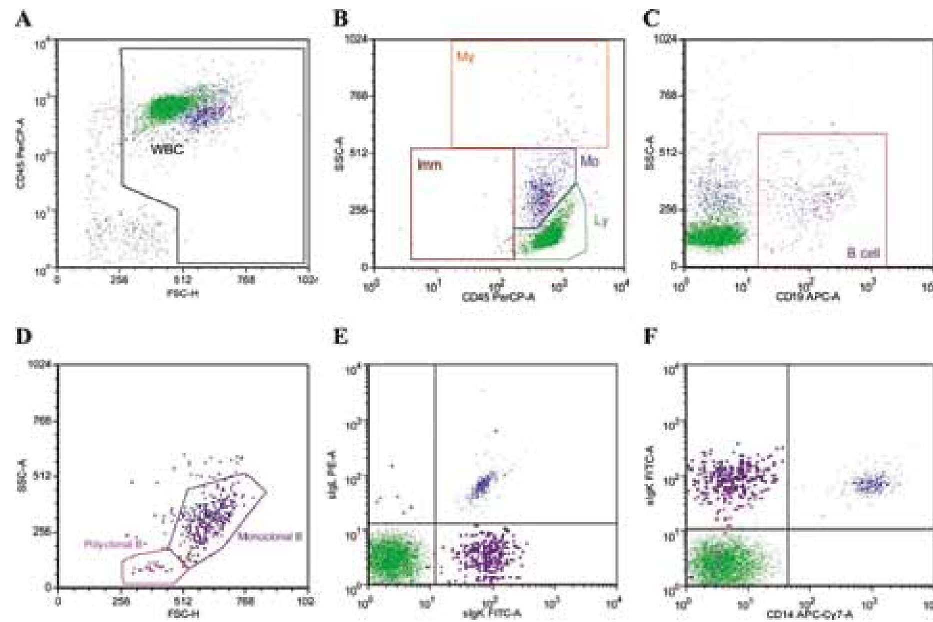


Figure 1 | Example of a 5-colour flow cytometric analysis for B-lymphocyte clonality in CSF

Each dot represents a single cell. For analysis, debris and non-leukocyte events were excluded by gating on forward scatter (FSC) and CD45 (gate 1, panel **A**). The leukocyte subsets (My = myeloid lineage; Imm = immature lineage; Mo = monocytes; Ly = lymphocytes) were defined with CD45 expression and side scatter (panel **B**) and show two major subsets: lymphocytes ($CD45^+$, SSC^{low}, $FSC^{intermediate}$; green dots) and monocytes ($CD45^+$, $SSC^{intermediate}$, FSC^{high}, $CD4^{dim}$; blue dots). B lymphocytes were gated using the lineage-specific marker CD19 and side scatter (SSC) (purple dots; gate 2, panel **C**). Next, the differential light scatter properties of the B-cell subpopulations as a function of their Ig light chain expression are shown in panels **D** and **E**. The combined analysis of clonality and light scatter revealed that the larger population of B lymphocytes have relatively high FSC and SSC signals with monoclonal expression of sIgK but not sIgL light chains (panel **E**, violet dots), compatible with B cell lymphoma, whilst the few B cells with relatively low FSC and SSC signals express either Ig kappa or lambda (panel **E**, blue dots). Note that the blue dots that express both sIgK and sIgL (panel E) are monocytes, as revealed by their CD14 reactivity (panel **F**). This result is caused by so-called cytophilic Ig binding (i.e., Ig bound through Fc receptors).

Inflammatory neurological diseases

Multiple sclerosis

Flow cytometry of CSF is also used as a research tool in various neuro-inflammatory diseases. The distribution of lymphocyte subpopulations in the CSF may be a consistent indicator of the type of immune response active in these diseases[252]. Several studies

have reported on flow cytometric analysis of lymphocytes and their subsets in CSF of patients with MS. The CSF cell populations in MS patients have been shown to consist of approximately 60% $CD4^+$ T lymphocytes[75] with a higher frequency of the regulatory phenotype[64, 65] and a higher CD4/CD8 ratio[73], while the frequency of NKT lymphocytes is lower[77] than in control patients with non-inflammatory neurological disorders (NIND). Compared to blood, CSF of MS patients showed a relative depletion of $CD8^+$ effector memory T lymphocytes[67]. In relation to disease activity, patients with active MS had higher percentages of activated $CD4^+$ T lymphocytes[78, 79, 81, 82] and lower percentages of activated $CD8^+$ T lymphocytes[78, 79, 82] in their CSF than inactive MS patients. Moreover, the percentage of naïve $CD45RA^+$, $CD50^+$ (ICAM-3) lymphocytes in CSF is significantly increased[80], while cell surface expression of CD54 (ICAM-1) on T lymphocytes in CSF is significantly decreased[83] in patients with relapses compared to patients in remission. Both are suggested to be used as markers of MS disease activity in CSF as well as blood[80, 83]. With regard to B lymphocytes in CSF of MS patients, a significant accumulation of mature B lymphocytes and plasma blasts is observed[66, 69]. Most B lymphocytes have a memory phenotype[68-71] and more B lymphocytes express CD80 (costimulatory molecule inducing T lymphocyte activation) than in NIND and healthy controls[74, 76]. Furthermore, the number of dendritic cells is elevated in CSF of MS patients[72]. CSF flow cytometry was also used in evaluation of MS treatment with immunosuppressive drugs. Both rituximab (anti-CD20 monoclonal antibody [mAb])[84] and natalizumab (anti-α4 integrin mAb)[85] reduced the number of B and T lymphocytes, while high-dose methylprednisolone induced changes in the expression of CD25, CD26 and HLA-DR on $CD4^+$ T lymphocytes[86].

Paraneoplastic neurological syndromes

We and others have reported on CSF lymphocyte subsets in patients with PNS. In PNS associated with anti-Hu antibodies, CSF is characterized by (i) a very substantial (20-fold) B-lymphocyte expansion and (ii) a 3-fold T-cell expansion (including both $CD4^+$ and $CD8^+$ subsets) compared to controls[231]. Children with paraneoplastic opsoclonus-myoclonus syndrome had normal CSF cell counts, but higher percentages of B lymphocytes[253, 254], activated T lymphocytes and $\gamma\delta$-T lymphocytes, lower percentages of $CD4^+$ T lymphocytes and a lower CD4/CD8 ratio[254] compared to NIND controls.

TECHNICAL PITFALLS

Low cellularity

The low number of cells in CSF (normal range: <5 leukocytes/μl) hampers the use of flow cytometry[255]. To analyze lymphocyte subsets reproducibly, measuring a sufficient cell

number is required. However, the minimal number of CSF cells required is not universally defined. In literature, the minimal CSF cell number varies between 100 gated lymphocytes in lymphocyte subset characterization[244] and 1000 cells in suspected CSF localization of lymphoma[249]. A subpopulation was reliably identified whenever 13 or more clustered events displaying identical features were present, whereas the presence of fewer than 5 clustered events could not be related to the presence of a specific cell population[255]. In another study, a minimum of 15 events is reported to ascribe them to a specific cell population with a high probability[256]. In leptomeningeal metastasis of haematological malignancies, one study describes the presence of at least 10 clustered events with abnormal patterns of antigen expression for diagnosis[211], while another publication prescribes to classify clusters of more than 25 events as positive, 10-25 events as suspicious and below 10 events as negative[63]. In our laboratory, we agree with the latter publication and consider 25 clustered events as positive.

To obtain a maximal number of cells for analysis, CSF samples have to be concentrated by low-speed centrifugation. No significant cell loss was observed in hypocellular samples (<10 leukocytes/µl) when CSF cells were enriched by centrifuging at 200*g* for 15 minutes at 4°C[244]. CSF samples containing >10 leukocytes/µl can be stained and analyzed without cell enrichment[232]. However, in case of rare event detection, e.g. in CNS involvement of lymphoproliferative disorders, CSF cells should also be concentrated in samples containing >10 leukocytes/µl to increase the sensitivity. Another way to deal with the low cell numbers in CSF, is the use of a two step approach[63, 241]. First, one third of the sample is analyzed with a screening tube, which in most cases will answer the clinical question. When this first staining is not conclusive, the process should be repeated with the remaining CSF and the same reagent combination. Combining the list mode data of the first and second staining will increase sensitivity by enabling analysis of a larger number of cells. Second, if a pathological population is identified in the first step, immunophenotyping may be extended.

Rapid decline of leukocyte counts upon lumbar puncture

Another difficulty of CSF studies is the rapid decay of leukocytes after sampling as described in several studies (Table 3). Within 30 minutes after sampling, the CSF cell number decreases significantly[206, 211, 230, 257-259]. Also, differences in survival rate between different leukocyte subsets were observed: monocytes and granulocytes showed a more rapid cell loss than lymphocytes[206, 230, 258] (Table 3). In flow cytometric analysis, selective cell losses may cause underestimation of cell counts[220]. These errors can affect clinical decisions. E.g., in CSF samples with a pleocytosis, underestimation of the cell number may result in a normal cell count and pathology, such as a neuro-inflammatory disease, may be considered ruled out.

Table 3. Decline of CSF cell yields as a function of storage time

CSF additive	First assessment	Second assessment	Temperature	Cell loss relative to first assessment (%)			
Reference	(hours)	(hours)		WBC	Lymphocytes	Monocytes	Granulocytes
None							
Chow, 1984[257]	0[a]	2 and 24	22°C	40 and 60	-	-	-
	0[a]	2 and 24	4°C	15 and 39	-	-	-
Steele, 1986[258]	0[b]	2 and 4	Ambient	27[g] and 44[g]	12 and 34	20 and 39	50 and 58
Dux, 1994[230]	0[c]	1.5	On ice	62[g]	35	90	
De Graaf, 2011[206]	0.5	1	Ambient	26	4	36	34
Saline							
Steele, 1986[258]	0[b]	4	Ambient	6[g]	0	0	18
Serum-containing buffer							
Veerman, 1985[259]	0.5[d]	24	Ambient	0	-	-	-
Dux, 1994[230]	0[c]	1.5	On ice	-	10	-	-
De Graaf, 2011[206]	0.5[e]	1	Ambient	9	2	18	0
Chemical stabilization							
Quijano, 2009[211]	0[f]	24 and 48	Unknown	21 and 40	-	-	-

[a]Spiking: homologous blood was added to cell-free supernatant of CSF samples.
[b]Spiking: lymphocytes, monocytes and neutrophils were obtained from peripheral blood and subsequently spiked into CSF samples that had been centrifuged to remove cells or into saline.
[c]Spiking: after CSF withdrawal, CSF cells were pelleted by centrifugation and resuspended in CSF or in PBS containing 5% fetal calf serum. The cell number at resuspension was set at 100%.
[d]Sterile physiologic medium (one part Earle's balanced salt solution and one part 20% human serum albumin) was added directly after CSF withdrawal.
[e]Serum-containing medium (RPMI-1640 with HEPES, L-Glutamine, Penicillin/Streptomycin, heat-inactivated fetal bovine serum and heparin) was added directly after CSF withdrawal.
[f]CSF directly collected into tubes containing EDTA and 0.2 ml of Transfix™.
[g]WBC loss is calculated by adding up the lymphocyte, monocyte and granulocyte loss.

This cell loss in native CSF can be reduced by addition of medium to CSF directly after sampling. In an earlier study, we showed that addition of serum-containing medium (RPMI-1640 with HEPES, L-Glutamine, Penicillin/Streptomycin, heat-inactivated fetal bovine serum and heparin) preserves CSF cells until at least five hours after sampling[206]. Another study showed that immediate addition of Earle's balanced salt solution with human serum albumin to CSF prevents total WBC loss until at least 24h after sampling[259]. Also, addition of TransFix™ (fixative) has been shown to reduce CSF cell loss[211]. Other previous reports that investigated CSF cell preservation methods, were more laboratory based than clinical. Spiking of CSF cells into 5% fetal calf serum[230] or saline[258] showed no significant cell loss, while spiking into acellular CSF did[230, 258] (Table 3). In addition, immediate cooling of the CSF sample[257], a minimum of centrifugation steps[230] and aspiration of the supernatant instead of decanting the sample[63] all reduce cell loss. Furthermore, due to the absence of free immunoglobulins (Ig) in CSF, washing CSF cells before surface-bound Ig staining is not necessary, which minimizes the wash steps in the protocol[63]. The function of media, in most studies serum-containing, in preserving leukocytes is probably a buffering one. Both increase in pH in CSF after removal from the body (due to diffusion of CO_2 out of the sample) and hypotonicity of CSF (causing movement of water and solutes from the extracellular to the intracellular compartments) have been hypothesized to contribute to cell death[258, 260, 261], although the effect of both factors has not been confirmed. It remains to be investigated which medium is the most effective in preventing CSF cell loss, being both practical for use in clinical settings and inexpensive. Evidently, CSF samples used for cell counting should be handled carefully by (i) collection in a buffering medium to prevent the rapid cell loss; (ii) a minimum of wash and centrifugation steps; (iii) aspirating instead of decanting; and (iv) data acquisition at least within 24h after sampling[259], although the maximal storage time has not been determined yet.

Nonspecific fluorescence

Nonspecific or "background" fluorescence may constitute a serious problem, especially in rare event detection in CSF samples. Its cause can be categorized into three groups[262]. First, autofluorescence by excitation of naturally occurring cellular components (e.g., granule-associated flavoproteins in granulocytes) other than the antibody bound fluorochrome[262]. This problem may be reduced by the use of a 532 nm laser[263] or specific tools like single laser excitation[264] and cell-by-cell autofluorescence correction[265]. Second, spectral overlap which becomes significant when more than four colours are used and can be minimized by choosing a combination of fluorochromes that have no or little overlap with each other[228]. Third, non-specific antibody binding may occur which can be eliminated by optimizing antibody concentration using titration assays[266].

Blood contamination

Red cells present in CSF reflect either CNS bleeding or a traumatic lumbar puncture in which peripheral blood contaminates the CSF. The possibility of blood contamination of CSF samples can be ruled out by absence of cell populations in CSF that are present in blood at normal or high numbers (e.g. neutrophils and erythrocytes). In flow cytometric absolute cell counting of blood contaminated CSF samples, correction of the number of leukocytes has to be performed. We prefer to use the leukocyte/erythrocyte ratio in peripheral blood for correction[267], because this method accounts for a patient's individual situation. Alternatively, the CSF leukocyte number may be arbitrarily adjusted by correction according to the CSF erythrocyte count, which results in a correction of 1 leukocyte per 500 erythrocytes present in CSF[268, 269]. This latter method is widely used in clinical practice, because information on peripheral blood is not needed.

In blood contaminated CSF samples, which are investigated for the presence of CNS involvement of haematological malignancies, detection of a small population of neoplastic cells is only diagnostic of CSF involvement if these cells are not detected in a simultaneously obtained blood sample[63]. In acute leukaemia, lumbar puncture should not be performed in an acute phase of the disease when the frequency of circulating malignant cells is high. In case of a traumatic lumbar puncture, malignant cells from the blood may become detectable in CSF leading to apparently false-positive CSF results. Moreover, iatrogenic contamination of CSF with malignant cells might be caused[270].

Detection of monoclonal B-cell populations

Detection of a monoclonal B-lymphocyte population in CSF diagnoses CNS localization of a B-cell lymphoma in patients with haematological malignancies[204, 238]. B-lymphocyte clonality can be investigated by flow cytometry by assessing surface immunoglobulin light chain expression on $CD19^+$ B-lymphocytes and comparison of the "light chain ratio (LCR)" or "kappa/lambda ratio", which is determined by dividing the percentage of cells with the dominant light chain by the percentage of cells with the minor light chain[247]. Normal ranges for the LCR differ between laboratories. A LCR threshold of 2 was reported to have a specificity of 92.3% and a sensitivity of 73.1%[271], while other studies proposed thresholds ranging from 2 to 6, with highest specificities and sensitivities around 70%[272-277]. This indicates that if e.g., a threshold of 2 is used, approximately 10% of patients with a LCR above 2 are reported to have a monoclonal B-cell population, but do not have a B-cell lymphoma. Therefore, a LCR shift is only suggestive for presence of a monoclonal B-cell population, and further analysis of the CSF has to be performed. Assessment of additional markers may increase specificity as abnormal light scatter patterns and abnormal intensities of CD19 and/or CD20 indicate the presence of a malignant cell population[211], although absence of such an abnormal pattern does not rule out malignancy. Preferably,

assessment of monoclonality and abnormal marker expression are combined, e.g. looking for monoclonality in a large forward scatter (FSC) or dim CD20 population, as presented in Figure 1. Furthermore, detection of clonally identical rearranged DNA sequences in malignant B lymphocytes by PCR is suggestive of the presence of B-cell lymphoma[222], but only a minority of clinical laboratories have this technique operational. Another important point of attention is that not every monoclonal B-cell population in CSF indicates symptomatic CNS disease[237]. In patients with indolent haematological disorders, including chronic lymphatic leukaemia (CLL), malignant B cells in the CSF may represent asymptomatic leptomeningeal involvement and may require treatment only when (new) symptoms arise[247, 278, 279]. At last, clinical follow-up will aid in diagnosing CNS disease.

PERSPECTIVES

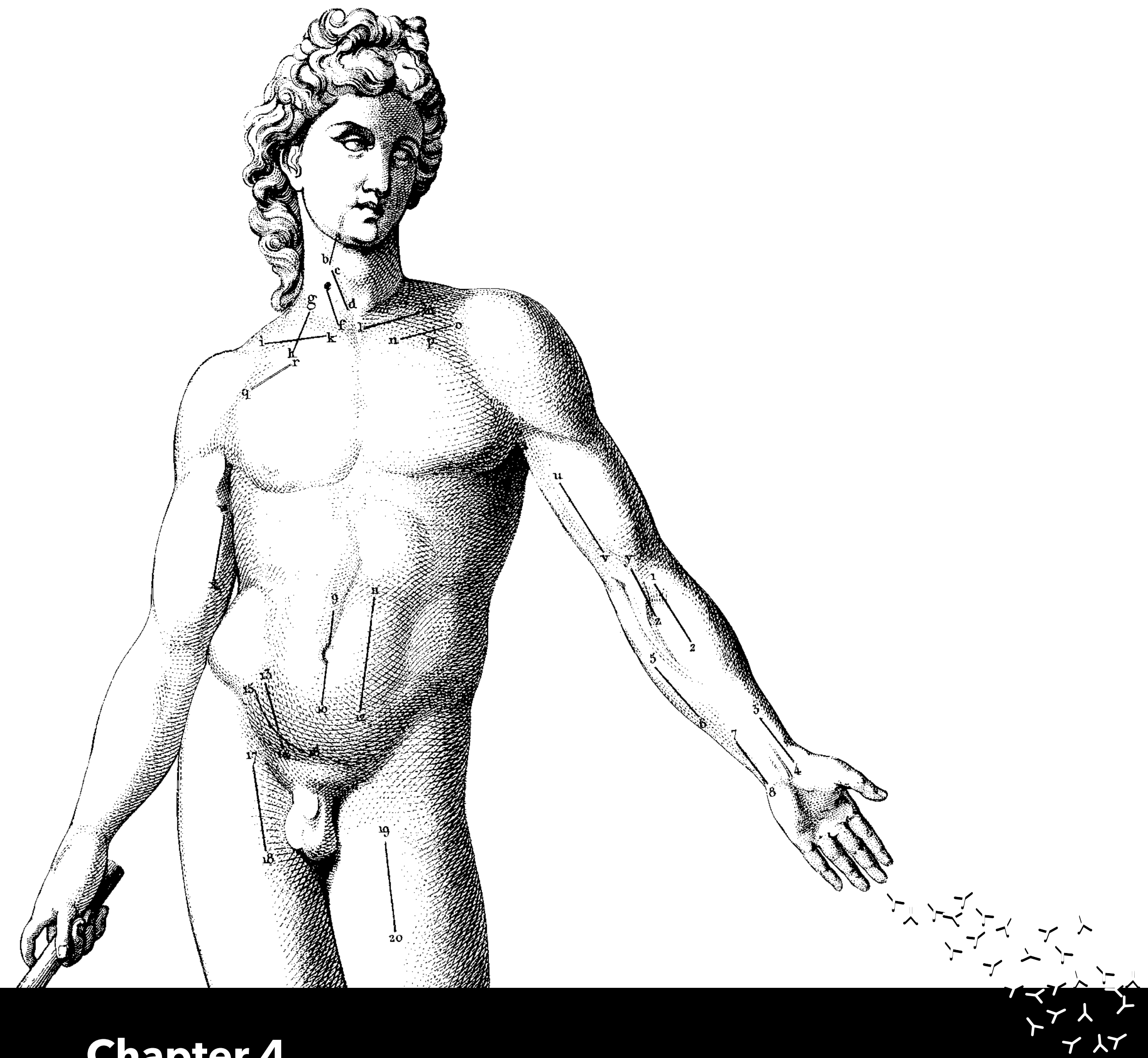
We expect that the expansion of the number of colours amenable to flow cytometry will enable the simultaneous study of more parameters within the same sample. Using this approach, more refined information on CSF cell subsets will become available and cell populations can be defined more accurately[280]. An ongoing challenge is the search for new fluorochromes that can be used in conjunction with current ones and yet do not contribute to significant spectral overlap[281]. Even without new fluorochromes or lasers, instruments will improve through advances in software for data processing. However, visualizing these data becomes more and more complex and would require multiple sequential analyses to provide information about each cell subset. Therefore, automated classification systems are being developed[282-285]. Additionally, optimization of storage conditions to preserve CSF cells should result in higher cell yields and thereby increase the detection rate of flow cytometry in CSF samples with low cellularity.

Importantly, flow cytometry can be combined with molecular techniques including PCR to improve sensitivity in detecting CSF involvement of lymphoma[286, 287]. Furthermore, broadspectrum tumour cell-specific antigens could be fluorescently labelled and used in flow cytometric detection of CNS malignancy[210]. In future, DNA clonality of the tumour might be identified on biopsy material and then followed by CSF assays along the course of the disease. This allows us to detect whether selection and development of new malignant clones occur in resistant or relapsing disease and will enable us to appraise the therapeutic and prognostic implications of molecular diagnostic testing of CSF[223]. However, these future techniques have to be internationally validated and standardized to be used in clinical practice.

In summary, these future advances will lead to a higher sensitivity and specificity to detect CNS localization of malignancies, while in neuro-inflammatory diseases (e.g., MS), CSF flow cytometry might become an important tool in the diagnosis, prognosis and follow-up of patients.

Acknowledgment

This work was supported by the Gratama Foundation, Teteringen, The Netherlands.



Chapter 4

Addition of serum-containing medium to cerebrospinal fluid prevents cellular loss over time

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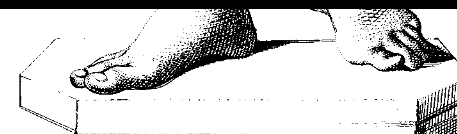
Journal of Neurology, 2011 March 12 (Epub)



ABSTRACT

Immediately after sampling, leukocyte counts in native cerebrospinal fluid (CSF) start to decrease rapidly. As the time lapse between CSF collection to analysis is not routinely registered, the clinical significance of decreasing cell counts in native CSF is not known. Earlier data suggest that addition of serum-containing medium to CSF directly after sampling prevents this rapid decrease in leukocyte counts, and thus may improve the accuracy of CSF cell counting and cell characterization. Here, we prospectively examined the effect of storage time after lumbar puncture on counts of leukocytes and their major subsets in both native CSF and after immediate addition of serum-containing medium, measured by flow cytometry and microscopy. We collected CSF samples of 69 patients in tubes with and tubes without serum-containing medium and determined counts of leukocytes and subsets at 30 minutes (30min), one hour (1h) and five hours (5h) after sampling. Compared to cell counts at 30min, no significant decrease in cell number was observed in CSF with serum-containing medium 1h and 5h after sampling, except for the granulocytes at 1h. In native CSF, approximately 50% of leukocytes and all their subsets were lost after 1h, both in flow cytometric and microscopic counting. In 6/7 (86%) samples with a mild pleocytosis (5-15x10^6 leukocytes/l), native CSF at 1h was incorrectly diagnosed as normocellular. In conclusion, addition of serum-containing medium to CSF directly after sampling prevents cell loss and allows longer preservation of CSF cells prior to analysis, both for microscopic and flow cytometric enumeration. We suggest that this protocol results in more accurate CSF cell counts and may prevent incorrect conclusions based on underestimated CSF cell counts.

INTRODUCTION

Cerebrospinal fluid (CSF) pleocytosis (>5x10^6 leukocytes/l) may indicate an infectious or inflammatory etiology of neurological symptoms[201]. Examples include viral, tuberculous and aseptic meningitis, neurosarcoidosis, neuro-Behçet and paraneoplastic neurological syndromes.

Earlier studies showed that leukocytes, derived from blood or CSF, disappear rapidly when spiked into CSF[230, 257, 258]. After two hours of storage in CSF, a leukocyte loss of 15%-62%[230, 257, 258] was observed, while after 24h storage only 39%-60% of leukocytes remained detectable[257]. Furthermore, the delay from sampling to processing has a differential effect on the various cell types in CSF, and affects the number of granulocytes most[230, 258].

However, the time from CSF collection to analysis is not routinely registered, and the clinical significance of decreasing cell counts in native CSF is not known. The rapid cellular loss in CSF suggests that CSF of patients with mild pleocytosis may be falsely diagnosed as normocellular depending on the time between lumbar puncture and analysis of the sample. Addition of serum-containing medium to CSF may prevent the rapid loss of leukocytes and their subsets[259] and thereby improve the accuracy of CSF cell counting and characterization.

In addition to the delay between sampling and analysis, CSF cell counts may also be affected by the counting method. Manual or microscopic analysis is the gold standard for determination of the (differential) white blood cell (WBC; leukocyte) and red blood cell (RBC; erythrocyte) counts in CSF. However, this technique is very time-consuming, costly and prone to inter-technician variability and low precision[288, 289]. Flow cytometry appears to improve accuracy and precision of CSF cell counting[290, 291]. Moreover, flow cytometry permits differentiation of leukocytes into lymphocytes and their subsets, monocytes and granulocytes by immunophenotyping, whereas microscopical analysis only differentiates between mononuclear cells (MNC; lymphocytes and monocytes) and polymorphonuclear cells (PMN; granulocytes).

In this study, we prospectively examined the effect of storage time after lumbar puncture on counts of leukocytes and their major subsets in both native CSF and after addition of serum-containing medium, as measured by flow cytometry and microscopy. The medium was added directly after lumbar puncture as it is known that cell decline starts immediately after CSF sampling[230, 257-259]. We determined the percentage of CSF samples with mild pleocytosis that would be incorrectly diagnosed as normocellular when only native CSF was examined. This study was conducted on CSF samples of 69 patients.

METHODS

Patients

A total of 61 patients with suspected neurological disease were included between January and August 2009 at the Department of Neurology, Daniel den Hoed Cancer Clinic, Erasmus MC, Rotterdam. We included patients with suspected or proven leptomeningeal localization of a hematological or solid malignancy undergoing diagnostic or therapeutic lumbar puncture (LP) in our in- or outpatient clinic. Patients were included only once. Additionally, 8 patients without neurological disease undergoing spinal anesthesia for orthopaedic, gynaecological or general surgery were included between May and August 2008 at the Department of Anesthesiology, Sint Franciscus Gasthuis, Rotterdam. The local ethical committees approved of the study design and written informed consent was obtained from all patients.

CSF collection

Up to 5 ml extra CSF was obtained from the 61 neurological patients during routine LP and aspirated before administration of the anesthetic drug in the 8 anesthesiological patients. Immediately after withdrawal, the CSF was split into different sterile tubes with or without serum-containing medium (1:1 dilution) and stored at room temperature. The medium consisted of RPMI-1640 with 25 mM HEPES, 1 mM L-Glutamine, 2% Penicillin/Streptomycin, 5% heat-inactivated fetal bovine serum (FBS) and 2500 IU heparin. This medium is routinely used in our laboratory for preservation of CSF cells before analysis[63, 230] and is stored in refrigerators in our in- and outpatient clinical wards where LPs are performed. All reagents were obtained from Cambrex Biosciences (Walkersville, MD), except for FBS (Greiner Bio-One, Frickenhausen, Germany) and heparin (Leo Pharma A/S, Ballerup, Denmark). In CSF of the 61 neurological patients, cell numbers were determined by flow cytometric and microscopic counting at 30 minutes (30min), one hour (1h) and five hours (5h) after sampling, while in CSF of the 8 patients who underwent spinal anesthesia, cell numbers were counted by flow cytometry only at 1h and 5h after sampling, due to smaller sample sizes in these patients.

Flow cytometric counting

Numbers of leukocytes, lymphocytes, monocytes and granulocytes were determined by 6-color flow cytometry. CSF cells were concentrated by centrifugation (8min, 450*g*) and resuspended in phosphate-buffered saline (PBS). Next, the sample was incubated for 15min at room temperature (RT) in the dark with 10 µl of each of the following monoclonal antibodies (mAb): CD3 conjugated with fluorescein isothiocyanate (FITC; clone SK7), CD56 conjugated with phycoerythrin (PE; clone C5.9), CD45 conjugated with peridinyl cholorophyllin (PerCP; clone 2D1), CD4 conjugated with PE-Cy7 (clone SK3), CD19 conjugated with allophycocyanin (APC; clone HIB19) and CD8 conjugated with APC-Cy7 (clone SK1). All mAb were obtained from BD Biosciences (San Jose, CA) with the exception of CD56-PE (Dako, Glostrup, Denmark) and CD19-APC (eBioscience, San Diego, CA). After incubation, cells were washed and subsequently resuspended in PBS. Immediately upon staining, list mode data were acquired on a 6-color FACSCanto flow cytometer (BD Biosciences, San Jose, CA). Analysis was performed using FCS Express software (De Novo Software, Los Angeles, CA). In the flow cytometric analysis of this study, we focused on leukocytes, lymphocytes, monocytes and granulocytes as microscopic enumeration could only differentiate between leukocytes, mononuclear cells (MNC; lymphocytes and monocytes) and granulocytes. The immunophenotypical definitions of leukocytes and their major subsets are shown in Table 1.

Table 1 | Immunophenotypical definitions of leukocytes and their subsets

Subset	Immunophenotypical definition
Leukocytes	$CD45^+$
Lymphocytes	$CD45^+$, SSC^{lo}, FSC^{im}
Monocytes	$CD45^+$, SSC^{im}, FSC^{hi}, $CD4^{dim}$
Granulocytes	$CD45^+$, SSC^{hi}

SSC = side scatter; FSC = forward scatter; hi = high expression; im = intermediate expression; dim = dim expression; lo = low expression.

Microscopic counting

Microscopic counting was performed before washing or concentration of the CSF. RBC and WBC were counted in a Fuchs-Rosenthal counting chamber at 400x magnification. First, RBC were counted in unstained CSF. Next, CSF was incubated with concentrated Türk reagent (crystal violet dissolved in acetic acid [both obtained from Merck, Darmstadt, Germany]) (10:1) for 5min to lyse the RBC and stain the WBC. After incubation, WBC were counted and visual differentiation between MNC and PMN was made. Subsequently, cell concentrations were calculated and corrected for dilution in medium.

Statistics

Because of the large variation in cell counts between samples, we used ratios to present the data clearly. Specifically, we calculated ratios for medium (cell count in *native CSF* divided by cell count in *CSF with medium)* in paired samples, for flow cytometry and for microscopy. These ratios were presented in box-and-whisker plots. To present the effect of storage time, the cell survival in native CSF and CSF with medium was determined ([i] cell count *at 1h* divided by cell count *at 30min,* and [ii] cell count *at 5h* divided by cell count *at 30min)* and graphically presented. Groups were compared with the Wilcoxon matched-pair test (SPSS version 15.0, SPSS Inc., Chicago, IL). Differences between groups with a *P*-value <0.05 were considered significant.

RESULTS

Patients

Patient characteristics are presented in Table 2. Due to limited volumes of CSF in some patients, not all counting methods could be performed at all three time points within each sample. Forty-eight CSF samples were investigated at 30min with both flow cytometry and microscopy, while at 1h flow cytometry was performed on 69 samples and microscopy on 61 samples. At 5h after sampling, 27 CSF samples were studied by flow cytometry and

19 by microscopy. Median and 25th-75th percentiles of paired flow cytometric enumerations are presented in Table 3.

Table 2 | Patient characteristics

	Patients
N	69
Age: median (range)	53 (20-77)
Gender (M/F)	42/27
Cerebrospinal fluid: median (range)	
Glucose (mmol/l)[a]	3.5 (1.8-9.5)
Protein (g/l)[a]	0.34 (0.05-2.47)
Lactate dehydrogenase (U/l)[a]	43 (10-116)
RBC ($x10^6$/l)	0 (0-1000)
WBC ($x10^6$/l)[a]	2 (0-356)
Reason for lumbar puncture	
Diagnostic	33
Therapeutic	28
Spinal anesthesia	8
Malignancy	
Hematological	43
Solid	18
No	8

[a]Reference ranges: glucose 2.5-3.7 mmol/l; protein 0.18-0.58 g/l; lactate dehydrogenase 0-69 U/l; WBC 0-5x10^6/l.
RBC = red blood cells; WBC = white blood cells.

Table 3 | Median and 25th-75th percentiles of paired flow cytometric cell counts (x10^6 cells/l)

	30min with	30min without	1h with	1h without	5h with	5h without
Leukocytes	1.13 (0.56-2.79)	0.97 (0.47-2.83)	1.12 (0.56-2.11)	0.49 (0.27-1.37)	1.02 (0.67-1.94)	0.50 (0.35-1.91)
Lymphocytes	0.46 (0.12-1.73)	0.32 (0.13-0.92)	0.35 (0.15-1.37)	0.21 (0.06-0.80)	0.48 (0.16-1.65)	0.25 (0.08-0.99)
Granulocytes	0.23 (0.13-0.49)	0.22 (0.11-0.48)	0.17 (0.10-0.34)	0.09 (0.06-0.22)	0.16 (0.09-0.41)	0.11 (0.06-0.26)
Monocytes	0.31 (0.14-0.73)	0.20 (0.09-0.68)	0.31 (0.12-0.64)	0.13 (0.05-0.29)	0.40 (0.20-0.91)	0.14 (0.06-0.39)

Effect of storage time on CSF cell counts

Because it is not feasible to count cells directly after sampling, we considered the cell count at 30min as a reasonable earliest measurement. Based on earlier data[63, 230, 259], we postulated that addition of serum-containing medium immediately after CSF sampling, would prevent cell loss and provide the most accurate estimation of the number of cells in CSF at the time of sampling. We approximated this situation by performing the first enumerations at 30min after sampling, a time lapse needed for transport of the CSF sample

to the laboratory and setting up the counting procedure.

First, the effect of storage time after the addition of serum-containing medium to CSF was evaluated. At 1h and 5h after sampling, no significant decrease in the number of lymphocytes or monocytes counted by flow cytometry was observed in CSF with serum-containing medium as compared to 30min after sampling (Figure 1A). However, granulocytes did decrease significantly (*P*<0.005), which may be due to their very low counts in our CSF samples (typically, CSF samples contained only 0.02-6.1 x10^6 granulocytes/l versus 0.01-43.3x10^6 lymphocytes/l). In native CSF (Figure 1B), the leukocyte number declined significantly over time (*P*<0.005), including the number of monocytes (*P*<0.005) and granulocytes (*P*<5x10^{-6}). The number of lymphocytes was stable at both time points.

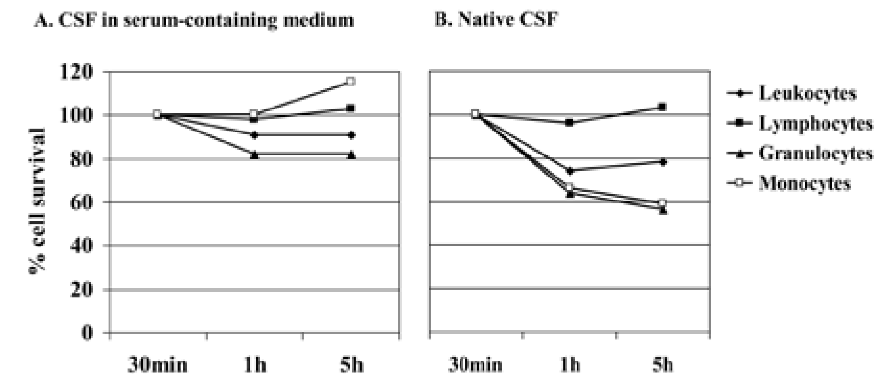


Figure 1 | Cell survival in CSF

Median percentage of cell survival of leukocytes and their subsets at 1h (N=48) and 5h (N=27) in CSF stored in serum-containing medium (Panel **A**) and native CSF (Panel **B**) as determined by flow cytometry. The cell count at 30min is set at 100%. In CSF with medium the number of granulocyted declined significantly (P<0.005) at 1h and 5h. In native CSF the number of leukocytes (P<0.005), monocytes (P<0.005) and granulocytes (P<5x10^{-6}) declined significantly at 1h.

Addition of serum-containing medium immediately after CSF sampling

Flow cytometry

At all time points, flow cytometric counts of leukocytes and their subsets in native CSF were significantly lower than in CSF with serum-containing medium, except for the granulocyte count 30min after sampling (Figure 2). Furthermore, the ratio between cell counts in CSF without and with serum-containing medium decreased over time. After 5h, only 56% of the lymphocytes were left in native CSF (Figure 2). The monocyte counts were affected even more: only 34% of cells was still detectable after 5h (Figure 2). Most of the cell loss in native CSF occurred during the first hour after sampling, while the differences between 1h and 5h were not significant (data not shown).

At 1h after sampling, 15 of the 69 patients (22%) had a pleocytosis (5-344x10^6 leukocytes/l) in the CSF sample with serum-containing medium. This pleocytosis was mild (5-15x10^6 leukocytes/l) in 7 patients. In the native samples, normal leukocyte counts were observed in 6 of the 15 (40%) samples with pleocytosis and even in 6 of 7 (86%) samples with a mildy increased leukocyte count.

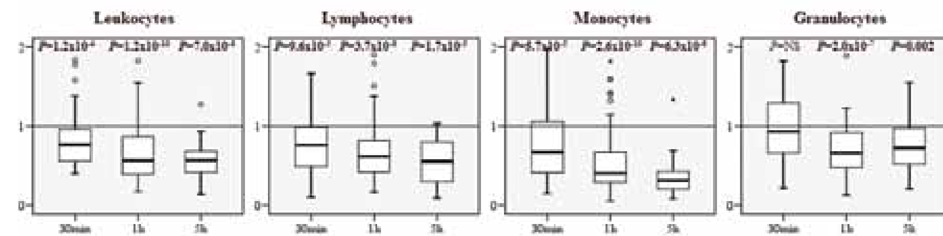


Figure 2 | Loss of cells in native CSF compared to CSF stored in serum-containing medium as determined by flow cytometry

Ratios of cell count in native CSF divided by cell count in CSF with serum-containing medium in paired samples for flow cytometric leukocyte, lymphocyte, monocyte and granulocyte counts at 30min (N=48), 1h (N=69) and 5h (N=27) after sampling. Box-and-whisker plots were used to present the data graphically, showing median and interquartile ranges; the whiskers extended to the adjacent values, i.e., 1½x the interquartile range rolled back to where there is data. Observed points more extreme than the adjacent values were considered outliers and have been plotted individually. P-values (Wilcoxon matched-pair test) for the difference in cell counts between CSF with and without serum-containing medium are given.

Microscopy

Microscopic counts of WBC, MNC and PMN (Figure 3) were also significantly lower in native CSF compared to CSF with serum-containing medium at all time points, except for the PMN 30min after sampling. When using microscopic counting, PMN showed the greatest loss in native CSF with a residual cell count of only 36% at 5h after sampling as compared to CSF stored in serum-containing medium.

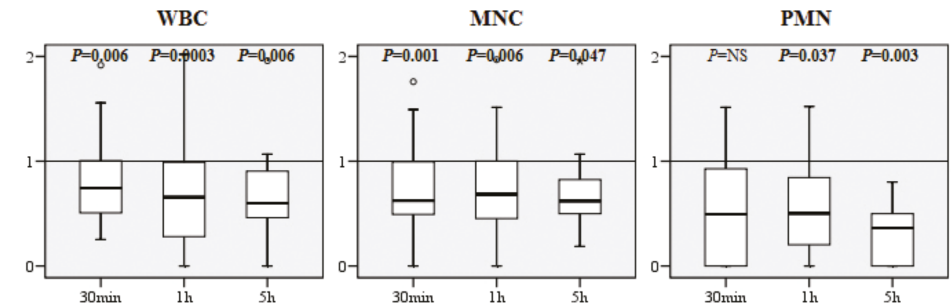


Figure 3 | Loss of cells in native CSF compared to CSF stored in serum-containing medium as determined by microscopy

Ratios of cell count in native CSF divided by cell count in CSF with serum-containing medium in paired samples for microscopic white blood cell (WBC), mononuclear cell (MNC) and polymorphonuclear cell (PMN) counts at 30min (N=48), 1h (N=61) and 5h (N=19) after sampling are presented in box-and-whisker plots. See further legend to Figure 2.

DISCUSSION

We prospectively investigated the effect of adding serum-containing medium to CSF directly after sampling on cell counts determined by flow cytometry and microscopy in CSF samples from 69 patients. Our findings show that addition of serum-containing medium to CSF directly after sampling not only prevents immediate cell loss, but also allows longer preservation of CSF cells. The latter is important for clinical practice. Especially after office hours, CSF samples may remain unattended at room temperature for considerable and unknown amounts of time: at a busy emergency room, during prolonged transportation towards the laboratory and on the laboratory bench pending analysis.

In CSF with a mild pleocytosis, WBC counting in samples without addition of medium may result in a normal cell count and pathology, e.g. neuro-inflammatory disease, may

incorrectly be excluded. In our study, this situation was observed in 40% of the samples with a pleocytosis and even in 86% of the samples with a mildly increased WBC count. This result indicates that addition of serum-containing medium to CSF samples may prevent false-negative CSF interpretation and thereby impacts on clinical decision-making.

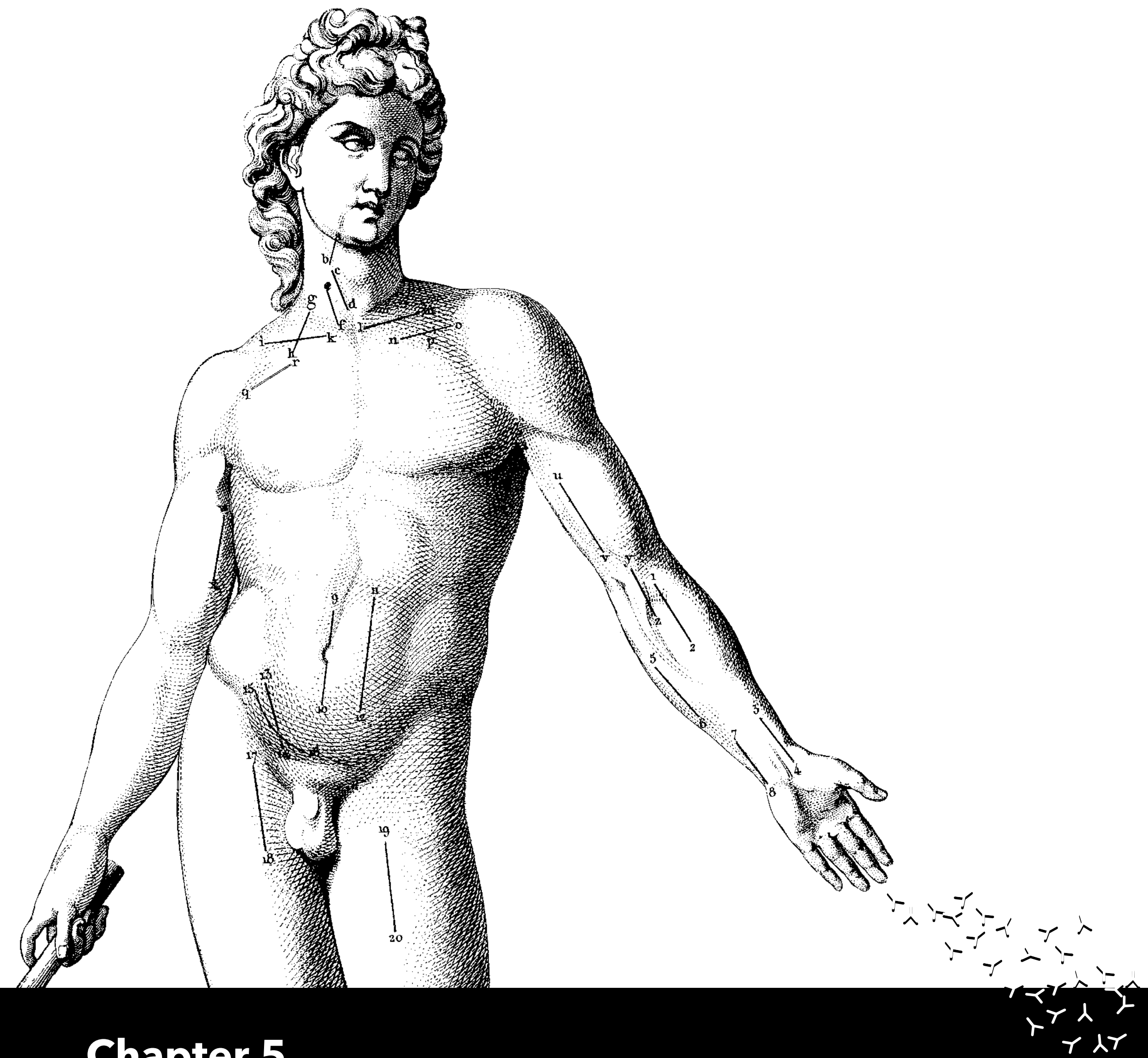
In addition to the total number of WBC, we also studied the major WBC subsets. The selective loss of granulocytes, in native clinical CSF samples as well as in CSF with medium, has not been described earlier but is not unexpected as even under ideal circumstances granulocytes remain viable for only 7h[258]. Knowledge of this selective cell loss is important, because it may result in distortion of the relative size of cell populations[230] and thereby affect clinical decisions (e.g. predominance of granulocytes in CSF may differentiate early bacterial from aseptic meningitis).

Different media can be added to CSF to reduce the cell loss. Here, we show that addition of serum-containing medium preserves CSF cells until at least five hours after sampling. An earlier study showed that immediate addition of Earle's balanced salt solution with human serum albumin to CSF prevents total WBC loss until at least 24h after sampling[259]. In this study, major WBC subsets were not determined[259]. Other previous reports that investigated CSF cell preservation methods, were more laboratory based than clinical. Spiking of CSF cells into 5% fetal calf serum[230] or saline[258] showed no significant cell loss, while spiking into acellular CSF did[230, 258]. In addition, refrigeration of the CSF sample[257] and a minimum of centrifugation steps [230] also reduce cell loss. The function of serum-containing media in preserving cells is probably a buffering one. Both increase in pH after removal of CSF from the body[260] and low osmolarity[258, 261] have been postulated to cause cell death. Also, it remains to be investigated which medium is the most effective in preventing CSF cell loss in clinical settings from practical and economic perspectives.

Based on the results of this study, we recommend adding serum-containing medium to CSF directly after collection, not only when CSF flow cytometry has to be performed, but also when cells are routinely counted by microscopy. Addition of serum-containing medium may prevent false negative reports of normal cell counts in CSF samples with a mild pleocytosis. Although the conditions and the maximal storage time to preserve CSF samples for optimal cellular analyses still have to be optimized, we may safely conclude that the use of native CSF samples for such analyses should be discouraged.

Acknowledgments

We would like to thank the neurologists and anesthesiologists for inclusion of the patients and the technicians of the Department of Clinical Chemistry in the Daniel den Hoed Cancer Clinic for their technical assistance. This work was supported by the Gratama Foundation, Teteringen, The Netherlands.



Chapter 5

Central memory CD4$^+$ T cells dominate the normal cerebrospinal fluid

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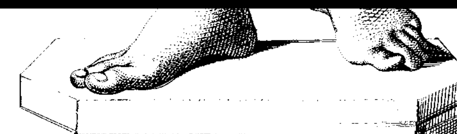
Cytometry Part B: Clinical Cytometry 2011;80:43-50



ABSTRACT

Background

In order to use cerebrospinal fluid (CSF) immune phenotyping as a diagnostic and research tool, we have set out to establish reference values of white blood cell (WBC) subsets in CSF.

Methods

We assessed the absolute numbers and percentages of WBC subsets by 6-colour flow cytometry in paired CSF and blood samples of 84 individuals without neurological disease who underwent spinal anaesthesia for surgery. Leukocyte (i.e., lymphocytes, granulocytes and monocytes), lymphocyte (i.e., T [$CD4^+$ and $CD8^+$], NK, NKT and B cells), T cell (i.e., naïve, central memory, effector memory and regulatory) and dendritic cell subsets (i.e., myeloid and plasmacytoid) were studied.

Results

CSF showed a predominance of T cells, while granulocytes, B and NK cells were relatively rare compared to blood. The majority of T cells in CSF consisted of $CD4^+$ T cells (~70%), most of them (~90%) with a central memory phenotype, while B cells were almost absent (<1%). Among the small population of dendritic cells in CSF, those of the myeloid subtype were more frequent than plasmacytoid dendritic cells (medians: 1.7% and 0.4% of leukocytes, respectively), whilst both subsets made up 0.2% of leukocytes in blood.

Conclusions

This study reports reference values of absolute numbers and percentages of WBC subsets in CSF, which are essential for further investigation of the immunopathogenesis of neuro-inflammatory diseases. Furthermore, the relative abundance of $CD4^+$ T cells, mainly with a central memory phenotype, and the presence of dendritic cells in CSF suggests an active adaptive immune response under normal conditions in the central nervous system (CNS).

INTRODUCTION

Patients with inflammatory disorders of the central nervous system (CNS) typically have increased white blood cell (WBC) counts in their CSF. Little information is yet available on the composition of these cells in normal CSF, as lumbar puncture without a clinical indication is generally considered unethical and flowcytometric studies in CSF mainly focus on detection of CSF involvement in hematologic malignancies[204]. Furthermore, the presence of relatively low numbers of cells with rapidly decreasing viability after sampling makes reliable identification and enumeration of leukocyte and lymphocyte subsets in CSF difficult[63]. Earlier studies have described the distribution of specific lymphocyte subsets in CSF of patients with neuro-inflammatory diseases such as multiple sclerosis, neuroborreliosis and paraneoplastic neurological syndromes (PNS) in order to investigate the immunopathogenesis of these diseases[231, 254, 292, 293]. In those studies, patients with other inflammatory or non-inflammatory neurological disorders were frequently included as controls instead of individuals without neurological disease. Svenningsson et al.[234, 235] studied normal CSF by assessing the *percentages* of lymphocyte subsets, but not absolute counts, in CSF of 34 healthy individuals with 2- or 3-colour flow cytometry. Here, a predominant presence of T cells, mostly with a central memory phenotype, and low frequencies of B and NK cells, were reported.

In the present study, we aimed to also study *absolute numbers* of WBC subsets in normal CSF, because percentages and absolute numbers can differ widely depending on the clinical setting[229]. In specific patients, enumeration of absolute levels of circulating cells and their subsets is of primary importance. E.g., in acquired immune deficiency syndrome (AIDS) absolute counting of $CD4^+$ T cells in peripheral blood is the major laboratory tool for staging human immunodeficiency virus (HIV) infected patients[294]. In candidates for autologous or allogeneic hematopoietic stem cell transplantation, enumeration of absolute levels of circulating $CD34^+$ hematopoietic progenitor cells is used as a marker for graft adequacy[295]. In our earlier work, PNS patients stood out by highly increased absolute counts of the major lymphocyte subsets in CSF, but above all, by B-cell counts that had increased more than 20-fold as compared to controls without neurological disease[231]. This indicates that assessment of absolute counts is also important in CSF and therefore knowledge of normal values is essential.

By using 6-colour flow cytometry, we were able to study multiple functionally different WBC subsets in paucicellular CSF samples, such as lymphocytes ($CD4^+$ and $CD8^+$ T cells, NK, NKT and B cells), granulocytes, monocytes and dendritic cells (myeloid and plasmacytoid). These subsets were assessed in CSF and blood samples of 84 individuals without neurological disease who underwent spinal anaesthesia for surgery. We considered these study persons normal with respect to the numbers and phenotypes of WBC in their CSF. In addition to description of normal values, we confirmed the predominant presence of central memory $CD4^+$ T cells and the paucity of B, NK and NKT cells in normal CSF. Additionally, we showed that dendritic cells, both myeloid as well as plasmacytoid are present in normal CSF. Comparison of our results with those in patients with neuro-inflammatory diseases may allow further studies of the immunopathogenesis of such diseases.

METHODS

Study persons

A total of 84 individuals without neurological disease undergoing spinal anaesthesia for surgery were included between May and August 2008 at the Department of Anaesthesiology, Sint Franciscus Gasthuis, Rotterdam. Individuals were not included when they had a history of (i) neurological disease; (ii) cancer; or (iii) treatment with corticosteroids or cytostatic drugs. The local ethical committee approved the study and written informed consent was obtained from all individuals.

Monoclonal antibodies

Properly titrated fluorochrome-conjugated monoclonal antibodies (mAb) were used for cell surface labelling. For enumeration of leukocyte and lymphocyte subsets in CSF, a single 6-colour mixture was used: CD3 conjugated with fluorescein isothiocyanate (FITC; clone SK7), CD56 conjugated with phycoerythrin (PE; clone C5.9), CD45 conjugated with peridinyl cholorophyllin (PerCP; clone 2D1), CD4 conjugated with PE-Cy7 (clone SK3), CD19 conjugated with allophycocyanin (APC; clone HIB19) and CD8 conjugated with APC-Cy7 (clone SK1). For blood two 4-colour mixtures were used: CD3-FITC, CD56-PE, CD45-PerCP and CD19-APC and CD4-FITC (clone HP2/6), CD8-PE (clone SK1), CD45-PerCP and CD3-APC (clone SK7). T cell subsets were assessed with a 6-colour panel: CD45RA-FITC (clone L48), CD127-PE (clone hIL-7R-M21), CD4-PerCP (clone SK3), CD25-PE-Cy7 (clone 2A3), CD27/28-APC (clone L128/CD28.2) and CD3-APC-eFluor780 (clone UCHT1). For dendritic cell subset immune phenotyping a 6-colour staining protocol described by Della Bella et al. [296] was used: LIN-1-FITC (Anti-Lineage 1 cocktail composed of CD3, CD14, CD16, CD19, CD20 and CD56 monoclonal antibodies), CD45-PerCP, CD123-PE-Cy7 (clone 6H6), CD11c-APC (clone S-HCL-3) and HLA-DR-APC-Cy7 (clone L243). All mAb were obtained from BD Biosciences (San Jose, CA) with the exception of CD56-PE (Dako, Glostrup, Denmark), and CD3-APC-eFluor780, CD19-APC and CD123-PE-Cy7 (eBioscience, San Diego, CA).

Cerebrospinal fluid

CSF was obtained during lumbar puncture performed by anaesthesiologists before administration of the anaesthetic drug. Lumbar puncture was done with a 27G atraumatic pencil-point Sprotte needle between the 2nd and 3rd or 3rd and 4th dorsal processes of the lumbar vertebrae in a sitting position. Approximately 5 ml CSF was carefully aspirated. Automated erythrocyte and leukocyte counts were obtained on a Sysmex XE-5000 (Sysmex, Hamburg, Germany). For immune phenotyping, 3-5 ml CSF was collected in tubes containing stabilization medium (RPMI-1640 with 25 mM HEPES, 1 mM glutamin, 2% Pen/Strep, 5% heat-inactivated BCS and 2500 IU heparin) to prevent direct cell lysis[63]. Within 6 hours after collection, cells were concentrated by centrifugation (8 min, 450*g*) and resuspended in phosphate-buffered saline (PBS). Next, 100 µl of the sample was incubated for 15 min (15') at room temperature (RT) in the dark with 10 µl of each of the above-mentioned mAb and after incubation the cells were washed in PBS. For enumeration of absolute numbers of leukocyte and lymphocyte subsets 100 µl PBS and 100 µl Cyto-Cal Count Control counting beads (Duke Scientific Corporation, Palo Alto, CA) were added[63], while for determination of T and dendritic cell subsets 250 µl PBS with 1% paraformaldehyde (PFA) was added. Immediately upon staining, list mode data were acquired on a 6-colour FACSCanto flow cytometer (BD Biosciences). Analysis was performed using FCS Express software (De Novo Software, Los Angeles, CA). Our gating strategy for leukocyte and lymphocyte subsets in CSF is presented in Figure 1. Absolute numbers were calculated as described previously [63]. First, the number of events for each of the lymphocyte and leukocyte subsets and the number of acquired counting bead events were defined. Next, the absolute number of each cell subset (x10^6 cells/l) was calculated by using the following formula: (events in cell subset gate / events in counting bead gate) x (counting beads added per tube / volume of CSF sample)[63]. This method was validated by comparing flow cytometric absolute cell numbers with microscopic cell numbers in 45 normocellular CSF samples of patients with haematological or solid malignancies (Table 1). Percentages were calculated out of total leukocytes for the leukocyte and dendritic cell subsets, out of total lymphocytes for the lymphocyte subsets or out of total T cells for the T cell subsets. When less than 25 leukocytes per sample were counted[63], individuals were excluded from the study.

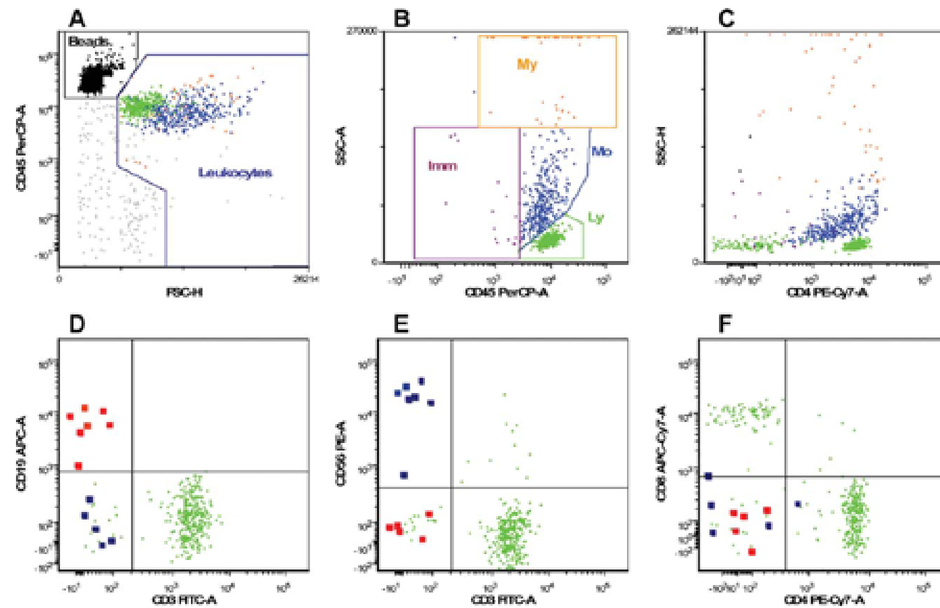


Figure 1 | Gating strategy for leukocyte and lymphocyte subsets in CSF

Example of a 6-color flow cytometric analysis of a normal CSF sample. Each dot represents a single cell. The units on the x- and y-axes are relative fluorescense intensity. For absolute counting, microspheres are used and defined as FSC^{lo}, FL^{+++} [black dots in panel **A**]. Debris, erythroytes and non-leukocyte events (gray) were excluded by defining leukocytes $CD45^{bright}$, $FSC^{im\text{-}hi}$ [panel **A**]. The leukocyte subsets (My = myeloid lineage; Imm = immature lineage; Mo = monocytes; Ly = lymphocytes) were defined with CD45 expression and side scatter [panel **B** and Table 3] and shows two major subsets: lymphocytes ($CD45^{+}$, SSC^{lo}, FSC^{im}; green dots) and monocytes ($CD45^{+}$, SSC^{im}, FSC^{hi}, $CD4^{dim}$; blue dots). In order to optimize resolution, side scatter area (SSC-A) is used. To visualize all granulocytes separately, a plot with side scatter height (SSC-H) is included [panel **C**]. Next, within the lymphocyte gate, T cells ($CD3^{+}$) and sporadic B cells ($CD19^{+}$; highlighted red dots [panel **D**]), sporadic NK ($CD3^{-}$,$CD56^{+}$; highlighted blue dots) and NKT cells ($CD3^{+}$,$CD56^{+}$ [panel **E**]), $CD4^{+}$ and $CD8^{+}$ T cells [panel **F**] were identified.

Table 1 | Comparison of microscopic and flow cytometric CSF absolute cell counts ($x10^{6}$/l) in 45 patients with haematological or solid malignancies

Subset	Microscopy Mean (SEM)	Flow cytometry Mean (SEM)	Pearson correlation (P^{a})
Leukocytes	1.34 (0.42)	1.35 (0.21)	0.78 (0.035)
MNC	1.01 (0.48)	1.09 (0.21)	0.75 (0.043)
PMN	0.34 (0.15)	0.18 (0.02)	0.37 (0.238)

[a]Correlation is significant at the 0.05 level (1-tailed).
SEM = Standard Error of Mean; MNC = mononuclear cells (lymphocytes and monocytes);
PMN = polymorphonuclear cells (granulocytes).

Blood

From all included individuals, approximately 14 ml blood was drawn in EDTA tubes. For the enumeration of absolute numbers of leukocyte and lymphocyte subsets, a single platform, whole blood, stain, lyse, no-wash method based on counting beads was used. Details of this method have been described elsewhere[297]. Briefly, 100 µl of EDTA anti-coagulated whole blood was stained with mixtures of the above-mentioned mAb. After incubation for 15' at RT, 2 ml NH_4Cl lysing buffer was added and 100 µl of Flowcount counting beads (Beckman Coulter, Miami, FL). After another 15' of incubation at RT, samples were acquired on a FACSCalibur flow cytometer (BD Biosciences). Analysis of list mode data was performed using CellQuestPro software (BD Biosciences). For quantification of T and dendritic cell subsets we used a lyse, stain and wash technique which required an additional washing step after red cell lysis in order to reduce background fluorescence due to unbound mAb. These samples were acquired on a 6-colour FACSCanto flow cytometer and analyzed using FCS Express software.

Statistical analysis

Results were analyzed with SPSS version 15.0 (SPSS Inc., Chicago, IL) and presented graphically in box-and-whisker plots. The boxes represent median and interquartile ranges; the whiskers extend to the adjacent values, i.e., 1.5x the interquartile range rolled back to where there is data. Observed values more extreme than the adjacent values were considered outliers and have been plotted individually. Because the absolute numbers and percentages of subsets in CSF were in general not normally distributed, non-parametric tests were used. Percentages of subsets in CSF and blood were compared with the Wilcoxon test. Earlier studies have shown a significant impact of age and gender on the numbers and phenotypes of lymphocyte subsets in blood[298-300]. Therefore, influences of these factors on the subsets in blood as well as CSF were analyzed with the Spearman correlation coefficient (age) or the Wilcoxon test (gender). Differences between groups with a P-value <0.05 were considered significant.

RESULTS

Study persons

We studied 84 paired CSF and blood samples from 84 individuals without neurological disease. Because all CSF samples contained a statistically sufficient number of leukocytes for the flowcytometric analysis (i.e., more than 25), no specimens were excluded from analysis. Clinical characteristics of the individuals are presented in Table 2. All individuals had normal CSF glucose and protein levels and a normal WBC count. No blood contamination was observed in any of the samples. After the first staining, enough CSF was left to perform additional staining for T cell subsets in 18 paired samples and dendritic cell subsets in 35 paired samples. There were no significant differences in the numbers of leukocyte and lymphocyte subsets between these 18 respectively 35 samples and the other samples. Table 3 outlines the different WBC subsets we studied, their immunological definition, function and absolute number in CSF.

Table 2 | Characteristics of 84 included individuals

	Individuals without neurological disease
N	84
Age: median (range)	53 (17 - 82)
Gender	
Male	39
Female	45
Cerebrospinal fluid: median (range)	
Volume (ml)[a]	2.86 (0.80 – 4.65)
Glucose (mmol/l)	3.3 (0.3 - 4.9)
Protein (g/l)	0.36 (0.19 - 0.71)
WBC (x10^6/l)	1.12 (0.40 – 3.17)
RBC (x10^6/l)	0 (0 - 0)
Reason for surgery	
Orthopaedic[b]	46
Gynaecological[c]	15
Urological[d]	9
Other[e]	14

[a]Volume of CSF used for determination of absolute numbers of leukocyte and lymphocyte subsets.
[b]Arthroscopy of hip or knee or hallux valgus correction.
[c]Hysteroscopy, endocervical currettage, abortion or labiaplasty.
[d]Orchidectomy, lithotripsy or spermatocele excision.
[e]Inguinal or abdominal wall hernia repair, dermatological or vascular surgery.
WBC = white blood cell; RBC = red blood cell.

Table 3 | Overview of WBC subsets, their immunological definition, function and absolute numbers in normal CSF

Subset	Immunological definition	Function	Absolute number[a]
Leukocytes	CD45$^+$	White blood cells	1.12 (0.40 – 3.17)
Granulocytes	CD45$^+$, SSC^{hi}	Involved in innate immunity	0.08 (0.02 – 0.43)
Monocytes	CD45$^+$, SSC^{im}, FSC^{hi}, CD4^{dim}	Precursors of macrophages	0.23 (0.08 – 1.11)
Lymphocytes	CD45$^+$, SSC^{lo}, FSC^{im}		0.66 (0.16 – 1.88)
T cells	CD3$^+$	Involved in adaptive immunity	0.62 (0.15 – 1.83)
CD4$^+$ T cells	CD4$^+$	Helper T cells	0.44 (0.08 – 1.43)
Naïve	CD45RA$^+$, 27/28$^+$	Have not yet encountered an antigen	0.02 (0.00 – 0.38)
Central memory	CD45RA$^-$, 27/28$^+$	Have seen or been primed with an antigen	0.43 (0.05 – 1.60)
Effector memory	CD45RA$^-$, 27/28$^-$	Present in settings of active antigenic stimulation	0.00 (0.00 – 0.02)
Late memory	CD45RA$^+$, 27/28$^-$	Control of viral reactivation and cancers	0.00 (0.00 – 0.02)
Regulatory	CD25^{++}, 127$^+$	Suppression of T cell responses	0.02 (0.00 – 0.12)
CD8$^+$ T cells	CD8$^+$	Cytotoxic T cells	0.13 (0.04 – 0.40)
Naïve	CD45RA$^+$, 27/28$^+$	Have not yet encountered an antigen	0.04 (0.01 – 0.22)
Central memory	CD45RA$^-$, 27/28$^+$	Have seen or been primed with an antigen	0.10 (0.05 – 0.46)
Effector memory	CD45RA$^-$, 27/28$^-$	Present in settings of active antigenic stimulation	0.02 (0.00 – 0.06)
Late memory	CD45RA$^+$, 27/28$^-$	Control of viral reactivation and cancers	0.02 (0.00 – 0.05)
NKT cells	CD56$^+$	Secretion of cytokines and regulatory function	0.01 (0.00 – 0.06)
B cells	CD3$^-$, 19$^+$	Differentiate into plasma cells and secrete antibodies	0.00 (0.00 – 0.03)
NK cells	CD3$^-$, 56$^+$	Release lytic granules that kill infected or tumor cells	0.01 (0.00 – 0.05)
Dendritic cells	CD45$^+$, LIN-1$^-$, HLA-DR$^+$	Specialized antigen-presenting cells	0.04 (0.01 – 0.18)
Myeloid	CD11c$^+$, 123$^-$	Derived from myeloid lineage	0.02 (0.00 – 0.13)
Plasmacytoid	CD11c$^-$, 123$^+$	Derived from lymphoid lineage	0.01 (0.00 – 0.03)

[a]Medians (5th-95th percentiles) of absolute numbers x10^6/l are given.
SSC = side scatter; FSC = forward scatter; LIN-1 = Anti-Lineage 1 cocktail composed of CD3, CD14, CD16, CD19, CD20 and CD56 monoclonal antibodies.

Leukocyte subsets

In CSF, the absolute number of leukocytes was approximately 5,000x lower than in blood (CSF: median $1.12x10^6$/l, blood: median $6048x10^6$/l). Lymphocyte (CSF: median $0.66x10^6$/l, blood: median $1716x10^6$/l) and monocyte counts (CSF: median $0.23x10^6$/l, blood: median $414x10^6$/l) were approximately 2,500x and 2,000x lower in CSF than in blood, while granulocyte counts were even ~45,000x lower (CSF: median $0.08x10^6$/l, blood: median $3568x10^6$/l) (Figure 2, panel A and B). This was also reflected in the distribution of the leukocyte subsets which showed major differences between both compartments: CSF contained relatively more lymphocytes and monocytes, but less granulocytes than blood (Figure 2, panel C and D).

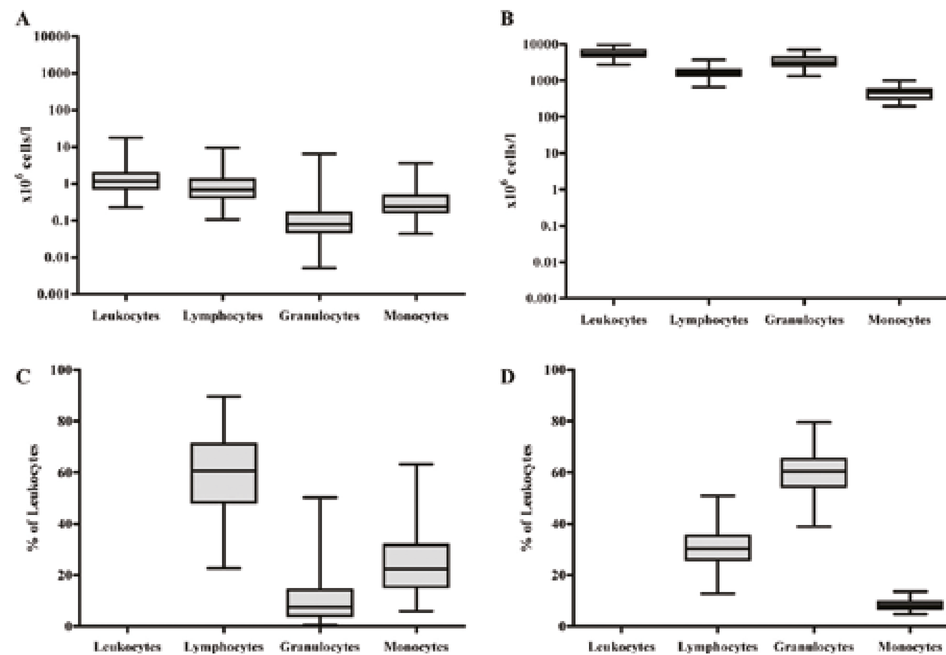


Figure 2 | Absolute numbers and percentages of leukocytes and leukocyte subsets in CSF and blood

Absolute numbers (i.e., $x10^6$ cells/l) of leukocytes, lymphocytes, granulocytes and monocytes in CSF (**A**) and blood (**B**). Percentages (expressed as percentage of leukocytes) of lymphocytes, granulocytes and monocytes in CSF (**C**) and blood (**D**). In paired analyses, all leukocyte subsets showed significant differences between the percentage in CSF and in blood ($P<1.0x10^{-15}$).

Lymphocyte subsets

As in blood, T cells were the most abundant lymphocyte subset in CSF with a predominance of $CD4^+$ over $CD8^+$ T cells, whilst absolute counts of NK, NKT and B cells were 10 to 100-fold lower than those of T cells (Figure 3, panel A and B). When looking at the percentages of the various lymphocyte subsets in CSF and blood (data not shown), the following differences were apparent: (i) the ratio between $CD4^+$ and $CD8^+$ T cells was shifted significantly in favour of $CD4^+$ T cells in CSF compared to blood (median: 3.0 [5th and 95th percentile: 1.1 and 6.5] versus 2.1 [0.9 and 6.0], respectively; $P<1.0x10^{-6}$); (ii) NK and NKT cells were <5% in most CSF samples; and (iii) B cells were by far the smallest subset in CSF, whilst NKT cells were the smallest subset in blood.

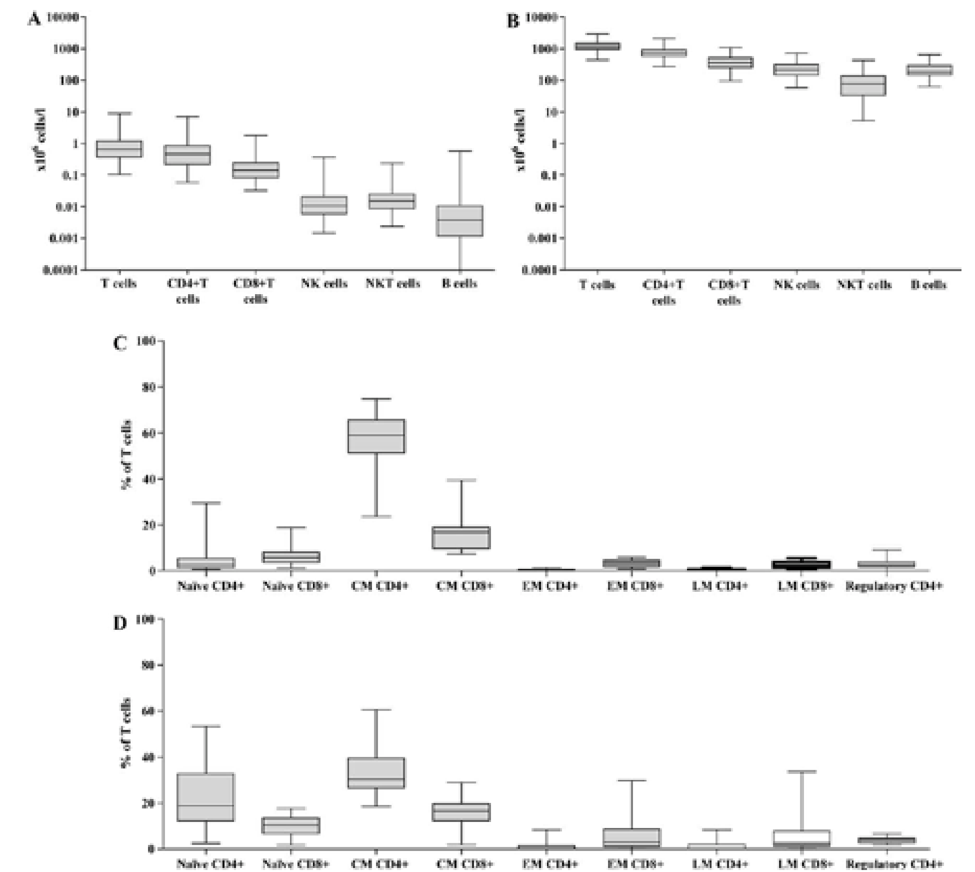


Figure 3 | Absolute numbers of lymphocyte subsets and percentages of T cell subsets in CSF and blood

Absolute numbers (i.e., $x10^6$ cells/l) of T cells, $CD4^+$ T cells, $CD8^+$ T cells, NK cells, NKT cells and B cells in CSF (**A**) and blood (**B**). Percentages (expressed as percentage of T cells) of naïve, central memory, effector memory, late memory and regulatory $CD4^+$ and $CD8^+$ T cells in CSF (**C**) and blood (**D**). CM = central memory; EM = effector memory; LM = late memory. For absolute B-cell counts, a minimum of 5 events was required to calculate an absolute count. In 13 of the 84 cases, this threshold was not reached. An indication of these missing data was given in the figure by placing the lower whisker of "B cells" just above the 100 cells/l level.

T cell differentiation

We then studied markers reflecting lymphocyte differentiation on T cells in paired CSF and blood samples of a subgroup of 18/84 individuals and determined the percentages of naïve, central memory, effector memory, late memory and regulatory phenotypes (Figure 3, panel C and D). The T cell subsets we describe here, are by and large similar to the subsets Kim et al.[301] described recently: the naïve phenotype corresponds with Kim's 'N compartment', the central memory phenotype with the 'M1 compartment', the effector memory phenotype with the 'M2 compartment' and the late memory phenotype with the 'M3 compartment'. We observed that the vast majority of T cells in CSF consisted of central memory $CD4^+$ T cells (~60% of total T cells and ~90% of $CD4^+$ T cells). Also, within the $CD8^+$ T cell subset the largest proportion was composed of cells with the central memory phenotype (~17% of total T cells and ~60% of $CD8^+$ T cells). This corresponds with earlier studies which showed that the majority of T cells in CSF had a memory phenotype[234, 235, 302, 303], while the proportion of naïve T cells was low [235, 303]. Compared to blood, we observed significantly higher percentages of central memory $CD4^+$ T cells ($P<5.0x10^{-4}$), while the percentages of the naïve phenotypes, both $CD4^+$ ($P<5.0x10^{-4}$) and $CD8^+$ ($P<5.0x10^{-2}$), and regulatory $CD4^+$ T cells ($P<5.0x10^{-2}$), as estimated by the coexpression of CD25 (bright fluorescence) and CD127 on $CD4^+$ T cells, were significantly lower in CSF.

Dendritic cells

The amount of CSF obtained allowed the additional characterization of dendritic cells in 35/84 samples. We detected a median of $0.04x10^6$ dendritic cells/l (5th and 95th percentiles: 0.01 and $0.18x10^6$ cells/l) in CSF, and a median of $31x10^6$ dendritic cells/l (14 and $52x10^6$ cells/l) in blood. Relatively, dendritic cells were 2.2% of leukocytes in CSF versus only 0.4% in blood ($P<5.0x10^{-7}$). We found a clear predominance of the myeloid subset over the plasmacytoid subset in CSF (medians: 1.7% and 0.4% of leukocytes, respectively), whilst both subsets had similar percentages in blood (median: 0.2%; Figure 4).

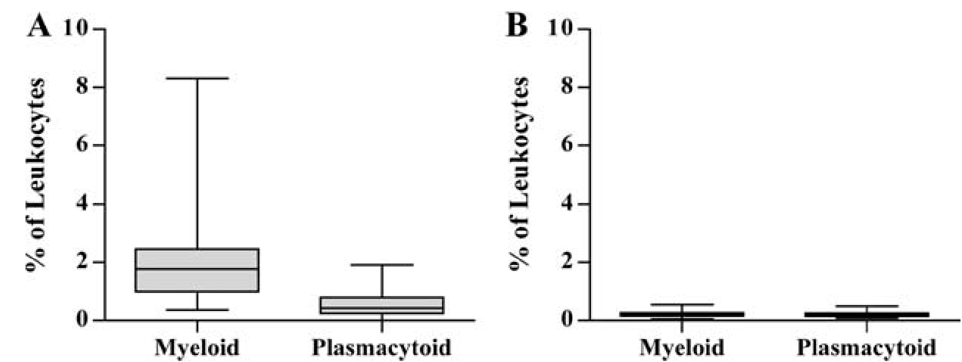


Figure 4 | Percentages of dendritic cell subsets in CSF and blood

Percentages (expressed as percentage of leukocytes) of myeloid and plasmacytoid dendritic cells in CSF (**A**) and blood (**B**).

Impact of age and gender

Age and gender had no significant impact on numbers and proportions of leukocytes and their major subsets in CSF and blood (data not shown). With regard to the lymphocyte subsets, absolute counts of $CD4^+$ T cells in CSF, but not in blood, were negatively correlated with age (r = -0.23, $P<0.05$), while absolute counts of $CD8^+$ T cells in blood, but not in CSF, were negatively correlated with age (r = -0.27, $P<0.05$). Additionally, absolute counts of B cells were negatively correlated with age both in CSF and blood (r = -0.23 and -0.25, respectively; $P<0.05$). However, multivariate analysis showed no confounding influence of age and gender on the CSF and blood results of this study (data not shown), which indicates that correction of these WBC reference values for age and gender is not necessary.

DISCUSSION

By using 6-colour flow cytometry, we studied absolute numbers and percentages of WBC subsets in paired CSF and blood samples of 84 individuals undergoing spinal anaesthesia before surgery. None of the patients had a history of neurological disease, cancer or treatment with immunosuppressive or cytostatic drugs, factors which may have influenced the numbers of WBC subsets. Furthermore, all CSF samples had normal levels of glucose and protein and normal WBC counts, without any blood contamination. Therefore, we propose that these CSF samples are useful as reference source for WBC subsets in that compartment.

Second, we confirmed the predominant presence of $CD4^+$ T cells with relatively low numbers of granulocytes, B, NK and NKT cells shown in earlier CSF studies[234, 235]. The predominance in CSF of cells of the adaptive immune system over those of the innate immune system may be related to the fact that, under normal circumstances, the CNS is much less exposed to pathogens (and auto-antigens) than peripheral organs.
Third, within the $CD4^+$ T cell subset in CSF, most cells have the central memory phenotype (~90%). This proportion is significantly higher than in blood (~58%), while the naïve and regulatory phenotypes were less abundant in CSF than in blood. This indicates a selective recruitment of central memory $CD4^+$ T cells into normal CSF[234, 235, 303-305]. Hypothetically, central memory T cells search for their specific antigen after migration into the CNS. If they encounter it, they accumulate, initiate inflammation and cause infectious or autoimmune reactions[303, 304, 306]; if not they will leave the CNS. This is considered immunological surveillance of the CNS as is also observed in other organs[307]. An alternative explanation for the high proportion of memory T cells in CSF, is the absence of migration of naïve T cells into normal CSF. Several studies have shown that activated lymphocytes more readily migrate into the CNS than resting lymphocytes[306-309]. It is hypothesized that in case of CNS inflammation, naïve T cells first have to be activated by antigen in perivascular spaces or lymph nodes draining the CNS, before they can enter the CSF[307].
Fourth, the number of B cells is hardly above detection limit (median: 0.004×10^6 cells/l) in normal CSF. This result corresponds with our earlier study in which we examined CSF lymphocyte subsets in healthy controls and PNS patients. Whilst CSF B-cell counts in the healthy controls were very low (median: 0.005×10^6 cells/l), PNS patients showed significantly elevated counts (median: 0.133×10^6 cells/l)[231]. This suggests that B cells are recruited to CSF in certain pathological conditions, rather than that they participate in immunosurveillance of the CNS, like the (central memory) T cells do.
Fifth, dendritic cells are relatively more abundant in CSF whilst being 'rare events' in blood. The absolute numbers we report here are comparable with the numbers Pashenkov et al.[72] observed in CSF of patients with non-inflammatory neurological diseases. In addition to the relatively high frequency of dendritic cells in CSF, we found a predominance of myeloid over plasmacytoid dendritic cells which may be explained by the origin of dendritic cells in CSF. Most myeloid dendritic cells arise in the CNS from blood monocytes[72], while plasmacytoid dendritic cells are all blood-borne[310]. Dendritic cells are the most potent antigen presenting cells[311]. In CSF they take up antigens that invade the CNS and present them to T cells in secondary lymphoid organs, such as deep cervical lymph nodes[310]. In this way, dendritic cells have an important role in the adaptive immune response in CSF. This role is also supported by the finding that in CSF of patients with neuroinflammatory diseases, such as multiple sclerosis, optic neuritis, neuroborreliosis and aseptic meningoencephalitis, numbers of both myeloid and plasmacytoid dendritic cells are significantly elevated[72].

In addition to serving as benchmark for WBC subsets in CSF, this study confirms the predominant presence of central memory $CD4^+$ T cells, which may have selectively been recruited to the CNS to function as immune surveillants. The presence of these cells in CSF would facilitate quick responses to antigens and induction of an adaptive immune response, aided by dendritic cells.

Acknowledgments

The authors are grateful to the anaesthesiologists of the Sint Franciscus Gasthuis for inclusion of individuals and Eric Brouwer for technical assistance. This work was supported by the Gratama Foundation, Teteringen, The Netherlands.

Chapter 6

B and T cell imbalances in CSF of patients with Hu-antibody associated PNS

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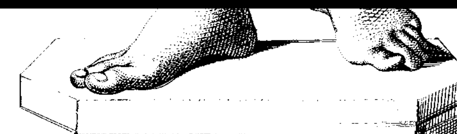
Journal of Neuroimmunology 2008;195:164-170



ABSTRACT

In paraneoplastic neurological syndromes associated with Hu-antibodies (Hu-PNS) an important role for cellular immunity is hypothesized. We characterized the cerebrospinal fluid (CSF) pleocytosis in Hu-PNS patients by assessing the major lymphocyte subsets by flow cytometry. The B cell subset in the CSF of Hu-PNS patients showed a significant absolute (~20x) and relative (~3x) expansion, while the numbers of $CD4^+$ T cells, $CD8^+$ T cells and NK cells only showed an absolute expansion (~4-7x) compared to the controls. On the other hand, the NKT cell subset showed a significant relative reduction in CSF and in blood of Hu-PNS patients. The relative B cell expansion is consistent with the intrathecal synthesis of Hu-antibodies, while the increased number of T and NK cells supports an additional role for cellular immunity in the pathogenesis of Hu-PNS. In addition, the autoimmune hypothesis of Hu-PNS is supported by the relative NKT cell deficiency.

INTRODUCTION

Paraneoplastic neurological syndromes (PNS) are a remote effect of cancer which causes significant neurological morbidity. High-titered Hu-antibodies are often found in the blood and cerebrospinal fluid (CSF) of PNS patients. Most patients with Hu-antibody associated PNS (Hu-PNS) have an underlying small cell lung carcinoma (SCLC).

The precise mechanisms responsible for neuronal damage in Hu-PNS remain poorly understood. Earlier publications have shown that the tumor produces the onconeuronal Hu antigens, normally solely expressed in the nervous system. Hypothetically, these antigens provoke an autoimmune response not only directed against the tumor but also against nervous tissue[1]. Although the Hu-antibody is a useful diagnostic marker[4], a direct pathogenic effect could not be established[33]. Pathological examination of affected areas of the nervous system in Hu-PNS shows loss of neurons with localized inflammatory cell infiltrates, containing B cells, $CD4^+$ T-helper cells and cytotoxic $CD8^+$ T cells[34, 39, 40, 44]. T cell receptor analysis supported a direct effector role of cytotoxic $CD8^+$ T cells, the same clones being likely operative in neuronal damage and immune-mediated tumor growth control[39, 44].

The CSF of Hu-PNS patients generally shows a pleocytosis, intrathecal synthesis of IgG and Hu-specific oligoclonal bands[2, 47]. In the blood increased numbers of activated $CD4^+$ and $CD4^+$ memory T cells have been reported[41] and in some publications the presence of Hu-specific T cells is suggested[38, 41]. Overall, these findings suggest the occurrence of both B and T cell mediated immune responses in these compartments[34, 40, 47].

As a detailed analysis of the CSF pleocytosis in Hu-PNS patients might provide further insight into the immune pathogenesis of this disease, we analysed lymphocyte subsets in paired CSF and blood samples of 12 untreated Hu-PNS patients and compared these results with those of two control groups. To differentiate between PNS and cancer-associated lymphocyte subset alterations, 15 patients with cancer (mostly with breast or prostate cancer, but without PNS) were included. The second control group consisted of 27 patients without cancer who donated CSF when undergoing spinal anaesthesia. We studied whether or not any major disturbance in the frequencies and proportions of the major lymphocyte subsets (i.e., B cells, NK cells, T cells, $CD4^+$ T cells, $CD8^+$ T cells and NKT cells) was related to PNS.

PATIENTS AND METHODS

Patients and controls

Twelve patients with (i) high-titered Hu-antibodies; (ii) a definite clinical diagnosis of PNS[38]; (iii) progressive neurological disease, defined by the increase of at least one point on the modified Rankin score in the 4 weeks prior to study entry; and (iv) no chemotherapy or immunosuppressive therapy prior to study entry were included. Hu-IgG antibody titers were determined on rat cerebellar sections by indirect immunofluorescence (IIF) and immunoreactivity was confirmed by western blotting using purified recombinant HuD in all cases[18]. Only patients with high-titered serum Hu-antibodies were eligible for the study (serum IIF titer ≥400). Modified Rankin scores were determined as described previously[6].

In addition, we included two groups of controls. The first group consisted of 15 patients with cancer (hereafter referred to as 'cancer controls'), who underwent lumbar puncture either because of neurological signs or symptoms or because of spinal anaesthesia during surgery with (i) no indication of PNS; (ii) no leptomeningeal metastases; and (iii) no chemotherapeutic treatment during testing. The second group consisted of 27 patients receiving spinal anaesthesia during surgery (hereafter referred to as 'non-cancer controls'). These patients had no clinical evidence of cancer and no neurological symptoms. Fresh CSF and blood samples were prospectively collected.

Written informed consent was obtained from all tested individuals and the local ethical review committee approved the study. Patient and CSF characteristics of Hu-PNS patients are listed in Table 1 and characteristics of both control groups are listed in Table 2.

Table 1 | Hu-PNS patients: patient and CSF characteristics at the time of study entry

Patient no.	Age/ gender	Hu-Ab titer serum	CSF									PNS	mRS[c]	Symptoms-diagnosis (months)	Symptoms-study (months)	Tumor-study[d] (months)	Tumor
			Hu-Ab titer	Protein (g/l)	IgG index[a]	Leuko-cytes[b]	CD4+ T cells[b]	CD8+ T cells[b]	B cells[b]	NK cells[b]	NKT cells[b]						
1	53/F	102,400	2,048	0.44	0.84	2.45	0.87	0.34	0.042	0.085	NT	PEM	3	8	8.5	17	SCLC
2	72/F	102,400	2,048	0.62	0.8	15.3	9.8	1.89	0.254	0.508	NT	PSN	3	1.3	1.5	0	SCLC
3	76/M	3,200	128	0.59	0.72	1.15	0.42	0.24	0.007	0.014	NT	PSN	4	2	4	3	SCLC
4	67/M	25,600	4,096	0.76	1.5	10.15	5.13	2.88	0.712	0.089	NT	PSN	3	2.5	3	-1	NSCLC
5	57/F	6,400	NT	2.15	1.1	1.85	1.18	0.13	0.105	0.045	NT	PSN	3	6	6.5	0.5	SCLC
6	54/F	51,200	512	0.66	0.59	6.85	2.06	1.36	0.041	0.165	<0.001	PSN	2	2.5	3	1	SCLC
7	65/F	204,800	2,048	0.38	2.5	2.45	0.86	0.51	0.179	0.061	0.003	PLE	3	1	1.5	4	Lymph[e]
8	70/F	51,200	512	0.65	1.3	3.6	1.58	0.9	0.162	0.047	0.001	PEM	3	6	6.5	-1	SCLC
9	67/M	102,400	256	0.33	0.73	1.15	0.5	0.31	0.034	0.019	0.005	PCD	3	2.5	3.5	0	SCLC
10	61/M	102,400	2,048	0.51	0.65	1.15	0.56	0.27	0.022	0.018	0.004	PEM	3	3.5	4.5	NA	NF
11	68/F	51,200	256	0.76	0.76	11.15	4.39	3.68	0.912	0.168	0.021	PSN	3	2.5	3	-2	Lung[f]
12	61/M	51,200	8,192	1.38	0.93	16.15	10.4	2.81	0.483	0.387	0.007	PSN	3	2.5	3	0	SCLC

[a]IgG index = (IgG CSF/IgG serum)/(albumin CSF/albumin serum); an index >0.6 indicates intrathecal IgG synthesis.
[b]Cells per microliter.
[c]modified Rankin Score at the time of study entry[6].
[d]Time between tumor detection and study entry; negative values indicate tumor detection after study entry.
[e]Mediastinal lymphadenopathy without a definite pathological diagnosis.
[f]Tumor mass visible on CT scan, no pathological diagnosis.
Hu-Ab = Hu-antibody; PNS = paraneoplastic neurological syndrome; PEM = paraneoplastic encephalomyelitis; PSN = paraneoplastic sensory neuronopathy; PLE = paraneoplastic limbic encephalitis; PCD = paraneoplastic cerebellar degeneration; mRS = modified Ranking Score; SCLC = small cell lung carcinoma; NSCLC = non small cell lung carcinoma; NT = not tested; NA = not applicable; NF = no tumor found.

Table 2 | Patient and control characteristics at the time of study entry

	Hu-PNS patients	Cancer controls	Non-cancer controls
N	12	15	27
Age: median (range)	63 (53-76)	67 (30-84)	57 (26-76)
Gender			
Male	5	7	18
Female	7	8	9
Cerebrospinal fluid: median (range)			
Protein (g/l)	0.64 (0.33-2.15)	0.41 (0.25-0.69)	0.34 (0.19-0.62)
Leukocytes (cells/µl)	3.0 (1.15-16.15)	1.0 (0.45-5)	1.0 (0.15-4)
Tumor			NA
Lung[a]	10	-	
Breast	-	7	
Prostate	-	4	
Melanoma	-	1	
Chondrosarcoma	-	1	
Renal cell	-	1	
Esophagus	-	1	
Unknown[a]	2	-	
Reason for lumbar puncture			
Neurological signs or symptoms	12	6	0
Spinal anaesthetics	0	9	27

[a]For further specification see Table 1.
NA = not applicable.

Monoclonal antibodies

Fluorochrome-conjugated monoclonal antibodies (mAb) were used for cell surface labelling. CSF samples were studied with a single 6-colour mixture: CD45 conjugated with peridinyl cholorophyllin (PerCP), CD3 conjugated with fluorescein isothiocyanate (FITC), CD56 conjugated with phycoerythrin (PE), CD4 conjugated with PE-Cy7, CD8 conjugated with allophycocyanin (APC) and CD19 conjugated with APC-Cy7. Blood samples were studied with two 4-colour mixtures: CD3-FITC, CD56-PE, CD45-PerCP and CD19-APC, and CD4-FITC, CD8-PE, CD45-PerCP and CD3-APC. All mAb were obtained from BD Biosciences (San Jose, CA, USA) with the exception of CD56-PE (Dako, Glostrup, Denmark).

Flow cytometric analysis of lymphocyte subsets in cerebrospinal fluid

Erythrocytes and leukocytes in CSF were enumerated within one hour after obtaining these samples. For immune phenotyping, cells were concentrated from 2 ml of fresh CSF by centrifugation (8 min, 450*g*) and resuspended in 100 µl phosphate-buffered saline (PBS). Next, samples were incubated for 15 min (15’) at room temperature (RT) in the dark with 10 µl of each of the mAb (see above). Subsequently, the cells were washed, resuspended in PBS containing 1% paraformaldehyde (PFA), and acquired on a 6-colour FACScanto

flow cytometer (BD Biosciences). Analysis was performed using FACSdiva software (BD Biosciences). The absolute number of each subset was determined by multiplying the percentage (expressed as fraction of total lymphocytes or in case of $CD4^+$, $CD8^+$ and NKT cells as fraction of T cells) by the absolute number of lymphocytes.

Flow cytometric analysis of lymphocyte subsets in peripheral blood

For the enumeration of B cells, T cells, NK cells, NKT cells, $CD4^+$ and $CD8^+$ T cells in blood based on 4-colour flow cytometry (FacsCalibur; BD Biosciences), a single platform, whole blood, stain, lyse, no-wash method based on counting beads was used. Details of this method have been described elsewhere [297]. Briefly, 100 µl of EDTA anti-coagulated whole blood was stained with mixtures of appropriately titrated mAb (see above). After incubation for 15' at RT, 2 ml NH4Cl lysing buffer was added and 100 µl of Flowcount counting beads (Beckman Coulter, Miami, FL). After another 15' of incubation at RT, samples were acquired on the flow cytometer. Analysis of list mode data was performed using CellQuestPro software from BD Biosciences. The absolute numbers of the subsets were calculated by multiplying the absolute lymphocyte numbers with the corresponding percentages of the cells expressing the markers of interest.

Statistical analysis

A linear regression analysis was used for making comparisons between the different patient groups. Because the lymphocyte subset data were in general not normally distributed, log-transformation was used for the lymphocyte counts in these models. Differences between groups with *P*-value <0.05 were considered significant. Box-and-whisker plots were used to present the data graphically, showing median and interquartile ranges; the whiskers extended to the adjacent values, i.e., 1.5x the interquartile range rolled back to where there is data. Observed points more extreme than the adjacent values were considered outliers and have been plotted individually.

RESULTS

Cerebrospinal fluid lymphocytes

Table 3 and Figure 1 give an overview of the absolute numbers of the lymphocyte subsets in CSF in the different patient groups. The CSF of the Hu-PNS patients shows a leukocytosis and lymphocytosis with increments in absolute numbers of the major subsets (B cells, NK cells, total T cells [$CD3^+$], $CD4^+$ T cells and $CD8^+$ T cells) in comparison with the non-cancer controls (Table 3). The absolute numbers of these five subsets were similar in both cancer and non-cancer controls. The absolute numbers of NKT cells were similar in all three groups. The lymphocytosis appears to be fully responsible for the pleocytosis, because analysis of the number of monocytes and granulocytes in the CSF showed similar results in the Hu-PNS patients as in the control groups (data not shown).

Among the CSF lymphocyte subsets, the increment of B cells was most striking: the median result was ~20x that of both control groups (i.e., 133 vs. 5-8 cells per microliter). The increments of the other subsets ranged from ~4x for $CD4^+$ T cells to ~7x for $CD8^+$ T cells and NK cells in comparison to both control groups (Table 3).

Table 3 | Absolute numbers of CSF and peripheral blood lymphocyte subsets in Hu-PNS patients and controls

Lymphocyte subset	Hu-PNS patients		Cancer controls		Non-cancer controls	
	Median	5th - 95th percentile	Median	5th - 95th percentile	Median	5th - 95th percentile
CSF						
T cells	1.93^b	0.67 - 13.38	0.4	0.16 - 1.8	0.46	0.2 - 2.02
$CD4^+$ T cells	1.38^b	0.42 - 10.4	0.28	0.12 - 1.39	0.34	0.12 - 1.36
$CD8^+$ T cells	0.705^b	0.13 - 3.68	0.07	0.03 - 0.7	0.13	0.06 - 1
B cells	0.133^b	0.007 - 0.912	0.008	0 - 0.059	0.005	0 - 0.034
NK cells	0.073^b	0.014 - 0.508	0.016	0.001 - 0.128	0.011	0.002 - 0.058
NKT cells	0.004	0 - 0.021	0.006	0 - 0.029	0.005	0 - 0.037
Peripheral blood						
T cells	1.034	0.654 - 1.672	1.208	0.559 - 1.527	1.272	0.4 - 2.1
$CD4^+$ T cells	0.755	0.473 - 1.288	0.793	0.367 - 1.072	0.832	0.309 - 1.448
$CD8^+$ T cells	0.278	0.094 - 0.495	0.321	0.128 - 0.49	0.372	0.086 - 0.741
B cells	0.179	0.088 - 0.43	0.181	0.06 - 0.497	0.178	0.087 - 0.354
NK cells	0.128^a	0.054 - 0.222	0.186	0.064 - 0.865	0.241	0.097 - 0.858
NKT cells	0.034^b	0.005 - 0.137	0.078	0.01 - 0.45	0.103	0.034 - 0.294

Median and ranges of absolute numbers of the different lymphocyte subsets in CSF and peripheral blood are given in Hu-PNS patients, cancer controls and non-cancer controls. Absolute cell counts are given in cells $x10^6/l$ for CSF and in cells $x10^9/l$ for peripheral blood. Groups were compared by linear regression analysis: Hu-PNS patients vs. non-cancer controls and cancer vs. non-cancer controls. Significant differences between groups were marked with ^{a}(P<0.01) and ^{b}(P<0.001).

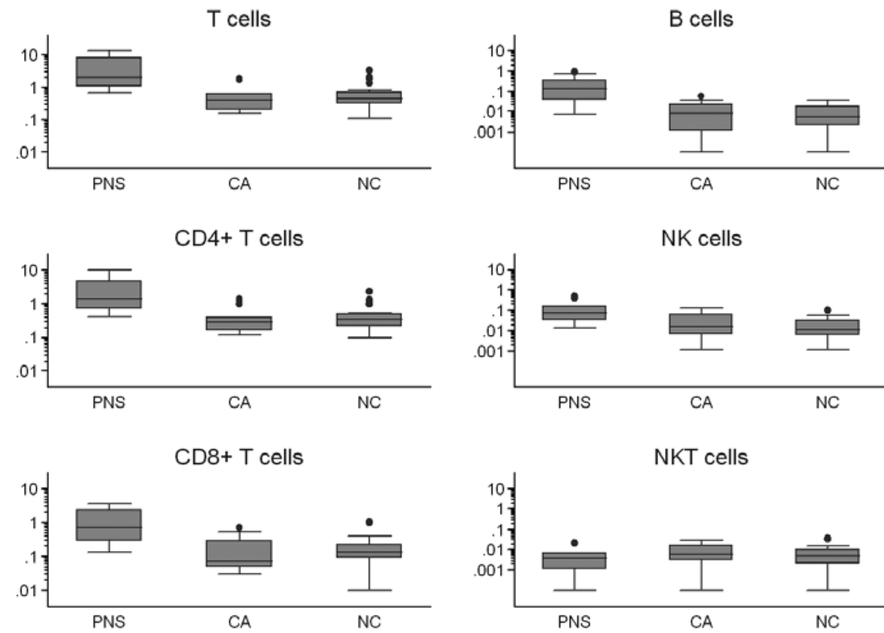


Figure 1 | CSF absolute cell numbers

CSF absolute numbers (i.e., cells per microliter) of total T cells (CD3⁺), CD4⁺ T cells, CD8⁺ T cells, B cells, NK cells and NKT cells are shown for Hu-PNS patients (abbreviated PNS), cancer controls (abbreviated CA) and non-cancer controls (abbreviated NC). See 'Patients and methods' for explanation of box-and-whisker plots. Logarithmic scales were used for the *y*-axes to compress the figures.

To highlight expansions or reductions of specific subsets within the lymphocytosis, we investigated their proportions expressed as fractions of total lymphocyte counts or, in case of $CD4^+$, $CD8^+$ T cells and NKT cells, as fractions of $CD3^+$ T cell counts (Figure 2). This analysis also showed a significant and selective increase of the B cells in the Hu-PNS patients (median: 3.6%; range: 1%-10.9%) compared to the cancer controls (1.3%; <0.1%-7.2%) and the non-cancer controls (1.2%; <0.1%-3.8%) (P<0.001). Thus, the proportions of B cells in the Hu-PNS patients were ~3x higher than those in the control groups. The NKT cells showed a significant and selective reduction in the Hu-PNS patients (0.2%; <0.1%-0.6%) compared to the cancer controls (1.3%; <0.1%-6.8%) and non-cancer controls (1.3%; <0.1%-5.6%) (P<0.001). Thus, the proportions of NKT cells were ~6x reduced in Hu-PNS patients as compared to both control groups.

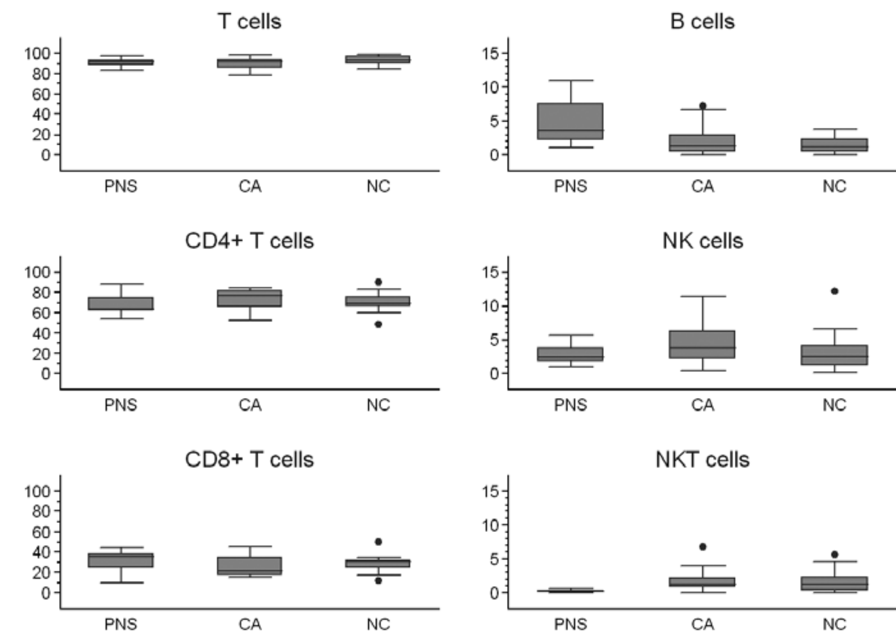


Figure 2 | Percentages of lymphocyte subsets in CSF

CSF percentages of lymphocyte subsets expressed as fractions of total lymphocyte counts (T cells, B cells and NK cells) or as fractions of CD3⁺ T cells (CD4⁺, CD8⁺ and NKT cells). See further legend to Figure 1.

Peripheral blood lymphocytes

The absolute numbers of the various lymphocyte subsets in the blood are shown in Table 3 and Figure 3. Hu-PNS patients showed similar counts of the absolute numbers of the major subsets as compared to non-cancer controls, except for a NK and NKT lymphopenia (P<0.005 and P<0.001 respectively). The proportions of NK and NKT cells (expressed as fractions of total lymphocytes and $CD3^+$ T cells respectively) were also lower in Hu-PNS patients than in non-cancer controls (data not shown). In the cancer controls the NK and NKT numbers were also lower than in the non-cancer controls (Figure 3), but these differences did not reach significance.

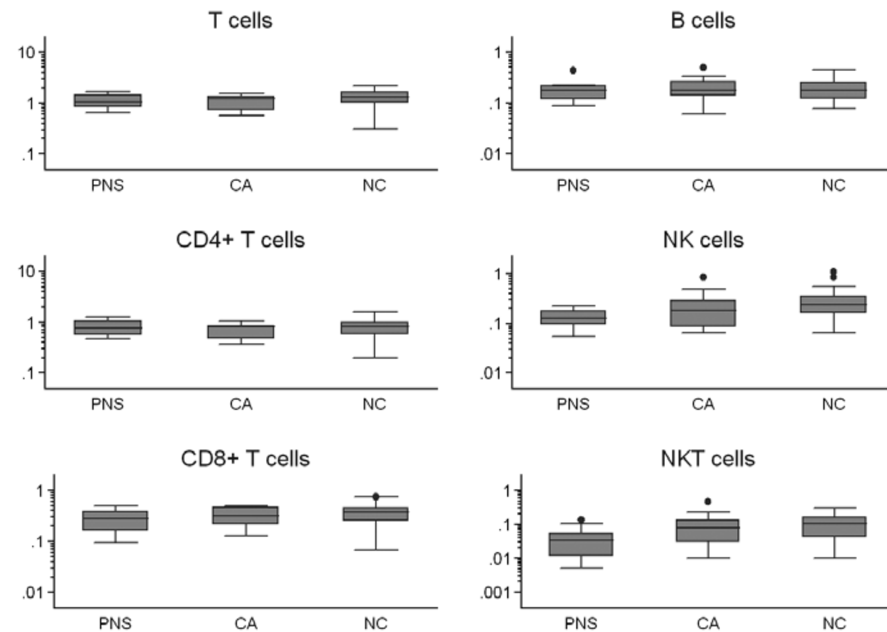


Figure 3 | Peripheral blood absolute cell numbers

Peripheral blood absolute numbers (i.e., cells x 10^3 per microliter) of total T cells ($CD3^+$), $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells and NKT cells in Hu-PNS patients, cancer controls and non-cancer controls. See further legend to Figure 1. Logarithmic scales were used for the *y*-axes to compress the figures.

DISCUSSION

The results of lymphocyte subset counts in CSF and peripheral blood of the cancer controls were, by and large, similar to the non-cancer controls. Therefore, we considered the data of both control groups as a reasonable approximation of reference values for the counts of CSF lymphocyte subsets. Our main question was to compare these values with the CSF lymphocyte subset counts of the Hu-PNS patients. This analysis showed us several striking imbalances of the CSF lymphocyte subsets of the Hu-PNS patients, which are partially in line with the pleocytosis and lymphocytosis in Hu-PNS reported in earlier publications[2, 47].

The most remarkable finding in the CSF of the Hu-PNS patients was the ~20-fold higher numbers of B cells compared to both control groups. When B cell results were expressed as fraction of the total number of CSF lymphocytes, Hu-PNS patients had still ~3x as much B cells as the controls, while the T and NK cell subset only showed an absolute (~4-7x) and no relative increase. This selective B cell expansion in the CSF may indicate a role for this subset in the pathogenesis of Hu-PNS. Although intrathecal synthesis of Hu-IgG produced by intrathecal B cell clones has been reported earlier[47], this striking expansion of B cells has never been observed. Under normal circumstances, CSF is nearly devoid of B cells[235]. B cell trafficking and proliferation within the CSF compartment have been found in neuroinflammatory disorders such as multiple sclerosis[307, 312]. In children with paraneoplastic opsoclonus-myoclonus syndrome a 5.1x higher B cell proportion is described[253]. Expansion of B cells in CSF indicates significant B cell recruitment to the CNS and the potential for autoantibody production[253]. The higher numbers of CSF B cells in our Hu-PNS patients may explain the intrathecal synthesis of Hu-IgG in PNS. Another role of autoreactive B cells in Hu-PNS may be enhancing antigen presentation to T cells as demonstrated for other autoimmune diseases[254].

Second, the CSF of Hu-PNS patients also contained higher numbers of total T cells ($CD3^+$), their $CD4^+$ and $CD8^+$ subsets, and NK cells. Here, the absolute numbers were ~4x to ~7x as high as in the controls. In paraneoplastic cerebellar degeneration (PCD) associated with Yo-antibodies expanded populations of tumor-specific cytotoxic T lymphocytes were found in blood[313] as well as increased proportions of activated T cells in CSF[52], suggesting a role of these cells in the neuronal degeneration. On the other hand, a non-specific inflammatory phenomenon may explain the lymphocytosis in the CSF of Hu-PNS patients as it affects most lymphocyte subsets.

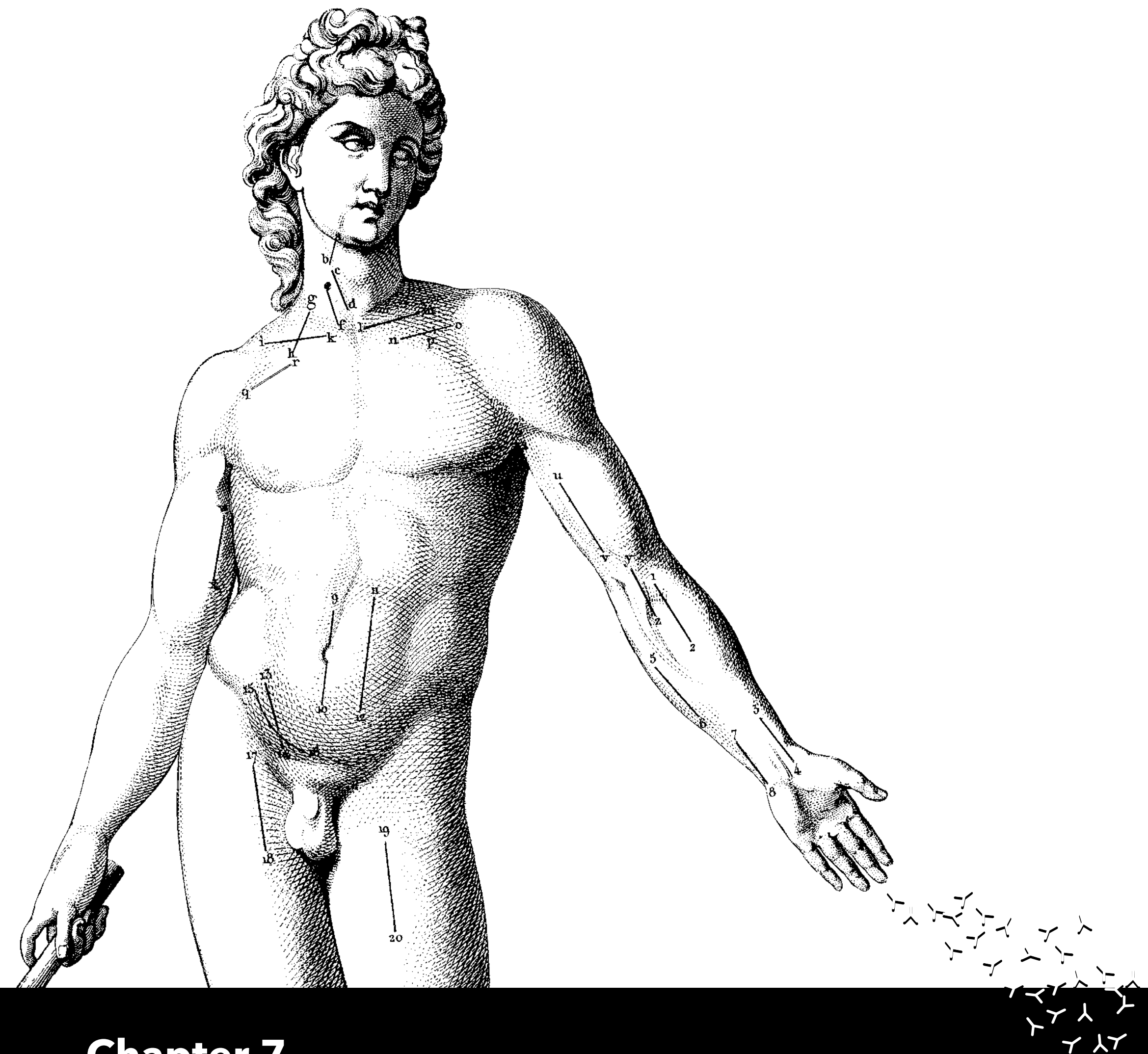
A third interesting finding in Hu-PNS patients is the relative deficiency of NKT cells in CSF as well as in blood. Although the absolute number of NKT cells in the CSF of Hu-PNS patients was similar to those of both control groups, their proportion was relatively low as all other lymphocyte subsets are present in CSF in significantly increased numbers. In the blood of Hu-PNS patients both absolute numbers and proportions of NKT cells were lower than in the controls. NKT cells are a heterogenous group of T cells that share properties of both T cells and NK cells and exert effector and regulatory functions. Upon activation, they produce large quantities of different cytokines, in the course of which they activate other immune cells and acquire the ability to kill tumor cells directly[314-316]. Dysfunction or deficiency of NKT cells has been associated with the development of autoimmune diseases, such as systemic lupus erythomatosus, multiple sclerosis and rheumatoid arthritis, although the basis for this involvement remains elusive[314, 315]. These autoimmune diseases are characterized by Th1-polarized T cell responses, which has also been proposed for Hu-PNS by Benyahia et al.[41] as Hu-specific lymphocytes in their study are predominantly of the Th1 subtype. We propose that the relative deficiency of NKT cells in the CSF and blood of our Hu-PNS patients, possibly in conjunction with Hu-specific Th1 cells[41], support the autoimmune hypothesis of Hu-PNS.

In conclusion, the pleocytosis in the CSF of Hu-PNS patients is caused by an increment of all major subsets (B, T [both $CD4^+$ and $CD8^+$] and NK cells). However, the B cells also show a relative expansion, while the NKT cells show a relative reduction. These findings suggest

not only a role for autoreactive B cells by producing Hu-antibodies and possibly enhancing antigen presentation, but also indicate the involvement of T cells in the pathogenesis of Hu-PNS. In addition, the autoimmune hypothesis of Hu-PNS is supported by the relative NKT cell deficiency.

Acknowledgments

The authors are grateful to the neurologists and anaesthesiologists for assistance in the inclusion of patients and controls. We thank Mrs. M. Kruger for her technical assistance. This study was supported by a 'Revolving Fund' grant from Erasmus MC (Rotterdam, The Netherlands) and by grant 06-026 from the Gratama foundation (Harlingen, The Netherlands).



Chapter 7

No evidence for the presence of HuD-specific T cells in the cerebrospinal fluid of patients with Hu-associated paraneoplastic neurological syndromes

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Journal of Neurology 2009;256:279-282

Sirs: 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[18]. Hu-antibodies are directed against a family of neuron specific proteins including HuD, HuC and Hel-N1; HuD is expressed in all SCLC tumor cells[18]. Despite high serum and cerebrospinal fluid (CSF) titers, a pathogenic role of Hu-antibodies was never proven in animal models[33]. The occurrence of oligoclonal CD8+ T cells in nervous and tumor tissues and HuD-specific CD8+ T cells in blood of Hu-PNS patients suggests their involvement in neuronal degradation[38, 39, 44, 58]. To investigate the putative role of HuD-specific cellular immunity in the pathogenesis of Hu-PNS, we determined the presence of HuD-specific T cells in the CSF of Hu-PNS patients. Based on our previous successful detection of virus-specific T cells using the HLA class I multimer technique and IFN-γ ELISPOT assays, we employed the same state-of-the-art techniques here[53, 60, 317, 318].

Thirteen patients with high-titer serum Hu-antibodies, a 'definite' diagnosis of PNS[3] and progressive neurological disease were included in the study. Their characteristics are shown in Table 1. Four patients with non-inflammatory neurological diseases (i.e., normal pressure hydrocephalus and herniated cervical disc) were included as controls (all male, aged between 52 and 79, Hu-antibody seronegative, no prior chemotherapy or immunosuppression, and expression of either the HLA-A*0101 [n=2], HLA-A*0201 [n=1] alleles, or both [n=1]). Our Institutional Review Board approved the study and all patients provided written informed consent. CSF-derived T cells of patients and controls were expanded[318, 319] and assayed for reactivity against a HuD-protein spanning peptide pool consisting of 15-mer peptides with 11 amino acids overlaps and HuD-derived 9-mer peptides that had been identified by others[38, 59] in IFN-γ ELISPOT assays. Additionally, fresh CSF samples (10 ml) and CSF-derived T cell lines (CSF-TCL) from HLA-matched Hu-PNS and control patients were stained with 9-mer HuD-peptide-loaded HLA class I multimers (based on HLA-A*0101, HLA-A*0201 and HLA-A*2403 alleles) to visualize HuD-specific CD8+ T cells.

The IFN-γ ELISPOT assays showed no HuD 15-mer or 9-mer specific responses in CSF-TCL from either Hu-PNS or control patients (Figure 1); neither were positive results observed in any of the fresh or expanded CSF samples tested with the multimers (data not shown).

Although the high titers of Hu-antibodies, their IgG1 isotype predominance[34] and the presence of intrathecal Hu-antibody production in the majority of Hu-PNS patients are indicative for the involvement of local Hu-specific CD4+ T cells[318], we were unable to detect HuD-specific T cells in CSF. There are several possible explanations for these negative results. First, the sensitivity of our assays may have been insufficient. Second, antigen-specific positive controls in the CSF are lacking as a HuD-specific T cell line is not available and T cells directed to other known antigens, such as Epstein Barr virus antigens, are not

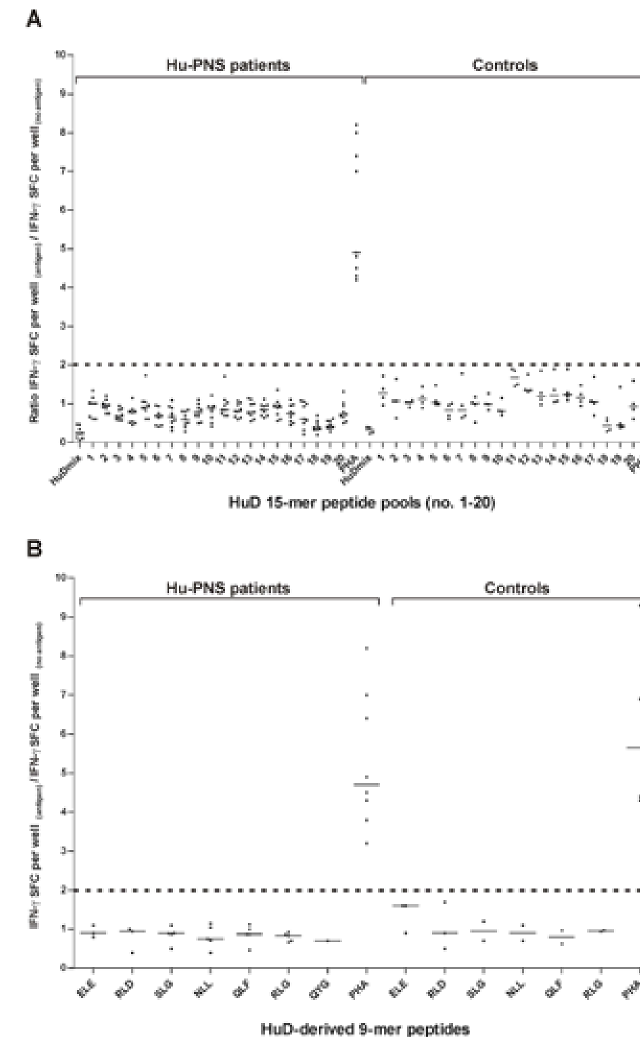


Figure 1 | HuD-peptide specific T cell responses

Expanded CSF-derived T cell cultures were stimulated with pools of HuD-derived 15-mer peptides or phytohemagglutinin (PHA) (**A**) or with individual 9-mer HuD-derived peptides (**B**), and assayed by INF-γ ELISPOT. Results of assays based on 1x10^4 T cells are shown. The ratios between the number of INF-γ spot-forming cells (SFC) following stimulation in the presence and 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 treshold for assay positivity, is shown as reference.

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

Table 1 | Patient and CSF characteristics at study entry

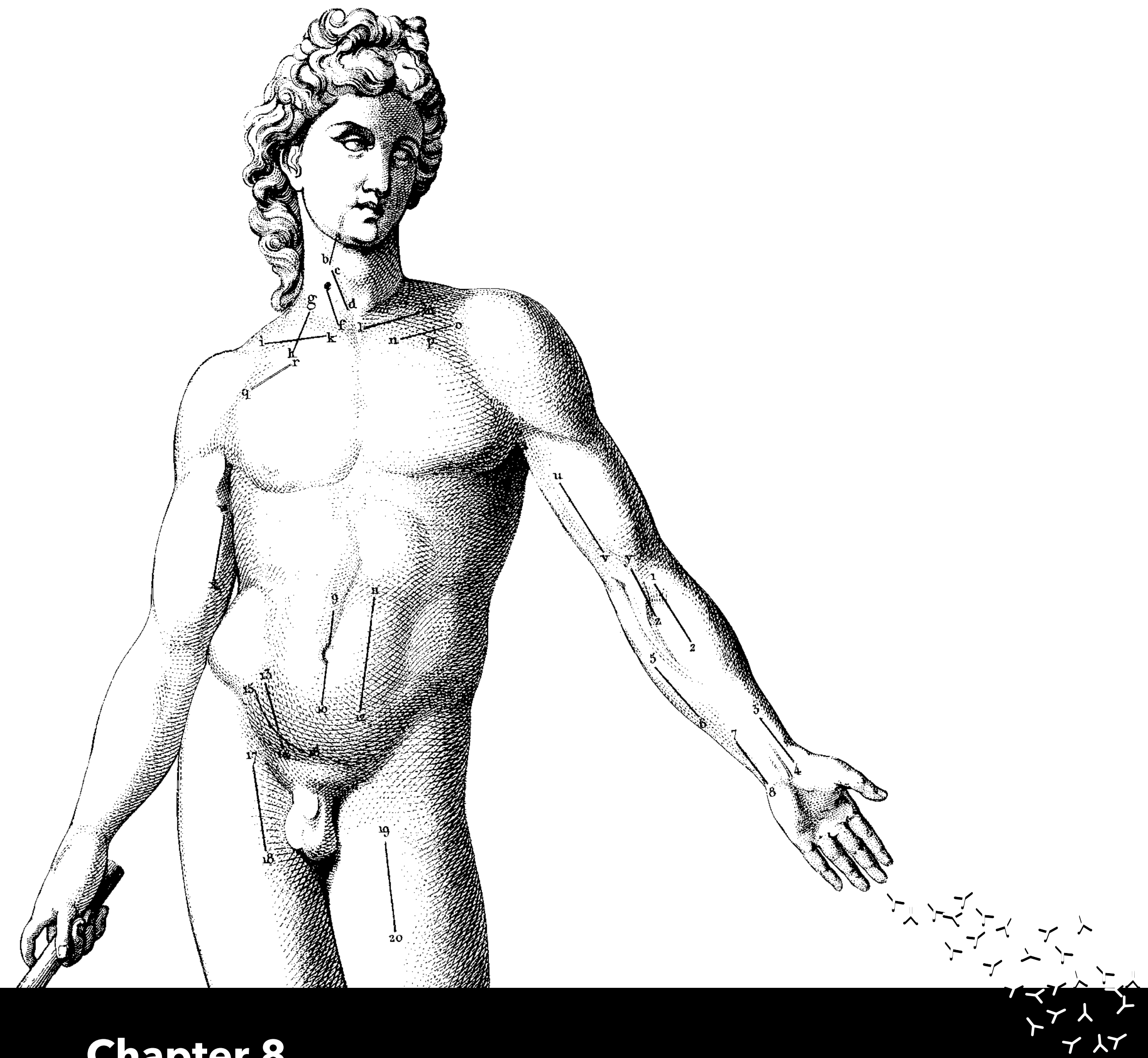
Hu-PNS patient	Age/ gender	Hu-Ab titer serum	CSF					PNS	Tumor	Therapy	Symptoms-diagnosis (months after onset)	Symptoms-assay (months after onset)	mRS	HLA
			Protein (g/l)	Hu-Ab titer	AI[a]	MNC (/µl)	PNC (/µl)							
Hu_1	64/F	102,400	0.38	2,048	2,5	2.3	<0.3	PLE	Lung[b]	None	1	1.5	3	A*0201
Hu_2	61/F	204,800	1.70	32,768	2,0	<0.3	<0.3	PLE	SCLC	Chemo	1	1.5	5	A*0101
Hu_3	66/M	102,400	0.33	256	0,6	1.0	<0.3	PCD	SCLC	None	2.5	3.5	3	*A11, A31*
Hu_4	69/F	51,200	0.65	512	0,7	3.3	<0.3	PLE, PSN	SCLC	None	6	6.5	3	A*0101
Hu_5	51/F	102,400	0.44	2,048	4,0	2.3	<0.3	PLE, PSN	No[c]	None	8	8.5	3	A*0101, A*0201
Hu_6	71/F	102,400	1.18	2,048	2,2	14.0	0.3	PSN	SCLC	None	1.3	1.5	3	A*2402
Hu_7	75/M	3,200	0.48	128	7,1	5.3	<0.3	PSN	SCLC	None	2	4	4	A*0201
Hu_8	66/M	25,600	0.76	4,096	7,0	10.0	<0.3	PSN	NSCLC	None	2.5	3	3	A*0101
Hu_9	53/F	51,200	0.66	512	1,6	6.7	<0.3	PSN	SCLC	None	2.5	3.0	2	A*0201
Hu_10	67/F	51,200	0.76	256	0,4	11.0	<0.3	PSN	No[c]	None	2.5	3	3	A*0201
Hu_11	56/F	6,400	1.89	256	1,9	3.0	1.0	PSN	SCLC	IVIg	6	6.5	3	A*0201
Hu_12	64/F	102,400	0.15	256	3,3	1.7	0.7	PSN	NSCLC	Chemo	12	15	2	A*0201
Hu_13	61/M	51,200	1.38	8,192	7,0	16.0	<0.3	PSN	Lung[b]	None	2.5	3	3	A*0101

[a]Antibody index as evidence of intrathecal Hu specific antibody synthesis. A ratio >1.3 indicates intrathecal Hu antibody synthesis.
[b]Tumor mass visible on CT scan.
[c]No tumor mass visible on CT or FDG-PET scan.
Hu-PNS patient = patient with Hu antibody associated PNS; Hu-Ab = Hu antibody; CSF = cerebrospinal fluid; AI = antibody index; 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; FDG-PET scan = fluorodeoxyglucose positron emission tomography scan.

detectable in unaffected patients. Third, failure of HuD-specific T cells to produce IFN-γ (i.e., anergic T cells) could be the cause of the negative results in the IFN-γ ELISPOT assays. Fourth, the preferential expansion of non-HuD reactive T cells may have hampered the detection of HuD-specific T cells. Finally, it could be possible that HuD-specific T cells are absent in the CSF or that T cells specific for other Hu-proteins (like HuC and Hel-N1) that do not crossreact with specific HuD-peptides are present. In conclusion, no HuD-specific T cells were detected in freshly isolated CSF T cells or CSF-TCL, although state-of-the-art techniques successful in detection of virus-specific T cells in CSF were used.

Acknowledgments

The authors are grateful to the neurological staff for assistance in the inclusion of patients. We thank Mrs. C. de Groot-van Ruyven and Mrs. M. Kruger for their technical assistance and J. Kraan for critical reading of the manuscript. This study was supported by a ‘Revolving Fund’ grant from Erasmus MC (Rotterdam, The Netherlands).



Chapter 8

HLA-DQ2+ individuals are susceptible to Hu-Ab associated paraneoplastic neurological syndromes

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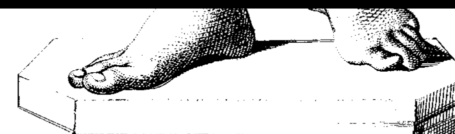
Journal of Neuroimmunology 2010;226:147-9



ABSTRACT

Background
Hypothetically, T cells are involved in the pathogenesis of paraneoplastic neurological syndromes associated with Hu-antibodies (Hu-PNS).

Objective
To identify genetic risk factors for Hu-PNS and investigate the role of T cells.

Methods
HLA-A, B, DRB1 and DQB1 alleles were compared in 53 Hu-PNS patients with 24 small-cell lung-cancer (SCLC) patients and 2440 healthy controls (HC).

Results
The frequency of both HLA-DQ2 and HLA-DR3 was significantly higher in Hu-PNS patients than in HC.

Conclusions
This study indicates an association between Hu-PNS and presence of HLA-DQ2 and HLA-DR3, which supports a role for $CD4^+$ T cells in the pathogenesis of Hu-PNS.

INTRODUCTION

Hu-antibody associated paraneoplastic neurological syndromes (Hu-PNS) are diagnosed in patients with severe and subacute neurological syndromes through detection of high-titer Hu-antibodies (Hu-Ab) in serum or CSF[2]. However, these antibodies have also been reported in 22.5% of SCLC patients without neurological symptoms[320]. Hu-Ab are directed against the neuronal HuD-antigen that is aberrantly expressed by all small-cell lung-cancers (SCLC)[7]. This ectopic expression hypothetically causes a strong autoimmune response, which has a positive antitumor effect[19], but may also cause devastating neurological damage. The precise immunopathogenic mechanism of the neurological damage remains unknown, although occurrence of both B- and T-cell mediated immune responses in Hu-PNS[34, 40, 47] indicates a role for cellular immunity. Although all SCLC express the onconeural HuD-antigen, Hu-PNS develops in less than 1% of patients.

In a wide range of autoimmune diseases, human leukocyte antigen (HLA) association is an important factor in an individual's susceptibility to the disease[321]. In this study we determined whether HLA association plays a role in Hu-PNS. Identification of disease-risk HLA-genes may lead to characterization of epitopes in the HuD-antigen and subsequent elucidation of the role of T cells in the pathogenesis of this disease. Moreover, HLA association might explain why only a small part of the SCLC patients develops Hu-PNS, despite the fact that the HuD-antigen is expressed in all SCLC. Therefore, we compared the frequency of the different HLA-alleles (HLA-A, B, DR and DQ) in healthy controls (HC) and SCLC patients either with or without Hu-PNS.

METHODS

Patients and controls
Hu-PNS patients were included in this study after detection of Hu-Ab at the Dutch national reference laboratory (Department of Immunology, Erasmus Medical Center, Rotterdam, The Netherlands) or the French national reference center for paraneoplastic antibodies (Centre de Référence Maladie Rare 'Syndromes neurologiques Paranéoplasiques', Hospices Civils de Lyon, Lyon, France) between June 2001 and January 2008. Fifty-three Caucasoid Hu-PNS patients were included because they met the following criteria: (i) high-titer serum Hu-Ab; (ii) histologically proven SCLC; (iii) a 'definite' diagnosis of PNS according to international guidelines[3]; and (iv) availability of leukocytes for HLA typing. We compared the Hu-PNS patients with 24 Caucasoid SCLC patients, who were included in collaboration with pulmonologists, when they (i) were Hu-antibody seronegative; and (ii) had no neurological signs or symptoms. Additionally, the Hu-PNS patients were compared with a cohort of 2440 healthy, unrelated, Dutch Caucasoid blood donors (HC)[322]. Written informed consent was obtained from all patients tested, and the local ethical review committee approved the study. Patient characteristics are presented in Table 1.

Table 1 | Characteristics of Hu-PNS patients, SCLC patients and healthy controls

Patient group (N)	Median serum Hu-antibody titer (range)	Type of PNS[a]		Median age (range)	Gender (M/F)
		PSN	PEM		
Hu-PNS (53)	6400 (400-204,800)	29	28	65 (46-85)	23/30
SCLC (24)	negative	not present		62 (40-79)	15/9
HC (2440)	not tested	unkown		27 (20-35)	1198/1242

[a]Four patients had both PSN and PEM.
PNS = paraneoplastic neurological syndrome; PSN = paraneoplastic sensory neuropathy; PEM = paraneoplastic encephalomyelitis; SCLC = small-cell lung cancer; M = male; F = female; HC = healthy controls.

HLA typing

In the Hu-PNS and SCLC patients, HLA typing was performed on DNA isolated from peripheral blood mononuclear cells by a molecular typing method using polymerase chain reaction with sequence-specific primers (PCR-SSP; Olerup-SSP™, Saltsjöbaden, Sweden)[323] at a 4-digit resolution level (Laboratory for Histocompatibility and Immunogenetics, Sanquin Blood Bank South West Region, Rotterdam, The Netherlands). Due to limited availability of DNA it was not possible to perform a complete HLA typing in 5 of the 53 Hu-PNS patients. In the HC, HLA typing was performed using standard serological methods (Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands). Regarding this study, the HLA types determined by PCR-SSP and by serological methods are comparable[324, 325].

Hu antibody detection

Hu IgG Ab titers were determined on rat cerebellar sections by indirect immunofluorescence (IIF)[326] and immunoreactivity was confirmed in all cases with western blotting using purified HuD in all cases[18]. Only patients with 'high-titer' serum Hu-Ab were eligible for the study (serum IIF titer ≤400[326]).

Statistical analysis

Odds ratios with 95% confidence intervals according to the Woolf test and two-sided Fisher exact test were used to compare the frequencies of the different HLA antigens in the patient groups and controls (Matlab version 6.5 [The MathWorks, Natick, MA])[327]. *P*-values were corrected for the number of alleles tested (HLA-A: 15 alleles; HLA-B: 21 alleles; HLA-DR: 13 alleles; HLA-DQ: 5 alleles) as described earlier[327] and were considered significant when $P<0.05$.

RESULTS

The HLA-antigen class II frequencies in the three groups are shown in Table 2 (for additional class I frequencies see Table 3). We found a significantly higher frequency of HLA-DQ2 in the Hu-PNS patients (33/53; 62%) than in the HC (881/2360; 37%) ($P=0.0015$). Although there was also a trend towards a higher prevalence of HLA-DQ2 in the Hu-PNS patients than in the SCLC patients (7/24; 29%), this difference did not reach statistical significance, probably because of the small size of the SCLC patient group. Additionally, the HLA-DR3 frequency was significantly higher in the Hu-PNS patients (25/53; 47%) than in the HC (599/2395; 25%) ($P=0.0082$).

Table 2 | HLA class II frequencies in the Hu-PNS patients, SCLC patients and healthy controls

Antigen	Hu-PNS (N=53)			SCLC (N=24)			HC (N=2440)			P value[a] PNS vs. SCLC	P value[a] PNS vs. HC
	Pos	Neg	Pf (%)	Pos	Neg	Pf (%)	Pos	Neg	Pf (%)		
DR1	14	39	26	3	21	13	437	1922	20	0.9717	0.9639
DR3	*25*	*28*	**47**	*5*	*19*	**21**	*599*	*1796*	**25**	*0.4330*	**0.0082**
DR4	11	42	21	11	13	46	679	1717	28	0.3399	0.9860
DR7	12	41	23	8	16	33	459	1937	19	0.9988	0.9998
DR8	3	50	6	2	22	8	128	2264	5	1.0000	1.0000
DR9	1	52	2	0	24	0	58	2328	2	1.0000	1.0000
DR10	2	51	4	2	22	8	100	2296	4	1.0000	1.0000
DR11	5	48	9	2	22	8	340	2054	14	1.0000	0.9993
DR12	3	50	6	0	24	0	108	2263	5	1.0000	1.0000
DR13	12	41	23	7	17	29	669	1686	28	1.0000	0.9995
DR14	1	52	2	3	21	13	127	2210	5	0.6942	0.9972
DR15	6	47	11	4	20	17	414	1208	26	0.9999	0.1871
DR16	2	51	4	0	24	0	43	2288	2	1.0000	0.9815
DQ2	*33*	*20*	**62**	*7*	*17*	**29**	*881*	*1479*	**37**	*0.0631*	**0.0015**
DQ3	19	34	36	14	10	58	1209	1156	51	0.3790	0.1687
DQ4	3	50	6	2	22	8	29	886	3	0.9943	0.9305
DQ5	18	35	34	8	16	33	300	567	35	1.0000	1.0000
DQ6	17	36	32	10	14	42	453	451	50	0.9487	0.0538

[a]After Bonferroni correction.
Pos = positive; Neg = negative; Pf = positive frequency.

Table 3 | HLA class I frequencies in the Hu-PNS patients, SCLC patients and healthy controls

Antigen	Hu-PNS (N=53)			SCLC (N=24)			HC (N=2440)			P value[a] PNS vs. SCLC	P value[a] PNS vs. HC
	Pos	Neg	Pf (%)	Pos	Neg	Pf (%)	Pos	Neg	Pf (%)		
A1	22	27	45	5	19	21	747	1692	31	0.6635	0.4696
A2	27	22	55	9	15	38	1284	1156	53	0.9730	1.0000
A3	10	39	20	12	12	50	700	1739	29	0.1966	0.9897
A11	6	43	12	4	20	17	281	2153	12	1.0000	1.0000
A23	1	48	2	1	23	4	60	2366	2	1.0000	1.0000
A24	11	38	22	4	20	17	403	2021	17	1.0000	0.9976
A25	1	48	2	0	24	0	46	2393	2	1.0000	1.0000
A26	1	48	2	1	23	4	107	2333	4	1.0000	1.0000
A29	3	46	6	3	21	13	119	2321	5	0.9994	1.0000
A30	0	49	0	1	23	4	85	2350	3	0.9975	0.9997
A31	4	45	8	1	23	4	146	2291	6	1.0000	1.0000
A32	5	44	10	2	22	8	149	2290	6	1.0000	0.9783
A33	1	48	2	0	24	0	32	2404	1	1.0000	1.0000
A66	0	49	0	1	23	4	11	2280	0	0.9975	1.0000
A68	2	47	4	1	23	4	244	2191	10	1.0000	0.9790
B7	6	42	13	7	17	29	668	1772	27	0.9094	0.3599
B8	18	30	38	2	22	8	554	1886	23	0.2132	0.3866
B13	2	46	4	1	23	4	109	2331	4	1.0000	1.0000
B14	1	47	2	1	23	4	70	2367	3	1.0000	1.0000
B15	7	41	15	6	18	25	386	2050	16	0.9998	1.0000
B18	4	44	8	1	23	4	158	2282	6	1.0000	1.0000
B22	2	46	4	2	22	8	142	2298	6	1.0000	1.0000
B27	3	45	6	0	24	0	157	2283	6	1.0000	1.0000
B35	3	45	6	2	22	8	429	2010	18	1.0000	0.6692
B37	2	46	4	2	22	8	99	2340	4	1.0000	1.0000
B38	5	43	10	0	24	0	98	2341	4	0.9755	0.6287
B39	3	45	6	1	23	4	79	2358	3	1.0000	0.9928
B40	5	43	10	8	16	33	435	2005	18	0.4072	0.9976
B41	0	48	48	1	23	4	28	2411	1	0.9998	1.0000
B44	16	32	32	5	19	21	586	1853	24	1.0000	0.9808
B45	0	48	48	1	23	4	36	2404	1	0.9998	1.0000
B49	1	47	47	1	23	4	25	2415	1	1.0000	1.0000
B51	8	40	40	1	23	4	274	2158	11	0.9980	0.9975
B52	0	48	48	1	23	4	15	2412	1	0.9998	1.0000
B57	2	46	46	5	19	21	133	2284	6	0.5486	1.0000
B58	0	48	48	0	24	0	26	2386	1	1.0000	1.0000

[a]After Bonferroni correction.
Pos = positive; Neg = negative; Pf = positive frequency.

DISCUSSION

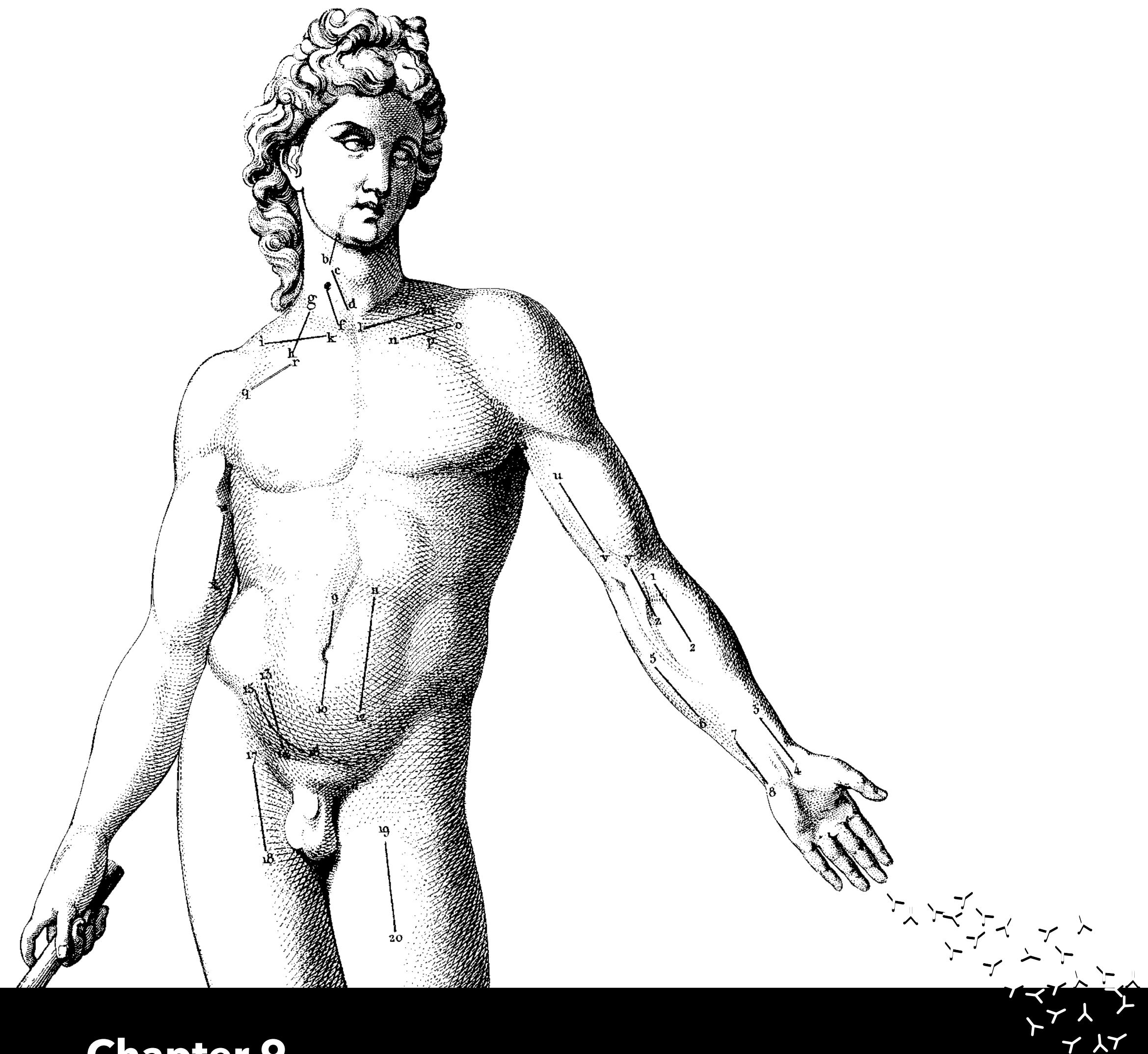
This study indicates an association between Hu-PNS and presence of the HLA-DQ2 and HLA-DR3 antigens. Until now, no HLA association in Hu-PNS has been reported. Two previous studies compared the HLA-A, B and DRB1 alleles in Hu-PNS patients. In the first study, 17 Caucasoid Hu-PNS patients were compared with 30 Caucasoid SCLC patients[328] and in the second study, 5 Japanese Hu-PNS patients have been studied[329], but no common antigen was identified in both studies. These negative findings can be explained by the fact that HLA-DQB1 alleles were not studied and patient groups were small.

HLA-DQ2 is associated with various autoimmune diseases including celiac disease, type I diabetes mellitus and myasthenia gravis[100-102]. The association in celiac disease is very strong, more than 90% of patients express HLA-DQ2[100]. This HLA class II association is in accordance with the central role for $CD4^+$ T cells in the pathogenesis of celiac disease[330]. As in our Hu-PNS patients, type 1 diabetes mellitus and myasthenia gravis have a weaker association with HLA-DQ2 than celiac disease[101, 102]. In general, HLA-associated diseases are usually the result of the combination of different HLA-antigens expressed at various loci rather than the result of one HLA variant only[330]. Both celiac disease and type 1 diabetes mellitus are associated with the 8.1 ancestral haplotype (HLA-A1, Cw7, B8, DR3, DQ2), which is a highly conserved HLA-complex associated with a wide range of autoimmune diseases[321]. We could not determine the haplotypes of our patients because we had no information on the HLA-types of their parents. However, of the 48 Hu-PNS patients with a complete class I and class II typing, 13 patients (27%) are HLA-$A1^+$, $B8^+$, $DR3^+$ and $DQ2^+$, while the frequency of the 8.1 ancestral haplotype in European populations is only around 10%[321]. This may indicate an association with the 8.1 ancestral haplotype in Hu-PNS, but has to be further investigated.

In Hu-PNS, the presence of HuD-specific T cells, both $CD4^+$ and $CD8^+$, is suggested[38, 41, 61]. Knowledge of the involved auto-antigen together with specific disease-associated HLA class II alleles which we describe here, may lead to detection of HuD-specific $CD4^+$ T cells in HLA-$DR3^+$/$DQ2^+$ Hu-PNS patients and subsequent epitope identification. However, the low incidence of Hu-PNS despite expression of the HuD-antigen in all SCLC cannot be explained with the results of this study only. As a substantial proportion of Hu-PNS patients do not express HLA-DR3 and -DQ2, we suggest that additional factors, such as dysfunction of regulatory T cells[51], must be involved in susceptibility to developing Hu-PNS.

Acknowledgments

We would like to thank Prof.dr. F. Koning and Prof.dr. F. Claas (Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands) for their expert advice and Neurobiotec bank for storage of the French patient samples. This work was supported by the Gratama Foundation, Teteringen, The Netherlands.



Chapter 9

Contamination of synthetic HuD protein spanning peptide pools with a CMV-encoded peptide

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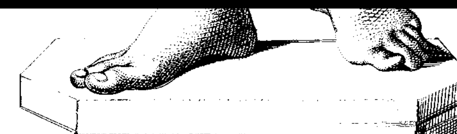
Cytometry Part A 2008;73:1079-1085



ABSTRACT

To detect HuD-specific T cells in patients with Hu-antibody associated paraneoplastic neurological syndromes (Hu-PNS), we used short-term stimulation assays with HuD protein spanning peptide pools (PSPP) with purities of at least 70% and found reproducible false-positive CD8$^+$ T-cell responses in three of 127 individuals (two healthy controls and one Hu-PNS patient), which all shared HLA-A*2402 and HLA-B*1801. After testing the 15-mer peptides of the HuD antigen separately, we discovered that the same three 15-mers yielded the CD8$^+$ T cell response in those three individuals. This highly unusual result could not be reproduced when using new batches of peptides with a higher level of purity (>82% and >95%). Therefore, we assumed this response was not directed against the HuD peptides and analyzed the HuD 15-mers by Fourier transform ion cyclotron resonance (FT-ICR) tandem mass spectrometry (MS/MS), which showed the presence of a cytomegalovirus (CMV)-encoded peptide. The three responding individuals all were CMV-seropositive and the contaminating peptide appeared to fit in the binding groove of HLA-B*18. Our data reveal that synthetic PSPP may contain immunogenic contaminations which may cause false-positive results in T-cell stimulation assays.

INTRODUCTION

Protein spanning peptide pools (PSPP) are mixtures of overlapping (mostly 15-mer) peptides, that can efficiently stimulate both CD4$^+$ and CD8$^+$ T-cell responses and are valuable for the detection of T-cell responses against proteins[331, 332]. In most cases, pools consisting of peptides of 70% purity are used in screening studies for antigen-specific T cells[331], because the preparation of peptides of higher purity requires a significant investment. We used HuD PSPP of 15-mer peptides with purities of at least 70% in an earlier study to detect circulating HuD-specific T cells in patients with Hu-antibody associated paraneoplastic neurological syndromes (Hu-PNS)[60, 333]. HuD peptides were pooled and tested in short-term stimulation of peripheral blood mononuclear cells (PBMC) of Hu-PNS patients, small cell lung carcinoma (SCLC) patients and healthy controls (HC) followed by intracellular detection of interferon (IFN)-γ[334]. The results showed reproducible positive CD8$^+$ T-cell responses in only three of 127 individuals (two HC and one Hu-PNS patient), which all shared HLA-A*2402 and HLA-B*1801. Next, we tested the 15-mer peptides separately in the two responding HC (the Hu-PNS patient was no longer available) and showed that the same three 15-mers (peptide #38, #40, and #86) yielded the CD8$^+$ T-cell responses in both HC. Subsequently, two newly synthesized batches of those 15-mers with a higher purity (>82% and >93%, respectively) were tested and elicited no responses at all. Therefore, we concluded that the earlier, probably HLA-restricted, CD8$^+$ T-cell response was not caused by the PSPP itself, but rather by an immunogenic contamination in the peptides[333].

Mass spectrometry (MS) is based on the precise measurement of the mass-to-charge ratio (*m/z)* and is considered to be a significant aid in protein and peptide structure determination. For characterization of by-products in synthetic peptides, liquid chromatography coupled to electrospray ionization mass spectrometry (LC/ESI-MS) is a sensitive method[335]. In the present study we identified the contamination in the HuD peptides by analyzing the three responsible 15-mers of the HuD antigen with Fourier transform ion cyclotron resonance (FT-ICR) MS and elucidated why only these three of 127 individuals responded. The HuD PSPP appeared to be contaminated with a cytomegalovirus (CMV)-encoded peptide, which elicited a T-cell response in CMV seropositive, HLA-B*18 positive individuals with a specific cellular immune response to CMV immediate early (IE)-1 peptide.

MATERIALS AND METHODS

Synthetic peptides

The 15-mer HuD and CMV IE-1 peptides were synthesized by custom peptide synthesis on an APEX 396 synthesizer (Advanced ChemTech, Giessen, Germany) at JPT Peptide Technologies (JPT, Berlin, Germany) using a standard Fmoc-based solid-phase synthesis protocol on a tricholorphenyl (TCP)-resin support. After lyophilization all peptides were shown to have a purity >70% according to analytical high-performance liquid chromatography (HPLC). The HuD peptides of this batch, batch 1, were used in the initial short-term stimulation assays. To see if the positive CD8$^+$ T cell responses in the three individuals could be reproduced with newly synthesized batches with a higher purity, the HuD 15-mers #38, #40, and #86 were produced with purities of >82% (batch 2) and >93% (batch 3) (JPT Peptide Technologies). The individual 15-mer peptides were dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, 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 and for the mass spectrometric analysis. The peptides were stored at -80°C.

Prior to mass spectrometric analysis, the peptides (0.2 mg/ml) were diluted 10-fold in deionized water (Milli-Q). Next 0.5 µl of matrix (2,5-dihydroxy benzoic acid [DHB] 10 mg/ml in 0.1% TFA/milliQ water) was pipetted onto a 400 µm anchorchip and 0.5 µl of the diluted peptide solution was pipetted on top of the peptide drop. The mixture was dried under vacuum in a dessicator to remove the DMSO. The amount of peptide spotted is 6 pmol.

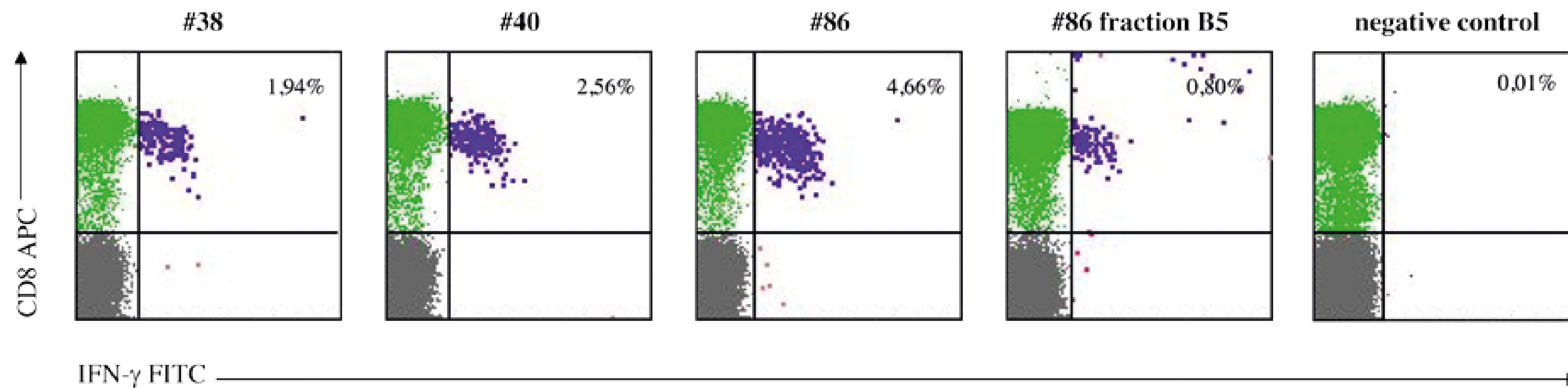


Figure 1 | CD8⁺ T-cell responses to HuD 15-mers

Assessment of CD8⁺ T-cell responses to HuD 15-mers #38, #40, #86, and fraction B5 of #86 in donor 1 (see Table 1). T cells were selected by gating on the CD3⁺ cells with low sideward light scatter. To calculate the percentages of IFN-γ-producing CD8⁺ T cells in response to peptide, thresholds were set on the unstimulated cells (negative control). A T-cell response was considered positive when the percentage of IFN-γ-producing CD8⁺ T cells in the peptide-stimulated sample was at least 2-fold the corresponding percentage in the unstimulated sample. According to these criteria, the T-cell responses toward all peptides shown here, were classified as positive.

Short-term stimulation assay

Written informed consent was obtained from all tested individuals and the local ethical review committee approved the study. PBMC were isolated within 12 h after venipuncture and stimulated as described elsewhere[331, 332]. Briefly, $2x10^6$ PBMC were incubated at 37°C in a CO_2 incubator for 18 h 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 h of stimulation, brefeldin A was added to detect intracellular accumulation of cytokines in activated T cells. After another 4 h of incubation, the stimulated PBMC were stored overnight at 4°C and stained and analyzed the next day using con IgG1 monoclonal antibody (mAb) conjugated with fluorescein isothiocyanate (FITC), IFN-γ mAb conjugated with FITC, con IgG1 mAb conjugated with phycoerythrin (PE), CD69 mAb conjugated with PE, CD8 mAb conjugated with PE, CD3 mAb conjugated with peridinyl chlorophyllin (PerCP), CD4 mAb conjugated with allophycocyanin (APC) and CD8 mAb conjugated with APC (all from BD Biosciences, San Jose, CA, USA; except for CD8-PE which was obtained from Dako, Glostrup, Denmark).

The detection limit of our short-term stimulation assay for CMV IE-1 peptide #94 was determined by stimulating PBMC with #94 in a concentration of 1 μg/ml titrated to 0.02 μg/ml.

Fourier transform ion cyclotron resonance mass spectrometry

The measurements were performed using a MALDI-FT-ICR mass spectrometer (Apex Q 9.4 Tesla equipped with a combi-source, Bruker Daltonics) [336]. A multishot accumulation was used as recommended[337-339]. Ten laser shots were accumulated in the storage hexapole, transferred to the FT-ICR cell, and scanned for 0.78 seconds (TD size 512 kB). Fifty scans were used for each mass spectrum. The acquisition mass range was 800–4000 *m/z*. Total accumulation time was 3 min. FT-MS spectra were processed with a Gaussian filter (line broadening [LB]=0, Gaussian broadening [GB]=0.7) and 2 zero fillings. A standard peptide calibration mix (Bruker Daltonics, Leipzig, Germany) 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.

RESULTS

The contamination and its properties

Since the HuD 15-mer #86 showed the largest CD8⁺ T-cell response (Figure 1, third panel), we decided to fractionate this sample by analytical HPLC. This procedure resulted in 39 fractions (A1 to A12, B1 to B12, C1 to C12, and D1 to D3). Next, these freeze-dried fractions were dissolved in 20 μl DMSO and tested in the short-term stimulation assay with PBMC of the two responding HC ($2x10^6$ PBMC with 5 μl of the dissolved fraction). Only fraction B5 appeared to yield an IFN-γ response in both HC. Figure 1 shows this response and the

responses elicited by HuD 15-mers #38, #40, and #86 in one of the HC. For stimulation, we used 1 μg/ml of each 15-mer, whereas the concentration of peptide in fraction B5 was unknown. Therefore, the percentages of IFN-γ-producing CD8^{+} T cells after stimulation with HuD #86 and fraction B5 are not quantitatively comparable.

Next, we analyzed HuD 15-mers #38, #40, and #86 and various fractions of #86, including B5, by FT-ICR MS and tandem mass spectrometry (MS/MS). The FT-ICR mass spectrum of fraction B5 is shown in Figure 2 and this spectrum contains 100 monoisotopic peaks having a signal-to-noise (S/N) ratio >10. MS/MS spectra for the most intense peaks were obtained and some of the found sequences are also shown in Figure 2. Some of these components (i.e., the omission peptides) had been identified in our previous study[333]. To ascertain which component was responsible for the IFN-γ response, we searched for peaks that were abundantly present in the mass spectrum of fraction B5 and absent or much weaker in the mass spectra of the surrounding fractions. One promising candidate appeared to be a peptide having a monoisotopic mass of 1638.7366 ± 0.024 Da (indicated by an asterisk in Figure 2) and whose first three isotopic peaks are shown in Figure 3C. Upon close inspection it appeared that this component could also be seen in the mass spectrum of the unfractionated HuD peptide #86 (Figure 3A). The corresponding mass region for peptide #86, batch 2, is given in Figure 3B and it can be seen that the contamination may also be present in this fraction, but to a far lesser extent. However, the fifth isotope peak of HuD #86 (monoisotopic mass 1633.7236) appears at *m/z* 1638.7403 and coincides with the mass of the impurity at mass 1638.7380. Based on an isotope intensity analysis, we conclude that mass 1638.7356 in batch 2 (indicated with an arrow in Figure 3B) corresponded to the fifth isotope of HuD #86, whereas mass 1638.7380 in batch 1 (Figure 3A) corresponded to the impurity which caused the T-cell response. The peak at mass 1638.7003 (Figure 3B) constituted another, unknown, impurity.

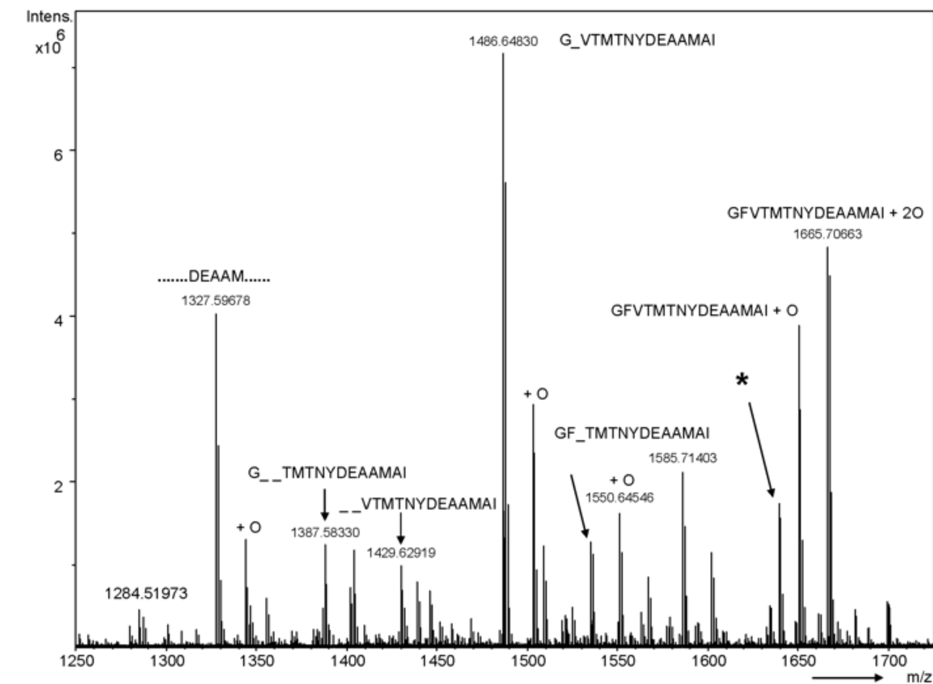


Figure 2 | Fraction B5 of the HuD peptide #86

MALDI-FT-ICR mass spectrum of fraction B5 of the HuD peptide #86 (GFVTMTNYDEAAMAI), batch 1, obtained by preparative HPLC. Sequences obtained by MS/MS as indicated. Underscores indicate missing amino acids. Oxidation is indicated by +O and +2O.

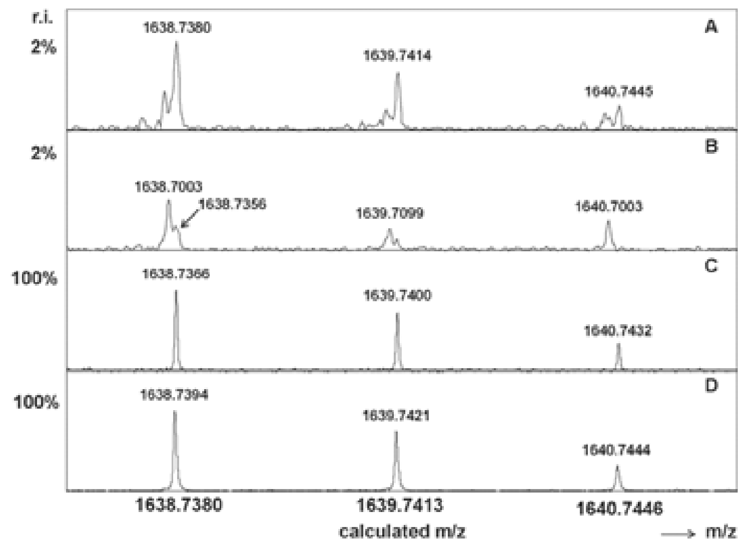


Figure 3 | FT-ICR mass spectra of HuD and HCMV peptides

Partial FT-ICR mass spectra of (**A**) HuD peptide #86 batch 1, (**B**) HuD peptide #86 batch 2, (**C**) preparative HPLC fraction B5 of HuD peptide #86 batch 1, and (**D**) the synthetic HCMV IE-1 peptide #94 AIAEESDEEEAIVAY. Spectra (A) and (B): internal calibration on peptide #86 (calc. for GFTNYDEAAMAI: 1633.7236, one sodium exchange [calc. 1655.7056] and two sodium exchanges [1677.6876]); intensity relative to m/z 1633.7236 = 100%. Spectra (A) and (B): intensity relative to m/z 1633.7236 = 100%. Spectra (C) and (D): acquired after external calibration.

To identify the contaminant, the FT-ICR MS/MS mass spectrum of *m/z* 1638.7366 in fraction B5 was obtained (Figure 4A). A search on a protein sequence database (Mascot) revealed this to be a CMV-encoded peptide, i.e., an HCMV IE-1 15-mer (#94) having sequence AIAEESDEEEAIVAY. (Six MS/MS masses searched, database MSDB, mass tolerance MS: 2 ppm, mass tolerance MS/MS: 0.005 Da, score 61, all scores >37. These data indicate extensive identity or extensive homology [P<0.05].) The MS/MS mass spectrum only contains the b series of ions, the y series being absent. The b-ions in Figure 4 fragmented further by loss of H_2O. The b_{13} ions, which terminate with the amino acid valine containing an isopropyl group, fragmented further by loss of C_3H_6.
We then selected IE-1 #94 (AIAEESDEEEAIVAY) from our HCMV IE-1 15-mer collection[340] and analyzed it by FT-ICR MS/MS (Figure 4B). This spectrum is virtually superimposable to that of the impurity found in HuD #86 (Figure 4A). Also the measured mass of peptide IE-1 #94 is within experimental uncertainty equal to that of the impurity (Figure 3D).

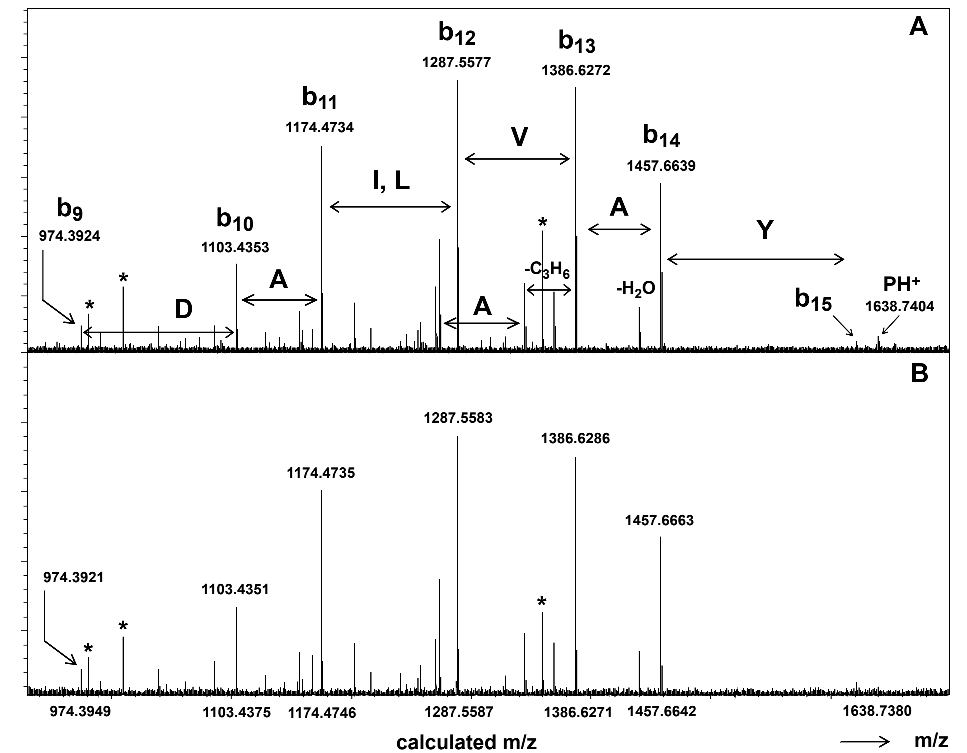


Figure 4 | Fraction B5 and IE-1 peptide #94

FT-ICR MS/MS spectra of (**A**) mass 1638.7366 in preparative HPLC fraction B5 of HuD peptide #86 batch 1, and (**B**) the synthetic HCMV IE-1 peptide #94 AIAEESDEEEAIVAY. Collision energy 65 eV. The synthetic peptide #94 itself contains an impurity peak at *m/z* 1642.8734 and so to avoid interference with the MS/MS experiments, the mass window was set at 3 Da. Asterisks indicate shot noise peaks. B-ions have the charge retained on the N-terminal fragment, whereas y-ions retain the charge on the C-terminal fragment. PH^+ (panel A) indicates protonated peptide #94.

In addition, HuD 15-mer #38 also yielded a weak signal with the same mass as the CMV-encoded peptide, while such a signal was undetectable in #40 (data not shown). Moreover, we determined the concentration of the contamination in the three HuD peptides by MS. In such quantitative experiments, differences in ionization efficiencies must be taken into account. Because the contaminant peptide ionized by a factor 7 less than HuD #38, we constructed a calibration curve. From this calibration curve we concluded that HuD #38 contained about 1% of the contaminant, although the intensity of the peak for the contaminant in HuD #38 was only about 0.15%. Similar experiments were performed for

the HuD peptides #86 and #40. In peptide #86, the concentration of the contamination was found to be 2% and in HuD peptide #40 the contaminant was below the S/N level. From the calibration curve and spiking experiments, we concluded that HuD #40 contains less than 0.4% of the contaminant (data not shown).

We also determined the detection limit of our short-term stimulation assay by titrating the concentration of IE-1 peptide #94. The $CD8^+$ T-cell response was still present at a concentration of IE-1 #94 of 1.5% (0.15 µg/ml), but absent at 0.7% (0.07 µg/ml), which means that the contamination in the HuD peptides should be present in a concentration exceeding 0.07 µg/ml to show a $CD8^+$ T-cell response.

Next, IE-1 peptide #94 was tested in the short-term stimulation assay with PBMC of the two responding HC to reproduce the $CD8^+$ T-cell response. In both donors this response could be reproduced (Figure 5: donor 1 and 2; donor 4 and 5: see below).

As mentioned previously, we observed that in our mass spectrometric quantification experiments, strong suppressive effects operated against the peptide IE-1 #94, i.e., against the contaminant. We attributed this to the preponderance of the acidic aminoacids aspartic acid (D) and glutamic acid (E) in AIAEESDEEEAIVAY, making the peptide very hydrophilic and difficult to cationize by protonation. Importantly, such peptides are expected to show more than average affinity towards chromatographic columns, i.e., the columns used in the lyophilisation procedures for the synthesis of peptides.

The responding individuals

The second aim of the present study was to discover the reason why only three out of 127 individuals showed the false-positive $CD8^+$ T-cell response. As mentioned earlier, the three responding individuals all share HLA-A*2402 and HLA-B*1801, while the HLA-C*, HLA-DRB1* and HLA-DQB1* typing was different between the individuals (Table 1: donors 1, 2, and 3). Another similarity between the 3 individuals is CMV seropositivity, which was expected after our discovery that a CMV-encoded peptide contaminated the PSPP. Because of the similarity in HLA type of the responding individuals, we performed a search using SYFPEITHY[341, 342], Bimas[343], NetMHC[344, 345], and IEDB[346] which revealed that IE-1 peptide #94 fitted in the binding groove of HLA-B*18[347] whereas there was no match with HLA-A*24.

Table 1 | HLA-type responding and non-responding CMV seropositive donors

Donor	Patient type	CMV serostatus	Response HuD	Response IE-1 #94	HLA-A*	HLA-B*	HLA-Cw*	HLA-DRB1*	HLA-DQB1*
1	HC	Positive	$CD8^+$ T cell	$CD8^+$ T cell	2402, 2501	0702, 1801	0702, 1203	15, --	06, --
2	HC	Positive	$CD8^+$ T cell	$CD8^+$ T cell	0101, 2402	0801, 1801	0701, --	01, 14	05, 05
3	Hu-PNS	Positive	$CD8^+$ T cell	$CD8^+$ T cell	0201, 2402	1801, 4402	0501, 0701	04, 11	03, --
4	Hu-PNS	Positive	No	No	25, 29[a]	18, 44[a]	NT	07, 15	02, 06
5	Hu-PNS	Positive	No	No	0201, 2501	18, 39[a]	NT	03, 04	02, 03
6	SCLC	Positive	No	No	01, 11[a]	18, 37[a]	NT	03, 04	02, 03

[a]Insufficient number of PBMC for molecular HLA-typing.
HC = healthy control; Hu-PNS = patient with Hu-antibody associated paraneoplastic neurological syndrome; SCLC = small cell lung carcinoma patient; NT = not tested;
-- = blank (i.e., no other HLA allele detected, donor probably homozygous).

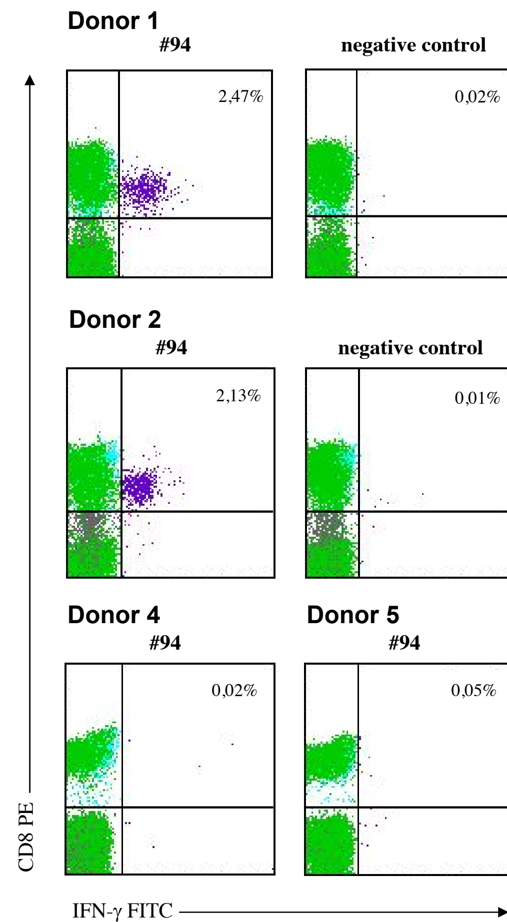


Figure 5 | HCMV-specific CD8+ T-cell responses

Assessment of CD8+ T-cell responses to HCMV IE-1 15-mer #94 in the two responding HC (donors 1 and 2) and the two non-responding Hu-PNS patients (donors 4 and 5). See further the legend to Figure 1. According to these criteria, the T-cell responses in donor 1 and 2 were classified as positive and the T-cell responses in donor 4 and 5 were classified as negative.

However, apart from the three responding individuals, three other patients (two Hu-PNS patients and one SCLC patient) of the 127 individuals we tested were CMV seropositive and HLA-B*18 (Table 1: donors 4, 5, and 6), but they did not show a CD8+ T-cell response against the HuD PSPP. We tested the CMV response of the two Hu-PNS patients (donors 4 and 5) in a short-term stimulation assay with thawed PBMC in order to compare the fine

specificity of their IE-1 response with those of the responding individuals. Both patients showed no CD8+ T-cell response against IE-1, i.e., the IE-1 peptide mix and IE-1 peptide #94 (Figure 5: donors 4 and 5), whereas both CMV lysate and PP65 peptide mix elicited a T-cell response (data not shown). This negative response against IE-1 may explain the lack of a CD8+ T-cell response against the HuD PPSP in these patients although they are CMV seropositive and HLA-B*18.

DISCUSSION

A reproducible IFN-γ CD8+ T-cell response was observed by coincidence in three of 127 individuals after stimulation with HuD PSPP. These individuals had identical HLA-A* and HLA-B* types. This response was directed against the same three HuD 15-mers (peptides #38, #40, and #86) and appeared to be caused by a contamination with a 15-mer derived from HCMV IE-1 (peptide #94). Recently, Currier et al.[348] also demonstrated the presence of contaminating HCMV peptides in synthetic peptides from two independent custom peptide suppliers. Therefore, it seems unlikely that the effects we have observed are spurious events or are specific to our laboratory.

The MS/MS spectra of HuD peptides #38 and #86 both showed the presence of the contamination. The contamination was not found in #40, although this peptide also yielded a positive response. There are two possible explanations for this finding. First, the contamination in HuD peptide #40 is present in such a low concentration that it is undetectable by MS/MS, but still causes a CD8+ T-cell response. The detection limit of our short-stimulation assay (0.7%) approximates the detection limit of the MS (0.4%), therefore a low concentration could explain why the contamination is not detected in HuD peptide #40. A second explanation could be that the CD8+ T-cell response against HuD peptide #40 is due to another, unrelated, immunogenic contamination.

An important question is where the contamination originated. There are two possible explanations. First, the peptides may have been contaminated during the peptide synthesis at the manufacturer's site; second, cross contamination may have occurred when the peptides were used in our laboratory. We do not have an unopened vial of the original stock peptide from the manufacturer left in order to check the presence of the contamination. Most impurities in synthetic peptides are considered to arise from deletion, additional residue incorporation, truncation and incomplete deprotection during solid phase peptide synthesis[349]. However, this explanation seems very unlikely in our case, because the amino acid sequences of the contaminated HuD peptides and the IE-1 peptide #94 are totally different. Another possibility for contamination during the peptide synthesis is that a peptide of an earlier procedure (e.g., generated during lyophilisation of the IE-1

peptides) has been left behind on the column and has come along with a peptide of the second procedure (here: lyophilization of the HuD peptides). This reasoning assumes that the same column has been used during multiple procedures. In addition, IE-1 #94 is a very hydrophilic peptide, which means that it may have a more than average affinity towards chromatographic columns. Reconstruction of the experiments in which the peptides, both the HuD and the IE-1 peptides, were used in our own laboratory, shows that it is very unlikely that the HuD peptides were contaminated by cross contamination. Both peptides were never used in the same experiment or on the same day, not even within the same month. Also, the fact that the spectra of the contaminating peptide (Figure 4, panel A) and IE-1#94 of our own collection (Figure 4, panel B) were identical, does not reveal the origin of the contamination.

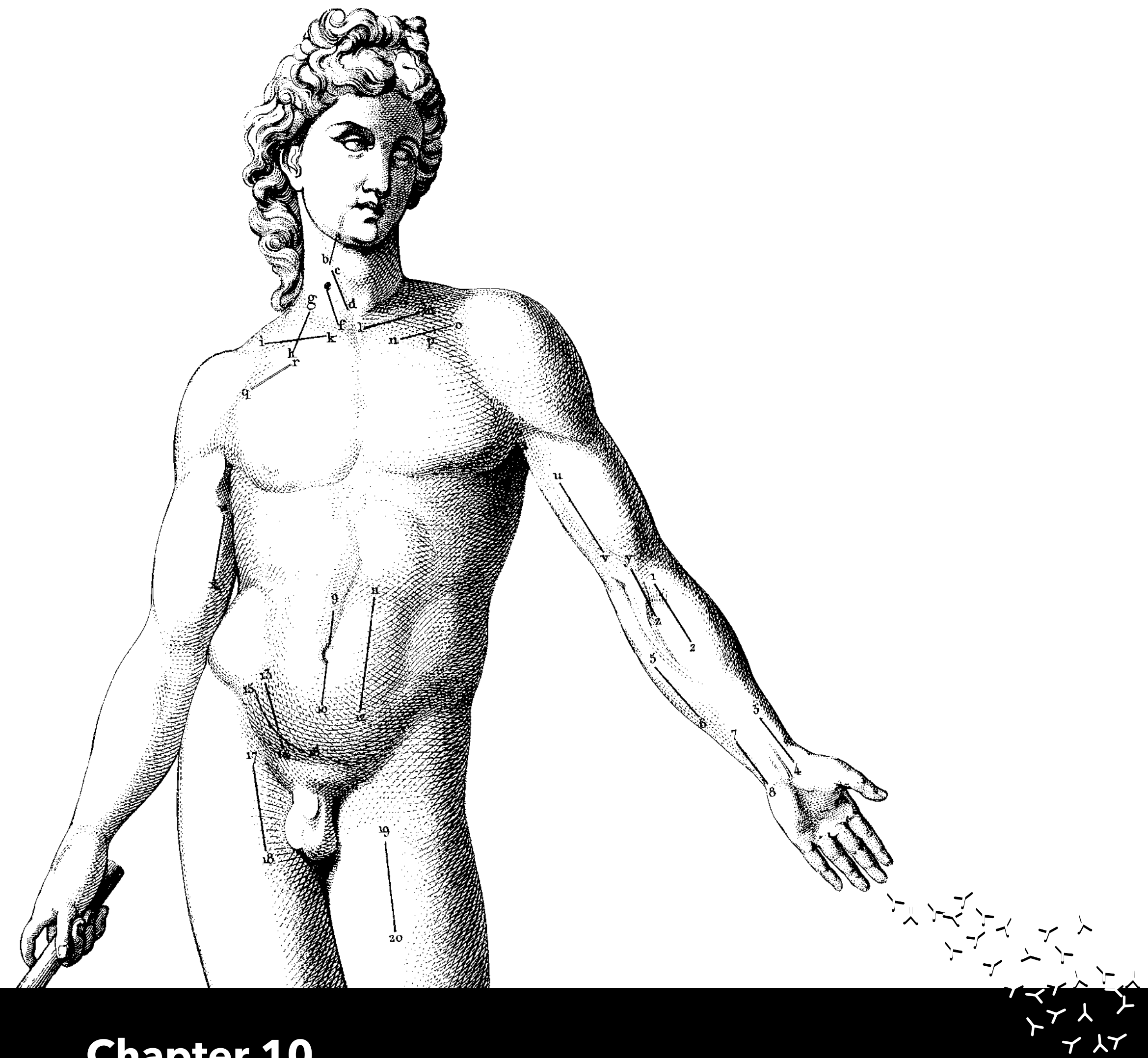
The $CD8^+$ T-cell response has only been observed in CMV seropositive, HLA-B*18 donors. Moreover, the HCMV IE-1 15-mer #94 fits in the binding groove of HLA-B*18. Therefore, we conclude that the $CD8^+$ T-cell response is HLA-B*18-restricted. A further three CMV seropositive, HLA-B*18 donors tested negative in the stimulation assays with the HuD PSPP, which could be explained by absence of a $CD8^+$ T-cell response to IE-1, a difference in fine specificity of the cellular immune response between individuals.

The HuD 15-mer peptides we used had a purity of >70%. Peptides with this level of purity are appropriate for screening studies for antigen-specific T cells and reduce costs significantly in comparison to the use of peptides which are at least 95% pure. For example, in the case of the HuD PSPP >70% purity costs around $18,000, while >95% purity is 2.3x more expensive, around $42,000. Therefore, taking account of the costs it is more reasonable to use peptides of >70% purity in T cell stimulation assays, whereas, in the case of the detection of a potentially relevant T-cell response, resynthesized peptides with a greater purity can be used to confirm this response.

In conclusion, we found false-positive HLA-B*18-restricted $CD8^+$ T-cell responses in a short-term simulation assay with HuD PSPP due to contamination of the PSPP with a CMV-encoded peptide. Therefore, we recommend critical assessment of T-cell responses after stimulation with PSPP. Specifically, relevant T-cell responses should be confirmed with the use of resynthesized peptides, possibly with a purity of 95% or greater.

Acknowledgments

We thank Dr. F. Kern (Department of Immunology, Brighton and Sussex Medical School, Brighton, UK) for helpful discussions and critical reading of the manuscript. We also thank our blood donors for their participation in this study, and Patricia van den Broek and Corrien Groot-van Ruijven for their technical assistance. This work was supported by the Gratama Foundation, Teteringen, The Netherlands.



Chapter 10

Human chorionic gonadotropin treatment of anti-Hu associated paraneoplastic neurological syndromes

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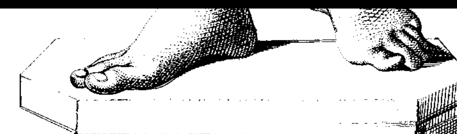
Journal of Neurology, Neurosurgery & Psychiatry 2010;81:1341-4



ABSTRACT

Objective
Paraneoplastic neurological syndromes associated with anti-Hu antibodies (Hu-PNS) are mediated by a T-cell immune response that is directed against the Hu antigens. In pregnancy, many Th1 mediated autoimmune diseases such as rheumatoid arthritis and multiple sclerosis regress. We hypothesized that this decreased disease activity during pregnancy may be related to high human chorionic gonadotropin (hCG) levels.

Methods
We treated 15 Hu-PNS patients in a prospective, uncontrolled and unblinded trial with 10,000 IU daily of hCG administered by intramuscular (i.m.) injection during 12 weeks. Primary outcome measures were functional improvement defined as a decrease of one or more points on the modified Rankin Scale (mRS) or stabilization in patients with mRS≤3 and improvement of neurological impairment assessed with the Edinburgh Functional Impairment Tests (EFIT). Secondary endpoints included the change in activities of daily living (ADL) as evaluated by means of the Barthel Index (BI).

Results
Seven out of 15 patients (47%) improved on the mRS or stabilized at mRS≤3. Four patients (27%) showed significant improvement of neurological impairment as indicated by an overall EFIT score of ≥1 point. Five patients improved on the BI (33%).

Conclusion
Comparison with previous studies suggests that hCG may have immunomodulatory activity and may modify the course of Hu-PNS although well established confounding factors may have contributed in this uncontrolled trial.

INTRODUCTION

The anti-Hu antibody (Hu-Ab) is the most prevalent paraneoplastic antibody[3]. The etiology of paraneoplastic neurological syndromes (PNS) associated with Hu-Ab (Hu-PNS) is believed to be autoimmune and the outcome is generally poor despite aggressive anti-tumor and immunosuppressive treatment[2, 6, 350]. Circumstantial evidence suggests that T-cell reactivity to the onconeural antigen HuD is pivotal for the development of PNS and the consensus in the field is that Hu-PNS can be classified as Th1 mediated organ specific autoimmune disorders, much like diabetes mellitus and rheumatoid arthritis[350].

In many patients with Th1 mediated autoimmune diseases such as rheumatoid arthritis[351] and multiple sclerosis[352], clinical symptoms or number of relapses regress during pregnancy. A prominent feature of early pregnancy is the presence in serum and urine of high levels of human chorionic gonadotrophin (hCG), a heterodimeric glycoprotein hormone that is secreted by syncytiotrophoblast cells of the chorion (an antecedent of the placenta). Because hormonal changes precede and accompany the immune changes in pregnant women, an immunomodulatory role for hCG was hypothesized. Khan et al.[353] tested the effect of hCG on the development of diabetes, a Th1 mediated autoimmune disease, in nonobese diabetic (NOD) mice. They showed that treatment of NOD mice with hCG lowered the increased blood glucose levels, reversed the established inflammatory infiltrate of pancreatic tissue, inhibited the development of diabetes for prolonged time and induced profound inhibition of the functional activity (i.e., production of IFN-gamma) of Th1 cells. The mechanism of action of hCG is probably related to its effects on the activation of NF-kappaB[354], a transcription factor that plays a central role in regulating immune responses, and is down-regulated in pregnancy[355].
We hypothesized that hCG induced inhibition of Th1 cells in Hu-PNS patients would result in clinical improvement or stabilization. hCG has been previously administered in clinical trials treating HIV-related Kaposi's sarcoma[356]. From these studies, we concluded that the daily intramuscular (i.m.) administration of 10,000 IU of hCG was safe. In this uncontrolled and unblinded trial, we treated 15 Hu-PNS patients with daily i.m. injections of 10,000 IU of hCG for 12 weeks.

MATERIALS AND METHODS

Patients
Inclusion criteria for this prospective uncontrolled and unblinded single center study included high serum titers of Hu-Ab (>400) and progression of neurological symptoms over the last three weeks, defined as new symptoms or progression of existing symptoms confirmed by objective neurological examination. From February 2005 through March 2007, we identified 46 new patients with high titer Hu-Ab, 15 of whom were included in the study. The main reasons for not including the remaining 31 patients were poor clinical condition (patient admitted to ICU for mechanical ventilation or patient already in nursing home or palliative unit), lack of progression of neurological symptoms over the last 3 weeks and declining participation by the patient or family. Clinical characteristics of the 15 included patients are summarized in Table 1. All patients were evaluated throughout the study by one of two clinical investigators (JEB, PSS). The study was approved by the Erasmus University Medical Center Institutional Review Board, and all participants gave written informed consent.

Table 1 | Patient characteristics

N°	Age/ Sex	Antibody	Syndrome	Symptoms-Diagnosis (months)	Tumor	Symptoms-Tumor (months)	Tumor treatment	Response tumor	Symptoms-last FU (months)	Dead/ Alive	Cause of Death
1	64/M	Hu	PSN	16	Prostate[a]	16	Chemo	PR	26	Dead	Tumor
2	51/F	Hu	PEM	11	SCLC	31	Chemo, RT	CR	52	Alive	-
3	70/F	Hu	PSN	1	SCLC	3	Chemo	CR	8	Dead	PNS
4	75/M	Hu, Amp	PSN	2	SCLC	1	Chemo, RT	CR	44	Alive	-
5	66/M	Hu	PSN	2	NSCLC	6	Chemo	CR	44	Alive	-
6	55/F	Hu, CV2	PSN, ON	7	SCLC	7	Chemo, RT	CR	25	Dead	Tumor
7	53/F	Hu	PSN	3	SCLC	4	Chemo, RT	CR	43	Alive	-
8	61/F	Hu	PEM	0	SCLC	0	Chemo	CR	1	Dead	Tumor
9	69/F	Hu	PEM	5	SCLC	7	Chemo	PR	11	Dead	PNS
10	66/M	Hu	PCD	3	SCLC	4	No	-	15	Dead	Tumor
11	66/F	Hu	PSN	11	NSCLC	14	Chemo, RT	CR	47	Alive	-
12	61/M	Hu	PCD	4	SCLC	11	Chemo, RT	PD	20	Dead	Tumor
13	68/F	Hu	PSN	2	SCLC	4	Chemo, RT	CR	23	Dead	Tumor
14	61/M	Hu	PSN	2	SCLC	4	Chemo, RT	CR	29	Dead	PNS
15	75/M	Hu	PCD	7	Lung[b]	7	No	-	8	Dead	PNS

[a]Small cell cancer of the prostate

[b]Large mediastinal mass on CT-scan

Amp = anti-amphiphysin; PSN = paraneoplastic sensory neuronopathy; PEM = paraneoplastic encephalomyelitis; ON = optic neuropathy; PCD = paraneoplastic cerebellar degeneration; SCLC = small cell lung cancer; NSCLC = non-small cell lung cancer; RT = radiotherapy; PR = partial remission; CR = complete remission; PD = progressive disease, FU = follow-up.

Treatment

The treatment consisted of hCG (Pregnyl, Organon, Oss, Netherlands; lot 685406) 10,000 IU daily administered by intramuscular (i.m.) injection during 12 weeks. Only Pregnyl batches that effectively suppressed IFN-γ production by $CD4^+$ T cell cultures of NOD mice following stimulation were used[353].

Outcome measures

The primary endpoints of the study were the functional and neurological improvement after 12 weeks of hCG. Functional outcome was considered 'successful' when a patient with mRS≤3 improved or stabilized (i.e. remained ambulatory) and when a patient with mRS≥4 (bedridden patient) improved to ≤3 (ambulatory), after the 12th week of hCG as compared to baseline, as defined by Keime-Guibert et al.[12].

Improvement of neurological impairment was assessed with the Edinburgh Functional Impairment Tests (EFIT) that incorporate objective measures of upper and lower limb function, memory and a rating scale for dysphasia[357]. An overall EFIT=0 indicates no change, EFIT>0 indicates significant neurological improvement and EFIT<0 indicates significant neurological deterioration.

Secondary endpoints included CSF protein and WBC, the Hu-Ab titers in serum and CSF, and the change in activities of daily living (ADL) as evaluated by means of the Barthel Index (BI)[358].

Laboratory evaluations

Serum and CSF were sampled at baseline and after four weeks of hCG treatment. Serum was additionally sampled at weeks 8, 12 and 20. The concentration of hCG was determined routinely in the clinical chemistry laboratory (normal value <1.9 IU/l). IgG titers of the Hu-Ab were determined, as described[6].

Statistical analysis

We compared WBC, total protein concentration and Hu-Ab titers in baseline CSF and CSF obtained after 4 weeks of treatment by means of the Wilcoxon matched pairs test. We used the same test to compare Hu-Ab serum titers at baseline and end of study. P-values were two-sided and a significance level α=0.05 was used. All statistical analyses were performed using GraphPad Prism version 5 software (GraphPad Software, Inc., San Diego, CA).

RESULTS

No adverse or serious adverse events related to hCG were reported. Eight patients received tumor treatment overlapping hCG treatment without complications (Table 2). None of

the patients received any other immunomodulatory or immunosuppressive drugs while on study treatment. Two patients received immunomodulatory treatment in the weeks preceding hCG administration. Both received one course of IVIg (2 g/kg over 5 doses), 6 weeks (patient 7) or 3 weeks (patient 14) prior to inclusion in the study. Both patients got worse both functionally and neurologically after IVIg. The serum and CSF levels of hCG were negligible at baseline. During the study, the CSF level increased to a median 7 IU/l (range 3.4 – 14 IU/l) while the serum level was median 365 IU/l (range 43 – 963 IU/l) at end of study. Eleven of the 15 patients received hCG treatment for the scheduled 12 weeks while four patients did not complete the treatment (Table 2).

Table 2 | Primary endpoints: functional and neurological outcome

N°	Weeks HCG	Chemo during HCG	Successful functional outcome[a]	mRS baseline	Change mRS	Neurological outcome[b]	EFIT baseline	EFIT overall
1	12	Yes	Yes	3	0	**Improve**	2	2
2	12	No	**Yes**	3	**-1**	**Improve**	2	2
3	12	Yes	No	3	1	Worse	2	-2
4	12	Yes	**Yes**	4	**-1**	**Improve**	2	2
5	12	No	Yes	3	0	Stable	2	0
6	12	Yes	Yes	3	0	Stable	2	0
7	12	Yes	No	2	1	Worse	2	-2
8	1	Yes	Died	5	1	N.e.	2	-
9	4	No	No	3	1	N.e.	3	-
10	4	No	Yes	3	0	Worse[c]	3	-1
11	12	No	Yes	2	0	**Improve**	2	1
12	12	No	No	2	1	Worse	2	-1
13	12	Yes	No	2	1	Worse	2	-2
14	12	Yes	No	3	1	Worse	2	-2
15	1	No	No	5	0	N.e.	4	-

[a]Functional outcome was successful when a patient with mRS≤3 improved or stabilized or when a patient with mRS≥4 improved to ≤3[12].
[b]Neurological outcome is improved when the overall EFIT>0; stable when overall EFIT=0; worse when overall EFIT<0[357]
[c]Overall EFIT score determined at week 4.
mRS = modified Rankin Scale; EFIT = Edinburgh Functional Impairment Tests; N.e. = not evaluable.
Improvement on mRS and EFIT in bold.

Primary endpoints

Functional outcome was 'successful' in seven out of 15 patients (47%). Six of twelve patients with mRS≤3 at baseline stabilized (n=5) or improved (n=1) while one of the three patients with a baseline mRS≥4 improved to ≤3 (Table 2).

Improvement of neurological impairment, as indicated by an overall EFIT score >0, was demonstrated in four patients (Table 2). The two patients who improved one point on the mRS also had improvement of neurological impairment.

Secondary endpoints

Improvement in ADL was seen in patients 1, 2, 4, 5 and 11. In all patients the change in BI was minimal (+5 points). The laboratory evaluations revealed a median of 2 WBC/µl (range 0.3 – 38) at baseline versus a median of 5 WBC/µl (range 0.3 – 31) after 4 weeks of treatment. The median CSF protein concentration was 0.6 g/l (range 0.13 – 2.15) at baseline and 0.7 g/l (range 0.14 – 1.89) after 4 weeks while the Hu-Ab titers changed in CSF from median 128 (range 8 – 4096) to median 528 (range 8 – 2048). None of these changes were significant. Also, the change in serum titer from baseline (median 6400, range 400 – 25600) to end of study (median 2400, range 800 – 25600) was not significant. Hu-Ab titers did not correlate with baseline mRS nor with the change in mRS during the study (data not shown).

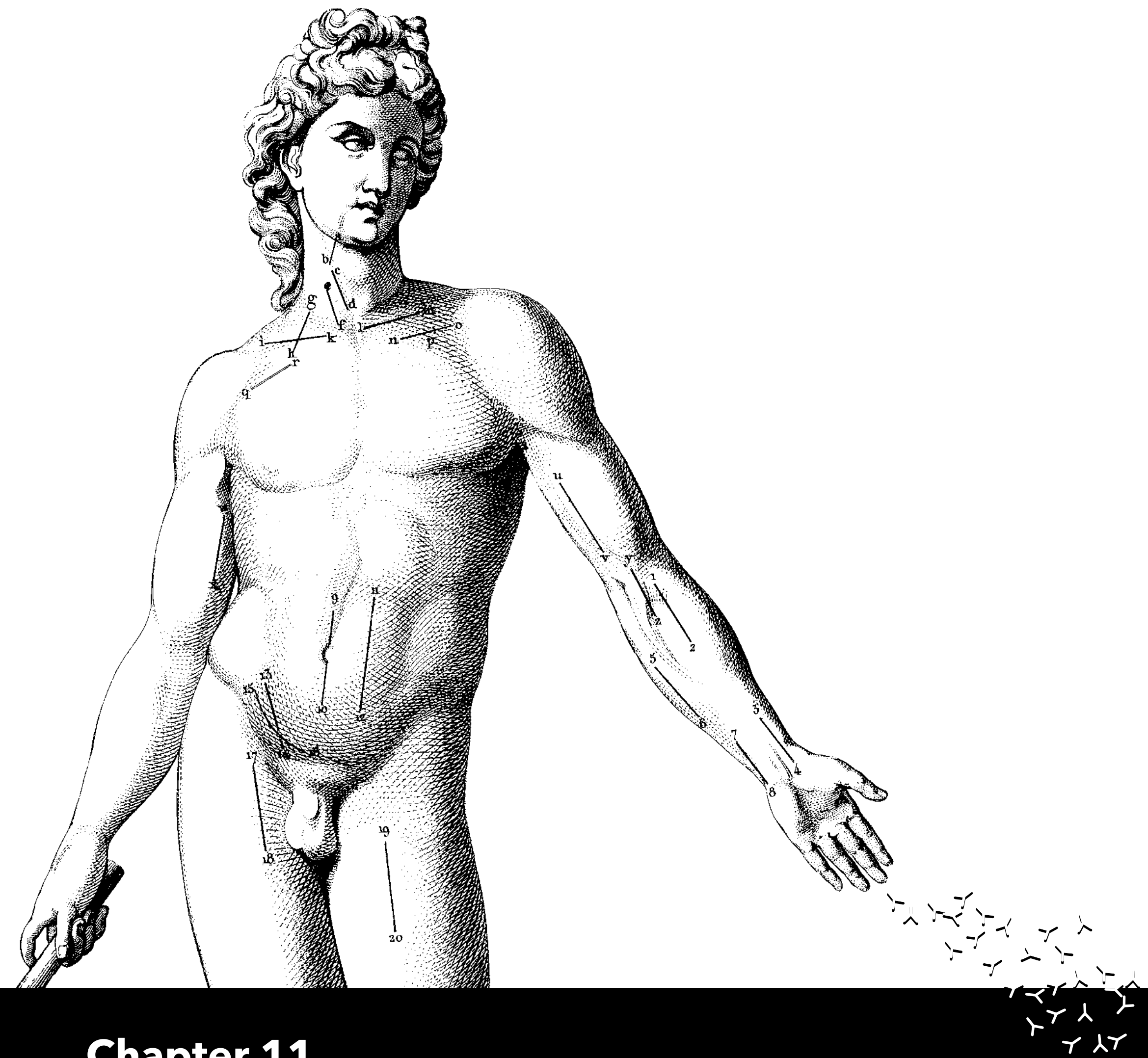
DISCUSSION

With a positive response defined as stabilization at mRS≤3 or improvement[12], treatment was 'successful' in seven out of 15 patients (47%). Four other studies have reported treatment results in Hu-PNS patients employing the same functional outcome criterion. In two retrospective studies, IVIg treatment was successful in six of 17 (35%)[181] while treatment with IVIg, cyclophosphamide and methylprednisolone was successful in two of nine (22%)[12] evaluable Hu-PNS patients. Vernino et al.[11] prospectively treated five Hu-PNS patients with plasma exchange combined with either cyclophosphamide or chemotherapy based on the absence or presence of an underlying tumor. Treatment was successful in three of these five Hu-PNS patients (60%)[11]. Shams'ili et al.[10] prospectively treated eight Hu-PNS patients with rituximab and observed improvement or stabilization at mRS≤3 in four of them (50%).
These studies suggest that immunomodulatory therapy, including hCG, may modify the course of Hu-PNS and obtain clinically useful stabilization in patients with Hu-PNS. However, the patient numbers are small and confounding factors may explain the favorable outcome in some of our patients in this uncontrolled study. Four patients were successful on both primary outcome measurements (patients 1, 2, 4 and 11). Several studies have demonstrated that effective treatment of the tumor is important to at least stabilize Hu-PNS[6, 7]. The successful outcome in patients 1 and 4 may have been confounded by the partial and complete tumor remissions that were achieved during the trial. Patient 2 presented with limbic encephalitis and sensory neuronopathy. During hCG treatment she

regained initiative and her memory subjectively improved. Spontaneous remission of Hu-Ab associated limbic encephalitis has been described and could also have occurred in this patient[6]. Finally, a long interval from symptoms to diagnosis, as observed in patients 1, 2 and 11 (11 -16 months), is also associated with a favorable outcome[6].
The pathogenesis of Hu-PNS is now believed to be mediated by cytotoxic T-cells[350]. Hu-Ab are considered a marker of the immune response rather than being pathogenic and Hu-Ab titers do not correlate well with disease severity nor with its course, as confirmed in this prospective study. In conclusion, seven out of 15 (47%) Hu-PNS patients either functionally improved or stabilized at mRS≤3 after treatment with hCG. Comparison with previous studies suggests that hCG may have immunomodulatory activity and may modify the course of Hu-PNS. However, well established confounding factors may have contributed in this uncontrolled trial.

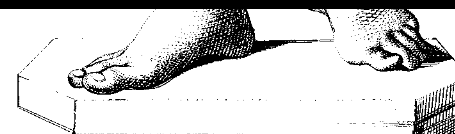
Acknowledgments

The authors gratefully acknowledge the physicians who referred patients and Irene van Heuvel for practical assistance.



Chapter 11

General Discussion



The primary aim of this thesis was to investigate the role of T lymphocytes in the pathogenesis of Hu-antibody associated paraneoplastic neurological syndromes (Hu-PNS) by studying cerebrospinal fluid (CSF) and blood of Hu-PNS patients.

CEREBROSPINAL FLUID

In this thesis, we used flow cytometric immunophenotyping to study CSF. This technique is mostly used for detection of leptomeningeal metastasis of haematological malignancies. Additionally, in research settings it is performed to study immune responses occurring in the CNS in neuro-inflammatory diseases, including PNS. CSF flow cytometry facilitates the detection of a large spectrum of cellular characteristics on one single cell, determination of absolute cell numbers and detection of rare events, with a high sensitivity as well as a high specificity (**Chapter 3**). By studying which leukocyte and lymphocyte populations are present in CSF, their frequency and activation status, more characteristics about the ongoing immune response in the CNS of Hu-PNS patients will be gathered.
However, the low CSF leukocyte concentration (normal range: ≤5 per µl; Hu-PNS patients: median 3, range 1-8 per µl[45]) with a rapidly decreasing viability after withdrawal from the body[211, 230, 257-259] is a huge challenge in CSF flow cytometry. To analyze leukocyte and lymphocyte subsets reproducibly, measuring a sufficient cell number is required[359]. CSF cell viability is increased by addition of serum-containing medium to CSF directly after sampling. This prevents immediate cell death and allows longer preservation of CSF cells (**Chapter 4**), which results in higher cell yields in order to optimally study leukocyte and lymphocyte subsets. Therefore, we added serum-containing medium to CSF in all our CSF studies.
Another important requirement to use CSF immunophenotyping as a research tool in Hu-PNS, is knowledge of the normal composition of CSF. Therefore, we generated reference values of absolute numbers and percentages of leukocyte and lymphocyte subsets by studying CSF of 84 individuals without neurological disease undergoing spinal anaesthesia for surgery (**Chapter 5**). In addition, we showed that normal CSF mainly contains central memory $CD4^+$ T lymphocytes, which may have selectively been recruited to the CNS to function as immune surveillants. The presence of these cells in CSF would facilitate quick responses to antigens and induction of an adaptive immune response, aided by dendritic cells, which were also present in normal CSF.
Subsequently, the lymphocyte subsets in CSF of Hu-PNS patients were compared to cancer and non-cancer controls, which showed different changes (**Chapter 6**). The significant absolute (~20x) and relative (~3x) B lymphocyte expansion indicates significant B-lymphocyte recruitment to the CNS and the potential for autoantibody

production[253], which may explain the intrathecal synthesis of Hu-IgG in PNS. Another role of B lymphocytes in CSF of Hu-PNS patients may be enhancing antigen presentation to T lymphocytes as demonstrated for other autoimmune diseases[254]. Also, the numbers of $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes and NK cells were absolutely increased (~4-7x) in Hu-PNS, suggesting either a role of these cells in the neuronal degeneration or merely a non-specific inflammatory phenomenon as it affects most lymphocyte subsets. Finally, the relative reduction of NKT lymphocytes in CSF of Hu-PNS patients supports the autoimmune hypothesis of Hu-PNS as dysfunction or deficiency of these cells is also described in other autoimmune diseases[314, 315]. All these findings together confirm the presence of both T- and B-lymphocyte mediated immune responses in Hu-PNS.
Consequently, the question arises if these higher numbers of T lymphocytes in CSF of Hu-PNS patients consist of or contain HuD-specific T lymphocytes. Although detection of HuD-specific T lymphocytes in blood of Hu-PNS patients appeared to be very difficult[38, 58-60], it seems worthwhile to focus on antigen-specific T lymphocytes in CSF of Hu-PNS patients. Because in patients with CNS viral infections and multiple sclerosis, the frequency of antigen-specific T lymphocytes was found to be higher in CSF than in blood[53-55, 360]. Unfortunately, we were not able to detect HuD-specific $CD8^+$ T lymphocytes with either IFN-γ enzyme-linked immunosorbent spot-forming assays (ELISPOT) or multimer staining (**Chapter 7**). These negative results are in contrast with a recent publication, in which the detection of HuD157-specific $CD8^+$ T lymphocytes in CSF of 1 HLA-A*0201 Hu-PNS patient by direct staining with tetramers is described[61]. Remarkably, these HuD157-specific $CD8^+$ T lymphocytes were shown to exhibit a different phenotype, producing type 2 cytokines including IL-13 and IL-5 instead of IFN-γ [61], which is an explanation for the failure to detect HuD-specific T lymphocytes in our IFN-γ ELISPOT assay. Furthermore, in our multimer staining we did not include this specific 9-mer peptide (HuD157). Also, our results could be false-negative due to insufficient sensitivity of our assays, lack of an antigen-specific positive control or preferential expansion of non-HuD reactive T lymphocytes. However, the HuD157-specific $CD8^+$ T lymphocytes were observed in only 1 Hu-PNS patient[61]. Therefore, this result has to be confirmed in a larger group of patients before our negative results can be considered false-negative.
Concluding, although CSF seems to be the right compartment to study in search for T lymphocytes involved in Hu-PNS as CSF reflects ongoing immune responses in the CNS, and the CSF of Hu-PNS patients showed T-lymphocyte imbalances compared to controls, the presence of HuD-specific $CD8^+$ T lymphocytes in CSF could not be demonstrated.

BLOOD

In blood of Hu-PNS patients, different imbalances are observed compared with SCLC patients and healthy controls. With regard to lymphocyte subsets, the number of B, $CD4^+$ and $CD8^+$ T lymphocytes were decreased[50], while the proportions of activated $CD4^+$ and $CD8^+$ T lymphocytes and $CD4^+$ memory T lymphocytes were increased[41, 50]. Moreover, reduced numbers of terminally differentiated effector $CD8^+$ T lymphocytes and lymphocytes with a cytotoxic phenotype ($CD56^+$ and $CD57^+$) were observed[50] and expression levels of FOX-P3, TGF-β and CTLA-4 mRNA in regulatory T lymphocyte subsets were down-regulated suggesting dysfunctional regulatory T lymphocytes[51]. Also, a NKT lymphopenia was observed (**Chapter 6**). Overall, these findings suggest the occurrence of T-lymphocyte mediated immune responses and presence of HuD-specific T lymphocytes in blood of Hu-PNS patients. Several groups have focused on detection of these HuD-specific T lymphocytes in blood of Hu-PNS patients, but were unsuccessful[38, 58-60]. However, a recent publication showed the presence of HuD-specific $CD8^+$ T lymphocytes in blood of 3/3 Hu-PNS patients[61].

Instead of T-lymphocyte stimulation assays, we used an alternative approach in the search for HuD-specific T lymphocytes, and looked for possible human leukocyte antigen (HLA)-association in Hu-PNS (**Chapter 8**). In a wide range of autoimmune diseases, HLA-association is an important factor in an individual's susceptibility to the disease[321] and knowledge of the involved auto-antigen (HuD) together with specific disease-associated HLA antigens, may lead to detection of HuD-specific T lymphocytes and subsequent epitope identification. In our study, we found an association of Hu-PNS with presence of HLA-DQ2 and -DR3 antigens, HLA class II antigens. This indicates a role for $CD4^+$ T lymphocytes in the pathogenesis of Hu-PNS.

The role of T lymphocytes in Hu-PNS is also supported by the results of our clinical trial, in which we treated 15 Hu-PNS patients with 10,000 IU daily of human chorionic gonadotropin (hCG) administered by intramuscular injection during 12 weeks (**Chapter 10**). Seven out of 15 patients (47%) improved on the modified Rankin Scale (mRS) or stabilized at mRS≤3, 4 patients (27%) showed significant improvement of neurological impairment as indicated by an overall Edinburgh Functional Impairment Tests (EFIT) score ≥1 point and 5 patients (33%) improved on the Barthel Index (BI). Although confounding factors, including additional anti-tumor therapy and spontaneous remission described in some of the PNS, may have contributed in this uncontrolled trial, these results indicate that hCG may have immunomodulatory activity and may modify the course of Hu-PNS. In mice, hCG induced inhibition of functional activity of Th1 lymphocytes[353], a subset of $CD4^+$ T lymphocytes involved in Th1-mediated autoimmune disease including diabetes and MS[352]. Although frequencies and functional activity of T lymphocytes were not determined in this clinical trial, possibly the inhibition of Th1 lymphocytes in the Hu-PNS patients after hCG treatment is responsible for clinical improvement or stabilization of the disease.

Another issue in Hu-PNS is the low frequency (less than 1%) of Hu-PNS despite expression of the HuD antigen in all SCLC[7]. One possible explanation is HLA-association[321]. Although we found an HLA class II association in Hu-PNS, this association was not absolute. Only 62% of Hu-PNS patients was HLA-$DQ2^+$ and 47% HLA-$DR3^+$ (**Chapter 8**). In general, HLA-associated diseases are usually the result of the combination of different HLA antigens expressed at various loci rather than the result of one HLA variant only[330]. To confirm if HLA type determines development of Hu-PNS in SCLC patients or not, further studies are warranted. An alternative explanation for the low frequency of Hu-PNS was recently proposed by the introduction of a novel hypothesis about the development of Hu-PNS, in which a natural immune response to the HuD antigen is absent and HuD tolerance has to be broken by some kind of tissue distress[21]. Apparently, this tissue distress is present in a minority of SCLC patients, given that only a small population of SCLC patients succumbs to neuronal degeneration. This hypothesis has to be further investigated.

In conclusion, both association of Hu-PNS with HLA-DQ2 and HLA-DR3, and neurological improvement or stabilization upon treatment with hCG confirm the involvement of T lymphocytes in Hu-PNS.

FUTURE STUDIES

First, CSF studies in Hu-PNS patients should be extended. The massive B-lymphocyte expansion in CSF of Hu-PNS patients (**Chapter 6**) has to be further characterized by determining the numbers of different B-lymphocyte subsets, including naïve and memory B lymphocytes, plasma blasts and plasma cells as is also performed in MS[69]. In addition, the presence and frequencies of various T-lymphocyte subsets, including regulatory, activated, suppressor and effector T lymphocytes can be studied. The characterization of B- and T-lymphocyte subsets in CSF will show us the differences in Hu-PNS patients compared to controls indicating which cells are involved in the neurological autoimmune response.

Second, the detection of HuD-specific $CD8^+$ T lymphocytes in 3/3 Hu-PNS patients[61] should be confirmed in a larger patient group both in blood as well as CSF. For blood, sorted $CD8^+$ T lymphocytes will be stimulated with autologous dendritic cells and either HuD133-peptide in HLA-A*0301^+ Hu-PNS patients or HuD157-peptide in HLA-A*0201^+ Hu-PNS patients. After 8 days of stimulation, HuD133- or HuD157-tetramer staining will be performed to identify the specific $CD8^+$ T lymphocytes, while CSF is directly stained with HuD133- or HuD157-tetramers. Although, it should be taken into account that different kinds of T lymphocytes may be present in different Hu-PNS patients[361], it should be

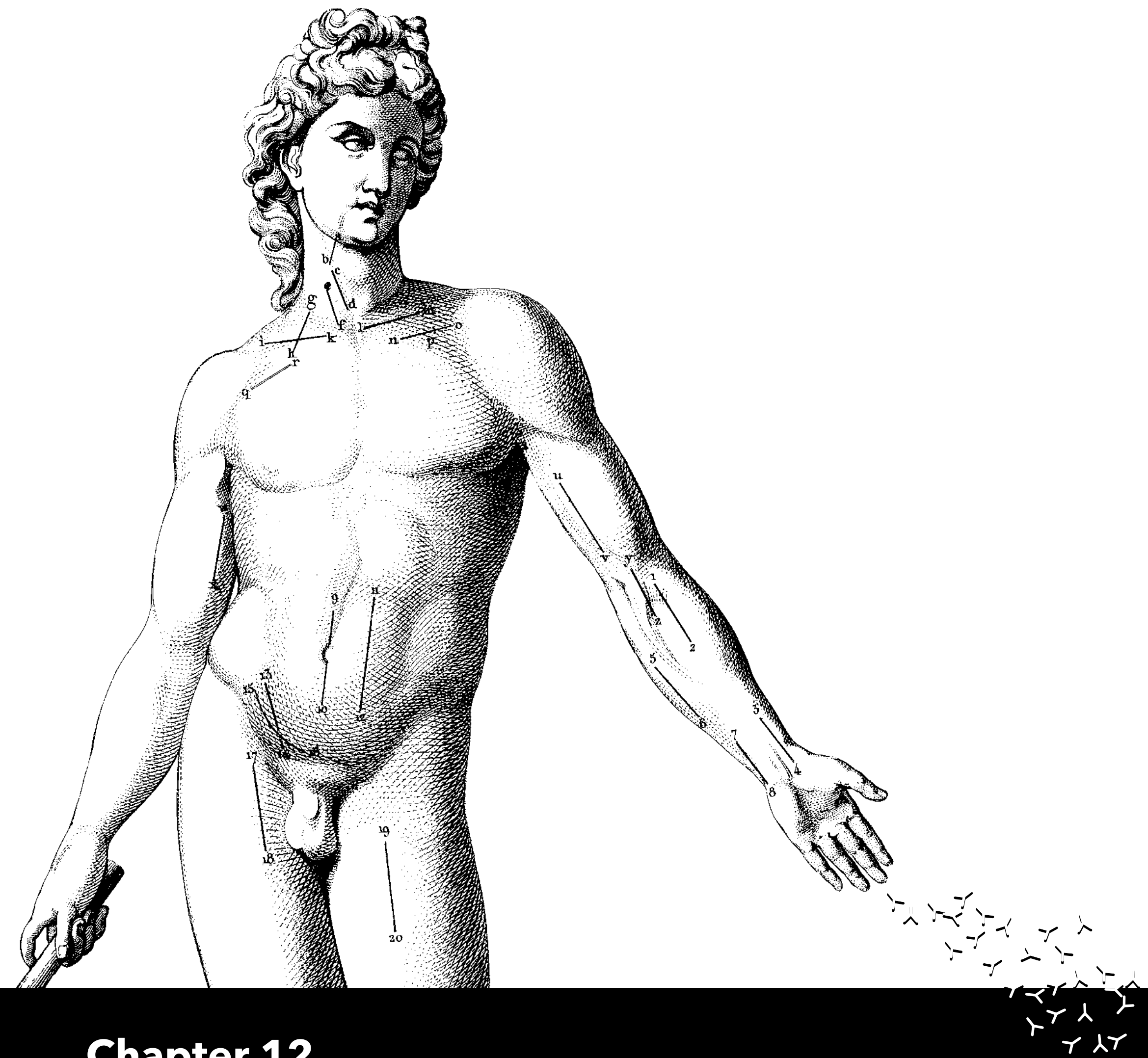
possible to detect HuD-specific $CD8^+$ T lymphocytes in at least a part of Hu-PNS patients as these cells were demonstrated in only 3/3 patients[61].

Third, the association of Hu-PNS with HLA-DQ2 and -DR3 suggests involvement of $CD4^+$ T lymphocytes in Hu-PNS. Additionally, the high titers of IgG1 isotype anti-Hu antibodies indicate help from HuD-specific $CD4^+$ T lymphocytes to B lymphocytes[34, 362, 363]. Furthermore, in an earlier study the presence of HuD-specific $CD4^+$ T lymphocytes was suggested by proliferation of $CD4^+$ T lymphocytes upon HuD protein stimulation[41]. To search for HuD-specific $CD4^+$ T lymphocytes, the 'reversed immunology approach' can be used. Because epitope immunogenicity is correlated with HLA binding[364], this method starts with identifying which HuD-derived 15-mer peptides have optimal binding to HLA-DQ2 and -DR3. By using iTopia™ Epitope Discovering System (Beckman Coulter, Fullerton, CA)[61, 365-367], peptide binding, affinity and dissociation rate of an overlapping peptide library of an antigen can be determined for certain HLA molecules. This method lead to the detection of HuD-specific $CD8^+$ T lymphocytes in Hu-PNS patients[61]. Also, in Yo-PNS with high titer anti-Yo antibodies directed against cdr2 proteins, a previously unidentified cdr2 epitope could be identified, which induced the detection of cdr2-specific cytotoxic T lymphocytes[365]. However, iTopia™ is now only available for class I HLA molecules, but will be developed for class II molecules in future. Alternatively, screening for HLA class II binding has been described with other peptide-binding assays[368, 369], which possibly could be used in Hu-PNS. After identification of HLA-DQ2 and -DR3-restricted HuD 15-mer peptides, these peptides can be used in stimulation assays with T lymphocytes of Hu-PNS patients with addition of autologous dendritic cells as antigen-presenting cells, because addition of these cells improves sensitivity[370]. Next, fluorescently labeled tetramers of HLA-DQ2 molecules presenting the corresponding HuD peptides can be used to detect HuD-specific T lymphocytes, although HLA class II tetramers have very limited success up till now[371-374]. Alternatively, intracellular cytokine staining with a cocktail of cytokines can be performed on stimulated T lymphocytes[375]. This cytokine cocktail should contain type 1 cytokines, e.g., IFN-γ, as well as type 2 cytokines, e.g., IL-5 and IL-13.

Fourth, the most important compartment to search for HuD-specific T lymphocytes is tumor or affected nervous tissue as indicated by observation of inflammatory T lymphocyte infiltrates in neuronal[34, 39, 40, 44], as well as tumor tissue[39, 44] in PNS. After identification and isolation of HuD-specific T lymphocytes in these tissues, they can be further characterized by detailed analysis of the T cell receptors (TCR)[39, 44]. Unfortunately, it is very difficult to obtain considerable amounts of tumor tissue, because the majority of SCLC patients is treated with chemotherapy following a diagnostic bronchoscopy[376], which yields only small amounts of tumor tissue. Brain biopsies are never routinely performed in Hu-PNS patients. Furthermore, post-mortem tissue of Hu-PNS patients could be studied. However, acquiring this tissue is also difficult due to the low incidence of Hu-PNS. Moreover, the

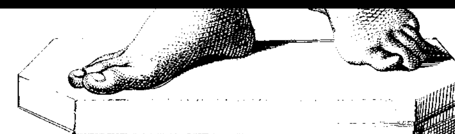
inflammatory process may be extinct in this tissue and HuD-specific T lymphocytes might not be detectable anymore.

The ultimate goal of Hu-PNS research is to develop an immune therapy modality against SCLC without harming the nervous system and, more specifically, contribute to development of targeted immunotherapy of Hu-PNS. Understanding the immunopathogenesis of neuronal damage in Hu-PNS and of tumor control in SCLC patients with anti-Hu antibodies is a first step towards this goal. This knowledge may also be applicable to other tumors, and other neurological and systemic autoimmune disorders.



Chapter 12

Summary/Samenvatting



SUMMARY

Paraneoplastic neurological syndromes (PNS) are remote effects of cancer, by definition not caused by invasion of the tumor or its metastases, nor by infection, ischemia, metabolic and nutritional deficits, surgery or other forms of tumor treatment. The diagnosis and clinical management of PNS are reviewed in **Chapter 2**. One of the most frequently involved 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 associated with Hu-antibodies (Hu-PNS).
In PNS, ectopic expression of onconeuronal antigens in the tumour has been suggested to trigger an immune response also reacting with structurally related antigens expressed in the nervous system. This immune response inhibits tumor growth. However, the price of tumor control is high, as PNS are devastating neurological syndromes leaving most of the patients severely disabled within a few months. Findings supporting a pathogenic role for Hu antibodies include the strong correlation between high titers of Hu antibodies in serum and CSF with development of PNS, the intrathecal synthesis and deposits of Hu antibodies in the central nervous system (CNS), and the highly restricted expression of Hu antigens by the nervous system and the tumor. However, a direct pathogenic role for Hu antibodies in the pathogenesis of Hu-PNS has never been proven. Alternatively, a role for the cellular immunity in the pathogenesis of Hu-PNS is suggested. Autopsy studies in Hu-PNS patients show extensive inflammatory infiltrates of $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes and plasma cells in affected nervous tissue. Oligoclonal and monoclonal cytotoxic T-lymphocyte clones are detected in nervous and tumour tissue and in 78% of Hu-PNS patients also in peripheral blood.
The primary aim of this thesis was to further investigate the role of T cells in the pathogenesis of Hu-PNS by studying CSF and blood of Hu-PNS patients with several immunological techniques, including flow cytometry, enzyme-linked immunosorbent spot-forming assay (ELISPOT) and an HLA-association study.

The first compartment we investigated was CSF, which we studied by using flow cytometry. In **Chapter 3** the use of flow cytometry in CSF is reviewed. Flow cytometry of CSF is mainly used in patients suspected of leptomeningeal localization of haematological malignancies. Additionally, in several neuro-inflammatory diseases including PNS, CSF flow cytometry is performed in order to investigate the immunopathogenesis of these diseases. This technique facilitates the detection of a large spectrum of cellular characteristics on one single cell, determination of absolute cell numbers and detection of rare events, with a high sensitivity as well as a high specificity. Technically, the low cellularity together with a rapidly declining viability make CSF flow cytometry challenging. To increase cellular viability in CSF in order to improve accuracy of CSF immunophenotyping and cell counting, we investigated the effect of adding serum-containing medium to CSF directly after sampling in **Chapter 4**. We showed that addition of serum-containing medium prevents immediate cell death and allows longer preservation of CSF cells prior to flow cytometric or microscopic analysis. Without addition of medium, immediate cell loss will result in underestimation of the number of cells present in CSF and may decrease the sensitivity of flow cytometry in the diagnosis of leptomeningeal localization of hematological malignancies.
In order to use CSF flow cytometry as a diagnostic and research tool in neuro-inflammatory diseases including PNS, reference values of white blood cell (WBC) subsets in CSF are needed. **Chapter 5** describes absolute numbers and percentages of leukocyte and lymphocyte subsets in 84 CSF samples of individuals without neurological disease determined by 6-colour flow cytometry. Beside generation of reference values, we showed that normal CSF predominantly contains T lymphocytes, with a central memory phenotype, while B lymphocytes are nearly absent. In addition, we observed the presence of dendritic cells, both myeloid and plasmacytoid subsets, in CSF. These findings suggest that under normal conditions, the adaptive immune system is active in CSF, ready to quickly respond to invading, potentially dangerous, antigens.
By comparing CSF lymphocyte subsets in 12 Hu-PNS patients with 15 cancer and 27 non-cancer controls in **Chapter 6**, we were able to analyze the CSF pleocytosis generally found in Hu-PNS patients. This provided further insight into the immune pathogenesis of Hu-PNS. We found several striking imbalances in the CSF lymphocyte subsets in Hu-PNS, including 20-fold higher B lymphocyte numbers, 4-fold higher T lymphocyte numbers and a NKT lymphocyte deficiency. This suggests not only a role for B lymphocytes in intrathecal Hu-antibody production and antigen presentation, but also the involvement of T lymphocytes is confirmed, while the autoimmune hypothesis in Hu-PNS is supported by the NKT cell shortage.
This T lymphocytosis in CSF indicates a role for T lymphocytes in the pathogenesis of Hu-PNS. In addition, T lymphocytes in CSF may reflect a T lymphocyte response in the CNS parenchyma. Therefore, we studied the presence of HuD-specific T lymphocytes in CSF of 13 Hu-PNS patients in **Chapter 7**. CSF was prospectively collected and CSF-derived T lymphocytes were expanded and assayed for HuD reactivity using HuD-derived peptides in IFN-γ ELISPOT assays. Additionally, fresh and expanded CSF-derived T lymphocytes 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 lymphocytes were detected with either method. This finding questions the hypothesized role of HuD-specific T lymphocytes in the immunopathology of Hu-PNS, although our results could be false-negative.

The other compartment we studied was blood. We further investigated the role of T lymphocytes in the pathogenesis of Hu-PNS by determining whether human leukocyte antigen (HLA) association plays a role in Hu-PNS. Additionally, we aimed to explain the low incidence of Hu-PNS. In **Chapter 8**, we compared the HLA types of 55 Hu-PNS patients with 24 SCLC patients and 2440 healthy blood bank donors. All patients and donors were Caucasian. The significant higher frequencies of HLA-DQ2 and HLA-DR3 in the Hu-PNS patients suggest that HLA-DQ2$^+$ and HLA-DR3$^+$ SCLC patients have a higher predisposition for development of Hu-PNS. Furthermore, a role for CD4$^+$ T lymphocytes in Hu-PNS is indicated. Knowledge of the involved auto-antigen (HuD) together with specific disease-associated HLA class II alleles (DQ2 and DR3) which we describe here, may lead to detection of HuD-specific CD4$^+$ T lymphocytes in HLA-DR3$^+$/DQ2$^+$ Hu-PNS patients and subsequent epitope identification. However, the low incidence of Hu-PNS despite expression of the HuD-antigen in all SCLC can not be explained with this study.
During our earlier search for HuD-specific T lymphocytes in blood, we encountered false-positive CD8$^+$ T-lymphocyte responses in a short-term simulation assay with HuD protein-spanning peptide pools (PSPP) that are analyzed in **Chapter 9**. Our data reveal that synthetic PSPP may contain immunogenic contaminations which may cause false-positive results in T-lymphocyte stimulation assays. Therefore, we recommend critical assessment of T-lymphocyte responses after stimulation with PSPP. Specifically, relevant T-lymphocyte responses should be confirmed with the use of resynthesized peptides, possibly with a purity of 95% or greater.

Finally, **Chapter 10** reports on the treatment of Hu-PNS patients with human chorionic gonadotropin (hCG). In this prospective, uncontrolled, unblinded trial, 15 Hu-PNS patients were treated with 10,000 IU daily of hCG administered by intramuscular injection during 12 weeks. Seven out of 15 patients (47%) improved on the modified Rankin Scale (mRS) or stabilized at mRS≤3. Four patients (27%) showed significant improvement of neurological impairment as indicated by an overall Edinburgh Functional Impairment Tests (EFIT) score ≥1 point. Five patients (33%) improved on the Barthel Index (BI). These results indicate that hCG may have immunomodulatory activity and may modify the course of Hu-PNS, although well established confounding factors may have contributed in this uncontrolled trial. Moreover, because hCG induces inhibition of Th1 lymphocytes, the results of this clinical trial further support the role of T lymphocytes in Hu-PNS.

In this thesis, the role of T lymphocytes in the pathogenesis of Hu-PNS is confirmed by (i) 4-fold higher T lymphocyte numbers in CSF of Hu-PNS patients compared to controls; (ii) association of Hu-PNS with HLA-DQ2 and HLA-DR3; and (iii) neurological improvement or stabilization upon treatment with hCG. However, the presence of HuD-specific CD8$^+$ T cells in CSF could not be demonstrated. In **Chapter 11**, we propose future studies directed at the continued search for HuD-specific T lymphocytes in Hu-PNS, as more research is required to either confirm or reject a role for T lymphocytes in these syndromes.

Samenvatting

Paraneoplastische neurologische syndromen (PNS) zijn bij-effecten van kanker die per definitie niet direct veroorzaakt worden door ingroei van de tumor zelf of door metastasen, noch door infectieuze, vasculaire, metabole of met de behandeling samenhangende oorzaken. De klinische benadering van PNS wordt besproken in **Hoofdstuk 2**. De meest voorkomende tumor bij PNS is kleincellig longcarcinoom (KCLC). Bij ongeveer 50% van de patiënten met PNS en KCLC kunnen Hu antistoffen worden aangetoond. Dit proefschrift richt zich op PNS geassocieerd met Hu antistoffen (Hu-PNS).
Door aberrante expressie van neuronale eiwitten in de tumor, zogenaamde onconeuronale antigenen, wordt in PNS een immuunrespons uitgelokt die niet alleen gericht is tegen de tumor, maar ook tegen neuronale eiwitten in het zenuwstelsel. Deze immuunrespons resulteert in een betere tumor gerelateerde prognose, maar naast dit gunstige effect veroorzaakt PNS ook ernstige neurologische syndromen waarbij patiënten binnen enkele maanden zeer geïnvalideerd kunnen raken. De pathogene rol van de Hu antistoffen wordt ondersteund door de sterke correlatie van hoge titers Hu antistoffen in serum en liquor met de ontwikkeling van PNS, de intrathecale synthese van Hu antistoffen in het centrale zenuwstelsel (CZS) en de expressie van Hu antigenen die beperkt is tot het zenuwstelsel en de tumor. Een direct pathogene rol voor Hu antistoffen in de pathogenese van Hu-PNS kon echter nooit bevestigd worden. Daarnaast wordt een rol voor de cellulaire immuniteit in de pathogenese van Hu-PNS verondersteld. Autopsie studies in Hu-PNS patiënten tonen uitgebreide inflammatoire infiltraten met $CD4^+$ en $CD8^+$ T lymfocyten, B lymfocyten en plasma cellen in aangedaan zenuwweefsel. Tevens zijn oligoclonale en monoclonale cytotoxische T-lymfocyten aangetoond in zenuw- en tumorweefsel en bij 78% van de Hu-PNS patiënten ook in bloed.
Het doel van de in dit proefschrift beschreven studies was meer inzicht te krijgen in de mogelijke rol van T cellen in de pathogenese van Hu-PNS door onderzoek van liquor en bloed van Hu-PNS patiënten met verschillende immunologische technieken, zoals flow cytometrie, enzyme-linked immunosorbent spot-forming assays (ELISPOT) en een HLA-associatie studie.

Allereerst hebben we liquor onderzocht met behulp van flow cytometrie. In **Hoofdstuk 3** wordt het gebruik van flow cytometrie in liquor beschreven. Flow cytometrie in liquor wordt voornamelijk gebruikt bij patiënten die verdacht worden van leptomeningeale lokalisatie van hematologische maligniteiten. Daarnaast wordt liquor flow cytometrie gebruikt voor onderzoek naar de immuunpathogenese van verschillende neuro-inflammatoire aandoeningen zoals PNS. Met deze techniek is het mogelijk om een groot aantal kenmerken van één enkele cel tegelijkertijd te bepalen. Daarnaast kunnen absolute cel aantallen bepaald worden en zeldzame cellen gedetecteerd worden met een hoge sensitiviteit en specificiteit. Flow cytometrie in liquor is echter een uitdagende techniek door het lage cel aantal met een snel dalende viabiliteit. Om de cel viabiliteit in liquor te verbeteren en daarmee de nauwkeurigheid van liquor immuunfenotypering en celtelling te vergroten, hebben we het effect van toevoeging van serumhoudend medium direct na liquor afname onderzocht in **Hoofdstuk 4**. Toevoeging van serumhoudend medium voorkomt directe celdood in liquor en maakt het mogelijk liquor cellen langer te bewaren totdat flow cytometrische of microscopische analyse plaatsvindt. Zonder toevoeging van medium kan directe celdood resulteren in onderschatting van het aantal cellen in liquor en hiermee wordt de sensitiviteit van flow cytometrie in de diagnostiek van leptomeningeale lokalisatie van hematologische maligniteiten lager.
Om liquor flow cytometrie te kunnen gebruiken voor diagnostiek en research doeleinden in neuro-inflammatoire aandoeningen zoals PNS, zijn referentiewaarden van leukocyten subsets in liquor nodig. **Hoofdstuk 5** beschrijft de absolute aantallen en percentages van leukocyten en lymfocyten subsets bepaald met 6-kleuren flow cytometrie in 84 liquor monsters van personen zonder neurologische aandoening. Deze studie laat zien dat normale liquor voornamelijk T lymfocyten met een central memory fenotype bevat, terwijl B lymfocyten zeer schaars zijn. Ook dendritische cellen, zowel myeloïde als plasmacytoïde, zijn aanwezig in liquor. Deze bevindingen suggereren dat onder fysiologische omstandigheden het adaptieve immuunsysteem actief is in liquor, klaar om direct te reageren op binnenkomende, potentieel gevaarlijke, antigenen.
In **Hoofdstuk 6** wordt de liquor pleiocytose die meestal voorkomt in Hu-PNS geanalyseerd door de liquor lymfocyten subsets in 12 Hu-PNS patiënten te vergelijken met 15 kankerpatiënten en 27 controles zonder kanker. Dit geeft meer inzicht in de immuunpathogenese van Hu-PNS. Verschillende verschuivingen binnen de liquor lymfocyten subsets in Hu-PNS waren zichtbaar, zoals een 20x hoger aantal B lymfocyten, een 4x hoger aantal T lymfocyten en een lager aantal NKT lymfocyten. Dit wijst op een rol voor B lymfocyten in de intrathecale Hu-antistof productie en antigeen presentatie. Tevens wordt de betrokkenheid van T lymfocyten bevestigd, terwijl het tekort aan NKT cellen de autoimmuun hypothese in Hu-PNS ondersteunt.
De T lymfocytose in liquor suggereert een rol voor T lymfocyten in de pathogenese van Hu-PNS. Daarnaast zijn de T lymfocyten in liquor mogelijk een afspiegeling van de T lymfocyt respons die plaats vindt in het CZS parenchym. In **Hoofdstuk 7** hebben we daarom gekeken naar de aanwezigheid van HuD-specifieke T lymfocyten in liquor van 13 Hu-PNS patiënten. Na prospectieve verzameling van liquor, werden T lymfocyten uit liquor aspecifiek geëxpandeerd en getest op reactiviteit met HuD peptiden in IFN-γ ELISPOT assays. Tevens werden zowel verse als geëxpandeerde T lymfocyten uit liquor

getest op binding met HuD-specifieke HLA klasse I multimeren. Hoewel bij 12 van de 13 Hu-PNS patiënten inflammatoire veranderingen in de liquor gezien werden, konden er geen HuD-specifieke $CD8^+$ T lymfocyten aangetoond worden. Deze bevinding trekt de veronderstelde rol van HuD-specifieke T lymfocyten in de immuunpathologie van Hu-PNS in twijfel, hoewel onze resultaten ook vals-negatief kunnen zijn.

Naast liquor hebben we ook bloed onderzocht. Door te bepalen of humaan leukocyten antigeen (HLA) associatie een rol speelt in Hu-PNS, werd de rol van T lymfocyten in de pathogenese van Hu-PNS verder bestudeerd. Daarnaast zochten we naar een verklaring voor de lage incidentie van Hu-PNS. In **Hoofdstuk 8** worden de HLA typeringen van 55 Hu-PNS patiënten vergeleken met 24 KCLC patiënten en 2440 gezonde bloedbank donoren, allen Kaukasisch. De significant hogere frequenties van HLA-DQ2 en HLA-DR3 in de Hu-PNS patiënten suggereert dat HLA-$DQ2^+$ en HLA-$DR3^+$ KCLC patiënten een hogere predispositie hebben voor het ontwikkelen van Hu-PNS. Bovendien wijst dit op een rol voor $CD4^+$ T lymfocyten in Hu-PNS. Kennis van het betrokken auto-antigeen (HuD) in combinatie met specifieke ziekte-geassocieerde HLA klasse II allelen (DQ2 and DR3), kan leiden tot detectie van HuD-specifieke $CD4^+$ T lymfocyten in HLA-$DR3^+$/$DQ2^+$ Hu-PNS patiënten en vervolgens identificatie van het betrokken epitoop. De lage incidentie van Hu-PNS ondanks expressie van het HuD-antigeen in alle KCLC kon echter niet verklaard worden met de resultaten van deze studie.
Tijdens onze eerdere zoektocht naar HuD-specifieke T lymfocyten in bloed, stuitten we in een stimulatie assay met HuD protein-spanning peptide pools (PSPP) op vals-positieve $CD8^+$ T-lymfocyt reacties. Deze vals-positieve reacties worden geanalyseerd in **Hoofdstuk 9**. Onze data laten zien dat synthetische PSPP immunogene vervuilingen kunnen bevatten die vals-positieve reacties kunnen veroorzaken in T-lymfocyt assays. Daarom adviseren we kritische beoordeling van relevante T-lymfocyt reacties na PSPP stimulatie door bevestiging van deze reacties met behulp van nieuw gesynthetiseerde peptides, bij voorkeur met een zuiverheid van 95% of meer.

Hoofdstuk 10 beschrijft de behandeling van Hu-PNS patiënten met humaan choriongonadotrofine (hCG). In deze prospectieve, niet-gecontroleerde, niet-geblindeerde trial werden 15 Hu-PNS patiënten behandeld met dagelijks 10.000 IU hCG intramusculair gedurende 12 weken. Zeven van de 15 patiënten (47%) toonden verbetering of stabilisatie op de modified Rankin Scale (mRS). Vier patiënten (27%) toonden significante neurologische verbetering in de Edinburgh Functional Impairment Tests (EFIT) en 5 patiënten (33%) verbeterden op de Barthel Index (BI). Deze resultaten wijzen op een immuunmodulerend effect van hCG waarmee mogelijk het beloop van Hu-PNS beïnvloed kan worden, hoewel andere factoren mogelijk ook een rol hebben gespeeld in deze niet-gecontroleerde trial.

Daarnaast ondersteunen de resultaten van deze trial de rol van T lymfocyten in Hu-PNS, aangezien hCG inhibitie van Th1 lymfocyten induceert.

Dit proefschrift bevestigt de rol van T lymfocyten in de pathogenese van Hu-PNS door (i) 4x hogere aantallen T lymfocyten in liquor van Hu-PNS patiënten vergeleken met controles; (ii) associatie van Hu-PNS met HLA-DQ2 en HLA-DR3; en (iii) neurologische verbetering of stabilisatie na behandeling met hCG. De aanwezigheid van HuD-specifieke $CD8^+$ T cellen in liquor kon echter niet aangetoond worden. In **Hoofdstuk 11** wordt de aanzet geleverd tot toekomstig onderzoek gericht op de voortdurende zoektocht naar HuD-specifieke T lymfocyten in Hu-PNS, aangezien verder onderzoek noodzakelijk is om de rol van T cellen in de pathogenese van deze syndromen op te helderen.

References

1. Darnell RB, Posner JB: Paraneoplastic syndromes involving the nervous system, N Engl J Med 2003, 349:1543-1554

2. de Beukelaar JW, Sillevis Smitt PA: Managing paraneoplastic neurological disorders, Oncologist 2006, 11:292-305

3. Graus F, Delattre JY, Antoine JC, Dalmau J, Giometto B, Grisold W, Honnorat J, Smitt PS, Vedeler C, Verschuuren JJ, Vincent A, Voltz R: Recommended diagnostic criteria for paraneoplastic neurological syndromes, J Neurol Neurosurg Psychiatry 2004, 75:1135-1140

4. Molinuevo JL, Graus F, Serrano C, Rene R, Guerrero A, Illa I: Utility of anti-Hu antibodies in the diagnosis of paraneoplastic sensory neuropathy, Ann Neurol 1998, 44:976-980

5. Shams'ili S, Grefkens J, de Leeuw B, van den Bent M, Hooijkaas H, van der Holt B, Vecht C, Sillevis Smitt P: Paraneoplastic cerebellar degeneration associated with antineuronal antibodies: analysis of 50 patients, Brain 2003, 126:1409-1418

6. Sillevis Smitt P, Grefkens J, de Leeuw B, van den Bent M, van Putten W, Hooijkaas H, Vecht C: Survival and outcome in 73 anti-Hu positive patients with paraneoplastic encephalomyelitis/sensory neuronopathy, J Neurol 2002, 249:745-753

7. Graus F, Keime-Guibert F, Rene R, Benyahia B, Ribalta T, Ascaso C, Escaramis G, Delattre JY: Anti-Hu-associated paraneoplastic encephalomyelitis: analysis of 200 patients, Brain 2001, 124:1138-1148

8. Gultekin SH, Rosenfeld MR, Voltz R, Eichen J, Posner JB, Dalmau J: Paraneoplastic limbic encephalitis: neurological symptoms, immunological findings and tumour association in 50 patients, Brain 2000, 123 (Pt 7):1481-1494

9. Keime-Guibert F, Graus F, Broet P, Rene R, Molinuevo JL, Ascaso C, Delattre JY: Clinical outcome of patients with anti-Hu-associated encephalomyelitis after treatment of the tumor, Neurology 1999, 53:1719-1723

10. Shams'ili S, de Beukelaar J, Gratama JW, Hooijkaas H, van den Bent M, van 't Veer M, Sillevis Smitt P: An uncontrolled trial of rituximab for antibody associated paraneoplastic neurological syndromes, J Neurol 2006, 253:16-20

11. Vernino S, O'Neill BP, Marks RS, O'Fallon JR, Kimmel DW: Immunomodulatory treatment trial for paraneoplastic neurological disorders, Neuro Oncol 2004, 6:55-62

12. Keime-Guibert F, Graus F, Fleury A, Rene R, Honnorat J, Broet P, Delattre JY: 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

13. Mason WP, Graus F, Lang B, Honnorat J, Delattre JY, Valldeoriola F, Antoine JC, Rosenblum MK, Rosenfeld MR, Newsom-Davis J, Posner JB, Dalmau J: Small-cell lung cancer, paraneoplastic cerebellar degeneration and the Lambert-Eaton myasthenic syndrome, Brain 1997, 120 (Pt 8):1279-1300

14. Uchuya M, Graus F, Vega F, Rene R, Delattre JY: Intravenous immunoglobulin treatment in paraneoplastic neurological syndromes with antineuronal autoantibodies, J Neurol Neurosurg Psychiatry 1996, 60:388-392

15. 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

16. Graus F, Vega F, Delattre JY, Bonaventura I, Rene R, Arbaiza D, Tolosa E: Plasmapheresis and antineoplastic treatment in CNS paraneoplastic syndromes with antineuronal autoantibodies, Neurology 1992, 42:536-540

17. 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

18. 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

19. Graus F, Dalmou J, Rene R, Tora M, Malats N, Verschuuren JJ, Cardenal F, Vinolas N, Garcia del Muro J, Vadell C, Mason WP, Rosell R, Posner JB, Real FX: 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

20. Dalmau J, Furneaux HM, Gralla RJ, Kris MG, Posner JB: 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

21. DeLuca I, Blachere NE, Santomasso B, Darnell RB: Tolerance to the neuron-specific paraneoplastic HuD antigen, PLoS One 2009, 4:e5739

22. Matzinger P: Tolerance, danger, and the extended family, Annu Rev Immunol 1994, 12:991-1045

23. Fuchs EJ, Matzinger P: Is cancer dangerous to the immune system?, Semin Immunol 1996, 8:271-280

24. Sun E: Cell death recognition model for the immune system, Med Hypotheses 2008, 70:585-596

25. Dunn GP, Old LJ, Schreiber RD: The immunobiology of cancer immunosurveillance and immunoediting, Immunity 2004, 21:137-148

26. Dalmau J, Graus F, Rosenblum MK, Posner JB: Anti-Hu-associated paraneoplastic encephalomyelitis/sensory neuronopathy. A clinical study of 71 patients, Medicine 1992, 71:59-72

27. Darnell RB, DeAngelis LM: Regression of small-cell lung carcinoma in patients with paraneoplastic neuronal antibodies, Lancet 1993, 341:21-22

28. Monstad SE, Drivsholm L, Storstein A, Aarseth JH, Haugen M, Lang B, Vincent A, Vedeler CA: Hu and voltage-gated calcium channel (VGCC) antibodies related to the prognosis of small-cell lung cancer, J Clin Oncol 2004, 22:795-800

29. 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

30. Carpentier AF, Rosenfeld MR, Delattre JY, Whalen RG, Posner JB, Dalmau J: DNA vaccination with HuD inhibits growth of a neuroblastoma in mice, Clin Cancer Res 1998, 4:2819-2824

31. Verschuuren JJ, Dalmau J, Hoard R, Posner JB: Paraneoplastic anti-Hu serum: studies on human tumor cell lines, J Neuroimmunol 1997, 79:202-210

32. Sillevis Smitt P, Manley G, Dalmau J, Posner J: The HuD paraneoplastic protein shares immunogenic regions between PEM/PSN patients and several strains and species of experimental animals, J Neuroimmunol 1996, 71:199-206

33. 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

34. 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

35. Hormigo A, Lieberman F: Nuclear localization of anti-Hu antibody is not associated with in vitro cytotoxicity, J Neuroimmunol 1994, 55:205-212

36. Dick DJ, Harris JB, Falkous G, Foster JB, Xuereb JH: Neuronal anti-nuclear antibody in paraneoplastic sensory neuronopathy, J Neurol Sci 1988, 85:1-8

37. Blumenthal DT, Salzman KL, Digre KB, Jensen RL, Dunson WA, Dalmau J: Early pathologic findings and long-term improvement in anti-Ma2-associated encephalitis, Neurology 2006, 67:146-149

38. Rousseau A, Benyahia B, Dalmau J, Connan F, Guillet JG, Delattre JY, Choppin J: T cell response to Hu-D peptides in patients with anti-Hu syndrome, J Neurooncol 2005, 71:231-236

39. Plonquet A, Gherardi RK, Creange A, Antoine JC, Benyahia B, Grisold W, Drlicek M, Dreyfus P, Honnorat J, Khouatra C, Rouard H, Authier FJ, Farcet JP, Delattre JY, Delfau-Larue MH: Oligoclonal T-cells in blood and target tissues of patients with anti-Hu syndrome, J Neuroimmunol 2002, 122:100-105

40. Bernal F, Graus F, Pifarre A, Saiz A, Benyahia B, Ribalta T: Immunohistochemical analysis of anti-Hu-associated paraneoplastic encephalomyelitis, Acta Neuropathol (Berl) 2002, 103:509-515

41. Benyahia B, Liblau R, Merle-Beral H, Tourani JM, Dalmau J, Delattre JY: Cell-mediated autoimmunity in paraneoplastic neurological syndromes with anti-Hu antibodies, Ann Neurol 1999, 45:162-167

42. Panegyres PK, Reading MC, Esiri MM: The inflammatory reaction of paraneoplastic ganglionitis and encephalitis: an immunohistochemical study, J Neurol 1993, 240:93-97

43. Graus F, Ribalta T, Campo E, Monforte R, Urbano A, Rozman C: Immunohistochemical analysis of the immune reaction in the nervous system in paraneoplastic encephalomyelitis, Neurology 1990, 40:219-222

44. Voltz R, Dalmau J, Posner JB, Rosenfeld MR: T-cell receptor analysis in anti-Hu associated paraneoplastic encephalomyelitis, Neurology 1998, 51:1146-1150

45. Psimaras D, Carpentier AF, Rossi C: CSF study in paraneoplastic syndromes, J Neurol Neurosurg Psychiatry 2010, 81:42-45

46. Stich O, Jarius S, Kleer B, Rasiah C, Voltz R, Rauer S: Specific antibody index in cerebrospinal fluid from patients with central and peripheral paraneoplastic neurological syndromes, J Neuroimmunol 2007, 183:220-224

47. 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

48. Dalmau J, Furneaux HM, Rosenblum MK, Graus F, Posner JB: 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

49. Furneaux HF, Reich L, Posner JB: Autoantibody synthesis in the central nervous system of patients with paraneoplastic syndromes, Neurology 1990, 40:1085-1091

50. de Beukelaar JW, Sillevis Smitt PA, Hop WC, Kraan J, Hooijkaas H, Verjans GM, Gratama JW: Imbalances in circulating lymphocyte subsets in Hu antibody associated paraneoplastic neurological syndromes, Eur J Neurol 2007, 14:1383-1391

51. Tani T, Tanaka K, Idezuka J, Nishizawa M: Regulatory T cells in paraneoplastic neurological syndromes, J Neuroimmunol 2008, 196:166-169

52. 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

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

54. Greten TF, Slansky JE, Kubota R, Soldan SS, Jaffee EM, Leist TP, Pardoll DM, Jacobson S, Schneck JP: 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

55. Sun JB, Olsson T, Wang WZ, Xiao BG, Kostulas V, Fredrikson S, Ekre HP, Link H: Autoreactive T and B cells responding to myelin proteolipid protein in multiple sclerosis and controls, Eur J Immunol 1991, 21:1461-1468

56. Olsson T, Zhi WW, Hojeberg B, Kostulas V, Jiang YP, Anderson G, Ekre HP, Link H: Autoreactive T lymphocytes in multiple sclerosis determined by antigen-induced secretion of interferon-gamma, J Clin Invest 1990, 86:981-985

57. Tanaka K, Tanaka M, Inuzuka T, Nakano R, Tsuji S: Cytotoxic T lymphocyte-mediated cell death in paraneoplastic sensory neuronopathy with anti-Hu antibody, J Neurol Sci 1999, 163:159-162

58. Tanaka M, Maruyama Y, Sugie M, Motizuki H, Kamakura K, Tanaka K: Cytotoxic T cell activity against peptides of Hu protein in anti-Hu syndrome, J Neurol Sci 2002, 201:9-12

59. Plonquet A, Garcia-Pons F, Fernandez E, Philippe C, Marquet J, Rouard H, Delfau-Larue MH, Kosmatopoulos K, Lemonnier F, Farcet JP, Gherardi RK, Langlade-Demoyen P: Peptides derived from the onconeural HuD protein can elicit cytotoxic responses in HHD mouse and human, J Neuroimmunol 2003, 142:93-100

60. 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, 56:1501-1506

61. Roberts WK, Deluca IJ, Thomas A, Fak J, Williams T, Buckley N, Dousmanis AG, Posner JB, Darnell RB: Patients with lung cancer and paraneoplastic Hu syndrome harbor HuD-specific type 2 $CD8^+$ T cells, J Clin Invest 2009, 119:2042-2051

62. Hausler M, Sellhaus B, Schweizer K, Ramaekers VT, Opladen T, Kleines M: Flow cytometric cerebrospinal fluid analysis in children, Pathol Res Pract 2003, 199:667-675

63. Kraan J, Gratama JW, Haioun C, Orfao A, Plonquet A, Porwit A, Quijano S, Stetler-Stevenson M, Subira D, Wilson W: Flow cytometric immunophenotyping of cerebrospinal fluid, Curr Protoc Cytom 2008, Chapter 6:Unit 6 25

64. Huang YH, Zozulya AL, Weidenfeller C, Metz I, Buck D, Toyka KV, Bruck W, Wiendl H: Specific central nervous system recruitment of HLA-G(+) regulatory T cells in multiple sclerosis, Ann Neurol 2009, 66:171-183

65. Venken K, Hellings N, Thewissen M, Somers V, Hensen K, Rummens JL, Medaer R, Hupperts R, Stinissen P: Compromised $CD4^+$ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level, Immunology 2008, 123:79-89

66. Kuenz B, Lutterotti A, Ehling R, Gneiss C, Haemmerle M, Rainer C, Deisenhammer F, Schocke M, Berger T, Reindl M: Cerebrospinal fluid B cells correlate with early brain inflammation in multiple sclerosis, PLoS One 2008, 3:e2559

67. Haegele KF, Stueckle CA, Malin JP, Sindern E: Increase of $CD8^+$ T-effector memory cells in peripheral blood of patients with relapsing-remitting multiple sclerosis compared to healthy controls, J Neuroimmunol 2007, 183:168-174

68. Harp C, Lee J, Lambracht-Washington D, Cameron E, Olsen G, Frohman E, Racke M, Monson N: Cerebrospinal fluid B cells from multiple sclerosis patients are subject to normal germinal center selection, J Neuroimmunol 2007, 183:189-199

69. Cepok S, Rosche B, Grummel V, Vogel F, Zhou D, Sayn J, Sommer N, Hartung HP, Hemmer B: Short-lived plasma blasts are the main B cell effector subset during the course of multiple sclerosis, Brain 2005, 128:1667-1676

70. Monson NL, Cravens PD, Frohman EM, Hawker K, Racke MK: Effect of rituximab on the peripheral blood and cerebrospinal fluid B cells in patients with primary progressive multiple sclerosis, Arch Neurol 2005, 62:258-264

71. Corcione A, Casazza S, Ferretti E, Giunti D, Zappia E, Pistorio A, Gambini C, Mancardi GL, Uccelli A, Pistoia V: Recapitulation of B cell differentiation in the central nervous system of patients with multiple sclerosis, Proc Natl Acad Sci U S A 2004, 101:11064-11069

72. Pashenkov M, Huang YM, Kostulas V, Haglund M, Soderstrom M, Link H: Two subsets of dendritic cells are present in human cerebrospinal fluid, Brain 2001, 124:480-492

73. Oreja-Guevara C, Sindern E, Raulf-Heimsoth M, Malin JP: Analysis of lymphocyte subpopulations in cerebrospinal fluid and peripheral blood in patients with multiple sclerosis and inflammatory diseases of the nervous system, Acta Neurol Scand 1998, 98:310-313

74. Sellebjerg F, Jensen J, Ryder LP: Costimulatory CD80 (B7-1) and CD86 (B7-2) on cerebrospinal fluid cells in multiple sclerosis, J Neuroimmunol 1998, 84:179-187

75. Wilson DB, Golding AB, Smith RA, Dafashy T, Nelson J, Smith L, Carlo DJ, Brostoff SW, Gold DP: Results of a phase I clinical trial of a T-cell receptor peptide vaccine in patients with multiple sclerosis. I. Analysis of T-cell receptor utilization in CSF cell populations, J Neuroimmunol 1997, 76:15-28

76. Svenningsson A, Dotevall L, Stemme S, Andersen O: Increased expression of B7-1 costimulatory molecule on cerebrospinal fluid cells of patients with multiple sclerosis and infectious central nervous system disease, J Neuroimmunol 1997, 75:59-68

77. Svenningsson A, Andersen O, Hansson GK, Stemme S: Reduced frequency of memory CD8+ T lymphocytes in cerebrospinal fluid and blood of patients with multiple sclerosis, Autoimmunity 1995, 21:231-239

78. Okuda Y, Okuda M, Apatoff BR, Posnett DN: The activation of memory CD4(+) T cells and CD8(+) T cells in patients with multiple sclerosis, J Neurol Sci 2005, 235:11-17

79. Matsui M, Araya S, Wang HY, Matsushima K, Saida T: Differences in systemic and central nervous system cellular immunity relevant to relapsing-remitting multiple sclerosis, J Neurol 2005, 252:908-915

80. Kraus J, Oschmann P, Engelhardt B, Stolz E, Kuehne BS, Laske C, Schaefer C, Traupe H, Kaps M: CD45RA+ ICAM-3+ lymphocytes in cerebrospinal fluid and blood as markers of disease activity in patients with multiple sclerosis, Acta Neurol Scand 2000, 102:326-332

81. Barrau MA, Montalban X, Saez-Torres I, Brieva L, Barbera N, Martinez-Caceres EM: CD4(+)CD45RO(+)CD49d(high) cells are involved in the pathogenesis of relapsing-remitting multiple sclerosis, J Neuroimmunol 2000, 111:215-223

82. Matsui M, Mori KJ, Saida T, Akiguchi I, Kameyama M: The imbalance in CSF T cell subsets in active multiple sclerosis, Acta Neurol Scand 1988, 77:202-209

83. Kraus J, Oschmann P, Engelhardt B, Schiel C, Hornig C, Bauer R, Kern A, Traupe H, Dorndorf W: Soluble and cell surface ICAM-1 as markers for disease activity in multiple sclerosis, Acta Neurol Scand 1998, 98:102-109

84. Cross AH, Stark JL, Lauber J, Ramsbottom MJ, Lyons JA: Rituximab reduces B cells and T cells in cerebrospinal fluid of multiple sclerosis patients, J Neuroimmunol 2006, 180:63-70

85. Stuve O, Marra CM, Jerome KR, Cook L, Cravens PD, Cepok S, Frohman EM, Phillips JT, Arendt G, Hemmer B, Monson NL, Racke MK: Immune surveillance in multiple sclerosis patients treated with natalizumab, Ann Neurol 2006, 59:743-747

86. Sellebjerg F, Christiansen M, Jensen J, Frederiksen JL: Immunological effects of oral high-dose methylprednisolone in acute optic neuritis and multiple sclerosis, Eur J Neurol 2000, 7:281-289

87. Kern F, LiPira G, Gratama JW, Manca F, Roederer M: Measuring Ag-specific immune responses: understanding immunopathogenesis and improving diagnostics in infectious disease, autoimmunity and cancer, Trends Immunol 2005, 26:477-484

88. Picker LJ, Singh MK, Zdraveski Z, Treer JR, Waldrop SL, Bergstresser PR, Maino VC: Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry, Blood 1995, 86:1408-1419

89. Manz R, Assenmacher M, Pfluger E, Miltenyi S, Radbruch A: Analysis and sorting of live cells according to secreted molecules, relocated to a cell-surface affinity matrix, Proc Natl Acad Sci U S A 1995, 92:1921-1925

90. Frentsch M, Arbach O, Kirchhoff D, Moewes B, Worm M, Rothe M, Scheffold A, Thiel A: Direct access to CD4+ T cells specific for defined antigens according to CD154 expression, Nat Med 2005, 11:1118-1124

91. Betts MR, Koup RA: Detection of T-cell degranulation: CD107a and b, Methods Cell Biol 2004, 75:497-512

92. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM: Phenotypic analysis of antigen-specific T lymphocytes, Science 1996, 274:94-96

93. Lyons AB: Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution, J Immunol Methods 2000, 243:147-154

94. Houck DW, Loken MR: Simultaneous analysis of cell surface antigens, bromodeoxyuridine incorporation and DNA content, Cytometry 1985, 6:531-538

95. Bloom BR: In vitro methods in cell-mediated immunity in man, N Engl J Med 1971, 284:1212-1213

96. Brunner KT, Mauel J, Cerottini JC, Chapuis B: Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs, Immunology 1968, 14:181-196

97. Roden MM, Lee KH, Panelli MC, Marincola FM: A novel cytolysis assay using fluorescent labeling and quantitative fluorescent scanning technology, J Immunol Methods 1999, 226:29-41

98. Engvall E, Perlmann P: Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G, Immunochemistry 1971, 8:871-874

99. Czerkinsky C, Andersson G, Ekre HP, Nilsson LA, Klareskog L, Ouchterlony O: Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells, J Immunol Methods 1988, 110:29-36

100. Wolters VM, Wijmenga C: Genetic background of celiac disease and its clinical implications, Am J Gastroenterol 2008, 103:190-195

101. Knip M, Siljander H: Autoimmune mechanisms in type 1 diabetes, Autoimmun Rev 2008, 7:550-557

102. Bartoccioni E, Scuderi F, Augugliaro A, Chiatamone Ranieri S, Sauchelli D, Alboino P, Marino M, Evoli A: HLA class II allele analysis in MuSK-positive myasthenia gravis suggests a role for DQ5, Neurology 2009, 72:195-197

103. Ghodke Y, Joshi K, Chopra A, Patwardhan B: HLA and disease, Eur J Epidemiol 2005, 20:475-488

104. Darnell JC, Posner JB: Paraneoplastic syndromes involving the nervous system, N Engl J Med 2003, 349:1543-1554

105. Graus F, Keime-Guibert F, Rene R, Benyahia B, Ribalta T, Ascaso C, Escaramis G, Delattre JY: Anti-Hu-associated paraneoplastic encephalomyelitis: analysis of 200 patients, Brain 2001, 124:1138-1148.

106. Sillevis Smitt P, Grefkens J, De Leeuw B, Van Den Bent M, Van Putten W, Hooijkaas H, Vecht C: Survival and outcome in 73 anti-Hu positive patients with paraneoplastic encephalomyelitis/sensory neuronopathy, J Neurol 2002, 249:745-753.

107. Shams'ili S, Grefkens J, de Leeuw B, van den Bent M, Hooijkaas H, van der Holt B, Vecht C, Sillevis Smitt P: Paraneoplastic cerebellar degeneration associated with antineuronal antibodies: analysis of 50 patients, Brain 2003, 126:1409-1418.

108. Rojas I, Graus F, Keime-Guibert F, Rene R, Delattre JY, Ramon JM, Dalmau J, Posner JB: Long-term clinical outcome of paraneoplastic cerebellar degeneration and anti-Yo antibodies, Neurology 2000, 55:713-715.

109. Darnell RB, DeAngelis LM: Regression of small-cell lung carcinoma in patients with paraneoplastic neuronal antibodies, Lancet 1993, 341:21-22

110. Byrne T, Mason WP, Posner JB, Dalmau J: Spontaneous neurological improvement in anti-Hu associated encephalomyelitis, J Neurol Neurosurg Psychiatry 1997, 62:276-278

111. Graus F, Dalmau J, Rene R, al. e: 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

112. Stich O, Graus F, Rasiah C, Rauer S: Qualitative evidence of anti-Yo-specific intrathecal antibody synthesis in patients with paraneoplastic cerebellar degeneration, J Neuroimmunol 2003, 141:165-169.

113. Stich O, Rauer S: Qualitative evidence of anti-Ri specific intrathecal antibody synthesis and quantification of anti-Ri antibodies in serial samples from a patient with anti-Ri syndrome, J Neurol Neurosurg Psychiatry 2006, 77:282-283

114. Sillevis Smitt P, Kinoshita A, DeLeeuw B, Moll W, Coesmans M, Jaarsma D, Henzen-Logmans S, Vecht C, De Zeeuw C, Sekiyama N, Nakanishi S, Shigemoto R: Paraneoplastic cerebellar ataxia due to autoantibodies against a glutamate receptor, New Engl J Med 2000, 342:21-27

115. Sillevis Smitt PAE, Manley GT, Posner JP: Immunization with the paraneoplastic encephalomyelitis antigen HuD does not cause neurologic disease in mice., Neurology 1995, 45:1873-1878

116. Tanaka M, Tanaka K, Tokiguchi S, Shinozawa K, Tsuji S: 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

117. De Beukelaar J, Verjans GM, van Norden Y, Milikan J, Kraan J, Hooijkaas H, Sintnicolaas K, Gratama JW, Sillevis Smitt P: No evidence for circulating HuD-specific $CD8^+$ T cells in patients with paraneoplastic neurological syndromes and Hu antibodies, Cancer Immunol Immunother 2007, 56:1501-1506

118. Yu Z, Kryzer TJ, Griesmann GE, Kim K, Benarroch EE, Lennon VA: CRMP-5 neuronal autoantibody: marker of lung cancer and thymoma-related autoimmunity, Ann Neurol 2001, 49:146-154.

119. Antoine JC, Cinotti L, Tilikete C, Bouhour F, Camdessanche JP, Confavreux C, Vighetto A, Renault-Mannel V, Michel D, Honnorat J: [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

120. Rees JH, Hain SF, Johnson MR, Hughes RA, Costa DC, Ell PJ, Keir G, Rudge P: The role of [18F]fluoro-2-deoxyglucose-PET scanning in the diagnosis of paraneoplastic neurological disorders, Brain 2001, 124:2223-2231.

121. Younes-Mhenni S, Janier MF, Cinotti L, Antoine JC, Tronc F, Cottin V, Ternamian PJ, Trouillas P, Honnorat J: FDG-PET improves tumour detection in patients with paraneoplastic neurological syndromes, Brain 2004, 127:2331-2338

122. Sommer C, Weishaupt A, Brinkhoff J, Biko L, Wessig C, Gold R, Toyka KV: Paraneoplastic stiff-person syndrome: passive transfer to rats by means of IgG antibodies to amphiphysin, Lancet 2005, 365:1406-1411

123. Vernino S, O'Neill BP, Marks RS, O'Fallon JR, Kimmel DW: Immunomodulatory treatment trial for paraneoplastic neurological disorders, Neuro-oncol 2004, 6:55-62

124. Henson RA, Urich H: Cancer and the nervous system: The neurologic complications of systemic malignant disease. Edited by Oxford, Blackwell Scientific, 1982

125. Lucchinetti CF, Kimmel DW, Lennon VA: Paraneoplastic and oncologic profiles of patients seropositive for type 1 antineuronal nuclear autoantibodies, Neurology 1998, 50:652-657

126. Lawn ND, Westmoreland BF, Kiely MJ, Lennon VA, Vernino S: Clinical, magnetic resonance imaging, and electroencephalographic findings in paraneoplastic limbic encephalitis, Mayo Clin Proc 2003, 78:1363-1368.

127. Gultekin SH, Rosenfeld MR, Voltz R, Eichen J, Posner JB, Dalmau J: Paraneoplastic limbic encephalitis: neurological symptoms, immunological findings and tumour association in 50 patients, Brain 2000, 123:1481-1494

128. Dropcho EJ: Antiamphiphysin antibodies with small-cell lung carcinoma and paraneoplastic encephalomyelitis, Ann Neurol 1996, 39:659-667

129. Chan KH, Vernino S, Lennon VA: ANNA-3 anti-neuronal nuclear antibody: marker of lung cancer-related autoimmunity, Ann Neurol 2001, 50:301-311.

130. Vernino S, Lennon VA: New Purkinje cell antibody (PCA-2): marker of lung cancer-related neurological autoimmunity, Ann Neurol 2000, 47:297-305.

131. Corsellis JAN, Goldberg GJ, Norton AR: "Limbic encephalitis" and its association with cancer., Brain 1968, 91:481-496

132. Ances BM, Vitaliani R, Taylor RA, Liebeskind DS, Voloschin A, Houghton DJ, Galetta SL, Dichter M, Alavi A, Rosenfeld MR, Dalmau J: Treatment-responsive limbic

encephalitis identified by neuropil antibodies: MRI and PET correlates, Brain 2005, 128:1764-1777

133. Dalmau J, Tuzun E, Wu HY, Masjuan J, Rossi JE, Voloschin A, Baehring JM, Shimazaki H, Koide R, King D, Mason W, Sansing LH, Dichter MA, Rosenfeld MR, Lynch DR: Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma, Ann Neurol 2007, 61:25-36

134. Vitaliani R, Mason W, Ances B, Zwerdling T, Jiang Z, Dalmau J: Paraneoplastic encephalitis, psychiatric symptoms, and hypoventilation in ovarian teratoma, Ann Neurol 2005, 58:594-604

135. Alamowitch S, Graus F, Uchuya M, Reñé R, Bescansa E, Delattre JY: Limbic encephalitis and small cell lung cancer. Clinical and immunological features, Brain 1997, 120:923-928

136. Voltz R: Neuropsychological symptoms in paraneoplastic disorders, J Neurol 2007, 254 Suppl 2:II84-II86

137. Kassubek J, Juengling FD, Nitzsche EU, Lucking CH: Limbic encephalitis investigated by 18FDG-PET and 3D MRI, J Neuroimaging 2001, 11:55-59.

138. Voltz R, Gultekin SH, Rosenfeld MR, Gerstner E, Eichen J, Posner JB, Dalmau J: A serologic marker of paraneoplastic limbic and brain-stem encephalitis in patients with testicular cancer [see comments], N Engl J Med 1999, 340:1788-1795

139. Dalmau J, Graus F, Villarejo A, Posner JB, Blumenthal D, Thiessen B, Saiz A, Meneses P, Rosenfeld MR: Clinical analysis of anti-Ma2-associated encephalitis, Brain 2004, 127:1831-1844

140. Vincent A, Buckley C, Schott JM, Baker I, Dewar BK, Detert N, Clover L, Parkinson A, Bien CG, Omer S, Lang B, Rossor MN, Palace J: Potassium channel antibody-associated encephalopathy: a potentially immunotherapy-responsive form of limbic encephalitis, Brain 2004, 127:701-712

141. Pozo-Rosich P, Clover L, Saiz A, Vincent A, Graus F: Voltage-gated potassium channel antibodies in limbic encephalitis, Ann Neurol 2003, 54:530-533.

142. 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.

143. Rosenfeld MR, Eichen JG, Wade DF, Posner JB, Dalmau J: Molecular and clinical diversity in paraneoplastic immunity to Ma proteins, Ann Neurol 2001, 50:339-348.

144. 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

145. Posner JB: Paraneoplastic cerebellar degeneration, Can J Neurol Sci 1993, 20:S117-S122

146. Bernal F, Shams'ili S, Rojas I, Sanchez-Valle R, Saiz A, Dalmau J, Honnorat J, Sillevis Smitt P, Graus F: Anti-Tr antibodies as markers of paraneoplastic cerebellar degeneration and Hodgkin's disease, Neurology 2003, 60:230-234.

147. Choi KD, Kim JS, Park SH, Kim YK, Kim SE, Smitt PS: Cerebellar hypermetabolism in paraneoplastic cerebellar degeneration, J Neurol Neurosurg Psychiatry 2006, 77:525-528

148. Furneaux HM, Rosenblum MK, Dalmau J, Wong E, Woodruff P, Graus F, Posner JB: Selective expression of Purkinje-cell antigens in tumor tissue from patients with paraneoplastic cerebellar degeneration, N Engl J Med 1990, 322:1844-1851

149. Fathallah-Shaykh H, Wolf S, Wong E, Posner JB, Furneaux HM: 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

150. Mason WP, Graus F, Lang B, Honnorat J, Delattre J-Y, Valldeoriola F, Antoine JC, Rosenblum M, Rosenfeld M, Newsom-Davis J, Posner JB, Dalmau J: Small-cell lung cancer, paraneoplastic cerebellar degeneration and the Lambert-Eaton myathenic syndrome, Brain 1997, 120:1279-1300

151. Bataller L, Wade DF, Graus F, Stacey HD, Rosenfeld MR, Dalmau J: Antibodies to Zic4 in paraneoplastic neurologic disorders and small-cell lung cancer, Neurology 2004, 62:778-782

152. Shams'ili S, Sillevis Smitt P: Paraneoplasia. Edited by Schiff D, O'Neill BP. New York, McGraw-Hill, 2005, p. pp. 649-679

153. Posner JB: Paraneoplastic syndromes. Edited by Philadelphia, FA Davis, 1995, p. pp. 353-385

154. Digre KB: Opsoclonus in adults. Report of three cases and review of the literature, Arch Neurol 1986, 43:1165-1175

155. Anderson NE, Budde-Steffen C, Rosenblum MK, Graus F, Ford D, Synek BJ, Posner JB: Opsoclonus, myoclonus, ataxia, and encephalopathy in adults with cancer: a distinct paraneoplastic syndrome, Medicine 1988, 67:100-109

156. Wirtz PW, Sillevis Smitt PA, Hoff JI, de Leeuw B, Lammers GJ, van Duinen SG, Verschuuren JJ: Anti-Ri antibody positive opsoclonus-myoclonus in a male patient with breast carcinoma, J Neurol 2002, 249:1710-1712.

157. Bataller L, Graus F, Saiz A, Vilchez JJ: Clinical outcome in adult onset idiopathic or paraneoplastic opsoclonus-myoclonus, Brain 2001, 124:437-443.

158. Altman AJ, Baehner RL: Favorable prognosis for survival in children with coincident opso-myoclonus and neuroblastoma, Cancer 1976, 37:846-852.

159. Rudnick E, Khakoo Y, Antunes NL, Seeger RC, Brodeur GM, Shimada H, Gerbing RB, Stram DO, Matthay KK: 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.

160. Hormigo A, Dalmau J, Rosenblum MK, River ME, Posner JB: Immunological and pathological study of anti-Ri-associated encephalopathy, Ann Neurol 1994, 36:896-902

161. 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

162. 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

163. 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.

164. Dalmau J, Graus F, Cheung NK, Rosenblum MK, Ho A, Canete A, Delattre JY, Thompson SJ, Posner JB: Major histocompatibility proteins, anti-Hu antibodies and paraneoplastic encephalomyelitis in neuroblastoma and small cell lung cancer, Cancer 1995, 75:99-109

165. Antunes NL, Khakoo Y, Matthay KK, Seeger RC, Stram DO, Gerstner E, Abrey LE, Dalmau J: Antineuronal antibodies in patients with neuroblastoma and paraneoplastic opsoclonus-myoclonus, J Pediatr Hematol Oncol 2000, 22:315-320.

166. Pranzatelli MR, Tate ED, Wheeler A, Bass N, Gold AP, Griebel ML, Gumbinas M, Heydemann PT, Holt PJ, Jacob P, Kotagal S, Minarcik CJ, Schub HS: Screening for autoantibodies in children with opsoclonus-myoclonus-ataxia, Pediatr Neurol 2002, 27:384-387.

167. Jongen JL, Moll WJ, Sillevis Smitt PA, Vecht CJ, Tijssen CC: Anti-Ri positive opsoclonus-myoclonus-ataxia in ovarian duct cancer, J Neurol 1998, 245:691-692

168. Dropcho EJ, Kline LB, Riser J: Antineuronal (anti-Ri) antibodies in a patient with steroid-responsive opsoclonus-myoclonus, Neurology 1993, 43:207-211

169. Nitschke M, Hochberg F, Dropcho E: Improvement of paraneoplastic opsoclonus-myoclonus after protein A column therapy, N Engl J Med 1995, 332:192

170. Hayward K, Jeremy RJ, Jenkins S, Barkovich AJ, Gultekin SH, Kramer J, Crittenden M, Matthay KK: 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

171. Mitchell WG, Davalos-Gonzalez Y, Brumm VL, Aller SK, Burger E, Turkel SB, Borchert MS, Hollar S, Padilla S: Opsoclonus-ataxia caused by childhood neuroblastoma: developmental and neurologic sequelae, Pediatrics 2002, 109:86-98

172. 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.

173. Chalk CH, Windebank AJ, Kimmel DW, McManis PG: The distinctive clinical features of paraneoplastic sensory neuronopathy, Can J Neurol Sci 1992, 19:346-351

174. Horwich MS, Cho L, Porro RS, al. e: Subacute sensory neuropathy: a remote effect of carcinoma, Ann Neurol 1977, 2:7-19

175. Graus F, Bonaventura I, Uchuya M, Valls-Sole J, Rene R, Leger JM, Tolosa E, Delattre JY: Indolent anti-Hu-associated paraneoplastic sensory neuropathy, Neurology 1994, 44:2258-2261

176. Molinuevo JL, Graus F, Serrano C, Rene R, Guerrero A, Illa I: Utility of anti-Hu antibodies in the diagnosis of paraneoplastic sensory neuropathy, Ann Neurol 1998, 44:976-980.

177. Antoine JC, Honnorat J, Camdessanche JP, Magistris M, Absi L, Mosnier JF, Petiot P, Kopp N, Michel D: 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.

178. Honnorat J, Antoine JC, Derrington E, Aguera M, Belin MF: 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

179. Pittock SJ, Lucchinetti CF, Parisi JE, Benarroch EE, Mokri B, Stephan CL, Kim KK, Kilimann MW, Lennon VA: Amphiphysin autoimmunity: paraneoplastic accompaniments, Ann Neurol 2005, 58:96-107

180. Saiz A, Dalmau J, Butler MH, Chen Q, Delattre JY, De Camilli P, Graus F: Anti-amphiphysin I antibodies in patients with paraneoplastic neurological disorders associated with small cell lung carcinoma, J Neurol Neurosurg Psychiatry 1999, 66:214-217

181. Uchuya M, Graus F, Vega F, Rene R, Delattre J-Y: Intravenous immunoglobulin treatment in paraneoplastic neurological syndromes with antineuronal autoantibodies, J Neurol Neurosurg Psychiatry 1996, 60:388-392

182. 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.

183. Wirtz PW, Willcox N, van der Slik AR, Lang B, Maddison P, Koeleman BP, Giphart MJ, Wintzen AR, Roep BO, Verschuuren JJ: HLA and smoking in prediction and prognosis of small cell lung cancer in autoimmune Lambert-Eaton myasthenic syndrome, J Neuroimmunol 2005, 159:230-237

184. O'Neill JH, Murray NM, Newsom-Davis J: The Lambert-Eaton myasthenic syndrome. A review of 50 cases, Brain 1988, 111:577-596

185. 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

186. Wirtz PW, Willcox N, Roep BO, Lang B, Wintzen AR, Newsom-Davis J, Verschuuren JJ: 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.

187. Hawley RJ, Cohen MH, Saini N, Armbrustmacher VW: The carcinomatous neuromyopathy of oat cell lung cancer, Ann Neurol 1980, 7:65-72

188. Motomura M, Johnston I, Lang B, Vincent A, Newsom-Davis J: An improved diagnostic assay for Lambert-Eaton myasthenic syndrome, J Neurol Neurosurg Psychiatry 1995, 58:85-87

189. Rosenfeld MR, Wong E, Dalmau J, Manley G, Posner JB, Sher E, Furneaux HM: Cloning and characterization of a Lambert-Eaton myasthenic syndrome antigen, Ann Neurol 1993, 33:113-120

190. Chalk CH, Murray NMF, Newsom-Davis J, O'Neill JH, Spiro SG: Response of the Lambert-Eaton myasthenic syndrome to treatment of associated small-cell lung carcinoma, Neurology 1990, 40:1552-1556

191. 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

192. 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.

193. Newsom-Davis J, Murray NM: Plasma exchange and immunosuppressive drug treatment in the Lambert-Eaton myasthenic syndrome, Neurology 1984, 34:480-485

194. Bird SJ: Clinical and electrophysiologic improvement in Lambert-Eaton syndrome with intravenous immunoglobulin therapy, Neurology 1992, 42:1422-1423

195. Buchbinder R, Forbes A, Hall S, Dennett X, Giles G: Incidence of malignant disease in biopsy-proven inflammatory myopathy. A population-based cohort study, Ann Intern Med 2001, 134:1087-1095.

196. Hill CL, Zhang Y, Sigurgeirsson B, Pukkala E, Mellemkjaer L, Airio A, Evans SR, Felson DT: Frequency of specific cancer types in dermatomyositis and polymyositis: a population-based study, Lancet 2001, 357:96-100.

197. Mastaglia FL, Garlepp MJ, Phillips BA, Zilko PJ: Inflammatory myopathies: clinical, diagnostic and therapeutic aspects, Muscle Nerve 2003, 27:407-425.

198. Targoff IN: Update on myositis-specific and myositis-associated autoantibodies, Curr Opin Rheumatol 2000, 12:475-481.

199. Griggs RC, Mendell JR, Miller RG: Evaluation and treatment of myopathies. Edited by Philadelphia, F.A. Davis Company, 1995

200. Straus SE, Thorpe KE, Holroyd-Leduc J: How do I perform a lumbar puncture and analyze the results to diagnose bacterial meningitis?, Jama 2006, 296:2012-2022

201. Fishman RA: Cerebrospinal fluid in diseases of the nervous system. Edited by Philadelphia, Pennsylvania, Saunders, 1992

202. Van der Meche FG, Van Doorn PA, Meulstee J, Jennekens FG: Diagnostic and classification criteria for the Guillain-Barre syndrome, Eur Neurol 2001, 45:133-139

203. Wasserstrom WR, Glass JP, Posner JB: Diagnosis and treatment of leptomeningeal metastases from solid tumors: experience with 90 patients, Cancer 1982, 49:759-772

204. Bromberg JE, Breems DA, Kraan J, Bikker G, van der Holt B, Smitt PS, van den Bent MJ, van't Veer M, Gratama JW: CSF flow cytometry greatly improves diagnostic accuracy in CNS hematologic malignancies, Neurology 2007, 68:1674-1679

205. Mahieu S, Vertessen F, Van der Planken M: Evaluation of ADVIA 120 CSF assay (Bayer) vs. chamber counting of cerebrospinal fluid specimens, Clin Lab Haematol 2004, 26:195-199

206. de Graaf M, van den Broek P, Kraan J, Luitwieler R, van den Bent M, Boonstra J, Schmitz P, Gratama J, Sillevis Smitt P: Addition of serum-containing medium to cerebrospinal fluid prevents cellular loss over time, J Neurol 2011, March 12 (Epub)

207. Kleine TO, Nebe CT, Lower C, Lehmitz R, Kruse R, Geilenkeuser WJ, Dorn-Beineke A: Modifications of haematology analyzers to improve cell counting and leukocyte differentiating in cerebrospinal fluid controls of the Joint German Society for Clinical Chemistry and Laboratory Medicine, Cytometry A 2009, 75:688-691

208. Boer K, Deufel T, Reinhoefer M: Evaluation of the XE-5000 for the automated analysis of blood cells in cerebrospinal fluid, Clin Biochem 2009, 42:684-691

209. de Jonge R, Brouwer R, de Graaf MT, Luitwieler RL, Fleming C, de Frankrijker-Merkestijn M, Sillevis Smitt PA, Boonstra JG, Lindemans J: Evaluation of the new body fluid mode on the Sysmex XE-5000 for counting leukocytes and erythrocytes in cerebrospinal fluid and other body fluids, Clin Chem Lab Med 2010, 48:665-675

210. Chamberlain MC, Glantz M, Groves MD, Wilson WH: Diagnostic tools for neoplastic meningitis: detecting disease, identifying patient risk, and determining benefit of treatment, Semin Oncol 2009, 36:S35-45

211. Quijano S, Lopez A, Manuel Sancho J, Panizo C, Deben G, Castilla C, Antonio Garcia-Vela J, Salar A, Alonso-Vence N, Gonzalez-Barca E, Penalver FJ, Plaza-Villa J, Morado M, Garcia-Marco J, Arias J, Briones J, Ferrer S, Capote J, Nicolas C, Orfao

A: Identification of leptomeningeal disease in aggressive B-cell non-Hodgkin's lymphoma: improved sensitivity of flow cytometry, J Clin Oncol 2009, 27:1462-1469

212. Glass JP, Melamed M, Chernik NL, Posner JB: Malignant cells in cerebrospinal fluid (CSF): the meaning of a positive CSF cytology, Neurology 1979, 29:1369-1375

213. DeAngelis LM, Cairncross JG: A better way to find tumor in the CSF?, Neurology 2002, 58:339-340

214. Straathof CS, de Bruin HG, Dippel DW, Vecht CJ: The diagnostic accuracy of magnetic resonance imaging and cerebrospinal fluid cytology in leptomeningeal metastasis, J Neurol 1999, 246:810-814

215. Freilich RJ, Krol G, DeAngelis LM: Neuroimaging and cerebrospinal fluid cytology in the diagnosis of leptomeningeal metastasis, Ann Neurol 1995, 38:51-57

216. Zeiser R, Burger JA, Bley TA, Windfuhr-Blum M, Schulte-Monting J, Behringer DM: Clinical follow-up indicates differential accuracy of magnetic resonance imaging and immunocytology of the cerebral spinal fluid for the diagnosis of neoplastic meningitis - a single centre experience, Br J Haematol 2004, 124:762-768

217. Oertel J, Huhn D: Immunocytochemical methods in haematology and oncology, J Cancer Res Clin Oncol 2000, 126:425-440

218. Tani E, Costa I, Svedmyr E, Skoog L: Diagnosis of lymphoma, leukemia, and metastatic tumor involvement of the cerebrospinal fluid by cytology and immunocytochemistry, Diagn Cytopathol 1995, 12:14-22

219. Hovestadt A, Henzen-Logmans SC, Vecht CJ: Immunohistochemical analysis of the cerebrospinal fluid for carcinomatous and lymphomatous leptomeningitis, Br J Cancer 1990, 62:653-654

220. Windhagen A, Maniak S, Heidenreich F: Analysis of cerebrospinal fluid cells by flow cytometry and immunocytochemistry in inflammatory central nervous system diseases: comparison of low- and high-density cell surface antigen expression, Diagn Cytopathol 1999, 21:313-318

221. Jaeger U, Kainz B: Monitoring minimal residual disease in AML: the right time for real time, Ann Hematol 2003, 82:139-147

222. Hug A, Storch-Hagenlocher B, Haas J, Vogt-Schaden ME, Goldschmidt H, Wildemann B: Single-cell PCR analysis of the immunoglobulin heavy-chain CDR3 region for the diagnosis of leptomeningeal involvement of B-cell malignancies using standard cerebrospinal fluid cytospins, J Neurol Sci 2004, 219:83-88

223. Ekstein D, Ben-Yehuda D, Slyusarevsky E, Lossos A, Linetsky E, Siegal T: CSF analysis of IgH gene rearrangement in CNS lymphoma: relationship to the disease course, J Neurol Sci 2006, 247:39-46

224. Rhodes CH, Honsinger C, Sorenson GD: PCR-detection of tumor-derived p53 DNA in cerebrospinal fluid, Am J Clin Pathol 1995, 103:404-408

225. Swinkels DW, de Kok JB, Hanselaar A, Lamers K, Boerman RH: Early detection of leptomeningeal metastasis by PCR examination of tumor-derived K-ras DNA in cerebrospinal fluid, Clin Chem 2000, 46:132-133

226. Rhodes CH, Honsinger C, Sorenson GD: Detection of tumor-derived DNA in cerebrospinal fluid, J Neuropathol Exp Neurol 1994, 53:364-368

227. Lugli E, Roederer M, Cossarizza A: Data analysis in flow cytometry: the future just started, Cytometry A 2010, 77:705-713

228. McLaughlin BE, Baumgarth N, Bigos M, Roederer M, De Rosa SC, Altman JD, Nixon DF, Ottinger J, Oxford C, Evans TG, Asmuth DM: Nine-color flow cytometry for accurate measurement of T cell subsets and cytokine responses. Part I: Panel design by an empiric approach, Cytometry A 2008, 73:400-410

229. Brando B, Barnett D, Janossy G, Mandy F, Autran B, Rothe G, Scarpati B, D'Avanzo G, D'Hautcourt JL, Lenkei R, Schmitz G, Kunkl A, Chianese R, Papa S, Gratama JW: Cytofluorometric methods for assessing absolute numbers of cell subsets in blood. European Working Group on Clinical Cell Analysis, Cytometry 2000, 42:327-346

230. Dux R, Kindler-Rohrborn A, Annas M, Faustmann P, Lennartz K, Zimmermann CW: A standardized protocol for flow cytometric analysis of cells isolated from cerebrospinal fluid, J Neurol Sci 1994, 121:74-78

231. de Graaf M, de Beukelaar J, Bergsma J, Kraan J, van den Bent M, Klimek M, van Norden Y, Kusuma A, Smitt PS, Gratama JW: B and T cell imbalances in CSF of patients with Hu-antibody associated PNS, J Neuroimmunol 2008, 195:164-170

232. Heinrich A, Ahrens N, Schmidt S, Khaw AV: Immunophenotypic patterns of T-cell activation in neuroinflammatory diseases, Acta Neurol Scand 2006, 113:248-255

233. Maxeiner HG, Rojewski MT, Schmitt A, Tumani H, Bechter K, Schmitt M: Flow cytometric analysis of T cell subsets in paired samples of cerebrospinal fluid and peripheral blood from patients with neurological and psychiatric disorders, Brain Behav Immun 2009, 23:134-142

234. Svenningsson A, Hansson GK, Andersen O, Andersson R, Patarroyo M, Stemme S: Adhesion molecule expression on cerebrospinal fluid T lymphocytes: evidence for common recruitment mechanisms in multiple sclerosis, aseptic meningitis, and normal controls, Ann Neurol 1993, 34:155-161

235. Svenningsson A, Andersen O, Edsbagge M, Stemme S: Lymphocyte phenotype and subset distribution in normal cerebrospinal fluid, J Neuroimmunol 1995, 63:39-46

236. de Graaf MT, Smitt PA, Luitwieler RL, van Velzen C, van den Broek PD, Kraan J, Gratama JW: Central memory $CD4^+$ T cells dominate the normal cerebrospinal fluid, Cytometry B Clin Cytom 2010, 80:43-50

237. French CA, Dorfman DM, Shaheen G, Cibas ES: Diagnosing lymphoproliferative disorders involving the cerebrospinal fluid: increased sensitivity using flow cytometric analysis, Diagn Cytopathol 2000, 23:369-374

238. Hegde U, Filie A, Little RF, Janik JE, Grant N, Steinberg SM, Dunleavy K, Jaffe ES, Abati A, Stetler-Stevenson M, Wilson WH: 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

239. Nuckel H, Novotny JR, Noppeney R, Savidou I, Duhrsen U: Detection of malignant haematopoietic cells in the cerebrospinal fluid by conventional cytology and flow cytometry, Clin Lab Haematol 2006, 28:22-29

240. Roma AA, Garcia A, Avagnina A, Rescia C, Elsner B: Lymphoid and myeloid neoplasms involving cerebrospinal fluid: comparison of morphologic examination and immunophenotyping by flow cytometry, Diagn Cytopathol 2002, 27:271-275

241. Subira D, Castanon S, Roman A, Aceituno E, Jimenez-Garofano C, Jimenez A, Garcia R, Bernacer M: Flow cytometry and the study of central nervous disease in patients with acute leukaemia, Br J Haematol 2001, 112:381-384

242. Finn WG, Peterson LC, James C, Goolsby CL: Enhanced detection of malignant lymphoma in cerebrospinal fluid by multiparameter flow cytometry, Am J Clin Pathol 1998, 110:341-346

243. Sayed D, Badrawy H, Ali AM, Shaker S: Immunophenotyping and immunoglobulin heavy chain gene rearrangement analysis in cerebrospinal fluid of pediatric patients with acute lymphoblastic leukemia, Leuk Res 2009, 33:655-661

244. Kleine TO, Albrecht J, Zofel P: Flow cytometry of cerebrospinal fluid (CSF) lymphocytes: alterations of blood/CSF ratios of lymphocyte subsets in inflammation disorders of human central nervous system (CNS), Clin Chem Lab Med 1999, 37:231-241

245. Dahmen UM, Boettcher M, Krawczyk M, Broelsch CE: Flow cytometric “rare event analysis”: a standardized approach to the analysis of donor cell chimerism, J Immunol Methods 2002, 262:53-69

246. Sancho JM, Orfao A, Quijano S, Garcia O, Panizo C, Perez-Ceballos E, Deben G, Salar A, Gonzalez-Barca E, Alonso N, Garcia-Vela JA, Capote J, Penalver FJ, Provencio M, Arias J, Plaza J, Caballero D, Morado M, Feliu E, Ribera JM: Clinical significance of occult cerebrospinal fluid involvement assessed by flow cytometry in non-Hodgkin’s lymphoma patients at high risk of central nervous system disease in the rituximab era, Eur J Haematol 2010, 85:321-328

247. Nowakowski GS, Call TG, Morice WG, Kurtin PJ, Cook RJ, Zent CS: Clinical significance of monoclonal B cells in cerebrospinal fluid, Cytometry B Clin Cytom 2005, 63:23-27

248. Almeida SM, Nanakanishi E, Conto AJ, Souza LP, Antonelli Filho D, Roda CD: Cerebrospinal fluid cytological and biochemical characteristics in the presence of CNS neoplasia, Arq Neuropsiquiatr 2007, 65:802-809

249. Urbanits S, Griesmacher A, Hopfinger G, Stockhammer G, Karimi A, Muller MM, Pittermann E, Grisold W: FACS analysis-a new and accurate tool in the diagnosis of lymphoma in the cerebrospinal fluid, Clin Chim Acta 2002, 317:101-107

250. Brem SS bP, Black P, Brem H, Chamberlain M, Chiocca EA, et al.: NCCN clinical practice guidelines in oncology. Edited by http://www.nccn.org/professionals/physician_gls/PDF/cns.pdf

251. Stetler-Stevenson M, Braylan RC: Flow cytometric analysis of lymphomas and lymphoproliferative disorders, Semin Hematol 2001, 38:111-123

252. Moser RP, Robinson JA, Prostko ER: Lymphocyte subpopulations in human cerebrospinal fluid, Neurology 1976, 26:726-728

253. Pranzatelli MR, Travelstead AL, Tate ED, Allison TJ, Verhulst SJ: CSF B-cell expansion in opsoclonus-myoclonus syndrome: a biomarker of disease activity, Mov Disord 2004, 19:770-777

254. Pranzatelli MR, Travelstead AL, Tate ED, Allison TJ, Moticka EJ, Franz DN, Nigro MA, Parke JT, Stumpf DA, Verhulst SJ: B- and T-cell markers in opsoclonus-myoclonus syndrome: immunophenotyping of CSF lymphocytes, Neurology 2004, 62:1526-1532

255. Subira D, Castanon S, Aceituno E, Hernandez J, Jimenez-Garofano C, Jimenez A, Jimenez AM, Roman A, Orfao A: Flow cytometric analysis of cerebrospinal fluid samples and its usefulness in routine clinical practice, Am J Clin Pathol 2002, 117:952-958

256. Gross HJ, Verwer B, Houck D, Hoffman RA, Recktenwald D: Model study detecting breast cancer cells in peripheral blood mononuclear cells at frequencies as low as 10(-7), Proc Natl Acad Sci U S A 1995, 92:537-541

257. Chow G, Schmidley JW: Lysis of erythrocytes and leukocytes in traumatic lumbar punctures, Arch Neurol 1984, 41:1084-1085

258. Steele RW, Marmer DJ, O’Brien MD, Tyson ST, Steele CR: Leukocyte survival in cerebrospinal fluid, J Clin Microbiol 1986, 23:965-966

259. Veerman AJ, Huismans L, van Zantwijk I: Storage of cerebrospinal fluid samples at room temperature, Acta Cytol 1985, 29:188-189

260. Cunniffe JG, Whitby-Strevens S, Wilcox MH: Effect of pH changes in cerebrospinal fluid specimens on bacterial survival and antigen test results, J Clin Pathol 1996, 49:249-253

261. Fry MM, Vernau W, Kass PH, Vernau KM: Effects of time, initial composition, and stabilizing agents on the results of canine cerebrospinal fluid analysis, Vet Clin Pathol 2006, 35:72-77

262. Hulspas R, O’Gorman MR, Wood BL, Gratama JW, Sutherland DR: Considerations for the control of background fluorescence in clinical flow cytometry, Cytometry B Clin Cytom 2009, 76:355-364

263. Perfetto SP, Roederer M: Increased immunofluorescence sensitivity using 532 nm laser excitation, Cytometry A 2007, 71:73-79

264. Alberti S, Parks DR, Herzenberg LA: A single laser method for subtraction of cell autofluorescence in flow cytometry, Cytometry 1987, 8:114-119

265. Roederer M, Murphy RF: Cell-by-cell autofluorescence correction for low signal-to-noise systems: application to epidermal growth factor endocytosis by 3T3 fibroblasts, Cytometry 1986, 7:558-565

266. Srivastava P, Sladek TL, Goodman MN, Jacobberger JW: Streptavidin-based quantitative staining of intracellular antigens for flow cytometric analysis, Cytometry 1992, 13:711-721

267. Mayefsky JH, Roghmann KJ: Determination of leukocytosis in traumatic spinal tap specimens, Am J Med 1987, 82:1175-1181

268. Novak RW: Lack of validity of standard corrections for white blood cell counts of blood-contaminated cerebrospinal fluid in infants, Am J Clin Pathol 1984, 82:95-97

269. Reske A, Haferkamp G, Hopf HC: Influence of artificial blood contamination of the analysis of cerebrospinal fluid, J Neurol 1981, 226:187-193

270. te Loo DM, Kamps WA, van der Does-van den Berg A, van Wering ER, de Graaf SS: Prognostic significance of blasts in the cerebrospinal fluid without pleiocytosis or a traumatic lumbar puncture in children with acute lymphoblastic leukemia: experience of the Dutch Childhood Oncology Group, J Clin Oncol 2006, 24:2332-2336

271. Chizuka A, Kanda Y, Nannya Y, Oshima K, Kaneko M, Yamamoto R, Suguro M, Hamaki T, Matsuyama T, Takezako N, Miwa A, Togawa A: The diagnostic value of kappa/lambda ratios determined by flow cytometric analysis of biopsy specimens in B-cell lymphoma, Clin Lab Haematol 2002, 24:33-36

272. Davidson B, Risberg B, Berner A, Smeland EB, Torlakovic E: Evaluation of lymphoid cell populations in cytology specimens using flow cytometry and polymerase chain reaction, Diagn Mol Pathol 1999, 8:183-188

273. Zardawi IM, Jain S, Bennett G: Flow-cytometric algorithm on fine-needle aspirates for the clinical workup of patients with lymphadenopathy, Diagn Cytopathol 1998, 19:274-278

274. 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

275. Morse EE, Yamase HT, Greenberg BR, Sporn J, Harshaw SA, Kiraly TR, Ziemba RA, Fallon MA: The role of flow cytometry in the diagnosis of lymphoma: a critical analysis, Ann Clin Lab Sci 1994, 24:6-11

276. Geary WA, Frierson HF, Innes DJ, Normansell DE: Quantitative criteria for clonality in the diagnosis of B-cell non-Hodgkin's lymphoma by flow cytometry, Mod Pathol 1993, 6:155-161

277. Witzig TE, Banks PM, Stenson MJ, Griepp PR, Katzmann JA, Habermann TM, Colgan JP, Gonchoroff NJ: Rapid immunotyping of B-cell non-Hodgkin's lymphomas by flow cytometry. A comparison with the standard frozen-section method, Am J Clin Pathol 1990, 94:280-286

278. Hanse MC, Van't Veer MB, van Lom K, van den Bent MJ: Incidence of central nervous system involvement in chronic lymphocytic leukemia and outcome to treatment, J Neurol 2008, 255:828-830

279. Cramer SC, Glaspy JA, Efird JT, Louis DN: Chronic lymphocytic leukemia and the central nervous system: a clinical and pathological study, Neurology 1996, 46:19-25

280. Pedreira CE, Costa ES, Barrena S, Lecrevisse Q, Almeida J, van Dongen JJ, Orfao A: Generation of flow cytometry data files with a potentially infinite number of dimensions, Cytometry A 2008, 73:834-846

281. Nomura L, Maino VC, Maecker HT: Standardization and optimization of multiparameter intracellular cytokine staining, Cytometry A 2008, 73:984-991

282. Habib LK, Finn WG: Unsupervised immunophenotypic profiling of chronic lymphocytic leukemia, Cytometry B Clin Cytom 2006, 70:124-135

283. Boddy L, Wilkins MF, Morris CW: Pattern recognition in flow cytometry, Cytometry 2001, 44:195-209

284. Costa ES, Arroyo ME, Pedreira CE, Garcia-Marcos MA, Tabernero MD, Almeida J, Orfao A: A new automated flow cytometry data analysis approach for the diagnostic screening of neoplastic B-cell disorders in peripheral blood samples with absolute lymphocytosis, Leukemia 2006, 20:1221-1230

285. Costa ES, Pedreira CE, Barrena S, Lecrevisse Q, Flores J, Quijano S, Almeida J, del Carmen Garcia-Macias M, Bottcher S, Van Dongen JJ, Orfao A: Automated pattern-guided principal component analysis vs expert-based immunophenotypic classification of B-cell chronic lymphoproliferative disorders: a step forward in the standardization of clinical immunophenotyping, Leukemia 2010, 24:1927-1933

286. Rhodes CH, Glantz MJ, Glantz L, Lekos A, Sorenson GD, Honsinger C, Levy NB: A comparison of polymerase chain reaction examination of cerebrospinal fluid and conventional cytology in the diagnosis of lymphomatous meningitis, Cancer 1996, 77:543-548

287. Gleissner B, Siehl J, Korfel A, Reinhardt R, Thiel E: CSF evaluation in primary CNS lymphoma patients by PCR of the CDR III IgH genes, Neurology 2002, 58:390-396

288. Aune MW, Sandberg S: Automated counting of white and red blood cells in the cerebrospinal fluid, Clin Lab Haematol 2000, 22:203-210

289. Aulesa C, Mainar I, Prieto M, Cobos N, Galimany R: Use of the Advia 120 hematology analyzer in the differential cytologic analysis of biological fluids (cerebrospinal, peritoneal, pleural, pericardial, synovial, and others), Lab Hematol 2003, 9:214-224

290. de Graaf MT, Smitt PA, Luitwieler RL, van Velzen C, van den Broek PD, Kraan J, Gratama JW: Central memory CD4(+) T cells dominate the normal cerebrospinal fluid, Cytometry B Clin Cytom 2011, 80:43-50

291. Kleine TO, Nebe CT, Lower C, Lehmitz R, Geilenkeuser WJ, Kruse R, Dorn-Beineke A: Evaluation of cell counting and leukocyte differentiation in cerebrospinal fluid controls using hematology analyzers by the German Society for Clinical Chemistry and Laboratory Medicine, Clin Chem Lab Med 2010, 48:839-848

292. Holub M, Kluckova Z, Beran O, Aster V, Lobovska A: Lymphocyte subset numbers in cerebrospinal fluid: comparison of tick-borne encephalitis and neuroborreliosis, Acta Neurol Scand 2002, 106:302-308

293. Feger U, Luther C, Poeschel S, Melms A, Tolosa E, Wiendl H: Increased frequency of $CD4^+$ CD25+ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients, Clin Exp Immunol 2007, 147:412-418

294. O'Gorman MR, Zijenah LS: CD4 T cell measurements in the management of antiretroviral therapy--A review with an emphasis on pediatric HIV-infected patients, Cytometry B Clin Cytom 2008, 74 Suppl 1:S19-26

295. Gratama JW, Orfao A, Barnett D, Brando B, Huber A, Janossy G, Johnsen HE, Keeney M, Marti GE, Preijers F, Rothe G, Serke S, Sutherland DR, Van der Schoot CE, Schmitz G, Papa S: Flow cytometric enumeration of CD34+ hematopoietic stem and progenitor cells. European Working Group on Clinical Cell Analysis, Cytometry 1998, 34:128-142

296. Della Bella S, Giannelli S, Taddeo A, Presicce P, Villa ML: Application of six-color flow cytometry for the assessment of dendritic cell responses in whole blood assays, J Immunol Methods 2008, 339:153-164

297. 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

298. 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

299. Nakamura H, Kawasaki N, Hagiwara M, Saito M, Konaka C, Kato H: Cellular immunologic parameters related to age, gender, and stage in lung cancer patients, Lung Cancer 2000, 28:139-145

300. Olsson J, Wikby A, Johansson B, Lofgren S, Nilsson BO, Ferguson FG: 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

301. Kim TK, St John LS, Wieder ED, Khalili J, Ma Q, Komanduri KV: Human late memory $CD8^+$ T cells have a distinct cytokine signature characterized by CC chemokine production without IL-2 production, J Immunol 2009, 183:6167-6174

302. Kivisakk P, Trebst C, Liu Z, Tucky BH, Sorensen TL, Rudick RA, Mack M, Ransohoff RM: T-cells in the cerebrospinal fluid express a similar repertoire of inflammatory chemokine receptors in the absence or presence of CNS inflammation: implications for CNS trafficking, Clin Exp Immunol 2002, 129:510-518

303. Provencio JJ, Kivisakk P, Tucky BH, Luciano MG, Ransohoff RM: Comparison of ventricular and lumbar cerebrospinal fluid T cells in non-inflammatory neurological disorder (NIND) patients, J Neuroimmunol 2005, 163:179-184

304. Engelhardt B, Ransohoff RM: The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms, Trends Immunol 2005, 26:485-495

305. Kivisakk P, Mahad DJ, Callahan MK, Trebst C, Tucky B, Wei T, Wu L, Baekkevold ES, Lassmann H, Staugaitis SM, Campbell JJ, Ransohoff RM: Human cerebrospinal fluid central memory $CD4^+$ T cells: evidence for trafficking through choroid plexus and meninges via P-selectin, Proc Natl Acad Sci U S A 2003, 100:8389-8394

306. Hickey WF: Basic principles of immunological surveillance of the normal central nervous system, Glia 2001, 36:118-124

307. Hickey WF: Leukocyte traffic in the central nervous system: the participants and their roles, Semin Immunol 1999, 11:125-137

308. Vora AJ, Perry ME, Hobbs C, Dumonde DC, Brown KA: Selective binding of peripheral blood lymphocytes to the walls of cerebral vessels in frozen sections of human brain, J Immunol Methods 1995, 180:165-180

309. Hickey WF: Migration of hematogenous cells through the blood-brain barrier and the initiation of CNS inflammation, Brain Pathol 1991, 1:97-105

310. Pashenkov M, Soderstrom M, Huang YM, Link H: Cerebrospinal fluid affects phenotype and functions of myeloid dendritic cells, Clin Exp Immunol 2002, 128:379-387

311. Press R, Nennesmo I, Kouwenhoven M, Huang YM, Link H, Pashenkov M: Dendritic cells in the cerebrospinal fluid and peripheral nerves in Guillain-Barre syndrome and chronic inflammatory demyelinating polyradiculoneuropathy, J Neuroimmunol 2005, 159:165-176

312. Cepok S, Jacobsen M, Schock S, Omer B, Jaekel S, Boddeker I, Oertel WH, Sommer N, Hemmer B: Patterns of cerebrospinal fluid pathology correlate with disease progression in multiple sclerosis, Brain 2001, 124:2169-2176

313. Albert ML, Darnell JC, Bender A, Francisco LM, Bhardwaj N, Darnell RB: Tumor-specific killer cells in paraneoplastic cerebellar degeneration, Nat Med 1998, 4:1321-1324

314. Lalazar G, Preston S, Zigmond E, Ben Yaacov A, Ilan Y: Glycolipids as immune modulatory tools, Mini Rev Med Chem 2006, 6:1249-1253

315. Linsen L, Somers V, Stinissen P: Immunoregulation of autoimmunity by natural killer T cells, Hum Immunol 2005, 66:1193-1202

316. Metelitsa LS, Naidenko OV, Kant A, Wu HW, Loza MJ, Perussia B, Kronenberg M, Seeger RC: Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells, J Immunol 2001, 167:3114-3122

317. Gratama JW, van Esser JW, Lamers CH, Tournay C, Lowenberg B, Bolhuis RL, Cornelissen JJ: 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

318. Verjans GM, Feron EJ, Dings ME, Cornelissen JG, Van der Lelij A, Baarsma GS, Osterhaus AD: 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

319. Milikan JC, Kuijpers RW, Baarsma GS, Osterhaus AD, Verjans GM: 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

320. Monstad SE, Knudsen A, Salvesen HB, Aarseth JH, Vedeler CA: Onconeural antibodies in sera from patients with various types of tumours, Cancer Immunol Immunother 2009, 58:1795-1800

321. Price P, Witt C, Allcock R, Sayer D, Garlepp M, Kok CC, French M, Mallal S, Christiansen F: The genetic basis for the association of the 8.1 ancestral haplotype (A1, B8, DR3) with multiple immunopathological diseases, Immunol Rev 1999, 167:257-274

322. Schipper RF, Schreuder GM, D'Amaro J, Oudshoorn M: HLA gene and haplotype frequencies in Dutch blood donors, Tissue Antigens 1996, 48:562-574

323. Olerup O: HLA-B27 typing by a group-specific PCR amplification, Tissue Antigens 1994, 43:253-256

324. Noreen HJ, Yu N, Setterholm M, Ohashi M, Baisch J, Endres R, Fernandez-Vina M, Heine U, Hsu S, Kamoun M, Mitsuishi Y, Monos D, Perlee L, Rodriguez-Marino S, Smith A, Yang SY, Shipp K, Hegland J, Hurley CK: Validation of DNA-based HLA-A and HLA-B testing of volunteers for a bone marrow registry through parallel testing with serology, Tissue Antigens 2001, 57:221-229

325. Mercier B, al Daccak R, Samaan A, David F, Carta A, Cracco P, Raguenes O, Dufosse F, Ferec C, Charron D, et al.: HLA-DRB and -DBQ typing by PCR amplification using sequence-specific primers (PCR-SSP): assessment after 1 year of routine use by three laboratories, Eur J Immunogenet 1994, 21:105-123

326. Moll JW, Antoine JC, Brashear HR, Delattre J, Drlicek M, Dropcho EJ, Giometto B, Graus F, Greenlee J, Honnorat J, et al.: Guidelines on the detection of paraneoplastic anti-neuronal-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

327. Svejgaard A, Ryder LP: HLA and disease associations: detecting the strongest association, Tissue Antigens 1994, 43:18-27

328. Uchuya M, Fleury A, Graus F, Costagliola D, Liblau R, Merle-Beral H, Theodorou I, Delattre JY: Lack of association between human leukocyte antigens and the anti-Hu syndrome in patients with small-cell lung cancer, Neurology 1998, 50:565-566

329. Tanaka M, Tanaka K, Nakano R, Inuzuka T, Tsuji S, Shinozawa K, Kojo T, Mastui T, Kumamoto T, Suzumura A: Lack of association between human leukocyte antigens and anti-Hu syndrome in patients with small-cell lung cancer, Neurology 1999, 52:431

330. Caillat-Zucman S: Molecular mechanisms of HLA association with autoimmune diseases, Tissue Antigens 2009, 73:1-8

331. Kern F, Faulhaber N, Frommel C, Khatamzas E, Prosch S, Schonemann C, Kretzschmar I, Volkmer-Engert R, Volk HD, Reinke P: Analysis of CD8 T cell reactivity to cytomegalovirus using protein-spanning pools of overlapping pentadecapeptides, Eur J Immunol 2000, 30:1676-1682

332. Maecker HT, Dunn HS, Suni MA, Khatamzas E, Pitcher CJ, Bunde T, Persaud N, Trigona W, Fu TM, Sinclair E, Bredt BM, McCune JM, Maino VC, Kern F, Picker LJ: Use of overlapping peptide mixtures as antigens for cytokine flow cytometry, J Immunol Methods 2001, 255:27-40

333. de Beukelaar JW, Gratama JW, Smitt PA, Verjans GM, Kraan J, Luider TM, Burgers PC: The impact of impurities in synthetic peptides on the outcome of T-cell stimulation assays, Rapid Commun Mass Spectrom 2007, 21:1282-1288

334. Kern F, Surel IP, Brock C, Freistedt B, Radtke H, Scheffold A, Blasczyk R, Reinke P, Schneider-Mergener J, Radbruch A, Walden P, Volk HD: T-cell epitope mapping by flow cytometry, Nat Med 1998, 4:975-978

335. Sanz-Nebot V, Benavente F, Toro I, Barbosa J: Liquid chromatography-mass spectrometry approach for the characterization and purification of crude synthetic peptide hormones, Anal Bioanal Chem 2003, 377:306-315

336. 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

337. O'Connor PB, Costello CE: Application of multishot acquisition in Fourier transform mass spectrometry, Anal Chem 2000, 72:5125-5130

338. Mize TH, Amster IJ: Broad-band ion accumulation with an internal source MALDI-FTICR-MS, Anal Chem 2000, 72:5886-5891

339. Moyer SC, Budnik BA, Pittman JL, Costello CE, O'Connor PB: Attomole peptide analysis by high-pressure matrix-assisted laser desorption/ionization Fourier transform mass spectrometry, Anal Chem 2003, 75:6449-6454

340. Gratama JW, Kern F: Flow cytometric enumeration of antigen-specific T lymphocytes, Cytometry A 2004, 58:79-86

341. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S: SYFPEITHI: database for MHC ligands and peptide motifs, Immunogenetics 1999, 50:213-219

342. Rammensee HG, Friede T, Stevanoviic S: MHC ligands and peptide motifs: first listing, Immunogenetics 1995, 41:178-228

343. Parker KC, Bednarek MA, Coligan JE: Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains, J Immunol 1994, 152:163-175

344. Buus S, Lauemoller SL, Worning P, Kesmir C, Frimurer T, Corbet S, Fomsgaard A, Hilden J, Holm A, Brunak S: Sensitive quantitative predictions of peptide-MHC binding by a 'Query by Committee' artificial neural network approach, Tissue Antigens 2003, 62:378-384

345. Nielsen M, Lundegaard C, Worning P, Lauemoller SL, Lamberth K, Buus S, Brunak S, Lund O: Reliable prediction of T-cell epitopes using neural networks with novel sequence representations, Protein Sci 2003, 12:1007-1017

346. Bakhtadze E, Borg H, Stenstrom G, Fernlund P, Arnqvist HJ, Ekbom-Schnell A, Bolinder J, Eriksson JW, Gudbjornsdottir S, Nystrom L, Groop LC, Sundkvist G: HLA-DQB1 genotypes, islet antibodies and beta cell function in the classification of recent-onset diabetes among young adults in the nationwide Diabetes Incidence Study in Sweden, Diabetologia 2006, 49:1785-1794

347. Gavin MA, Gilbert MJ, Riddell SR, Greenberg PD, Bevan MJ: Alkali hydrolysis of recombinant proteins allows for the rapid identification of class I MHC-restricted CTL epitopes, J Immunol 1993, 151:3971-3980

348. Currier JR, Galley LM, Wenschuh H, Morafo V, Ratto-Kim S, Gray CM, Maboko L, Hoelscher M, Marovich MA, Cox JH: Peptide impurities in commercial synthetic peptides and their implications for vaccine trial assessment, Clin Vaccine Immunol 2008, 15:267-276

349. De Spiegeleer B, Vergote V, Pezeshki A, Peremans K, Burvenich C: Impurity profiling quality control testing of synthetic peptides using liquid chromatography-photodiode array-fluorescence and liquid chromatography-electrospray ionization-mass spectrometry: The obestatin case, Anal Biochem 2008, 376:229-234

350. Dalmau J, Rosenfeld MR: Paraneoplastic syndromes of the CNS, Lancet Neurol 2008, 7:327-340

351. Nelson JL, Hughes KA, Smith AG, Nisperos BB, Branchaud AM, Hansen JA: Maternal-fetal disparity in HLA class II alloantigens and the pregnancy-induced amelioration of rheumatoid arthritis, N Engl J Med 1993, 329:466-471

352. Confavreux C, Hutchinson M, Hours MM, Cortinovis-Tourniaire P, Moreau T: Rate of pregnancy-related relapse in multiple sclerosis. Pregnancy in Multiple Sclerosis Group, N Engl J Med 1998, 339:285-291

353. Khan NA, Khan A, Savelkoul HF, Benner R: Inhibition of diabetes in NOD mice by human pregnancy factor, Hum Immunol 2001, 62:1315-1323

354. Manna SK, Mukhopadhyay A, Aggarwal BB: Human chorionic gonadotropin suppresses activation of nuclear transcription factor-kappa B and activator protein-1 induced by tumor necrosis factor, J Biol Chem 2000, 275:13307-13314

355. McCracken SA, Hadfield K, Rahimi Z, Gallery ED, Morris JM: NF-kappaB-regulated suppression of T-bet in T cells represses Th1 immune responses in pregnancy, Eur J Immunol 2007, 37:1386-1396

356. Tavio M, Nasti G, Simonelli C, Vaccher E, De Paoli P, Giacca M, Tirelli U: Human chorionic gonadotropin in the treatment of HIV-related Kaposi's sarcoma, Eur J Cancer 1998, 34:1634-1637

357. Clyde Z, Chataway SJ, Signorini D, Gregor A, Grant R: Significant change in tests of neurological impairment in patients with brain tumours, J Neurooncol 1998, 39:81-90

358. Mahoney FI, Barthel DW: Functional Evaluation: the Barthel Index, Md State Med J 1965, 14:61-65

359. Hoy T: Red cells and reticulocytes. Edited by Macey M. Oxford, Blackwell Scientific Publication, 1994, p. 193

360. Zhang J, Markovic-Plese S, Lacet B, Raus J, Weiner HL, Hafler DA: 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

361. Roberts WK, Darnell RB: Neuroimmunology of the paraneoplastic neurological degenerations, Curr Opin Immunol 2004, 16:616-622

362. 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

363. Zeng G, Wang X, Robbins PF, Rosenberg SA, Wang RF: 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

364. Wentworth PA, Vitiello A, Sidney J, Keogh E, Chesnut RW, Grey H, Sette A: Differences and similarities in the A2.1-restricted cytotoxic T cell repertoire in humans and human leukocyte antigen-transgenic mice, Eur J Immunol 1996, 26:97-101

365. Santomasso BD, Roberts WK, Thomas A, Williams T, Blachere NE, Dudley ME, Houghton AN, Posner JB, Darnell RB: A T-cell receptor associated with naturally occurring human tumor immunity, Proc Natl Acad Sci U S A 2007, 104:19073-19078

366. Weichold FF, Mueller S, Kortsik C, Hitzler WE, Wulf MJ, Hone DM, Sadoff JC, Maeurer MJ: Impact of MHC class I alleles on the M. tuberculosis antigen-specific CD8^{+} T-cell response in patients with pulmonary tuberculosis, Genes Immun 2007, 8:334-343

367. Bachinsky MM, Guillen DE, Patel SR, Singleton J, Chen C, Soltis DA, Tussey LG: Mapping and binding analysis of peptides derived from the tumor-associated antigen survivin for eight HLA alleles, Cancer Immun 2005, 5:6

368. Stepniak D, Wiesner M, de Ru AH, Moustakas AK, Drijfhout JW, Papadopoulos GK, van Veelen PA, Koning F: Large-scale characterization of natural ligands explains the unique gluten-binding properties of HLA-DQ2, J Immunol 2008, 180:3268-3278

369. Vader W, Stepniak D, Kooy Y, Mearin L, Thompson A, van Rood JJ, Spaenij L, Koning F: The HLA-DQ2 gene dose effect in celiac disease is directly related to the magnitude and breadth of gluten-specific T cell responses, Proc Natl Acad Sci U S A 2003, 100:12390-12395

370. 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

371. Wooldridge L, Lissina A, Cole DK, van den Berg HA, Price DA, Sewell AK: Tricks with tetramers: how to get the most from multimeric peptide-MHC, Immunology 2009, 126:147-164

372. Cecconi V, Moro M, Del Mare S, Dellabona P, Casorati G: Use of MHC class II tetramers to investigate CD4^{+} T cell responses: problems and solutions, Cytometry A 2008, 73:1010-1018

373. Kwok WW, Ptacek NA, Liu AW, Buckner JH: Use of class II tetramers for identification of CD4^{+} T cells, J Immunol Methods 2002, 268:71-81

374. Ferlin W, Glaichenhaus N, Mougneau E: Present difficulties and future promise of MHC multimers in autoimmune exploration, Curr Opin Immunol 2000, 12:670-675

375. Li Pira G, Kern F, Gratama J, Roederer M, Manca F: Measurement of antigen specific immune responses: 2006 update, Cytometry B Clin Cytom 2007, 72:77-85

376. Jackman DM, Johnson BE: Small-cell lung cancer, Lancet 2005, 366:1385-1396

List of abbreviations

’ minutes
9-mer peptides consisting of 9 amino acids
15-mer peptides consisting of 15 amino acids
µl microliter
µm micrometer
Ab antibody
AChR acetylcholine receptor
ACTH adrenocorticotropic hormone
ADL activities of daily living
AFP alpha-fetoprotein
AI antibody index
AIDS acquired immune deficiency syndrome
Amp anti-amphiphysin
anti-Hu antibodies against the onconeural HuD antigen
ANNA antineuronal nuclear antibody
APC allophycocyanin
BCS bovine calf serum
BI Barthel Index
BrdU bromo-deoxy-uridine
CA cancer controls
CC conventional cytomorphology
CD cluster of differentiation
cdr calcium-dependent regulator
CFSE carboxyfluoroscein diacetate succinimidyl ester
CLL chronic lymphatic leukemia
CM central memory
CMV cytomegalovirus
CNS central nervous system
CR complete remission
CRMP collapsin response mediator protein
CSF cerebrospinal fluid
CSF-TCL cerebrospinal fluid-derived T cell lines
CT computed tomography
CTLA cytotoxic T-lymphocyte antigen
CV coefficient of variation
CV crossveinless
D aspartic acid
Da Dalton
DAP 3,4-diaminopyridine
dim dim expression
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid
E glutamic acid
EDTA ethyleendiamine tetra-acetaat
EFIT Edinburgh Functional Impairment Tests
e.g. exempli gratia
ELISA enzyme-linked immunosorbent assay
ELISPOT enzyme-linked immunosorbent spot-forming assay
EM effector memory
eV elektronvolt
F female
FBS fetal bovine serum
FC flow cytometry
FDA food and drug administration
FDG-PET fluorodeoxyglucose positron emission tomography
FITC fluorescein isothiocyanate
FLAIR fluid attenuated inversion recovery
FoxP3 forkhead/winged helix transcription factor
FSC forward light scatter
FT-ICR Fourier transform ion cyclotron resonance
FU follow-up
g centripetal acceleration
g gram
h hours
HA hematology analyzer
HC healthy controls
hCG human chorionic gonadotropin
HCMV human cytomegalovirus
Hel-N1 neuronspecific RNA binding protein
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hi high expression
HIV human immunodeficiency virus
HLA human leukocyte antigen
HPLC high-performance liquid chromatography

Hu antigen called after the first patient's initials
Hu-Ab Hu-antibody
HuC neuronspecific RNA binding protein
HuD neuronspecific RNA binding protein
HuDmix HuD protein spanning 15-mer peptide pool
Hu-PNS Hu-antibody associated paraneoplastic neurological syndromes
Hz Hertz
ICU intensive care unit
IE cytomegalovirus immediate early
i.e. id est
IFN interferon
Ig immunoglobulin
IIF indirect immunofluorescence
IL interleukin
im intermediate expression
i.m. intramuscular
imm immature lineage
IU international units
IVIg intravenous immunoglobulin
l liter
LCR light chain ratio
LC/ESI-MS liquid chromatography coupled to electrospray ionization mass spectrometry
LEMS Lambert-Eaton myasthenic syndrome
LM late memory
lo low expression
LP lumbar puncture
Ly lymphocytes
M male
m/z mass-to-charge ratio
Ma antigen called after the first patient's initials
mAb monoclonal antibody
Maldi-FT-ICR matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance
mg milligram
mGluR1 metabotropic glutamate receptor
MHC major histocompatibility complex
Mi antigen called after the first patient's initials
min minutes

ml milliliter
mM millimolar
mmol millimol
MNC mononuclear cells
Mo monocytes
MRI magnetic resonance imaging
mRNA messenger ribonucleic acid
mRS modified Rankin Scale
MS mass spectrometry
MS multiple sclerosis
MS/MS tandem mass spectrometry
My myeloid lineage
MysB myasthenic syndrome antigen B
N number
NA not applicable
nAChR nicotinic acetylcholine receptor
NC non-cancer controls
N.e. not evaluable
Neg negative
NF not found
ng nanogram
NIND non-inflammatory neurological diseases
NK natural killer
NMDA *N*-methyl-D-aspartate
NOD nonobese diabetic
NSCLC non small cell lung cancer
NT not tested
ON optic neuropathy
PBMC peripheral blood mononuclear cells
PBS phosphate buffered saline
PCA Purkinje cytoplasmic antibody
PCD paraneoplastic cerebellar degeneration
PCR polymerase chain reaction
PCR-SSP polymerase chain reaction with sequence-specific primers
PD progressive disease
PE phycoerythrin
PEM paraneoplastic encephalomyelitis
PerCP peridinyl chlorophyllin

PET	positron emission tomography
Pf	positive frequency
PFA	paraformaldehyde
PHA	phytohemagglutinin
PLE	paraneoplastic limbic encephalitis
PMA	phorbol myristate acetate
PMN	polymorphonuclear cells
pmol	picomol
PNC	polynuclear cells
PNS	paraneoplastic neurological syndromes
Pos	positive
PP65	cytomegalovirus phosphoprotein 65
PR	partial remission
PSN	paraneoplastic sensory neuronopathy
PSPP	protein spanning peptide pools
RBC	red blood cell
Ri	antigen called after the first patient's initials
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	radiotherapy
RT	room temperature
RT-PCR	real time polymerase chain reaction
SCLC	small cell lung cancer
SEM	standard error of mean
SFC	spot-forming cells
SLE	systemic lupus erythematosus
S/N	signal-to-noise
SPECT	single-photon emission computed tomography
SSC	sideward light scatter
Ta	antigen called after the first patient's initials
TCP	tricholorphenyl
TCR	T cell receptor
Temp	temperature
TGF	transforming growth factor
Th	T helper
TNF	tumor necrosis factor
Tr	antigen called after the first patient's initials
U	unit

VGCC	voltage-gated calcium channels
VGPC	voltage-gated potassium channels
vs.	versus
WBC	white blood cell
Yo	antigen called after the first patient's initials
Zic	zinc finger protein

Dankwoord

Velen hebben dit proefschrift mogelijk gemaakt. Hierbij wil ik iedereen hartelijk danken en een aantal bij naam noemen.

Mijn grootste dank gaat uit naar alle patiënten en controlepersonen die deel hebben genomen aan onze studies. Ook alle artsen en paramedici die betrokken waren bij de inclusie van deze personen wil ik danken.

Mijn promotor, Prof.dr. P.A.E. Sillevis Smitt. Beste Peter, dank voor je inspirerende begeleiding en altijd kritische betrokkenheid bij dit onderzoek. Ik vond en vind het erg prettig om met je samen te werken.

Dr. J.W. Gratama, copromotor. Beste Jan Willem, ook jou dank ik voor je nauwe betrokkenheid. Je altijd aanwezige nieuwe ideëen en uitgebreide kennis van de immunologie waren zeer waardevol voor dit onderzoek.

Prof.dr. M.J. van den Bent, lid van de kleine commissie. Beste Martin, dank voor deelname in de kleine commissie, bijdrage aan de manuscripten en hulp bij de inclusie van patiënten. Ook Dr. J.E.C. Bromberg, Dr. W. Taal en de arts-assistenten van de afdeling Neuro-Oncologie in de Daniel den Hoed kliniek wil ik danken voor de inclusie van patiënten.

Prof.dr. H. Hooijkaas, lid van de kleine commissie, dank voor deelname in de kleine commissie. Ook wil ik u, samen met de andere immunologen van de afdeling Immunologie, danken voor het snel doorbellen van de ‘positieve’ patiënten, wat ervoor gezorgd heeft dat we zoveel PNS patiënten in heel Nederland konden includeren.

Prof.dr. F. Claas, lid van de kleine commissie, dank voor deelname in de kleine commissie en voor de samenwerking bij de HLA studie.

De leden van de grote commissie, Prof.dr. R.Q. Hintzen, Dr. C.H. Lamers, Dr. A.W. Langerak, Prof.dr. J. Lindemans en Prof.dr. J.J.G.M. Verschuuren dank ik voor het plaatsnemen in deze commissie.

Patricia van den Broek, mijn steun en toeverlaat bij het pipetteren en vandaag bij de verdediging. Lieve Patricia, zonder jou was dit nooit gelukt! Bedankt voor je enthousiasme, inzet, deskundigheid en gezelligheid. Ik vind het jammer dat onze samenwerking voorbij is en wens je alle goeds voor de toekomst.

Jaco Kraan, dank voor je uitgebreide technische kennis. Altijd wist jij weer een antwoord te geven op mijn vragen.

Alle overige collega's van de afdeling Medische en Tumor Immunologie dank ik voor de goede samenwerking. In het bijzonder dank ik Cor Lamers en Reno Debets voor de interesse en betrokkenheid in onze studies.

Irene van den Heuvel, dank voor je hulp bij de klinische trials. Ria van Straten, dank voor alle organisatorische werkzaamheden.

Dr. P.C. Burgers en Dr. T.M. Luiders. Beste Peter en Theo, dank voor de samenwerking bij de contaminatie studie. Jullie enthousiasme om dit tot op de bodem uit te zoeken was aanstekelijk!

Esther Hulsenboom en Eric Brouwer, dank voor jullie hulp bij de diverse studies. Ook alle andere collega's van de afdeling Neuro-Oncologie dank ik voor de samenwerking.

Ronald Luitwieler en Chris van Velzen, hartelijk dank voor de mogelijkheid liquor te verzamelen van gezonde controles op de afdeling Anesthesiologie in het SFG. Het was geen straf om na een ochtendje epiduralen prikken terug te fietsen in de zon naar de Daniel met de liquor monsters in mijn fietstas!

Dr. J.G. Boonstra en alle analisten van de afdeling Klinische Chemie, hartelijk dank voor jullie samenwerking bij de liquormedium studie. Beste Joke, dank ook voor je bijdrage aan de manuscripten.

Wilfried Levering, hartelijk dank voor je bijdrage aan het HLA manuscript.

Geert Haasnoot, dank voor je statistische analyse van de HLA data.

Linda Bralten en Linda IJsselstijn, dank voor jullie afleiding met bikram-yoga op de maandagavond en onze gezellige etentjes waarbij we onze onderzoekservaringen konden delen. Jullie promoties zullen zeker snel volgen! Linda, dank voor het terzijde staan vandaag.

Bianca Mostert en Michiel Strijbos, mijn kamergenoten in de Daniel den Hoed kliniek, dank voor de goede en (meestal) rustige werkomgeving. Bianca, tijdens de vele Nespresso's die we samen gedronken hebben was er altijd wel wat te bespreken over de afdeling of onze klinische ervaringen. Ik hoop je in de kliniek weer tegen te komen.

Arjen de Jongste, je bent inmiddels al een tijdje bezig met het vervolgonderzoek, ik wens je veel succes met het voltooien ervan!

Al mijn lieve vrienden en vriendinnen buiten het ziekenhuis, dank voor de interesse voor mijn onderzoek de afgelopen jaren en de zeer nodige afleiding met borrels, etentjes, weekendjes weg, vakanties en andere gezellige activiteiten.

Lieve pap en mam, dank voor jullie interesse in mijn onderzoek. Al blijft het moeilijk te begrijpen wat ik precies gedaan heb, ik weet dat jullie trots zijn!

Ook Pieter en Maaike, Reinier en Mirjam, Jet en Freek, Jan en Luisa wil ik danken voor hun steun en afleiding tijdens de afgelopen jaren.

Allerliefste Freek, zonder jou was dit proefschrift er zeker ook gekomen, maar jouw nimmer aflatende motivatie, enthousiasme en positivisme hebben het wel veel makkelijker gemaakt! Ik hoop dat we samen nog veel mooie dingen mee mogen maken!

PhD Portfolio

PHD TRAINING

General courses

- English Writing (2008)
- Biostatistics for Clinicians (2009)
- Basiscursus Didactiek (2009)

Specific courses

- Infections and Host Response (2007)
- Antigen Specific Flow Cytometry (Brighton, UK; 2007)
- Post-Infectious Diseases (2007)
- Molecular Immunology (2008)
- Basic and Translational Oncology (2008)

Seminars and workshops

- Seminar Gennaro De Libero: Dendritic cells (2007)
- Werkgroep Neuro-Immunologisch onderzoek: Anti-MAG antistoffen (2007)
- Werkgroep Neuro-Immunologisch onderzoek: Lymfocyten subsets in CSF bij PNS (2007)
- Seminar Pierre Coulie: Cancer vaccination (2008)
- Seminar Ian Cressie: Publishing and acceptance criteria for scientific journals (2008)
- Seminar Ruth Huizinga: Autoimmunity to neuronal antigens in multiple sclerosis (2008)
- Symposium: 10 years Neuro-Oncology Laboratories (2008)
- Symposium: 10 years Josephine Nefkens Institute (2008)
- Wetenschapsdag Translational Tumor Immunology (2009)
- IKR/IKW Symposium: Paraneoplastic Neurological Syndromes (2009)
- Scientific Meeting Department of Medical Oncology (2009)
- Netherlands Neuro-Ophthalmological Society (NeNOS) (2010)
- LWNO Wetenschappelijke dag (2010)

Presentations

- IKR/IKW symposium Paraneoplastic Neurological Syndromes: The role of T cells in anti-Hu associated paraneoplastic neurological syndromes (oral presentation; Rotterdam; 2009)

- 9th Euroconference on Clinical Cell Analysis: Normal ranges of white blood cell subsets in cerebrospinal fluid (oral presentation; Saint Étienne, France; 2009)
- Joint Meeting Belgian Society for Analytical Cytology (BVAC/ABCA) and Dutch Society for Cytometry (NVC): Memory CD4^{+} T cells predominate the normal cerebrospinal fluid (oral presentation); Addition of serum-containing medium to native cerebrospinal fluid prevents cellular loss over time (poster presentation; Antwerp, Belgium; 2009)
- Netherlands Neuro-Ophthalmological Society (NeNOS): Paraneoplastische Neurologische Syndromen (oral presentation; Utrecht; 2010)
- 9th Meeting of the European Association for Neuro-oncology (EANO): HLA-DQ2^{+} individuals are susceptible to Hu-antibody associated paraneoplastic neurological syndromes (poster presentation; Maastricht; 2010)
- Landelijke Werkgroep Neuro-Oncologie (LWNO) Wetenschappelijke dag: HLA-DQ2 en -DR3 associatie in paraneoplastische neurologische syndromen met Hu-antistoffen (oral presentation; Rotterdam; 2010)

(Inter)national conferences

- 7th Euroconference on Clinical Cell Analysis (Rotterdam; 2007)
- 3rd Measuring Antigen Specific Immune Responses (MASIR) conference (La Plagne, France; 2008)
- Dutch Tumor Immunology Meeting (Breukelen; 2008)
- 9th Euroconference on Clinical Cell Analysis (Saint Étienne, France; 2009)
- Joint Meeting Belgian Society for Analytical Cytology (BVAC/ABCA) and Dutch Society for Cytometry (NVC) (Antwerp, Belgium; 2009)

TEACHING

Lecturing

- Keuze onderwijs 2e jaars Geneeskunde: Paraneoplastische syndromen (2007, 2008, 2009)

Curriculum Vitae

Marieke Theodora de Graaf werd geboren op 16 april 1979 in Alkmaar. De lagere school doorliep zij op de J.D. van Arkelschool te Broek op Langedijk. In 1997 behaalde zij haar Gymnasium diploma aan de CSG Jan Arentsz te Alkmaar. In datzelfde jaar begon zij met Medische Biologie aan de Vrije Universiteit te Amsterdam, na uitloting voor Geneeskunde. Na 2 jaar Medische Biologie, werd ze in 1999 ingeloot voor Geneeskunde aan de Vrije Universiteit te Amsterdam. Tijdens haar studie was zij lid en bestuurslid van de studentenvereniging V.C.S.A. (Vereniging van Christelijke Studenten te Amsterdam). Haar wetenschappelijke stage voor Geneeskunde liep zij op de afdeling Neonatologie van het VU Medisch Centrum te Amsterdam, waar zij onderzoek deed naar de motorische ontwikkeling van prematuren onder supervisie van Dr. J.F. Samsom. In november 2003 behaalde zij het Doctoraalexamen Geneeskunde en begon met haar coschappen. Na o.a. keuze coschappen op de afdelingen Kinderneurologie en Neonatologie in het VU Medisch Centrum te Amsterdam en een oudste coschap op de afdeling Neurologie in het Spaarne Ziekenhuis te Hoofddorp, behaalde zij in december 2005 het Artsexamen. Direct aansluitend ging zij als ANIOS (arts-niet-in-opleiding-tot-specialist) werken op de afdeling Neurologie in het Onze Lieve Vrouwe Gasthuis te Amsterdam (opleider Prof.dr. P. Portegies). In december 2006 is zij begonnen als AGIKO (assistent-geneeskundige-in-opleiding-tot-klinisch-onderzoeker) op de afdeling Neurologie (opleider Prof.dr. P.A.E. Sillevis Smitt) in het Erasmus MC te Rotterdam, waar zij in januari 2007 een promotie onderzoek startte op de afdeling Neurologie (Prof.dr. P.A.E. Sillevis Smitt) in samenwerking met de afdeling Interne Oncologie (Dr. J.W. Gratama) op de locatie Daniel den Hoed kliniek. Dit proefschrift is hiervan het resultaat. Tijdens haar promotie onderzoek heeft zij in 2008 een Erasmus MC Grant (€50.000) en een ZonMW AGIKO Stipendium (€63.530) ontvangen. Sinds januari 2010 vervolgt zij haar opleiding Neurologie op de afdeling Neurologie in het Erasmus MC te Rotterdam.

List of publications

De Graaf MT, De Jongste AHC, Kraan J, Boonstra JG, Sillevis Smitt PAE, Gratama JW. Flow cytometric characterization of cerebrospinal fluid cells. *Cytometry Part B: Clinical Cytometry, 2011 May 12 (Epub)*

De Graaf MT, De Tollenaere C, Schrijvers D, Sillevis Smitt PAE. Paraneoplastische syndromen. *In: Van de Velde CJH: Oncologie; Hoofdstuk 11. Houten, Bohn Stafleu van Loghum, 2011: pp 219-34*

De Graaf MT, Van den Broek PDM, Kraan J, Luitwieler RL, Van den Bent MJ, Boonstra JG, Schmitz PIM, Gratama JW, Sillevis Smitt PAE. Addition of serum-containing medium to cerebrospinal fluid prevents cellular loss over time. *Journal of Neurology, 2011 March 12 (Epub)*

De Graaf MT, Sillevis Smitt PAE, Luitwieler RL, Van Velzen C, Van den Broek PDM, Kraan J, Gratama JW. Central memory $CD4^+$ T cells dominate the normal cerebrospinal fluid. *Cytometry Part B: Clinical Cytometry 2011;80:43-50*

Van Broekhoven PCA, **De Graaf MT**, Bromberg JE, Hooijkaas H, Van den Bent MJ, De Beukelaar JWK, Khan NA, Gratama JW, Van der Geest JN, Frens MA, Benner R, Sillevis Smitt PAE. Human chorionic gonadotropin treatment of anti-Hu associated paraneoplastic neurological syndromes. *Journal of Neurology, Neurosurgery & Psychiatry 2010;81:1341-4*

De Graaf MT, De Beukelaar JWK, Haasnoot GW, Levering WHBM, Rogemond V, Didelot A, Honnorat J, Gratama JW, Sillevis Smitt PAE. HLA-$DQ2^+$ individuals are susceptible to Hu-Ab associated paraneoplastic neurological syndromes. *Journal of Neuroimmunology 2010;226:147-9*

De Jonge R, Brouwer R, **De Graaf MT**, Luitwieler RL, Fleming C, De Frankrijker-Merkestijn M, Sillevis Smitt P, Boonstra J, Lindemans J. Evaluation of the new body fluid mode on the Sysmex XE-5000 for counting leukocytes and erythrocytes in cerebrospinal fluid and other body fluids. *Clinical Chemistry and Laboratory Medicine 2010;48(5):665-75*

De Beukelaar JW, Milikan JC, Verjans GM, **De Graaf MT**, Van Norden Y, Lamers CL, Van den Bent MJ, Bromberg JE, Hulsenboom E, Sintnicolaas K, Gratama JW, Sillevis Smitt PA. No evidence for the presence of HuD-specific T cells in the cerebrospinal fluid of patients with Hu-associated paraneoplastic neurological syndromes. *Journal of Neurology 2009;256:279-82*

De Graaf MT, De Beukelaar JW, Burgers PC, Luider TM, Kraan J, Sillevis Smitt PA, Gratama JW. Contamination of synthetic HuD protein spanning peptide pools with a CMV-encoded peptide. *Cytometry Part A 2008;73:1079-85*

Wick, MR, **De Graaf MT**, Sillevis Smitt PAE. Nonneurological, nondermatologic paraneoplasias. *In: Wick MR: Metastatic carcinomas of unknown origin; Chapter 2: Paraneoplastic syndromes associated with MCUOs. New York, Demos, 2008: pp 73-103*

De Graaf MT, Sillevis Smitt PAE. Neurological paraneoplasias. *In: Wick MR: Metastatic carcinomas of unknown origin; Chapter 2: Paraneoplastic syndromes associated with MCUOs. New York, Demos, 2008: pp 27-41*

De Graaf MT, De Beukelaar JW, Bergsma J, Kraan J, Van den Bent MJ, Klimek M, Van Norden Y, Kusuma A, Sillevis Smitt PA, Gratama JW. B and T cell imbalances in CSF of patients with Hu-antibody associated PNS. *Journal of Neuroimmunology 2008;195:164-70*

De Graaf MT, Samsom JF, Pettersen EM, Schaaf VA, Van Schie PE, De Groot L. Vestibulospinal component of postural control (vestibular function) in very preterm infants (25 to 27 weeks) at 3, 6, and 12 months corrected age. *Journal of Child Neurology 2004;19:614-8*