Paraneoplastic Syndromes of the Central Nervous System

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Paraneoplastic Syndromes of the Central Nervous System

Paraneoplastische Syndromen
van het
Centrale Zenuwstelsel

Proefschrift

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
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Johan Wim Berend Moll

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"De meeste dingen in het leven doet men,
ook al doet men het anders voorkomen,
vanwege de vrouwen"

"De wetenschap leeft door de liefde ervoor,
de liefde des te minder door de wetenschap erover"

naar H.Hesse

Aan mijn familie en vrienden

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1.1 Immune diagnosis of paraneoplastic neurological disease

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Abstract

In recent years a continuous stream of new information on clinical, pathological and immunological aspects of paraneoplastic neurological syndromes has been published. In this survey, we will discuss current opinions on the value of anti-neuronal antibody detection for establishing a diagnosis of one of the paraneoplastic syndromes of the central nervous system.

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We thank Dr. Jerome B. Posner for his valuable comments.

Introduction

Paraneoplastic neurological syndromes (PNS) of the central nervous system consist of a variety of clinical neurological disorders that include encephalomyelitis, paraneoplastic cerebellar degeneration (PCD), subacute sensory neuronopathy (SNNP), retinal paraneoplastic syndrome, stiff-man syndrome and motor neuron disease.
For the clinician, it is often difficult to arrive at a correct diagnosis and to identify the underlying tumor. In two-third of cases a paraneoplastic neurological syndrome precedes the discovery of underlying cancer. Neurological signs and ancillary investigations are usually aspecific and the tumor is frequently of small volume. However, demonstration of specific anti-neuronal antibodies has created new possibilities for establishing a reliable diagnosis of PNS.
In 1965, Wilkinson and Zeromski identified a specific anti-neuronal antibody in serum of patients who suffered from sensory neuronopathy and small cell lung cancer[1], but it took almost twenty years before the diagnostic value of these antibodies became clear[2-4]. Since, a number of well-characterized autoantibodies against neuronal antigens have been recognized in association with specific neurological syndromes and certain types of cancer[5-12].
The involved antigens have for the larger part been identified and cDNAs have been cloned from neural and tumor expression libraries[13-17]. Recombinant proteins are now available for diagnostic assays and the precise nature of the target antigens is currently subject of study.
Nevertheless, questions remain because autoantibodies against central nervous system (CNS) antigens have been identified in a variety of other clinical situations and occasionally in normal controls. These uncertainties include the exact diagnostic value of the antibodies, the minimum titer-height in serum and the nature of reactive antigens.
These points need further clarification in order to provide the clinician with practical guidelines that enable appropriate interpretation of the detection of anti-neuronal antibodies. Posner and coworkers defined the requirements for recognition of anti-neuronal antibodies that are clinically valuable[18].

1. The antibody must be present in more than one patient, with a similar neurological disorder and corresponding tumor, and the presence of both false-negative and false-positive autoantibodies should be rare.
2. The concentration of antibodies in serum must be relatively high.
3. A higher titer in cerebrospinal fluid (CSF) than in serum would suggest intrathecal synthesis and would provide evidence for a neurologically relevant antibody.
4. The antibodies should react with a symptomatic part of the nervous system and the nature of the antigen must be identified by both indirect immunofluorescence and Western blots on neuronal proteins.

Using these criteria, several antibodies have been identified that appear to mark specific paraneoplastic syndromes each of which is related to a narrow range of tumors (table 1).

Based on these requirements, we will review the most common PNS-associated anti-neuronal antibodies, including the anti-Hu, anti-Purkinje cell, anti-Yo, anti-Ri, anti-128 kD and anti-CAR antibodies. For each of these antibodies the associated clinical syndrome, the underlying malignancy and the nature of the reactive antigen will be discussed. Lambert-Eaton myasthenic syndrome will not be mentioned here, although it is clearly paraneoplastic and antibody-mediated. Other paraneoplastic disorders of muscle and peripheral nerve are usually not antibody-related and will also not be discussed. At the end, the clinical consequences of the detection of anti-neuronal antibodies in patients suspected of PNS will be summarized as guidelines.

Paraneoplastic Encephalo-Myelitis/Sensory-Neuronopathy: Anti-Hu Antibody

In patients with paraneoplastic encephalomyelitis (PEM) more than one part of the CNS can be involved leading to a combination of cerebral, cerebellar, brain stem or spinal cord signs. In many cases, one affected part of the CNS causes the majority of neurological signs like limbic encephalitis, brain-stem encephalitis or cerebellar syndrome. Besides, a paraneoplastic or subacute paraneoplastic sensory neuronopathy (PSN) can be the only clinical phenomenon or it can accompany the encephalomyelitis. Both PEM and PSN are regarded as different clinical representations of the same disease entity, that is also designated as the anti-Hu syndrome if associated with the presence of anti-Hu antibodies[12,18,19].
The most frequently associated tumor is a small-cell lung cancer (SCLC) that occurs in about three-quarter of patients with PEM/PSN[12]. Next frequent comes non-small cell cancer of the lung although other tumors may be encountered including gynaecological tumors, neuroblastoma and Hodgkin's disease.

Antibodies
The first antibody reported was found in serum of four patients with SNNP and SCLC, although others could initially not repeat these findings[1,20]. In 1985, Graus et al. detected by indirect immunofluorescence an antibody that predominantly reacted with nuclei of neurons in serum of two patients with small-cell lung cancer[2]. This antibody was neither found in normal subjects nor in neurological patients with or without cancer[2]. By means of Western blotting on nuclear extracts of rat and human brain it was shown that this antibody reacted with a 35-38 kilo Dalton protein. The antibody was designated anti-Hu, after the initials of the patient in whom it was first discovered[21]. Others called this antibody anti-neuronal nuclear antibody or "ANNA-1"[22,23].
In about 80% of patients who harbour anti-Hu antibodies, SCLC can be identified[12]. Other tumors include non-small cell lung cancer, breast carcinoma, seminoma, chondro-myxosarcoma, prostate carcinoma, neuroblastoma and adenocarcinoma of the colon. In 13% of a series of 71 anti-Hu positive patients no tumor was found. In anti-Hu positive patients with PNS and SCLC, 95% of patients have tumor activity limited to the chest[12].

Table 1 Overview of the different PNS and associated antibodies with clinical and immunological features

Antibody	Paraneoplastic Neurological Syndrome	Most frequently Associated Cancers	Immunohistochemistry	Western blot
Anti-Hu/ ANNA-1	Encephalomyelitis, Sensory-Neuronopathy	Small cell lung, non- small cell lung	Strong staining of all neuronal nuclei and weaker staining of cytoplasm	38-40 kD reactive triplet band on extracts of crude or isolated CNS neurons/nuclei and SCLC, Recombinant protein of 43 kD (HuD)
Purkinje cell antibodies:				
Anti-Yo/ APCA-1/ PCA-1	Acute to subacute pan-cerebellar syndrome, dysarthria and upbeat nystagmus	Ovarian, breast, uterus	Purkinje cell cytoplasm and axons, coarse granular staining	34 and 62 kD band from Purkinje cell extracts and breast and ovary tumors of patients, reactive with recombinant CDR 34 and CDR 62 proteins
APCA-2/ PCA-2	More slowly developing cerebellar syndrome, less frequent dysarthria and nystagmus	M. Hodgkin	Purkinje cell cytoplasm, fine granular staining	No reactive protein bands detected as yet
Anti-Ri/ ANNA-2	Opsoclonus-Myoclonus	Breast, small cell lung	All nuclei of CNS	55 kD and 80 kD neuronal nuclear protein, 55kD recombinant protein
Anti-CAR	Visual loss, retinal degeneration	Small cell lung, breast, non-small cell lung	Retinal neurons, rods and cones	23 kD and 48 kD protein of retinal extracts, 26 (23)kD recombinant protein
Anti-128kD	Stiff-man syndrome	Breast	Synapsis of CNS neurons	128 kD protein on neuronal extracts

n.k. = not known

Neurologically asymptomatic patients with SCLC can harbour low titers of anti-Hu antibodies and in these patients smaller tumors are present as compared to SCLC without anti-Hu antibodies[24]. This antibody is found almost exclusively in patients with a smoking history. PEM/PSN has also been associated with other tumors in which no anti-Hu antibody could be identified, including breast, ovarian, uterus, stomach, oesophagal cancer, testicular carcinoma, synovioma, Hodgkin's lymphoma and non-small cell lung cancer. Five patients have been described who suffer from PEM/PSN with SCLC and who carry no anti-Hu antibody. Two of these cases with SNNP showed relatively mild symptoms compared to anti-Hu positive patients[12,15].

Table 1 Overview of the different PNS and associated antibodies with clinical and immunological features (continued)

Antigen	Estimated Diagnostic Sensitivity	Estimated Diagnostic Specificity	Total no. of patients	Total no. of false positive patients	References
Neuron specific RNA/DNA binding protein, role in RNA processing of neurons	30-40%	95-100%	>150	13	5,10,12
DNA binding, gene transcription regulators, carry leucine-zipper and zinc-finger motifs	40%	70-100%	>100	7	8,9,35,3738,89
n.k.	n.k.	100%	35	0	3,35,40
RNA-binding, role in post migratory maturation of neurons	n.k.	90-100%	23	1	48,63,66,90
Visinin like, calmodulin family, calcium binding domains	n.k.	80-100%	6	1	70-72,91-93
Amphiphysine, synaptic vesicle protein, function unknown	n.k.	100%	5	0	94-96

False positivity

Two studies have indicated a low specificity of anti-Hu for PEM/PSN. In one series anti-neuronal antibodies were observed in 43% of SCLC patients without any correlation to the neurological symptoms[25]. In another study, anti-Hu antibodies were observed in high titers (i.e. > 1:800) in 27 of 81 patients with SCLC without clear relationship to the neurological disorder. Of the 27 antibody positive patients only two suffered from PNS[26]. However, in both these series anti-Hu detection was based only on immunohistochemistry or indirect immunofluorescence and was not confirmed by Western blots. Under these circumstances an overreporting of the presence of paraneoplastic antibodies will easily occur as many different autoantibodies can bind to either neuronal nuclei or its cytoplasm. Dalmau et al. reported

anti-Hu antibodies in low titers in 15% of all patients with SCLC by using the combination of immunohistochemistry and Western blotting on SCLC protein extracts, indeed indicating that detection of these antibodies is not only limited to neurologically symptomatic patients[27].

Using both techniques together, a total of 13 patients have been reported carrying positive anti-Hu titers with concomitant neurological disease and no tumor[12]. These observations may thus be interpreted as false-positive as they carry no firm relation with cancer. Another possible explanation is suggested by the remarkable finding that two patients with anti-Hu positive PNS and SCLC showed spontaneous regression of their tumor[12,24,27]. This fascinating observation may indicate that anti-neuronal antibodies in patients without cancer might be related to the presence of an undetectable tumor-load or even to regression of tumor.

Another reason for false-positivity is cross-reactivity with other autoantibodies. One such reactivity has been suggested in a subset of patients with primary Sjögren's syndrome who seemingly contained anti-Hu antibodies that however did not react with the HuD fusion protein[28,29]. These observations provide one answer for the variance in diagnostic significance of these antibodies and simultaneously emphasize the necessity for improving laboratory techniques. Most investigators would agree that the finding of anti-Hu antibodies indicates specificity approaching 100% for the diagnosis of PNS and the associated neoplasm being SCLC in over 80% of cases, if detected in titers of above 1:500 - 1:5000. The sensitivity is $\approx$ 40%[5,10,12] if both immunohistochemistry and Western blotting are utilized.

Antigen

Graus established that anti-Hu antibodies react with a 38-40 kD protein band on Western blot of protein extracts of cerebral cortex[21]. Further experiments have revealed that the antigen consists of a triple band that can be identified using whole cerebral or cerebellar protein extracts. Most anti-Hu positive cases show the strongest reactivity with the lower part of this triplet. Incidentally anti-Hu sera show a stronger reaction with the upper part [unpublished data]. So far, these small differences seem to bear no clinical significance.

The Hu antigen is expressed by all neuronal nuclei of the CNS, by dorsal root ganglion cells and less strongly in the cytoplasm of these cell groups[27].

A cDNA has been isolated from a cerebellar and a SCLC expression library that produced a recombinant protein of 43 kD, designated as HuD and reactive with all anti-Hu positive sera [13]. The gene coding for the Hu antigen has been mapped to the human chromosome 1p34[30]. By amino acid sequencing and assessment of gene homology, the presence of three RNA binding sites were recognized on the protein showing a strong homology to the Drosophila proteins Elav and Sex-lethal, that play a role in the early maturation of neurons and sex determination of Drosophila. HuD is uniquely expressed by neuronal cells and certain tumor cells and may be involved in neuron-specific RNA processing[13].

All SCLC cell lines that have been studied did express Hu. However, there is no satisfactory explanation for the fact that there is only a small minority of patients with SCLC that develops anti-Hu antibodies and PEM/PSN. One may speculate that this could be related to a genetically determined specific immunological make-up as indicated by the presence of

systemic autoantibodies in up to 52% of all PNS patients as compared to 15% in control subjects (chapter 3). Another explanation for the rarity of PNS may be a mutation of the Hu protein causing subtle changes in its amino-acid sequence that would make the antigen more immunogenic[12].

Paraneoplastic Cerebellar Degeneration: Anti-Purkinje cell Antibodies

Paraneoplastic cerebellar degeneration (PCD) is a rare complication of a variety of cancers, particularly ovarian and breast cancer, small cell lung cancer and Hodgkin's disease, although other malignancies may be involved[3,8,31-33]. Clinically, the disorder is characterized by subacute development of pan-cerebellar dysfunction and pathologically by selective loss of Purkinje cells without inflammatory infiltrates. At the other hand, one can estimate that at least 50% of middle-aged patients who suddenly develop a pan-cerebellar disorder would go on to carry a tumor within a few years[34].

Antibodies

The first report on antibodies against cytoplasm of Purkinje cells (PCab) was from a patient with cerebellar degeneration and Hodgkin's disease[31]. A second patient with similar antibodies was described in 1981[3]. In 1983, an antibody specifically staining the cytoplasm of Purkinje cells and deep cerebellar nuclei was independently found by Greenlee and by Jaeckle and coworkers in patients with a gynaecological tumor and PCD but not in control series[8,35]. On Western blot the autoantibody reacted with a 34-38 kD and 62-64 kD protein using protein extracts of isolated Purkinje cells[36]. The antibody was identified in 18 out of 42 in one series and in 7 out of 21 in another series of patients with PCD using protein extracts of gradient isolated Purkinje cells[9,37]. This antibody has been designated anti-Yo or PCA-1.

In a recent study by Peterson and others 55 patients were reported with anti-Yo positive PCD. Ovarian cancer was found in 26, breast cancer in 13 and seven patients had other malignancies (fallopian tube, endometrial, mesovarium and adenocarcinoma of the lung)[38]. Anti-Yo antibodies were not seen in 13 patients with PCD and Hodgkin's disease, nor in 45 patients with breast cancer, 24 with ovarian cancer with or without a non-paraneoplastic cerebellar syndrome nor in 325 patients with non-paraneoplastic cerebellar syndromes and neither in 50 normal subjects[38].

In a review on 62 patients with PCD and breast cancer, PCab's were found in 30 patients, but not all were verified by immunoblot for harbouring definitely anti-Yo antibodies. Nine patients were PCab negative and the others were not tested. In 77% of patients the cerebellar degeneration antedated the discovery of their breast cancer with a median of 16 months (range 2-41 months)[39].

One can estimate that a total of about 40% of patients with PCD would harbour autoantibodies against Purkinje cells, and consequently 60% of patients with clinically proven PCD are PCab-negative.
The specificity for anti-Yo detection in patients with cerebellar syndrome ranges from 70% to nearly 100% for establishing a diagnosis of PCD. Patients with anti-Yo antibody positive PCD differ from patients with anti-Yo negative PCD both clinically and in gender[38]. In anti-Yo negative PCD, dysarthria and nystagmus appear far less frequent and are absent in 50% of cases. The neurological disorder is less severe, develops more slowly and usually follows rather than precedes a diagnosis of cancer. The majority of anti-Yo negative PCD patients are males predominantly suffering from lung cancer or from Hodgkin's disease[38].
The PCab's found in PCD associated with Hodgkin's disease react with a different antigen and is designated PCA-2[8]. It yields a more fine-speckled cytoplasmic staining of cerebellar Purkinje cells on immunohistochemistry and does not produce a reactive band on Western blot[8,11,32]. In one series of clinically proven PCD and Hodgkin's disease, only six out of 21 patients had PCab antibodies[40]. Until 1992 a total of 43 patients with Hodgkin's disease and PCD have been reported. Of 31 patients tested for PCab's only 10 were positive. One explanation for the infrequent occurrence of this antibody may be the labile nature of the involved antigen that would be deformed using conventional tissue processing.

False positivity
Only incidental cases of false positive anti-Yo antibodies have been reported in patients with PCD and no tumor, or in patients with tumor and no PCD. One series identified 13 patients with PCab positive PCD and no cancer despite intensive searching[22]. PCab antibodies with ovarian cancer and no PCD have been reported in three patients[7]. Recently, PCab's were identified in two patients with chronic sensory neuronopathy without cancer. However, it is doubtful if this observation really represents anti-Yo since the antibody did not solely react with Purkinje cell cytoplasm but also with cytoplasm of other neurons[41].
In summary, a total of 7 patients with anti-Yo positive PCD without tumor have been reported. Like for the Hu syndrome, one possible explanation for false positivity of anti-Yo might be that the tumor-load is minor or that the tumor would have regressed spontaneously by effective antibody-mediated immune surveillance. If true, such observations are intriguing as they provide clues for further research on the immunotherapy of cancer.

Antigens
Immuno-electronmicroscopy studies have revealed that the anti-Yo antibody binds to clusters of ribosomes, to the granular endoplasmic reticulum and to Golgi complex vesicles of Purkinje cells[32,42]. Three antigens reactive with anti-Yo antibodies have been cloned. The minor 34 kD antigen is designated CDR34 (cerebellar degeneration related) and the gene has been mapped to the upper boundary of the FRAX marker on the human X-chromosome (Xq24-q27)[15,43]. The major 62 kD protein gene is designated CDR62 and has been mapped to the autosome 16 (16p12-p13.1)[17,44]. CDR proteins are uniquely expressed in Purkinje cells and some neuroectodermal cell lineages. Unlike Hu, which is expressed in all SCLC, the CDRs are only expressed in tumors of patients with anti-Yo positive PCD.

A cDNA encoding a 52 kD protein has been cloned and shows extensive homology to the other CDR62 protein. This protein was not only detected in cerebellum and brainstem but also in the intestines[16]. Both CDR proteins have a leucine-zipper motif and a highly repetitive sequence of a hexapeptide. Using deletion fragments of the recombinant 52 kD protein, the major epitope of anti-Yo was localized at the site of the leucine-zipper motif at the amino-acid residues 94-133[16].
One has suggested that these proteins are DNA-binding proteins and may have a prominent role in regulating gene transcription, if coupled to an as yet unknown partner protein. Binding of anti-Yo antibodies after uptake in Purkinje cells to these proteins may interfere with gene transcription by inhibiting the binding of the partner protein[16,42].

Paraneoplastic Opsoclonus-Myoclonus Syndrome: Anti-Ri Antibody

Opsoclonus is a disorder of saccadic eye movements consisting of involuntary arhythmic and multidirectional conjugated saccades. The opsoclonus is often associated with truncal ataxia, dysarthria, myoclonus, vertigo or encephalopathy and may accompany PCD[18]. Probably a lesion of the pontine paramedian reticular formation is the site of the lesion responsible for the opsoclonus[45].
Opsoclonus-myoclonus syndrome (OMS) occurs primarily in children as a self-limiting disorder and can be the result of a viral infection of the brainstem. In about 2% of children suffering from neuroblastoma, the disorder occurs as a paraneoplastic syndrome, and 50% of children who present with OMS would harbour a neuroblastoma[46,47].
In adults the OMS occurs less frequently as a paraneoplastic disorder. In a review on 58 cases, there were only 11 believed to be of paraneoplastic origin[48]. The two most commonly associated tumors are lung and breast cancer, although many other tumors can be involved[49].
Paraneoplastic OMS differs from other paraneoplastic syndromes in that remissions occur following anti-tumor therapy, after administration of thiamine, clonazepam or spontaneously[50,51].

Antibodies
Children with neuroblastoma and OMS have an excellent prognosis for long-time survival as compared to neuroblastoma patients without OMS. This would suggest a possible immune surveillance although no specific anti-neuronal antibodies have been detected to date [52-55].
Antibodies of different specificities have been observed in total of 9 infants with OMS without neuroblastoma and were partially directed against neurofilamentous structures, although the meaning of these observations is still uncertain[56-58].
In adults, some studies have failed to demonstrate antibodies in paraneoplastic OMS, including patients with SCLC[49,59]. In cases with antibodies against Purkinje cells, the occurrence of OMS is likely to be an accompanying symptom of more widespread PCD and would represent a different entity of disease compared to a distinct paraneoplastic OMS with

neuroblastoma or with other tumors[48,60-63]. In association with breast carcinoma, a specific anti-neuronal nuclear antibody in patients with paraneoplastic OMS has been identified by the Posner group. This antibody was designated anti-Ri and resembles immunohistochemically the Hu antibody by reacting with virtually all neuronal nuclei of the CNS. Western blotting however would clearly distinguish it from anti-Hu antibodies by reacting with two separate bands of 53-61 kD and 75-84 kD[59]. This antibody is also referred to as "ANNA-2"[23].

These findings have been confirmed in one series in which anti-Ri antibodies were recognized in serum and CSF of eight women with severe ataxia of which six had opsoclonus. Breast carcinoma was found in five patients, adenocarcinoma in one and carcinoma of the fallopian tube in another patient; in one subject no cancer could be found. As of now a total of 21 anti-Ri seropositive cases with paraneoplastic OMS have been reported. Of these, cancer was diagnosed in 19 patients (16 with breast, 2 with gynaecological cancer, 1 with lung cancer) and no cancer could be identified in two patients[59,63-66]. Anti-Ri was neither detected in one series of 23 patients with OMS and other types of cancer, nor in one series of 87 patients with breast cancer and no OMS[63].

False positivity

Two anti-Ri positive cases were identified in OMS without cancer[63,66]. There is one description of an anti-Ri positive patient with breast cancer and gait ataxia, vertigo, eye-movement disturbances and without true opsoclonus[65]. A recent observation on antibodies against a 58-59 kD neuronal protein in a patient with ovarian cancer and a patient with small cell lung cancer and paraneoplastic OMS would suggest that apart from anti-Ri, different antibodies may occasionally be associated with paraneoplastic OMS[67].

Antigen

The gene that encodes a protein recognized by anti-Ri sera has been cloned and has been called Nova. This highly conserved protein seems to play a role in the post-migratory maturation of neurons and is homologous to other RNA-binding proteins[68]. In mice, its expression is restricted to the brain stem and spinal cord[14].

Other paraneoplastic syndromes

Retinal Paraneoplastic Syndrome: Anti-Retinal Antibodies

Although distinctly uncommon, visual loss due to retinal degeneration has been associated with the presence of cancer. If present it is usually seen with SCLC but occasionally with breast, uterus, non-small cell lung carcinoma or melanoma. The retinal paraneoplastic syndrome was first described in 1979 by Sawyer et al. in three patients with visual loss, associated with SCLC in two, and endometrial sarcoma in one patient[69].

Antibodies against a 23-26 kD CAR (Cancer Associated Retinopathy, Recoverin[70]) retinal protein have been found in patients with retinal paraneoplastic syndrome and SCLC[31,71].

When anti-CAR antibodies are found in high titers in patients with unexplained visual loss, a search for an underlying malignancy is warranted. This is specially true when one realizes that in patients with retinal paraneoplastic syndrome, improvement of visual perception has been reported following either immuno-suppressant or anti-tumor therapy[72-74].

Stiff-man syndrome: Anti-GAD and Anti-128kD antibodies

Stiff-man syndrome is a rare and severe disease of the central nervous system characterized by progressive rigidity and painful spasms of the body musculature that was first described in 1956[75]. In about two-thirds of patients, the syndrome is associated with diabetes mellitus and these patients harbour antibodies against pancreatic islet-cells and GAD (glutamic acid decarboxylase). A distinct group is formed by paraneoplastic stiff-man syndrome[76]. Breast cancer is often the underlying malignancy and an antibody recognizing a 128 kD protein (amphiphysin) has been isolated[77].

Paraneoplastic Motor Neuron Disease

It is uncertain whether motor neuron disease may occur as a paraneoplastic disorder in association with a solid tumor, apart from cases when it is the predominant or presenting symptom of encephalomyelitis associated with the anti-Hu syndrome (about 14-20% of PEM/PSN cases)[12].

A separate form exists as a subacute motor neuronopathy in association with haemato-oncological disorders, like Hodgkin's and non-Hodgkin's lymphoma or leukemia[78-81]. In a survey on 422 patients with motor neuron disease, eight patients had lymphoma[82]. Younger and colleagues have suggested that in these patients sometimes the presence of upper motor neuron signs would establish a diagnosis of paraneoplastic amyotrophic lateral sclerosis. In three of the seven patients examined, the lymphoma was accompanied by a paraprotein.

Guidelines for the clinical usefulness of anti-neuronal antibody detection

Encephalomyelitis / sensory neuronopathy

In clinical practice, patients with an acute or subacute developing encephalomyelitis or sensory neuronopathy should be examined on the presence of anti-Hu antibody. If this antibody is found, a search for an underlying tumor should primarily be directed towards detection of small cell lung cancer. If the initial search is negative, the tumor may become manifest in the months after the onset of neurological symptoms. One would preferably screen sera for anti-Hu by using indirect immunohistochemical assays on rat and human cerebellar cortex. Positive immunohistochemical reactivity must subsequently be confirmed by Western blot using human proteins as substrate and preferably by use of the recombinant protein as well. Immunohistochemically determined titers of anti-Hu antibodies of 1:500 or higher can safely be interpreted as positive and would yield a diagnostic specificity of 98%.

Cerebellar syndrome
Patients with clinical suspicion on paraneoplastic cerebellar syndrome should be screened on the presence of anti-Purkinje cell antibodies. Anti-Purkinje cell antibodies consist of a variety of antibodies reactive with different antigens. These can be used as diagnostic markers for diagnosing a paraneoplastic origin of the cerebellar syndrome and if positive would prompt a search for an underlying tumor. It is important to differentiate between anti-Yo antibodies versus non-Yo PCab's that can be associated with Hodgkin's disease. Patients with PCD and positive anti-Yo antibodies determined by immunohistochemistry and Western blot on extracts of isolated Purkinje cells or recombinant Yo-antigens constitute a distinct group. Apart from rare exceptions, they carry gynaecological cancer and a focused search for an often occult tumor seems warranted. Presently, the major role for the identification of anti-Yo antibody is an early tumor diagnosis that would enable initiating treatment and hopefully leads to arresting the neurological progression.
When other PCab's are detected in a patient suspected of PCD the search for cancer should focus on gynaecological tumors, breast carcinoma, Hodgkin's disease or lung cancer. Detection of antibodies is performed by immunohistochemical detection and Western blot analysis using both human and rat tissue as substrate or recombinant Yo-protein. Immunohistochemically determined titers of PCab above 1:500 can be regarded as positive, yielding specificities approaching 100%.

Opsoclonus Myoclonus Syndrome
Detection of anti-Ri antibodies seems useful in adults with OMS either with or without other accompanying neurological symptoms. Anti-Ri antibodies are immunohistochemically difficult to differentiate from anti-Hu antibodies and reliable identification should be based on results from Western blotting on both cortical protein extracts and the recombinant protein. The specificity of anti-Ri detection for a diagnosis of paraneoplastic OMS is nearly 100%. The detection of anti-Ri antibodies should prompt a careful search for an underlying tumor, especially breast cancer. Occasionally no tumor can be found, although the presence of an occult tumor cannot be ruled out and this makes close follow-up advisable.
The finding of anti-Yo or other PCab's antibodies associated with paraneoplastic OMS indicates that this syndrome can be part of a more widespread PCD and under these circumstances a tumor search should also be directed to other gynaecological malignancies than breast cancer.
The significance of other anti-neuronal specific antibodies in patients with clinically suspected paraneoplastic OMS remains uncertain as yet. The sensitivity of the presence of these antibodies is low, as in many cases of OMS and cancer no antibodies can be found.

Conclusion

Except for motor neuron disease, the paraneoplastic disorders described here meet the requirements for possessing relevant antibodies. In each syndrome, autoantibodies should be

found in more than one patient at high titer. Each antibody thus designates a group of patients having a similar neurological disorder associated with specifically associated cancers. Usually the antibody reacts with a symptomatic part of the nervous system tissue, although a more widespread reactivity with clinically unaffected areas of CNS can be present. False positive antibodies are rare and this observation makes the detection of these antibodies of significant clinical value, not only for establishing a diagnosis of PNS, but also by indicating the presence and type of underlying neoplasm.

Nevertheless, there remains a substantial group of patients who are suspected of PNS and harbour no specific antibodies. Based on recent findings in patients with probable PNS, it seems likely that future studies may reveal the recognition of other specific antibodies that meet the criteria mentioned above[64,83-88]. Obviously, the clinician should remain alert, particularly when no specific antibodies are found and the possibility of a non-paraneoplastic cause should be kept high on the differential diagnostic list. We believe that this story is not complete and that over the next few years a number of other autoantibodies that are specifically associated with PNS will be identified.

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1.2 Aim of the thesis

Introduction

Although the first report on the detection of an anti-neuronal antibody in paraneoplastic neurological disease dates from 1965, it was only in the mid-eigthies that the presence of anti-neuronal antibodies in serum was used as a diagnostic marker for PNS[1-4].
In 1989 we introduced the assay to determine paraneoplastic antibodies as a diagnostic assay available to neurologists and other physicians in The Netherlands. At that time the classification of paraneoplastic syndromes (PNS) of the central nervous system was mainly based on clinical criteria. Although it was noticed that in more than 50% of cases the neurological symptoms precede the finding of underlying neoplasm, establishing a definite diagnosis of PNS during life was difficult[5]. Findings based on ancillary investigations were not specific and the diagnosis was based on clinical symptoms and on exclusion of other causes[5].
The indirect immunofluorescence assay (IIF) and the Western blotting technique to identify anti-neuronal antibodies were developed at the Departments of Pathology and Neuro-oncology of the Daniel den Hoed Cancer Center. This enabled us to provide a reference laboratory in The Netherlands for the detection of these antibodies in patients suspected of paraneoplastic neurological disorders. The above mentioned developments and the availability of patient sera created the base for the work described in this thesis.
The overall aim of the study described in this thesis was the development of methods for the identification of anti-neuronal specific antibodies and their antigens, to establish a diagnosis of paraneoplastic neurological disorders and the analysis of the relation of these antibodies with the clinical syndrome and the underlying malignancy.

Diagnostic value

When setting up an assay with the purpose of it being of clinical diagnostic use, the first question that rises is that of sensitivity and specificity of that assay. The aim of the first study, described in ***chapter 2***, was ***to establish the sensitivity and specificity of the anti-neuronal antibodies (at that time anti-Hu and anti-Yo) for making the diagnosis of PNS, when detected by IIF.*** Although it was known at that time, that the anti-Hu and anti-Yo antibodies were specifically related to paraneoplastic encephalomyelitis/sensory neuronopathy(PEM/PSN) and paraneoplastic cerebellar degeneration (PCD), respectively, and not found in control groups[2,3,6], no systematical study has been described reporting on the diagnostic value of the detection of these antibodies.

Genetic predisposition

Several observations suggest that a paraneoplastic neurological syndrome may in fact constitute a model of a human disease in which an immune response is directed against both the nervous system and the tumor[2,7-10]. Antibodies primarily directed against a tumor antigen and cross-reacting with similar neuronal antigens provide the common link. However, little information is available on the immunological mechanisms underlying this model. Whether the antibodies play a pathogenic role in causing neurological damage and tumor control or that their presence is merely an epiphenomenon remain to be established. Another crucial question is why only a few of all cancer patients develop this immune response and why other patients with the same tumors do not. This question is intriguing, if one realizes that the antibodies are directed against antigens which are expressed by each small cell lung cancer (SCLC)[7,8].

The aim of the study described in ***chapter 3*** was ***to provide data supporting the hypothesis that the rare occurrence of PNS may possibly be explained by a genetic predisposition of PNS patients to develop autoimmune disease.*** We describe 23 patients with paraneoplastic disease of the nervous system that are examined for the presence of systemic autoantibodies, including antibodies against DNA, Ro(SS-A), La(SS-B), mitochondria and IgG (rheumatoid factor). The presence of these autoantibodies may indicate a genetic susceptibility to develop autoimmune phenomena and may explain its relatively rare occurrence.

Clinical heterogeneity and IgG subclasses

Paraneoplastic encephalomyelitis/sensory neuronopathy (PEM/PSN) is a rare neurological syndrome, occurring in less than 1% of patients with SCLC[11]. It is therefore not surprising that mainly case reports or small series have been published on this subject. As recently as 1989 the largest series published on PEM/PSN still consisted of 18 patients[2,3,12-14]. The patients reported presented with a wide variety of clinical signs indicating the presence of limbic encephalitis, sensory neuronopathy, motor neuron dysfunction, cerebellar ataxia or brainstem dysfunction. The finding of the anti-Hu antibody in these patients in association with the presence of SCLC, suggests that these clinical signs are all manifestations of the same pathogenic process. It is, however, unknown why the Hu-syndrome shows major differences in clinical symptoms. The pathological findings also differ greatly in the site of inflammation and IgG depositions in the central nervous system[15,16]. It has been suggested that the clinical variety of the Hu-syndrome may be explained by different properties of anti-Hu antibodies like fine specificity of the antibody against different antigens of the Hu family (HuD, HuC, Hel-N1), antibody titer[17], complement binding capacity, accessibility of the central nervous system for antibodies or by differences in intrathecal anti-Hu synthesis[15,16,18]. Western blot analysis shows that anti-Hu of some patients primarily recognizes the upper or the lower band of the Hu protein complex (unpublished data). Another possibility is that the variety of anti-Hu IgG subclasses are responsible for the clinical heterogeneity. In an earlier

study involving a small number of patients, however, no correlation was found between IgG subclass and clinical presentation[16]. Until now there seems no satisfactory explanation for the variation in clinical symptoms or involved areas of the central nervous system in PEM/PSN. We studied series of 29 patients with the Hu-syndrome in relation with the anti-Hu IgG subclasses and tried ***to explain the variety in clinical course of the anti-Hu associated PEM/PSN (chapter 4).***

Novel antibodies

Because of the low incidence of PNS in patients with cancer, it is unlikely that the oncologist will see one patient with a paraneoplastic syndrome every year. If a patient with cancer complains of neurological symptoms, the likelihood of such a patient to have a paraneoplastic neurological syndrome is low, and non-paraneoplastic causes should primarily be sought. On the other hand, if a patient without cancer develops neurological symptoms and the cause of the neurological disability is not immediately obvious, the likelihood that such a patient has cancer is considerable[19]. This is especially true when the patient presents with one of the classic paraneoplastic syndromes. Although the likelihood in such patients of having cancer has not well been established, Posner gives an estimation of these changes to occur (table 1). The specificity of the antibodies for a diagnosis of PNS is high, but their sensitivity is relatively low[12,20]. Many patients clinically suspected of a PNS (probably > 50%) remain in whom no antibodies can be found[11,14]. At the other hand, many studies, mostly case-reports, indicate that other anti-neuronal antibodies against until as yet uncharacterized antigens may be present in some "sero-negative" PNS patients[21-23]. Other studies have not been able to detect other antibody reactivities in PEM/PSN patients with SCLC[24]. These differences may be explained by heterogeneity in patient groups or in detection techniques. Not all antibodies can be detected by routine immunohistochemical or Western blot assays. Methods of fixation can modify the antigenic epitopes and interfere with antibody detection[25]. Recently guidelines for the detection of clinically relevant antibodies associated with PNS have been published[26,27]. It is possible that other antibodies, although not fulfilling these guidelines are present in sera of patients with PNS. For Hu it has been shown that the antigen can occasionally be expressed by other tumors and corresponding antibodies can be found in serum[28]. Given the low sensitivity so far and isolated reports on incompletely characterized antibodies, it seems relevant ***to try to identify new anti-neuronal reactivity in relation to the neurological symptoms and thus increase the overall sensitivity of the detection of anti-neuronal antibodies for the diagnosis of PNS (chapters 5, 6 and 7).***

Table 1 Estimated likelihood of a given neurological disorder to be a paraneoplastic syndrome

Syndrome	% Paraneoplastic
Lambert-Eaton myasthenic syndrome	60
Subacute cerebellar degeneration	50
Opsoclonus/myoclonus (children)	50
Sensory neuronopathy	20
Opsoclonus/myoclonus syndrome	20
Myasthenia gravis	15
Sensorimotor peripheral neuropathy	10
Encephalomyelitis	10
Dermatomyositis	10

Source: J.B. Posner. Neurologic complications of cancer. Contemporary neurology series, Vol.45, p357. F.A. Davis Company, Philadelphia. 1995.

Guidelines

Since the discovery of the anti-Hu, -Yo and -Ri antibodies, identification of the paraneoplastic anti-neuronal antibodies rests on indirect immunofluorescence and Western blot analysis[26]. However, not all laboratories use Western blot analysis to identify the molecular weight of detected antigens[25,29,30]. A major problem in comparing results of different investigators has been that individual laboratories employ different techniques for the detection of these antibodies. Material from several animal species is used, methods of preparing substrates for Western blot are not always clearly described and concentrations of serum and end-point staining are not similar. Sometimes this results in substantial differences in sensitivity and specificity of the techniques used for paraneoplastic antibody detection[30]. Some early and recent publications on incompletely characterized novel anti-neuronal antibodies[21-23] have complicated the situation and seem to make it difficult for the clinician to interpret the significance of these antibodies.

Approach to consensus

In view of these uncertainties and controversies between research groups on the techniques and on the terminology of anti-neuronal antibodies, a workshop on this topic was organized during an international symposium on Paraneoplastic Neurological Disease, held on 22-23 October 1994 in Rotterdam, The Netherlands. The primary aim of this workshop was ***to formulate a report containing generally accepted guidelines to make laboratory testing of anti-neuronal antibody detection more uniform and easily interpretable and besides, to***

clarify the terminology. The resulting consensus described in ***chapter 8*** reports on the work from 11 international research groups that formed the consensus group to discuss and define the terminology, the methodology and interpretation of the finding of paraneoplastic anti-neuronal antibodies.

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2 Diagnostic value of anti-neuronal antibodies for paraneoplastic disorders of the nervous system

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Abstract

The diagnostic value of the presence of anti-neuronal antibodies in serum was examined in 21 patients suspected of paraneoplastic disorders of the nervous system (group 1) and was compared to three control groups; group 2: 25 patients with a neurological disease, without cancer and not signs of paraneoplastic disorder; group 3: 27 patients with neurological disease and cancer and no signs of a paraneoplastic disorder; group 4: 94 patients with cancer and without neurological disease. In group 1, anti-neuronal nuclear antibodies were detected in eight patients (38%), in titres from 1:1000 to 1:32000. A small cell lung cancer was present in six patients, ovarian cancer in one patient and in one patient no tumour could be detected. The neurological symptoms preceded a diagnosis of cancer in five out of eight patients. Anti-neuronal antibodies were found in the serum of two out of 94 patients (2%) from control group 3 but not in serum from any of the other control groups. These data indicate a moderate sensitivity of 38%, but a high specificity of 98.6% (95% confidence interval 95.5-99.8%) for the presence of anti-neuronal nuclear antibodies if a paraneoplastic nervous system disorder is suspected.

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Introduction

Paraneoplastic disorders of the nervous system can be divided mainly into limbic encephalitis, brainstem encephalitis, subacute cerebellar degeneration and a sensory or motor neuronopathy. In more than 50% of cases, they precede the finding of the underlying neoplasm[1]. Diagnosis during life can be difficult because clinical symptoms and findings on ancillary investigations are not specific. In recent years, several anti-neuronal autoantibodies have been identified in the serum of patients with a paraneoplastic disorder of the nervous system[2-4]. The antinuclear nucleoprotein antibody (anti-Hu) is mainly associated with small cell lung carcinoma (SCLC) and binds to nuclei of neurons[2-5]. The anti-Purkinje cell antibody (PCA) binds to the cytoplasm of Purkinje cells and has primarily been identified in patients with breast and ovarian cancer[3,6]. Apart from its interest in aetiology of paraneoplastic neurologic syndromes, the finding of these autoantibodies can be used for diagnostic purposes. We investigated the diagnostic value of the presence of anti-neuronal antibodies in patients with neurological signs suspected of paraneoplastic origin and compared it with three control groups.

Patients and Methods

Indirect immunofluorescence technique

The presence of anti-neuronal autoantibodies was investigated by the indirect immunofluorescence method (IIF). Snap frozen tissue blocks of human neuronal tissue (obtained at necropsy within six hours after death, from individuals without neurological disease) and with fresh neuronal tissue obtained from healthy Wistar rats, were used as test tissue. Unfixed frozen sections of human and rat cortex, cerebellum, spinal cord and vagal and optic nerve were incubated with serum diluted with phosphate-buffered saline (PBS, pH 7.2) 1:50 up to 1:6400, for one hour at room temperature. After rinsing three times in PBS for five minutes the sections were incubated with anti-human Ig and IgG (Fab)2, IgM (Fab)2 and IgA (Fab)2 monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) for 30 minutes (DAKO, Glostrup, Denmark). The dilution for the second antibody was 1:100 for Ig, IgG (Fab)2 and 1:50 for IgM (Fab)2. Sections were rinsed three times in PBS for five minutes, overlaid with a coverglass using a 10% glycine/PBS solution as mounting fluid, and evaluated under a Leitz fluorescence microscope with a FITC outfit.

As negative controls, sections were also incubated with PBS alone. To differentiate anti-neuronal antibodies from non-neuronal specific anti-nuclear antibodies, sera were also tested against frozen sections of normal human and rat liver. Stained sections were reviewed by the investigator and technician without previous knowledge of the patient's clinical status. Sera were considered positive for anti-neuronal antibodies only when distinct fluorescence staining was present in a titre of 1:500 or higher. Antibody-positive sera were also incubated with frozen sections of normal human and rat cerebral cortex, cerebellum, spinal cord, optical and vagal nerve.

Table 1 Patients and controls

Groups of patients		No.
1	Neurological disease with suspicion of paraneoplastic neurologic disease	21
	organic brain syndrome	5
	cerebellar ataxia	6
	sensory neuronopathy	5
	myelopathy	4
	brainstem encephalitis	1
2	Neurological disease without cancer and no suspicion of paraneoplastic neurologic disease	25
3	Cancer with neurological disease but no suspicion of paraneoplastic neurologic disease	27
4	Cancer without neurological disease	94
	small cell lung cancer	38
	breast cancer	9
	ovarian cancer	47

Four different categories of patients were distinguished: Group 1: patients with or without a verified cancer, in whom a reasonable or strong suspicion of a paraneoplastic neurological disorder was presented. Three case reports from this group have been described in detail[7]; Group 2: control group of 25 patients with neurological disease not related to a paraneoplastic neurological disorder and without cancer; Group 3: control group of 27 patients with neurological disease not related to a paraneoplastic neurological disorder and with cancer; Group 4: control group of 94 patients with cancer and no known neurological disease. Control sera of group 4 were collected over a period of five years and stored at -70°C. All patients in this group were prospectively studied and followed as part of ongoing chemotherapy trials. All sera of groups 1, 2, 3 and 4 were negative for anti-nuclear antibodies (ANF), when tested on normal human and rat liver or muscle tissue. In peripheral nerve or non-neuronal tissue, no reactivity could be observed using the same sera. No differences were seen in staining patterns on human or rat tissue.

Results

The number of patients in each group and a listing of the neurological diagnoses are summarized in table 1. The individual clinical and IIF data in patients with positive titres can be found in table 2. Anti-neuronal antibodies were detected in eight out of 21 patients

from group 1 (38%); in six patients with SCLC, in one patient with ovarian carcinoma and in one patient no tumour could be found. A bright speckled fluorescence of nuclei of cerebellar granular and Purkinje cells and cortical neurons with sparing of the nucleolus was observed in six patients (fig 1 and fig 2). This anti-neuronal nuclear antibody was of the IgG type (titres from 1:1000 to 1: 32 000), except one patient with an IgM antibody (titre 1:1000). Neurological symptoms preceded the diagnosis of a neoplasm in five out or the eight positive patients from group 1 with a median time interval of four months. An organic brain syndrome, compatible with a diagnosis of limbic encephalitis was seen in three patients with SCLC.

Extensive investigations including CT scan of the brain, bacterial and viral cultures of the cerebrospinal fluid (CSF) were negative. Causes for metabolic encephalopathies could be excluded and there were no indications of toxic exposure or drug abuse. Psychiatric evaluation did not reveal a cause for the mental abnormalities. A subacute cerebellar degeneration consisting of a truncal and appendicular ataxia together with dysarthria was seen in two patients with SCLC. A sensory neuronopathy in association with abnormal plantar reflexes was found in one patient with SCLC and in one patient in whom no tumour could be detected. A brain stem encephalitis consisting of a peripheral facial palsy, vestibular vertigo, glossopharyngeal and vagal nerve dysfunction, dysarthria and pyramidal signs was observed in a patient with ovarian carcinoma.

In the controls, only two patients of group 3 had increased titres of anti-neuronal nuclear antibodies, and both had a SCLC. One of these had recurrent headache with equivocal signs of pyramidal tract lesion, but no other neurological signs. The other patient had brain metas-

Table 2 Clinical and IIF data of patients with anti-neuronal nuclear antibodies

	Tumor	Onset (months)	Main clinical feature
Group 1			
1	SCLC	-0.5	Organic brain syndrome
2	SCLC	-2.5	Organic brain syndrome
3	SCLC	-24	Ataxia, dysarthria
4	SCLC	-2	Ataxia, dysarthria, sensory neuronopathy
5	SCLC	+4	Organic brain syndrome
6	Ovarian cancer	+9	Brain stem encephalitis
7	No tumour	-6	Sensory neuronopathy
8	SCLC		Sensory neuronopathy
Group 3			
9	SCLC		Brain metastasis
10	SCLC		Pyramidal tract lesion

Onset: time of onset of symptoms before detection of tumour, SCLC: small cell lung cancer

tases, treated by radiotherapy and was neurologically asymptomatic at the time serum was taken. When we consider group 2, 3 and 4 as one control group, the presence of anti-neuronal nuclear antibodies in patients suspected of a paraneoplastic neurolological disorder (group 1) indicate a sensitivity of 38% and a specificity of 98.6% (95% confidence interval 95.5-99.8%), (table 3).

Discussion

Anti-neuronal IgG antibodies in serum of patients with a paraneoplastic disorder of the nervous system have been identified, and can mainly be divided in anti-neuronal nuclear or cytoplasmic antibodies[2,4]. Antibodies binding to neuronal nuclei have been observed in Purkinje, granular, basket and molecular cells of the cerebellum as well as to nuclei of neurons in the cerebral cortex, brain stem, spinal cord and dorsal root ganglion[2,5]. These anti-neuronal nuclear antibodies have primarily been recognised as binding to neuronal nucleoprotein antigens with a molecular weight of 35-40 Kd and have been designated as anti-Hu[2,5]. The majority of anti-Hu antibodies are associated with SCLC[2-5]. We found anti-neuronal nuclear antibodies in three patients with an organic brain syndrome and these were compatible with a diagnosis of limbic encephalitis, in two patients with cerebellar ataxia and in one patient with a sensory neuronopathy, all with SCLC. An isolated IgM antibody in a patient with SCLC and suspected of limbic encephalitis is a new observation.

Table 2 Clinical and IIF data of patients with anti-neuronal nuclear antibodies (continued)

		Antibody specificity						
Titre	Class	CN	PC	GC	BC	SC	MLC	AHC
Group 1								
1:1600	IgG	+	-	+	-	-	-	-
1:1000	IgM	+	+	+	+	+	+	-
1:4000	IgG	+	+	+	+	+	+	-
1:32000	IgG	-	+	+	-	-	-	-
1:2000	IgG	-	+	+	+	+	-	-
1:6400	IgG	+	+	+	+	+	+	-
1:1600	IgG							
1:25000	IgG	-	+	+	-	-	-	-
Group 3								
1:800	IgG	-	+	+	-	-	-	-
1:6400	IgG	-	+	+	+	+	+	-

CN: Cerebral cortical neuron, PC: Purkinje cell, GC: Granular cell, BC: Basket cell, SC: Stellate cell, MLC: Molecular layer cell, AHC: Anterior horn cell.

Table 3 Diagnostic value of anti-neuronal antibodies for the presence of a PNS*

	No. of positive tests in control patients	No. of negative tests in control patients	Sensitivity	Specificity
Anti-neuronal nuclear antibodies				
Anderson et al.[5] 1988	14/87	303/303	14.5%	100%
This series	8/21	144/146	38%	98%
Anti-neuronal cytoplasmic antibodies				
Jaeckle et al.[4] 1985	6/12	167/167	50%	100%
Anderson et al.[6] 1988	18/42	317/319	43%	99%
Smith et al.[8] 1988	6/26	125/125	23%	100%

* In this table only series in which a titre of 1:500 or higher was considered to be positive have been included.

An anti-neuronal nuclear antibody in a patient with brainstem encephalitis and ovarian cancer has also not been reported before. The cause of this variation in clinical syndromes is unknown, but it may relate to differences in binding to one group of neurons or another.

In contrast, anti-neuronal cytoplasmic antibodies have primarily been associated with binding to Purkinje cells together with the occurrence of cerebellar ataxia and have been designated as PCA (anti-Purkinje cell antibodies)[3,4,6]. The clinical picture is usually designated as a paraneoplastic subacute cerebellar degeneration and has been encountered in gynaecological cancer, mainly ovarian, in breast and small cell lung cancer or in lymphoma[3,4,6]. In more than 50% of cases the paraneoplastic neurological disorder precedes the finding of a malignancy[1], which we also observed here. Until recently a final diagnosis of paraneoplastic disorder of the NS could only be confirmed at necropsy since the results of ancillary investigations are often negative or aspecific. Viral and post-viral inflammatory disease, Sjögren's syndrome or an idiopathic syndrome may give rise to similar clinical pictures[8,9]. The detection therefore of anti-neuronal autoantibodies in serum of patients suspected of a paraneoplastic NS syndrome could be of great help[5,6]. For this purpose information is needed about the sensitivity and specificity of the presence of these autoantibodies.

For anti-Hu antibodies, Anderson et al. found a specificity of almost 100% in a series of 18 patients with paraneoplastic NS disorder together with various control groups up to a total of 303 patients with or without cancer or neurological abnormalities[5]. They found false positive antibodies in two patients with a possible paraneoplastic neurological syndrome, but without the presence of a malignancy[5]. We found positive autoantibodies in two control patients of group 3. Two of these had a small cell lung cancer of the lung and positive titres of anti-neuronal nuclear antibodies. Neither of them had signs which are usually compatible with a paraneoplastic disorder of the NS. When we restrict ourselves to patients with positive titres

of anti-neuronal nuclear antibodies in serum, we found a specificity of 98% for the association of these antibodies with a suspected paraneoplastic disorder of the NS.
The presence of PCA has also been shown to be highly specific for a diagnosis of a paraneoplastic cerebellar degeneration[6,10]. In contrast to its specificity, the diagnostic sensitivity for the presence of either anti-Hu or PCA-antibodies was rather low in the few reported series, but this may be partly due to differences in opinion on a sufficiently high titer in serum[11,12]. A cut-off point of a titre of 1:500 or higher prevents a false positive interpretation of non-specific background stain[5,6,10]. Despite this precaution, the sensitivity may further differ from one series to another as the degree of clinical suspicion and the interpretation of subtle neurological findings is open to variation. Nevertheless, its high specificity makes the indirect immunofluorescence test a clinical useful screening test for the detection of anti-neuronal antibodies in serum of patients suspected of paraneoplastic disorder of the NS.

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3 Systemic and anti-neuronal autoantibodies in patients with paraneoplastic neurological disease

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Abstract

Sera from 23 patients with paraneoplastic disease of the central nervous system (PNS) were examined for the presence of anti-neuronal (anti-Hu, anti-Yo or anti-Ri) and systemic autoantibodies, including antibodies against DNA, centromeres, nRNP, Sm antigen, Scl-70, Ro(SS-A), La(SS-B), mitochondria, thyroid antigens, parietal cells, brush border antigen and rheumatoid factor. As controls, 33 patients with small cell lung cancer, 33 with ovarian cancer and 7 with breast cancer were examined. Systemic autoantibodies were found in 52% patients with paraneoplastic neurological syndromes compared to only 25% in control series of patients with solid tumors (p=0.013).

We conclude that this high percentage of systemic autoantibodies in PNS indicates a genetic susceptibility to develop autoimmune phenomena. One assumes that another prerequisite to develop paraneoplastic neurological disease is the occurrence of cross-reactivity against antigens expressed in both tumor and nervous tissue. Both conditions are probably necessary to develop PNS and may explain its relatively rare occurrence.

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Introduction

Anti-neuronal antibodies can be found in serum and cerebro-spinal fluid (CSF) of patients with paraneoplastic syndromes of the central nervous system[1-4]. The etiology of PNS is unknown but may be explained by an autoimmune reaction directed against certain specific antigens or epitopes shared by the underlying neoplasm and the nervous system[4-8]. Until now, three types of antibody reactivity have been demonstrated in association with PNS and their underlying tumors. These include the anti-Hu, anti-Yo and anti-Ri antibody which are connected to small cell lung cancer, ovarian cancer and breast cancer, respectively[9-11].
Other anti-neuronal autoantibody reactivities have been identified in individuals or small groups of patients with PNS, but their precise characteristics and clinical value remain yet to be determined[12-15].
An unresolved question is why a small percentage (<1%) of patients with cancer do develop this type of immune response and associated neurological disease. This problem is particularly intriguing if one realizes that the Hu antigen is expressed by all small cell lung cancers, and not only in patients suffering from PNS[11,16]. One answer could be that these patients have a genetic susceptibility to develop autoimmune reactivities. We examined wether in sera of patients with PNS, besides anti-neuronal autoantibodies, also systemic (non-neuronal) autoantibodies could be identified.

Patients and Methods

Sera of 23 consecutive patients (mean age: 59.7 $\pm$13.8 years, male/female ratio 14/9) with a clinical diagnosis of PNS and positive test for anti-neuronal antibodies (15 anti-Hu and 6 anti-Yo and 2 anti-Ri; titers > 1:1000) were tested for both organ and non-organ specific autoantibodies associated with other autoimmune disorders. Sera were tested for IgG antibodies against nuclear antigens including: native n-DNA, centromeres and the ENA group (nRNP, Sm antigen, Scl-70, Ro(SS-A), La(SS-B)). Other non-nuclear antibodies were tested against: mitochondria, smooth muscle, thyreoglobulin, thyroid microsomal antigen, gastric parietal cells, brush-border antigen and rheumatoid factor.
Serological determinations were performed without prior knowledge of clinical data. All tests were performed on one serum sample per patient. Samples were stored at -80 °C prior to testing.
Indirect immunofluorescence tests (IIF) were performed on all sera using various substrates as antigenic source including rat liver, culture preparations of Hep-2 cells (Inova, St. Diego, USA), Crithidia luciliae (DNA), rat kidney (mitochondria and brush border antigen), thyroid (thyroglobulin and microsomal fraction), stomach (parietal cells and smooth muscle)[17-19]. Antibodies to Sm, nRNP and Scl 70 were determined by immuno-precipitation reaction in agar gel[20,21]. Presence of anti-Ro/SS-A and anti-La/SS-B antibodies were tested by an ELISA method using affinity purified antigens[22]. Rheumatoid factor (IgM and IgA) was determined by an ELISA[23]. Antibodies to ganglioside GM1 were determined using an ELISA[24]. Anti-

neuronal antibody detection was performed using the indirect immunofluorescence assay and Western blotting techniques essentially as described by Graus[11] and slightly modified as described before[25].

Briefly, Hu fusion proteins (obtained from H. Furneaux, Memorial Sloan-Kettering Hospital, New York) and protein samples of both rat and human cerebral and cerebellar neurons, liver and spleen were separated by electrophoresis on a 10% polyacrylamide gel. Gels were loaded with 20 µmg of protein per lane. After electrophoretically being transferred to Hybond nitrocellulose membrane (Amersham, Buckinghamshire, United Kingdom), separate lanes were incubated with patient's and control serum in a 1:2000 dilution. After washing, lanes were incubated with alkaline phosphatase labeled anti-human IgG. To visualize reactive bands membranes were developed in 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad, Hercules, California).

In order to prove or exclude the presence of cross-reactivity of the anti-Hu with other antigens, sera were also pre-absorbed to the Hu fusion-proteins before testing. Sera from healthy controls and 33 patients with small cell lung cancer, 33 with ovarian cancer and 7 with breast cancer were used. Also reference values of our laboratory in which these tests were performed, including prevalence of autoantibodies in the normal population were used as controls.

Results

Of 23 sera from patients with PNS, 12 (52%) contained non-neuronal specific antibodies against one or more of the tested antigens, compared to 18 of the 73 (25%) control sera from patients with cancer only (p=0.013; see tables 1 and 2;). In 6 patients (25%) two or more antibodies could be identified. Anti-nuclear antibodies (ANA, centromeres, nRNP, Sm and La(SS-B) antibodies) were seen in 12 patients (52%). Antibodies against the ENA group were seen in 5 patients (22 %): two against La(SS-B), one against nRNP alone and two against both Sm antigen and nRNP. Antibodies against centromeres were seen in sera of two patients. In two sera anti-mitochondrial activity was observed and three sera showed antibodies against brush-border antigen. Three patients showed antibodies against smooth muscle antigen and two against parietal cells. No antibodies could be found to thyroid antigens, rheumatoid factor or Scl-70. Patients had no clinical signs of any other auto-immune disease than the paraneoplastic neurological syndrome.

Frequency of individual autoantibodies are listed in comparison with antibody frequency in the normal and diseased population (table 2). Pre-absorption to the Hu fusion proteins did not change the results of the systemic antibody testing.

Table 1 Systemic autoantibodies in patients with paraneoplastic neurological syndromes

			ANSA						
Pat.no.	Age	PNS	Type	Ig class / titer		Tumor	ANA	DNA	Cent
1	31	CS	PCA	IgG	>1:1600	M. Hodgkin	-	-	-
2	61	CS	PCA	IgG	>1:1600	Ovarian	-	-	++
3	78	CS	PCA	IgG	>1:1600	Renal cell	-	-	-
4	78	CS	PCA	IgG	>1:1600	M. Hodgkin	-	-	-
5	37	CS	PCA	IgG	>1:1600	Ovarian	-	-	-
6	39	CS	PCA	IgG	1:1600	M. Hodgkin	-	-	-
7	72	SNNP	Hu	IgG	>1:1600	SCLC	S40	-	-
8	70	CS	Hu	IgG	1:1600	SCLC	-	-	-
9	65	SNNP	Hu	IgG	1:500	SCLC	-	-	-
10	60	SNNP	Hu	IgG	1:1600	SCLC	S40	-	-
11	68	SNNP	Hu	IgG	1:1600	SCLC	-	-	-
12	46	CS	Hu	IgG	1:800	Breast	H160	-	-
13	81	CS	Hu	IgG	>1:1600	SCLC	-	-	-
14	83	CS	Hu	IgG/IgM	>1:1600	SCLC	-	-	-
15	59	SNNP	Hu	IgG/IgM	1:32 (CSF)	SCLC	H160	-	++
16	59	LE	Hu	IgG	>1:1600	SCLC	S160	-	-
17	45	ANP	Hu	IgG	>1:1600	SCLC	-	-	-
18	60	SNNP	Hu	IgG	1:1600	SCLC	S80	-	-
19	61	SNNP	Hu	IgG	1:1600	SCLC	-	-	-
20	56	SNNP	Hu	IgG	1:1600	SCLC	S80	-	-
21	48	SNNP	Hu	IgG	>1:800	SCLC	-	-	-
22	58	CS	Ri	IgG	>1:1600	Breast	-	-	-
23	58	SNNP	Ri	IgG	1:800	SCLC	S80	-	-

PNS = Paraneoplastic neurological syndrome
CS = Cerebellar syndrome
SNNP = Sensory neuropathy
LE = Limbic encephalitis
ANP = Autonomic neuropathy
ANSA = Anti-neural specific antibodies
PCA = Anti-Purkunje cell antibody
CSF = Cerebrospinal fluid
SCLC = Small lung cell cancer
ANA = Anti-nuclear antibodies
DNA = Anti-DNA antibodies
Cent = Anti-centromere antibodies

Table 1 Systemic autoantibodies in patients with paraneoplastic neurological syndromes (continued)

	ENA									
Pat.no.	RNP	Sm	SS-A	SS-B	Scl	AMA	ASMC	ASPA	BB	RF
1	-	-	-	-	-	-	-	-	-	-
2	++	++	-	-	-	-	-	-	-	-
3	++	++	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	++	++	-
5	++	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	++	++	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	++	-	-	++	-
14	-	-	-	++	-	-	-	-	-	-
15	-	-	-	-	-	-	++	-	-	-
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	-	-	-	++	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	++	-	-	-	-
22	-	-	-	-	-	-	++	-	-	-
23	-	-	-	-	-	-	++	-	-	-

S = Speckled anti-nuclear antibodies
H = Homogenous anti-nuclear antibodies
ENA = Extractable nuclear antibodies
RNP = Anti-ribonucleo protein antibodies
Sm = Anti-Sm antibodies
AMA = Anti-mitochondrial antibodies
ASMC = Anti-smooth muscle antibodies
ASPA = Anti-parietal cell antibodies
BB = Anti-Brush border antibodies
RF = Rheumatic factor
++ = positive

Table 2 Total number and prevalence (%) of systemic autoantibodies in paraneoplastic neurological syndromes and cancer- and normal controls*

	No. of patients	ANA	DNA	Cent	ENA		
					RNP	Sm	SS-A
PNS	23	8 (34)	0 (0)	2 (9)	3 (13)	2 (9)	0 (0)
Subgroups							
1. Anti-Hu positive	15	7 (46)	0 (0)	1 (7)	0 (0)	0 (0)	0 (0)
2. PCA positive	6	0 (0)	0 (0)	0 (0)	3 (50)	2 (33)	0 (0)
3. Anti-Ri positive	2	1 (50)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)
Solid tumors	73	11 (15)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Subgroups							
1. Small cell lung carcinoma	33	7 (21)	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)
2. Ovarian carcinoma	33	5 (15)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3. Breast carcinoma	7	1 (14)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Normal individuals	107	7 (7)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)

* For legend see table 1; Numbers indicate absolute value and percentage of total is given between brackets.

Discussion

We studied the presence of a spectrum of systemic autoantibodies in 23 patients with anti-neuronal antibody proven PNS. The overall frequency of systemic autoantibodies was significantly higher than in the control population (52% vs < 25%, table 2).

Since the discovery of anti-neuronal specific antibodies in patients with sensory neuronopathy and small cell lung cancer by Wilkinson et al. in 1965[2], more data have become available indicating that PNS may be caused by an autoimmune mechanism[5,7].

Paraneoplastic neurological syndromes partially fulfil Witebsky's criteria to characterize an autoimmune disease[26]. These criteria include the demonstration of relevant antibodies or cell-mediated immunity and the isolation of responsible antigens capable to induce an immune response in animals with corresponding pathological changes following either active or passive immunization.

Autoantibodies have been detected in both serum and cerebrospinal fluid of PNS patients, indicating intrathecal antibody production[27]. These antibodies can bind to neurons in brain regions affected by PNS[6,7]. In vitro experiments have pointed to a cytolytic effect of these antibodies against neuronal and tumor cell-lines[28].

Table 2 Total number and prevalence (%) of systemic autoantibodies in paraneoplastic neurological syndromes and cancer- and normal controls* (continued)

ENA							Total		p-value	
SS-B	Scl	AMA	ASMC	ASPA	BB	RF	I	II	I	II
2 (9)	0 (0)	2 (9)	3 (13)	2 (9)	3 (13)	0 (0)	17 (74)	12 (52)	<0.001	<0.001
2 (13)	0 (0)	2 (13)	2 (13)	0 (0)	1 (7)	0 (0)	10 (67)	5 (33)	0.010	0.081
0 (0)	0 (0)	0 (0)	0 (0)	2 (33)	2 (33)	0 (0)	5 (83)	5 (83)	0.005	0.001
0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	2 (100)	0.023	0.003
0 (0)	0 (0)	1 (1)	1 (1)	2 (3)	0 (0)	6 (8)	18 (25)	10 (14)	--	--
0 (0)	0 (0)	1 (3)	1 (3)	1 (3)	0 (0)	2 (6)	9 (27)	4 (12)	--	--
0 (0)	0 (0)	0 (0)	0 (0)	2 (6)	0 (0)	4 (12)	8 (24)	6 (18)	--	--
0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (14)	0 (0)	--	--
1 (1)	0 (0)	0 (0)	0 (0)	7 (7)	0 (0)	5 (5)	16 (15)	14 (13)	<0.001	<0.001

#=Total number and percentage () of patients having one or more autoantibodies; I=including ANA; II=excluding ANA.

Antigens have been isolated and cloned from both neural and tumor tissue, and expression ofthe Hu antigen can be found in nearly each small cell lung cancer, irrespective whether patients suffer from associated PNS or not[3,5,29]. However, neither by passive or active immunization, one has as yet been able to reproduce the disease in experimental animals[28].

Inspite of the rapid progress in detecting and isolating the involved antibodies with their corresponding antigens, the question remains why only a relatively small number of patients with cancer develop PNS.

Our data show that PNS patients, besides anti-neuronal antibodies, frequently harbor systemic autoantibodies that are usually associated with autoimmune diseases and of which some only rarely occur in the normal population.

Humans frequently develop autoantibodies without actual disease and this autoantibody formation appears to be related to both age and sex, the highest prevalence being observed in older females and the lowest in younger males[30].

Autoimmune disorders develop predominantly in women and it is no exception that more than one autoimmune disorder occurs in the same individual. The overlaps are generally seen within the same category of disease, but a higher incidence of organ-unrelated antibodies can be found in patients with an autoimmune disease[30]. One obvious explanation

for this aggregation of autoimmune responses may be a genetically determined predisposition of the individual for autoimmune reactivity[30].
One alternative explanation for the rarity of PNS may be the presence of a mutated antigen expressed by only a few small cell lung cancers. Dalmau et al. established a deleted region in the cDNA coding for Hu in SCLC of one PNS patient[31]. One argument against this theory holds that it seems less likely that antibodies raised against a mutated altered protein, would recognize the unmutated antigen as expressed by the central nervous system.
The finding of systemic antibodies in our patients cannot exclude a possible mutation of the Hu antigen as the underlying cause for the induction of PNS, but would make it a less probable explanation.
We conclude that the detection of a high percentage of autoantibodies usually associated with a spectrum of systemic autoimmune diseases provides indirect evidence for a genetic susceptibility in patients with PNS for autoimmune reactivity. Another prerequisite may be a cross-reactivity against identical antigens or epitopes by both tumor and nervous tissue. Both conditions are probably indispensable to develop PNS and may also explain the relative rarity of PNS in patients with cancer. As mainly small cell lung cancer shares certain antigens with neurons, this would explain the particular association of PNS with this type of cancer.

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4 Clinical presentation and IgG subclasses in anti-Hu positive paraneoplastic encephalomyelitis/sensory neuronopathy

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Abstract

It is unknown whether the wide clinical variety of the anti-Hu syndrome relates to differences in anti-Hu IgG subclasses. Therefore we examined the correlation between the anti-Hu IgG subclass (IgG_{1-4}) and clinical data in a group of 29 anti-Hu positive patients with paraneoplastic encephalomyelitis/sensory neuronopathy (PEM/PSN). The clinical course and onset of anti-Hu positive patients with PEM/PSN was divided according to: (1) presenting symptoms, (2) spectrum of neurological disease, (3) onset of disease, (4) time from neurological to tumor diagnosis, and (5) type of underlying tumor. Anti-Hu reactivity was determined by both the indirect immunofluorescence assay and immunoblot using the recombinant HuD protein. The anti-Hu IgG subclasses were tested by indirect immunohistochemistry, using HRP-labelled mouse anti-human IgG_{1-4} on sections of rat cerebellum. Seventeen patients (59%) presented with sensory, 3 (10%) with cerebellar, 4 (14%) with motor, 4 (14%) with mental and 1 (4%) with autonomic symptoms. Seven patients (24%) showed signs and symptoms of unifocal involvement. Fourteen patients had involvement of two areas. In seven patients three areas were involved. In eighteen patients (62%) the onset of neurological symptoms was subacute, in six acute and in five a more chronic course of disease was seen. Fourteen patients (48%) had a SCLC, 5 patients (17%) had a probable SCLC, 4 patients had a non-SCLC and three patients had a breast cancer. In 4 patients (14%) no tumor was found after follow-up between 20 to 78 months. Seven patients had only IgG_1 antibodies, 12 IgG_1 in combination with IgG_2 and 6 with IgG_3. IgG_4 was seen in four patients. Two patients showed only IgG_2. Pearson's correlation analysis indicated that a unifocal involvement is associated with absence of IgG_1 antibodies ($p=0.01$). This may imply that patients with IgG_1 anti-Hu antibodies may have more often multifocal involvement of the central nervous system.

Introduction

PEM/PSN is an infrequent neurological syndrome, that occurs in less than 1% of patients with small cell lung cancer (SCLC)[1]. Most patients with PEM/PSN and SCLC develop an immune response against the 35-40 kD neuronal protein (Hu antigen) which is expressed on neuronal cells and SCLC cells[2,3]. In 80% of patients neurological symptoms antedate the tumor diagnosis and the detection of the anti-Hu antibodies may lead to an early diagnosis of a still unidentified SCLC. Thus, anti-Hu antibodies, when found in high titer in serum and cerebrospinal fluid (CSF) of patients with PEM/PSN, may serve as a diagnostic marker for SCLC[4-6].

The detection of the anti-Hu antibody in patients with a variety of clinical signs as limbic encephalitis, sensory neuronopathy, motor neuron dysfunction, cerebellar ataxia or brainstem dysfunction suggests that these may all be different manifestations of one pathogenic process. However, the role of the anti-Hu antibody in causing clinical manifestations of PEM/PNS is unclear. Both in vivo and in vitro studies could not demonstrate a direct pathogenic role for the anti-Hu antibody [7-10]. It is unknown why patients show major differences in clinical symptoms or histopathological findings regarding site of inflammation and IgG depositions in the central nervous system (CNS).

One large study showed that 59% of the patients with PEM/PSN presented with sensory symptoms, 14% with motor weakness, 21% with cerebral symptoms, 13% with cerebellar dysfunction, 11% with brainstem dysfunction and 10% with orthostatic hypertension caused by autonomic nerve dysfunction [11]. Other clinical studies involved only small numbers of patients [2,12]. Until now no satisfactory explanation is known for the variation in clinical presentation and involved areas of the CNS in PEM/PSN.

The clinical variety of the Hu-syndrome may be caused by modulation of the immune response at the level of the blood brain barrier or differences in intrathecal anti-Hu[13,14]. On Western blot analysis of the human neuronal protein extracts, the anti-Hu IgG of some patients primarily recognizes either the upper or the lower band of the Hu protein complex. These differences in reactivity of the anti-Hu antibodies may partially explain the clinical heterogeneity. Another possibility is that anti-Hu IgG subclasses are responsible for the clinical variety. In an earlier study no correlation was found between IgG subclass and clinical presentation[15]. This study, however, was carried out in a small number of patients. In order to find an explanation for the wide variety in clinical course and onset of the Hu-syndrome, we examined the relationship between clinical data and anti-Hu IgG subclasses.

Patients and Methods

Patient selection

Over a five years period more than 2500 sera of patients with a clinical suspicion of a paraneoplastic neurological syndrome were sent to our laboratory for the detection of anti-neuronal antibodies (including anti-Hu,-Ri,-Yo). All sera were tested for anti-neuronal

antibodies using both the indirect immunofluorescence assay (IIF) and Western blot as described below. In total 30 patients were found positive for high titer anti-Hu antibodies (> 1:500 on IIF) and were selected for this study[16].
The distribution of clinical signs and symptoms was based on (1) presenting symptoms, (2) spectrum of neurological disease, (3) course at onset, (4) time from onset of neurological symptoms to tumor diagnosis and (5) type of underlying malignancy. Furthermore, clinical symptoms were scored based on possible affected parts of the entire nervous system including sensory-, motor-, cerebral-, cerebellar-, brainstem- and autonomic dysfunction, when they were not caused by metastatic or iatrogenic causes. The clinical presence of nystagmus was scored separately.

Immunofluorescence and determination of IgG subclass
Serum samples were drawn before anti-tumor therapy was initiated and stored at -80°C prior to testing. All tests were performed on one serum sample of each patient. Serological determinations were performed without prior knowledge of clinical data.
As antigenic substrate human and rat cerebellar and cerebral cortex, liver and kidney were used. Human tissue was obtained at autopsy performed between 0-16 hours post mortem.
Rat tissue was obtained immediately after decapitation. The indirect immunofluorescence assay was performed as described before[5]. Determination of IgG subclasses was performed as described below. Tissue was snap frozen in chilled isopentane and stored between -80 ^{0}C and -120 ^{0}C. Sections of 5 µm were cut on a cryostate, air dried for 20 minutes at room temperature and fixed in cold acetone for 10 minutes. After rinsing sections one time in phosphate buffered saline (PBS)(pH 7.4) sections were incubated with 0.1% hydrogen peroxide/PBS to avoid endogenous peroxidase activity and subsequently for 20 minutes with PBS containing 5-10% of bovine serum albumin (BSA) in order to block non-specific binding. Next, sections were incubated with patient serum (1:100 PBS) or CSF (1:5 PBS) for 1 hour at room temperature. After washing with PBS three times for five minutes sections were incubated with horseradish peroxidase (HRP) labeled rabbit anti-human pan-IgG-F(ab)2 (1:100)(DAKO, Glostrup, Denmark) and a panel of HRP labeled mouse monoclonal antibodies against human IgG_1 to IgG_4 (1:50) (Southern Biotechnology Associates, Inc. Birmingham, USA). After washing three times with PBS for 3 minutes bound IgG was visualized by developing the HRP with diaminobenzidine tetrahydrochloride (Sigma, St.Louis, USA).

Western blot
Western blotting was essentially performed following method described by Towbin in 1979 and slightly modified as described below[17]. As protein samples extracts of human and rat cerebellar cortical neurons and the HuD recombinant protein (obtained from H.M. Furneaux, Memorial Sloan-Kettering Hospital, New York, USA) were used. Protein samples were prepared by homogenizing frozen tissue pieces of 0.5 - 1.0 grams in a bullet homogenizer and dissolving the powder in PBS. After centrifugation at 11.000g the supernatant was recovered and protein contents measured by an MBradford protein assay. Protein samples

Table 1 Clinical presentation and IgG subclasses in anti-Hu positive PEM/PSN

Pat.no.	M/F	Age	S	Western blot		Immunohistochemistry					Tumor	Diagn. after onset of neur sympt. in months
				CB	HuD	Pan IgG	IgG1	IgG2	IgG3	IgG4		
1	F	62	+	++	++	1:1600	++	++	-	+	scle	6
2	M	48	+	++	++	>1:1600	+++	++	+	-	scle	8
3	F	59	+	++	++	>1:1600	++++	-	+	-	scle	3
4	M	81	-	-	+	<1:1600	+	+	-	+	n.t.f.	24
5	M	65	+	+	+	<1:1600	++	++	-	-	scle	3
6	F	45	+	-	+	<1:1600	++	-	+	-	scle	3
7	M	60	+	++	++	1:1600	++	++	-	-	p scle	24
8	F	73	-	-	+	<1:1600	+	-	-	-	n.t.f.	60
9	F	70	+	+	+	1:1600	-	-	-	-	scle	6
10	M	83	+	-	+	<1:1600	-	+++	-	-	p scle	4
11	F	73	-	+	++	1:1600	++	-	-	-	n-scle	1
12	M	80	+	+	++	1:1600	++	+	-	-	scle	0
13	M	72	+	++	++	>1:1600	++	-	-	-	scle,	11
14	M	59	+	++	++	>1:1600	++	++	-	-	scle	6
15	F	68	n.k.	+	+	1:1600	-	-	-	+	breast	0
16	M	70	-	++	++	1:1600	+++	+++	+	-	breast	78
17	M	68	+	-	++	1:1600	-	+++	-	-	scle	4
18	F	70	+	+	++	1:1600	++	-	-	-	p scle	2
19	M	56	+	++	+	1:1600	++	-	-	++	p scle	2
20	F	59	+	+	+	1:1600	+	-	-	-	scle	4
21	F	64	-	++	++	1:1600	+	-	+	-	n-scle	1
22	F	66	-	++	++	1:1600	+	+	-	-	n.t.f.	26
23	F	71	+	+	++	1:1600	+	-	-	-	n.t.f.	20
24	M	61	+	++	++	>1:1600	+	++	-	-	scle	24
25	M	68	+	++	++	>1:1600	+	++	-	-	p scle	11
26	F	70	+	++	++	>1:1600	+	-	+	-	scle	8
27	F	51	n.k.	++	++	1:1600	+	-	+	-	n-scle	1
28	F	69	+	++	++	1:1600	+	+	-	-	scle	2
29	F	58	+	++	++	>1:1600	+	-	-	-	breast	1
		avg 65.5	21				25	14	7	4		avg 11.8

CB: cerebellar protein extract; HuD: HuD fusion protein; >1:600: ranging from 1:3200 to 1:128.000; n.k.: not known; n.t.f.: no tumor found; scle: small cell lung cancer; n-scle: non small cell lung cancer; p scle: probable small cell lung cancer, avg: average. Numbers at bottom indicate average or total number of positives per column.

Table 1 Clinical presentation and IgG subclasses in anti-Hu positive PEM/PSN (continued)

Pat.no.	Survival after onset of neurol. sympt. in months	Course at onset	Presenting neurological symptoms	Total spectrum of neurological symptoms per patient					
				sensory	motor	cerebral	cerebellar	brainstem	autonomic
1	still alive	subacute	sensory	+	-	-	+	-	-
2	still alive	subacute	sensory	+	+	-	+	-	-
3	n.k.	subacute	motor	+	+	-	-	-	-
4	6	subacute	sensory	+	-	-	-	-	-
5	n.k.	acute	sensory	+	+	-	-	-	-
6	n.k.	acute	cerebral	-	-	+	-	-	+
7	36	chronic	sensory	+	-	+	-	-	-
8	still alive	chronic	sensory	+	+	-	-	-	-
9	40	chronic	motor	-	+	-	-	-	-
10	n.k.	acute	cerebral	-	-	+	-	-	-
11	3	acute	cerebellar	-	-	-	+	-	-
12	36	subacute	autonomic	-	-	-	+	+	+
13	still alive	subacute	sensory	+	-	-	-	-	-
14	n.k.	subacute	sensory	+	+	-	-	+	-
15	still alive	subacute	motor	-	+	-	-	-	-
16	still alive	subacute	sensory	+	-	-	+	-	-
17	still alive	subacute	sensory	+	-	-	-	-	+
18	1.5	acute	cerebellar	-	-	-	+	+	-
19	n.k.	subacute	sensory	+	-	-	+	-	-
20	3.5	subacute	sensory	+	+	-	-	-	-
21	14	subacute	cerebral	-	-	+	-	-	-
22	still alive	chronic	motor	+	+	+	-	-	-
23	still alive	chronic	sensory	+	+	-	-	-	-
24	still alive	subacute	sensory	+	+	-	-	-	-
25	11	subacute	cerebellar	-	-	+	+	+	-
26	10	subacute	sensory	+	+	-	-	-	+
27	n.k.	subacute	cerebral	+	-	+	-	-	-
28	24	acute	sensory	+	+	-	-	-	-
29	still alive	subacute	sensory	+	-	-	+	-	+
				20	13	7	9	4	5

were boiled in 1:5 Laemli's buffer (0.0625 M Tris hydrochloric acid (pH 6.8), 2% sodiumdodecylsulfate, 0.001% bromophenol blue and 5% 2-mercaptoethanol for 5 minutes. Protein samples were then separated by electrophoresis on a 10% SDS-polyacrylamide gel. Gels were loaded with 20 ug of protein per well or 150 ug per whole gel. After being transferred electrophoretically to Hybond nitrocellulose membrane (Amersham, Buckinghamshire, United Kingdom), separate lanes were incubated with patient's serum in a 1:1000 dilution. After washing, lanes were incubated with alkaline phosphatase labeled anti-human IgG $F(ab)_2$ (DAKO). To visualize reactive bands, lanes were then incubated with developing substrate for 10 minutes. Molecular weight of reactive bands was estimated by comparing to a pre-stained protein standard (SeeBlue™, Novex, San Diego, California).

Results

An overview of clinical features is given in table 1. All sera from the 29 patients with paraneoplastic neurological dysfunction showed characteristic anti-Hu staining pattern on immunofluorescence with bright staining of neuronal nuclei sparing the nucleolus and a diffuse fine granular staining of cytoplasm of all neurons in cerebellar and cerebral cortex. Titers ranged from 1:800 to 1:128.000 (table 1). All sera showed positive reactivity on Western blots with the HuD recombinant protein. Four sera, all with titers less then 1:1600, did not show anti-Hu reactivity against the human cerebellar cortex extract. Sixteen patients (55%) were female, age 45 to 73 (mean age 64.3) and 13 (45%) were men, ages 48 to 83 (mean age 66.4). From 27 patients data were available on smoking habits and 21 patients (72.4%) smoked more than 5 cigarettes per day.

Features relating to the tumor

Fourteen patients (48%) had a SCLC including one patient with a double tumor (SCLC and non-SCLC). Nine of these patients had a histologically proven SCLC and five a cytologically proven SCLC. Five patients (17%) had a probable SCLC, not histologically proven SCLC but the diagnosis was based on an X-ray or a CT-scan of the thorax. Four patients had a non-SCLC, including the one with the double tumor. Three patients had a breast cancer, of which one male. In the remaining 4 patients (14%) no tumor was found yet in spite of a follow up of 20 to 78 months. At the time the SCLC was diagnosed the tumor was limited to the chest in 13 (45%) patients and metastases outside the chest were found in 6 (21%) patients. In 23 (79%) patients the neurological symptoms antedated the finding of the tumor. In 6 the symptoms coincided with the tumor diagnosis. Mean time from onset of neurological symptoms until diagnosis of the underlying tumor was 11.8 months ranging from 0 to 6 years, including those patients in which no tumor had been found yet.

Presenting symptoms

The presenting neurological symptoms are summarized in figure 1. *Sensory symptoms* were the first complaint in 17 patients (59%), most of these complaints involved paresthesias or

dysaesthesias in the hands and feet. Four of these patients complained also about pain in hands or feet. *Motor weakness* as the presenting symptom was seen in four patients (14%). One patient (no. 3) showed a proximal paresis of both legs with muscle wasting. Another patient (no. 9) had a clinical picture starting with loss of strength in both hands.

In eight patients the clinical presentation was dominated by symptoms of disease outside the dorsal root ganglia or anterior horn cells. Three patients (10%) presented with complaints of *cerebellar dysfunction*, including both axial and limb ataxia, eventually developing to a pancerebellar and multifocal neurologic dysfunction. Four patients (14%) presented with symptoms of *limbic involvement* including seizures, confusion and memory loss as signs of cortical dysfunction. One of these patients (no. 10) developed subsequently the picture of a full-blown encephalomyelitis including sensory neuronopathy, cerebellar ataxia and autonomic dysfuntion. Presentation by *autonomic dysfunction* was only seen in patient no. 4 (3%) and was characterized by a severe orthostatic hypotension. Symptoms of *brainstem dysfunction* were not seen as the first presenting sign of PEM/PSN.

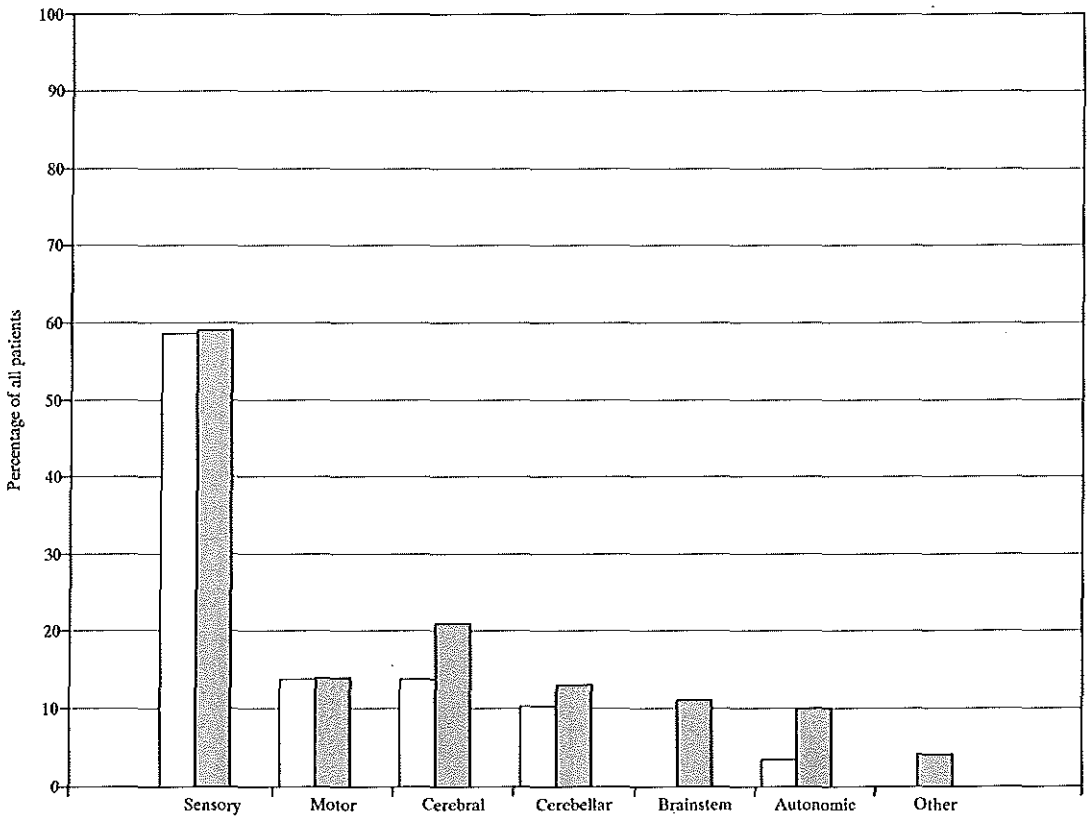

Fig 1 Presenting symptoms in 29 patients with PEM/PSN (%). Open columns represent data of the present study. Shadowed columns represent data from Dalmau et al.[11]

Spectrum of neurological disease

Figure 2 shows the areas of the nervous system involved that occurred during the disease. Seven patients (24%) showed signs and symptoms of unifocal involvement. All other patients developed multifocal neurologic dysfunction during the disease. Of the patients with unifocal involvement two patients showed cerebral dysfunction characterized by epileptic insults, together with confusion and memory loss, two patients motor neuron dysfunction characterized by muscle weakness and muscle wasting. In two patients the symptoms were limited to a pure sensory dysfunction and in one only cerebellar dysfunction was noticed. In the group of patients with multifocal involvement 14 patients had one other area involved. In seven patients three areas were involved. Sixteen patients had symptoms of a sensory neuronopathy accompanied by symptoms of involvement of other areas of the nervous system. The combination of sensory neuronopathy and motor weakness was seen eleven times. Other had cerebellar symptoms (5), cerebral/limbic symptoms (3), brainstem (1), or autonomic dysfunction (3). The combination of cerebellar- and brainstem dysfunction was seen three times (10%). Other combinations can be read from table 1. During the disease in total 20 patients (69%) finally showed signs of *sensory neuronopathy*, 13 of *motor neuron involvement* (45%), 7 of *limbic or cerebral cortical dysfunction* (24%), 9 of *cerebellar ataxia* (31%), 4 of *brainstem dysfunction* (14%) and 5 of *autonomic nerve dysfunction* (17%).

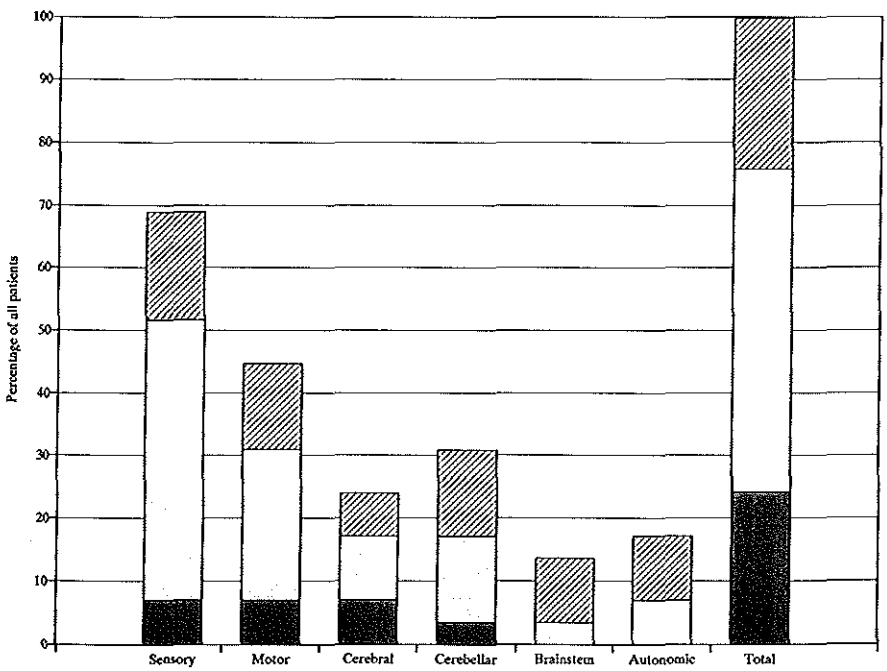

Fig 2 Number of involved areas of CNS in 29 patients with PEM/PSN (%). Shadowed parts represent patients with 1 area involved, open parts patients with 2 areas involved, and hatched parts patients with 3 or more areas involved.

Course at onset

In 18 patients (62%) the onset of the neurological syndrome followed a subacute course with symptoms developing over a few days to weeks. For the majority there was a rather rapid progression in the first period of disease followed by a more slow progression followed by stabilization in the months following. Most patients with a subacute course had progressive complaints of sensory disturbances as presenting symptoms.

A more acute course at onset was seen in six patients (20%). Two of these patients presented with seizures, two with rapidly progressing dysesthesia and pain and two others with an acute onset of gait ataxia.

A more chronic progressive course of the neurological disorder was seen in five patients (17%), involving three patients with sensory complaints and two with motor neuron dysfunction.

IgG subclasses

Anti-Hu antibodies of IgG_1 subclass were present in 25 (86%) cases, IgG_2 in 14 (48%), IgG_3 in 7 (24%) and IgG_4 in 4 (14%) cases (table 1). Seven patients showed IgG_1 reactivity only. Two patients showed only IgG_2 reactivity and one patient had only IgG_4 reactivity.

The combination of IgG_1 and IgG_2 was seen in eight patients, IgG_1 and IgG_3 in five patients and two patients showed reactivity of IgG_1, IgG_2 and IgG_3. Two other patients had IgG_1, IgG_2 and IgG_4 reactivity. Only one patient showed IgG_1 and IgG_4 reactivity. For patient number 9 it was not possible to determine the anti-Hu IgG subclass(es).

Cerebrospinal fluid was positive for anti-Hu antibodies in four of the six patients from whom it was available (data not shown). The anti-Hu antibodies in the CSF of three patients (no. 4,17,25) were of the same IgG subtype as in the serum, although IgG_1 could not be demonstrated in the CSF of patient number 4. Anti-Hu IgG_2 and IgG_4 were found in the CSF of patient number 13, while the serum showed IgG_1 reactivity only.

Correlation between IgG subclasses and clinical presentation

The only statistically significant correlation that could be found, indicated that a unifocal involvement was associated with the lack of IgG_1 antibodies. Three of the seven patients with only unifocal involvement had no IgG_1 antibodies, compared to 1 out of 21 patients with multifocal involvement ($p=0.01$)). No further statistically significant correlation was found between the anti-Hu IgG subclass and items on which clinical presentation was scored when arranged in two by two contigency tabels using Pearson's chi^2 or Fisher's two tailed exact test.

Discussion

In 1965 Henson and Urich introduced the term encephalomyelitis in patients with cancer who showed signs of damage in different parts of the nervous system[18]. In post mortem studies they recognized the variable distribution of inflammatory sites in the central nervous

system or dorsal root ganglia that could occur alone or concomitantly. In 1982 they reviewed 69 patients with PEM/PSN and recognized that the dorsal root ganglion was the most frequently involved area of inflammation and SCLC the most common type of malignancy[19].
Since the detection of the anti-Hu antibody in patients with a paraneoplastic neurological syndrome presenting with a variety of clinical signs of limbic encephalitis, sensory neuronopathy, motor neuron dysfunction, cerebellar ataxia or brainstem dysfunction it was suggested that these symptoms may all be different manifestations of one common pathogenic process. Consequently the term "Hu-syndrome" has been applied as a diagnostic entity to this group of symptoms next to the term PEM/PSN[11].
There is only one larger series of 71 patients reported on the Hu-syndrome[11]. Other descriptions can only be found in case-reports or series of less than 20 patients.
We report a second large series on the clinical spectrum of 29 patients with PEM/PSN and studied the relation between anti-Hu IgG subclass and variability in clinical course and presentation.

Patient selection and anti-Hu detection
Sera in our study were considered to be anti-Hu positive when both the results of IIF and Western blot were positive[20]. The sera of three patients did not react with the Hu complex in human cerebellar extract, but did react with the HuD recombinant protein. These observations illustrate the importance of using the recombinant protein in the detection of the anti-Hu antibody[21,22]. Earlier studies showed the importance of using both immunohistochemistry and Western blot, which has been adopted in a recent consensus report on guidelines for the proper detection and interpretation of paraneoplastic anti-neuronal antibodies[20].

Tumor type
In the majority of patients studied here the underlying tumor was SCLC (66%), a similar percentage to what others have found (77.5%)[11]. The tumor usually remains localized to the chest with limited metastatic spread. In our study, SCLC was limited to the chest in 13 (45%) patients. Earlier reports found limited disease in 96% of PEM/PSN patients with SCLC at the time of diagnosis. They hypothesized that the anti-Hu immune response may be effective in tumor growth control. Our data do not support this hypothesis, and are in agreement with data for SCLC in anti-Hu negative patients (limited disease in 42%)[23]. However, some patients have been in complete remission for more than 6 years after diagnosis.
We identified 4 (14%) patients in whom no tumor was found after a follow up of 20 to 78 months. In some patients the tumor was identified only after a long period of follow up. For example, in patient number 7 the tumor was found two years after onset of symptoms. In our study as well as in that of Dalmau et al. some tumors were only detected at autopsy[11]. We would advise regular screening of every patient with no tumor at presentation, as this may lead to the detection of the tumor up to several years later.
Many other tumor types have been reported to be associated with PEM/PSN including: breast, ovarian, endometrial, gastric and testicular cancer, synovioma, Hodgkin's disease

and undifferentiated or anaplastic carcinoma of the bronchus[11]. Most of these cases, however, did not harbor the anti-Hu antibody. Tumors other than SCLC, found in anti-Hu positive PEM/PSN have been found to express the Hu antigen (prostate carcinoma, adrenal carcinoma, chondromyxosarcoma, adenocarcinoma of the lung and neuroblastoma) mostly in individual cases. Next to SCLC, four patients had non-SCLC, including one with a double tumor (SCLC and non-SCLC). Interestingly, we observed three patients with breast cancer and anti-Hu antibodies. Breast cancer has been reported before in patients with PEM/PSN, however, not in association with anti-Hu antibodies. Whether breast cancer can express the Hu antigen is not known. Another possibility may be that these patients had an as yet not identified SCLC next to breast cancer.

Neurological findings

In 23 patients (79%) neurological symptoms antedated and in 6 the symptoms coincided with tumor diagnosis. The mean time from onset of neurological symptoms until diagnosis of the underlying tumor was 11.8 months ranging from 0 to 6 years.

In our study, 7 patients (24%) showed unifocal involvement, 48% had two areas involved and in 24% three areas were involved. Dalmau et al. found 20% with unifocal involvement, 39% with two areas of involvement and 54% with multifocal involvement. There seems to be no major difference in the clinical spectrum between our series and that of Dalmau et al. (fig 1)[11].

IgG subclasses

Jean et al. described nine patients with anti-Hu antibodies subdivided in IgG subclasses. All patients had IgG_1 antibodies, 5 IgG_2, 4 IgG_3 and none had IgG_4. All IgG bound to neurons was of the IgG_1 subclass. They found no correlation between the specific IgG subclass and neurological symptoms. They found no clear evidence that anti-Hu of different IgG subclasses recognized different epitopes limited to different brain regions. In another study, IgG_4 was, although in low titers, identified in serum of four patients[15]. The frequent presence of IgG_1 and IgG_2 cannot simply be explained by the fact that IgG_1 and IgG_2 represent 65% and 23%, respectively, of the total amount of IgG in serum in general. The anti-Hu IgG_1 and IgG_2 are clearly present in excess compared to the normal distribution. The predominance of anti-Hu IgG_1 suggests that fixation of complement and antibody-dependent cell-mediated cytotoxicity (ADCC) probably play a role in the immune response against the tumor and the nervous system.

The reason for this remains unclear although one may speculate that IgG_1 may pass the the blood brain barrier more easily. This may also explain why patients without IgG_1 have more often multiple areas of the CNS involved as is indicated by the finding that patients with IgG_1 have a statistically significant higher percentage of multifocal involvement.

In a direct immunohistochemical autopsy study, Graus et al. did not identify natural killer (NK) cells or deposits of complement in the nervous system of a patient with PEM/PSN, indicating that the ADCC mediated by NK cells and complement mediated cytotoxicity plays no or only a minor causative role in the damaging of neurons[24]. However, small amounts of complement have been observed in a few areas of the nervous system of four

anti-Hu positive patients[15]. IgG_4, if present, may play a protective role by masking the epitopes to prevent binding of the pathogenic, complement binding IgG_1 [15]. In our study the one patient that harboured IgG_4 antibodies had also IgG_1 antibodies. As he suffered from both sensory neuronopathy and cerebellar ataxia, this suggests that IgG_4, at least in his case, seems not to prevent damage of multiple areas of the CNS.

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5 Serum from four patients with paraneoplastic encephalomyelitis/ sensory neuronopathy (PEM/PSN) identifies a novel 60-64 kD protein in neurons and small cell lung cancer*

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Abstract

Serum of four patients with paraneoplastic encephalomyelitis/sensory neuronopathy contained a novel anti-neuronal antibody, designated anti-St. Anti-St recognizes a 60-64 kD molecular weight cytoplasmic protein complex in neurons of cerebellar and cerebral cortex. Indirect immunofluorescence studies showed diffuse homogeneous cytoplasmic staining of all neurons in cerebral and cerebellar cortex and in small cell lung cancer cell lines.
In three of the four patients the underlying malignancy was a small cell lung cancer. In the fourth patient no tumor was detected. The anti-St antibody was not found in control sera, including sera of 52 patients with cancer with neurological complications of non-paraneoplastic nature, 98 cancer patients without neurological complications, 51 patients with immune mediated neurological disease without cancer and 50 healthy controls.
We conclude that next to the anti-Hu antibodies, anti-St seems another specific anti-neuronal antibody associated with paraneoplastic encephalomyelitis/sensory neuronopathy and small cell lung cancer.

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Introduction

Patients with a clinically suspected paraneoplastic encephalomyelitis or sensory neuronopathy (PEM/PSN) harbour anti-Hu antibodies in 38-50% of cases[1-5]. This antibody was first described by Graus et al. in patients with sensory neuronopathy and small cell lung cancer (SCLC)[6]. Since then the detection of the anti-Hu antibody in patients with SCLC and encephalomyelitis, sensory neuronopathy or autonomic neuronopathy has lead to the suggestion that these clinical syndromes are different manifestations of one underlying process[7-9].

Previous studies have shown that the neurological symptoms can antedate the detection of the underlying malignancy in 70-80% of cases with PEM/PSN by several months to years[9,10,11]. The detection of the anti-Hu antibody precedes the diagnosis of the tumor in 60% and serves as a specific diagnostic marker for the presence of SCLC[1,9]. In one series of 71 patients, 78% of anti-Hu positive cases harboured a SCLC[9]. In 10% other tumors were present and in 13% no tumor could be found. The presence of the anti-Hu antibody seems to counteract tumor progression and even spontaneous regression of SCLC has been reported[12]. Only 4% of patients had metastasis outside the chest at time of diagnosis[9].

As in other paraneoplastic neurological syndromes (PNS), including paraneoplastic cerebellar degeneration (PCD) (associated with anti-Yo antibodies and gynaecological cancers)[13,14] and opsoclonus-myoclonus syndrome (associated with anti-Ri antibodies and breast cancer)[15,16] a commonly stated hypothesis says that the immune response is initiated by expression of neuronal antigens by the tumor subsequently cross-reacting with similar antigens in the CNS[17-19]. Darnell et al. have suggested that this immune response may be effective in tumor growth control of the underlying malignancy[20]. However, until now, neither in vivo nor in vitro experiments have lead to direct evidence for a pathogenic or immune modulating role of anti-Hu or other antibodies[20-24].

In spite of the specific association between anti-Hu related PEM/PSN and SCLC, 38-50% of patients with a clinical picture of PEM/PSN do not harbour anti-Hu antibodies[5]. Some studies, mostly consisting of individual case-reports, indicate that other as yet not characterized anti-neuronal antibodies can be found in some of these serologically negative PEM patients[5,24-26]. Others were unable to detect any other antibody reactivity in patients with PEM and SCLC[5]. These differences might be explained by the heterogeneity of patients and the possible nature of the antigen. It may well be that not all antigens can be detected by routine immunohistochemical or Western blot assays. Methods of fixation can greatly influence the antigenic epitopes and thus interfere with antibody detection[25].

In this paper we report four patients with PEM and SCLC in whom a novel anti-neuronal antibody against a 60-64kD neuronal antigen complex was detected by indirect immunofluorescence (IIF) and Western blot.

Material and Methods

Patient selection

Over the past five years more than 2000 sera of patients from our institution and from physicians throughout The Netherlands and Belgium, with a clinical suspicion of a paraneoplastic neurological syndrome were sent to our laboratory for the detection of anti-neuronal antibodies associated with PNS.

Sera were screened for the presence of anti-neuronal antibodies by IIF. Anti-neuronal antibody detection was performed using the IIF and Western blotting technique essentially as described before[17,28] and slightly modified as described here. Sera showing positive antibody reactivity on the IIF assay, regardless of the staining pattern, were tested for antibodies by Western blotting.

As controls were used sera of 52 patients with cancer with neurological complications of non-paraneoplastic origin, 98 cancer patients without neurological complications, 71 patients with immune mediated neurological disease without cancer (multiple sclerosis, myasthenia gravis and chronic inflammatory demyelinating polyneuropathy) and 50 healthy controls.

Anti-neuronal antibody testing

Tissue substrates and SCLC: As tissue-substrate, sections of human and rat central nervous system were used including cerebellar and cerebral cortex, dorsal root ganglia and spinal cord. Control tissue sections of liver and kidney and Hep-2 cells (Inova, St. Louis, USA) were used. Normal human neuronal tissue was obtained at autopsy within 6 hours after death, through the Netherlands Brain Bank (Amsterdam) from individuals without neurological disease. Rat tissue was obtained immediately after decapitation. Both human and rat tissues were snap frozen in chilled isopentane and stored between -80 ^{0}C and -120 ^{0}C before use. Aceton fixed cytospins of the GLC8-SCLC cell-line were used for determination of antibody reactivity against SCLC on IIF.

Immunofluorescence assay: Cytospins and sections of 5-7 µm were cut on a cryostate, air dried for 20 minutes at room temperature and fixed in cold acetone for 10 minutes. Tissue sections were incubated with 5% bovine serum albumin (BSA) in phosphate buffered saline, pH 7.4 (PBS) for one hour to block non-specific binding. Subsequently sections were incubated with patients sera in dilutions from 1:100 to 1:6400 in PBS. After washing, sections were incubated with FITC-labeled anti-human IgG-F(ab)$_2$ (1:40 PBS, DAKO, Glostrup, Denmark) for one hour. After washing sections were mounted with PBS/Glycerol (1:6) and read under a fluorescence microscope.

Western blotting: Western blotting was essentially performed following methods as described below[6]. Protein samples of cerebellar cortex and liver were prepared by homogenizing 0.5-1.0 gram of frozen tissue in a bullet homogenizer and dissolving the resulting powder in PBS. After centrifugation at 11000 g for 10 min the supernatant was recovered and protein contents measured by an MBradford protein assay. Protein samples were boiled in 1:5 Laemli's buffer (0.0625 M Tris-hydrochloric acid (pH 6.8), 2% sodium dodecyl sulfate, 0.001% bromophenol blue and 5% 2-ß-mercaptoethanol) for 5 minutes. Protein samples

were then separated by electrophoresis on a 10% SDS-polyacrylamide gel. Gels were loaded with 20 μg of protein per well or 150 μg per whole gel. After being transferred electrophoretically to Hybond nitrocellulose membrane (Amersham, Buckinghamshire, United Kingdom), separate lanes were incubated with patient's serum in a 1:1000 dilution. After washing, lanes were incubated with alkaline phosphatase labeled anti-human IgG $F(ab)_2$ (DAKO).
To visualize reactive bands, lanes were then incubated with developing substrate (5-bromo-4-chloro-3-indolyl-phosphate and nitroblue-tetrazolium (Bio-Rad, Hercules, California) in developing buffer (100mM Tris/HCl, 100mM NaCl, 5 mM MgCl, pH 9.5) for 10-30 minutes. Molecular weight of reactive bands was estimated using a prestained protein sample (Bio-Rad) as reference. Reactivity was scored positive when distinct bands were visible in neuronal extracts and not seen on liver extracts.

Control assays for systemic autoantibodies
In order to exclude false positive reactivity or cross-reactivity, all sera showing reactivity in one of the assays were tested for the presence of systemic autoantibodies (ANA, ANCA, anti-SSA, anti-SSB, RNP, Sm, Scl70, Jo1, IgM and IgA rheumatic factor, anti-mitochondrial, anti-parietal cell, anti-smooth muscle, anti-thyroglobulin and microsomal antibodies) using standardized routine assays as described before[29]. Positive reference sera against ANA, ANCA, SS-A, SS-B, RNP, Scl70, Sm and mitochondria were tested in both IIF and Western blot assay in order to determine reactivity patterns of these antisera against neuronal sections and protein extracts.

Results

Of all patients, 55 showed a characteristic clinical diagnosis of PEM/PSN. In sera of 30 patients anti-Hu antibodies were detected. In twelve anti-Hu positive patients small cell lung cancer was diagnosed at onset of neurological symptoms. In the anti-Hu negative group 68 patients had SCLC. In four of these patients, of whom three had SCLC, an antibody was found against a 60-64 kD reactive protein complex on Western blot (fig 1, appendix and table 1). Clinical features of the anti-Hu positive patients are described elsewhere (see chapter 6).

Anti-St reactivity
Immunofluorescence: On IIF, the antibody yielded a faint diffuse staining of the cytoplasm of all neurons on sections of cerebellar and cerebral cortex. Both Purkinje cells, granular layer cells and cerebral cortical neurons stained equally intensive in a homogeneous pattern, clearly different from anti-Yo cytoplasmic staining (figs 2a and 2b, appendix). The cytoplasm of dorsal root ganglia neurons (fig 2c, appendix) and motor neurons stained in an identical pattern. There was a low level of diffuse staining throughout the grey matter in sections of cerebellar and cerebral cortex. The antibody was of IgG isotype and present in

Table 1 Clinical and serological data in four patients harbouring anti-St antibodies

Patient no. M/F and age	Neurological symptoms	Course	Tumor	Tumor diagnosis in months after onset	Isotype and titer of Ab	Ancillary investigations	Serological reactivity in IIF and Western blot
1. M 65	Cerebellar syndrome	Subacute, stabilized	SCLC	0	IgG >1:800	CT normal CSF normal	Cytoplasmic, all neurons; 60-64 kD
2. M 64	Sensory neuronopathy and encephalo-myelitis	Subacute, slowly progressive	SCLC	6	IgG >1:800	CT normal CSF protein ↑ IgG ↑	Cytoplasmic, all neurons; 60-64 kD
3. F 80	Sensory neuronopathy and limbic encephalitis	Slowly progressive	SCLC	12	IgG >1:3200	CT normal CSF normal	Cytoplasmic, all neurons; 60-64 kD
4. F 69	Limbic ence-phalitis	Subacute, slowly progressive	no tumor	-	IgG >1:1600	CT normal CSF not done	Cytoplasmic, all neurons; 60-64 kD

titers from 1:800 to 1:3200 maximally when titrated in the IIF assay (table 1). In two sera also anti-nuclear antibodies were present when tested on both neuronal and liver tissue and the Hep-2 assay (patient 3 and 4). Sera of patient 1 and 2 yielded no reactivity on control tissue. This antibody reactivity was seen in none of the control sera. The serum of patient 1 was tested on cytospin preparations of the SCLC-cell line GCL8 resulting in a fine granular cytoplasmic staining, indicating that the antigenic epitopes are present in SCLC also (fig 2d, appendix).

Anti-St reactivity

Western blots: Reactivity of anti-St antibody on Western blots of extracts of human and rat cerebellar and cerebral cortex is shown in fig 1, appendix. A protein complex of 60-64 kD was identified by all four sera. The complex consisted of four separate bands of which the lower sharp band showed strongest reactivity in all four sera. The middle band was stronger than the upper and third band which yielded a weaker diffuse staining.

The protein complex in the rat cerebellar extracts comigrated with that in the human extracts but yielded a stronger (fig 1, appendix) more diffuse reactivity of the upper and third band. The complex was not identified in control extracts of rat and human liver. No immunoreactivity against this complex was seen using normal human sera and sera of the control groups.

Description of clinical findings

Case 1
A 65-year-old man with mild diabetic retinopathy developed severe cerebellar ataxia over a two days period, resulting in impossibility of walking or of eating independently. He experienced no dizzines or dysarthria. On neurological examination there was a horizontal nystagmus to the right. Strength was normal and sensory examination revealed hypoaesthesia of the distal extremities with asymmetrically lowered tendon-reflexes in the upper extremities and loss of tendon-reflexes in the lower extremities. There were no signs of cognition defects. A CT scan of the brain revealed no abnormalities. CSF examination showed normal cell counts, protein level and IgG index without signs of malignant cells. Serum anti-Hu antibodies were negative. On evaluation a SCLC was found four weeks after onset of neurological symptoms. The patient had been smoking cigarettes for over 30 years. A presumptive diagnosis of paraneoplastic cerebellar syndrome was made.

Case 2
A 64-year-old man suffered from paraesthesias and burning feet, increasing over a period of two-three weeks. After six weeks he experienced weakness in his legs and difficulties in walking. He had problems with bowel movements and suffered from hiccups. He had a dry mouth. Complaints of double vision, slurred speech and difficulties swallowing developed two months later. On neurological examination we observed an alert, healthy looking patient with intact cognition. There was mild distal sensory loss with disturbed pin-prick sensation and two-points discrimination. A moderate weakness of the extremities with preserved tendon-reflexes was found. There was moderate limb ataxia.
Examination of the CSF showed mild pleocytosis and increased IgG index. CT-scan of the brain and EEG showed no abnormalities. EMG examination revealed a mild axonal sensory polyneuropathy. Work-up for cancer resulted in a diagnosis of SCLC with multiple metastases in liver and mediastinum without malignant infiltration of the central nervous system, two months after onset of neurological symptoms. Anti-Hu antibodies in serum could not be detected. A presumptive diagnosis of paraneoplastic sensory neuronopathy and encephalomyelitis with brainstem, autonomic and motor involvement was made.

Case 3
This patient was an 80-year-old female with an unremarkable history. She developed progressive sensory loss over a period of one year with mild signs of organic brain syndrome, including disorientation in time, place and loss of short memory and cognition. There was no loss of strength with preservation of tendon reflexes. Clear hypoaesthesia of the lower limbs was found with disturbed pin-prick sensation and loss of vibration sense. Further examination revealed a SCLC one year after onset of neurological symptoms. A presumptive diagnosis of paraneopastic encephalomyelitis/sensory neuronopathy was made.

Case 4
A 69-year-old female with an otherwise unremarkable history developed signs of a severe organic brain syndrome over a six months period. There was severe loss of short memory functions and disorientation in time, place and person. Her relatives indicated that she could not be left alone since she would leave the gas turned on and ran out of the house leaving the doors open.
Further neurological examination revealed no signs of motor or sensory involvement. There was a clear loss of cognition. Ancillary investigations showed normal findings on CT-scan of the brain and normal CSF findings. Extensive search for an occult tumor did not reveal any tumor. She smoked 10-15 cigarettes a day. A presumptive diagnosis of paraneoplastic limbic encephalitis was made.

Discussion

We found a novel anti-neuronal antibody in four patients with a clinical picture of PEM/PSN of whom three harboured SCLC. The antibody was designated anti-St after the initials of the first patient. The anti-St antibody recognized a 60-64 kD cytoplasmic neuronal protein complex expressed in cerebellar, cerebral, dorsal root ganglion and motor neurons. At IIF the antibody recognized a diffusely expressed antigen which was present on virtually all neurons examined.
The serum of one of the patients recognized a cytoplasmic antigen in a SCLC cell line, indicating that the antigen was expressed by SCLC cells. However, this is no definite proof that this is an identical antigen as expressed by neurons, also because the reactive antigen in SCLC seems to be expressed in a more granular way than in neurons. Nonetheless, our data suggest that the anti-St sera recognize neuroectodermal antigens distinct from those described previously[25]. Since the anti-St antibody reactivity was neither found in sera of both normal and cancer groups nor in sera of patients with other neurological disease, this suggests that anti-St is a novel marker for PEM/PSN in patients who are anti-Hu antibody negative.
The low frequency and the diffuse staining pattern seen on IIF using frozen aceton fixed tissue substrates, may be the reason why this antibody reactivity has not been recognized before. Also the diffuse type of IIF staining together with the presence of other non-organ specific autoantibodies may have interfered with IIF recognition of the anti-St antibody. Recently both organ- and non-organ-specific antibodies have been identified in patients with other PNS, suggesting a genetic susceptibility of these SCLC patients to develop autoimmune reactivity[29]. The importance of appropriate methods of tissue-fixation and preparation of protein samples for Western blots, in order to identify novel antibodies or antigens involved in PNS has been recognized[25-27]. Recently PNS with novel anti-neuronal antibodies have been reported[25-27,30].
Darnell et al. identified antibody reactivity (anti-Nd) in one patient with cerebellar degeneration that reacted with a 150 kD protein expressed in Purkinje cells, cerebral neurons and

neuro-ectodermal tumors[27]. In their patient, however, no tumor could be detected. Antoine et al. identified antibody reactivity against 45, 60, 70 and 135 kD neuronal proteins in a patient with paraneoplastic encephalomyelitis, posterior uveitis and undifferentiated carcinoma. Nemni et al. reported anti-neuronal cytoplasmic antibody reactivity against a 62 kD cytoplasmic neuronal protein in patients with chronic sensory neuropathy without cancer[30].
When compared to the St antibody, the serum of the patient described by Antoine et al. showed a comparable but not identical immunohistochemical reactivity. The reactive bands on Western blot were of different molecular weight (57, 68 and 135 kD towards 60-64 kD in our patients) and their patient suffered from PEM in the presence of undifferentiated carcinoma[25]. The differences in antibody reactivity and molecular weights of reactive proteins may be due to differences in fixation methods (paraformaldehyde perfusion on paraffin sections versus aceton fixation on frozen sections). However, it seems more likely that this antibody recognized different neuronal antigens. The antibody described by Nemni et al. seems to recognize another neuronal antigen (personal communication) and is not associated with cancer.
The clinical picture of the patients described by us largely resembles the clinical picture of anti-Hu positive PEM/PSN and shows a similar variation. However, the onset of neurological symptoms in our four patients seems more protracted and less severe as compared to anti-Hu positive patients.
One patient presented with cerebellar symptoms, two with sensory signs and one with mental changes. In anti-Hu positive PEM/PSN, 59% present with isolated sensory symptoms and 21% with mental changes. Motor weakness is the presenting symptom in 14% and cerebellar symptoms in 13% of anti-Hu positive patients[9].
Except for patient no. 1 with a subacute cerebellar syndrome, the other three showed slowly progressive development of neurological symptoms over several months to one year. In anti-Hu positive patients, presentation of neurological symptoms is usually acute to subacute, with severe symptoms developing over a period of one night to two weeks, followed by slow progression over the next months. However, recently more indolent courses of anti-Hu positive PSN have been reported with moderate sensory loss developing over one or two years[31,32].
Another difference of our patients as compared to anti-Hu positive PEM/PSN concerns the nature of the underlying tumor. In 83% of anti-Hu positive PEM/PSN, neurological symptoms appear before a tumor diagnosis has been made and progression of neurological symptoms is often more severe than tumor-progression. Dalmau et al., Manley et al. and Delattre et al. have indicated that anti-Hu positive patients with SCLC have a better tumor survival than patients with SCLC without anti-Hu antibodies[10,19,33]. Although the median survival of both groups was not different, 68% anti-Hu positive patients had a tumor too small or localized to cause death, indicating that anti-Hu antibodies may be effective in tumor-growth control.
In our patients progression of the tumor was the main cause of death. This suggests that the anti-St immune response may not be as effective in tumor-growth control as the anti-Hu immune response. Obviously the number of patients with the anti-St antibody is small yet

and data on a possible role in the pathogenesis or in tumor-growth control by the antibody or anti-St reactive T-cells are scarce.

The pathogenic role of the anti-St antibody in the development of PNS remains to be established. This however, also holds for the other known paraneoplastic antibodies, like anti-Hu, anti-Yo and anti-Ri. As yet it has not been possible to demonstrate a definite pathogenic role for these antibodies, both in vivo and in vitro. A strict correlation between affected areas of the CNS in PNS patients and those neurons reactive with the associated anti-neuronal antibody is not always clear.

From a neurological point of view the paraneoplastic antibodies have led to remarkable findings. The antigens identified by the paraneoplastic antibodies play key roles in the development and differentiation of neurons. Most of the analysed antigens have RNA binding capacities. For the Hu antigen it has been suggested that it may be involved in oncogenesis. The Hu binds to the AU-rich elements of mRNA of c-myc, c-fos and GM-CSF, all proteins that regulate cell proliferation[33]. These findings stress the importance of the nature of the identified antigens involved in PNS. They support the hypothesis that antigens associated with autoimmune diseases are evolutionary conserved antigens playing key-roles in cell differentiation and maintenance[33].

Although the St antigen has only been partially characterized so far, further research on the exact structure of the St antigen is warranted and may lead to identification of another novel protein that is potentially important for neural and tumor development.

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6 Novel anti-neuronal antibodies in paraneoplastic neurological syndromes

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Abstract

Background. Detection of anti-Hu, -Yo, -Ri antibodies serves as highly specific diagnostic markers for paraneoplastic neurological syndromes. The sensitivity is limited, and this implies that many sera of patients with a paraneoplastic neurological syndrome remain antibody-negative. Some studies have indicated that other as yet incompletely characterized antibodies may be found in patients with paraneoplastic neurological disease. We performed a systematical study to determine the nature and potential diagnostic value of newly identified anti-neuronal antibodies.

Methods. We examined sera from 1600 patients suspected of paraneoplastic neurological disease and a total of 221 sera from four control-groups on the presence of anti-neuronal specific antibodies. Sera were screened by an indirect immunofluorescence assay, Western blots of neuronal protein extracts and in other routine autoantibody assays.

Results. We found autoantibodies in 18% (287/1600) patients. Systemic autoantibodies were identified in 6.4% of sera tested and in 7.3% of patients a range of other autoantibodies, often of low titer. Anti-neuronal antibodies were detected in 67 (4.2%) of patients. Forty-six (2.8%) patients had one of the well known paraneoplastic antibodies. In 21 patients, 5 newly identified anti-neuronal antibodies could be identified. An anti-60-64 kD antibody staining neuronal cytoplasm, was detected in 4 patients with paraneoplastic disease of whom 3 suffered from small-cell lung carcinoma. An anti 55 kD antibody showed a nuclear neuronal staining pattern in 4 neurological patients harboring a urogenital tumor. An anti-37 kD antibody reacting with all neuronal nuclei was identified in 7 patients with a variety of tumors and severe neurological complications. Of two other newly identified anti-neuronal antibodies, one (anti-210 kD) showed less consistent associated paraneoplastic features and of one (anti-40-200 kD) the nature of the antigen is uncertain.

Conclusions. This study shows the spectrum of autoantibodies that may be encountered in paraneoplastic neurological disease. Systemic antibodies were found in a relatively high percentage. Apart from anti-Hu, we identified novel anti-neuronal antibodies associated with paraneoplastic disease. A specific anti-neuronal 60-64 kD antibody associated with paraneoplastic neurological disease in small cell lung cancer. A 55 kD antibody was found to be associated with urogenital cancers, and a 37 kD antibody with a variety of cancers.

Introduction

Paraneoplastic neurological syndromes (PNS) consist of a variety of clinical neurological disorders that include: paraneoplastic encephalomyelitis (PEM), paraneoplastic sensory neuronopathy (PSN), paraneoplastic cerebellar degeneration (PCD), opsoclonus-myoclonus syndrome, and Lambert-Eaton myasthenic syndrome (LEMS)[1-3]. In 80% of PNS, the neurological symptoms antedate the detection of the underlying malignancy and frequently the progression of the tumor is slower than commonly seen in these patients[4-6].
Although the etiology of most PNS is unknown, it may be explained by an autoimmune response against similar antigens or epitopes shared by the tumor and the nervous system. Specific anti-neuronal antibodies, reacting with both neuronal and tumor antigens, have been recognized (table 1)[7-13]. In the Hu-syndrome (PEM/PSN) and in LEMS, both associated with small cell lung cancer (SCLC), antibodies against the Hu antigen and voltage-gated calcium channels (VGCC) have been identified[5,7,14]. Another example is PCD associated with gynaecological tumors in which antibodies can be found against the cytoplasm of Purkinje cells, designated anti-Yo[15,16]. In the opsoclonus-myoclonus syndrome associated with breast cancer, anti-Ri antibodies have been identified[11]. The presence of these anti-neuronal antibodies serves as a highly specific marker for a diagnosis of PNS and leads frequently to detection of the underlying tumor[17].
Next to their diagnostic value, PNS-associated antibodies have led to the detection of "onco-neural" antigens, which may play key roles in the regulation of gene expression or mRNA processing and thus in cell maintenance and proliferation[18-22].
Despite their specificity, the limited diagnostic sensitivity of the antibodies implies that many patients with PNS are antibody negative[16]. In almost 50% of PEM/PSN and SCLC, no anti-Hu antibodies can be identified. Some studies indicated that other as yet not well characterized antibodies can be found in sera of patients with PNS[23-28]. Others, however, were unable to detect a non-Hu antibody reactivity in PEM/PSN and SCLC[29,30]. We performed a systematical study on sera of 1600 patients to determine the existence and nature of newly identifiable anti-neuronal antibodies in PNS.

Patient and Methods

Patient Selection
Over the past five years more than 2500 sera of patients with a clinical suspicion of PNS were sent to our laboratory for the detection of anti-neuronal antibodies: anti-Hu, -Ri, -Yo and anti-Purkinje cell antibodies. From 1600 patients sufficient neurological and oncological data were available to be selected for this study.
Suspicion on PNS was scored in three grades based on clinical data. Grade 1: no suspicion; grade 2: possible PNS; and grade 3: clinically definite PNS. Patients had grade 1 when at the time of testing no tumor was diagnosed and neurological symptoms were not characteristic for PNS, or could well be explained by another cause. Grade 1 was also applied to patients

with a known tumor but in whom brain metastasis or therapy-induced neurological symptoms were present. Grade 2 was designated to patients suffering from a characteristic paraneoplastic neurological syndrome (e.g. encephalomyelitis/limbic encephalitis, sensory neuronopathy, sensory-motor neuropathy, cerebellar ataxia, bulbar encephalitis, opsoclonus-myoclonus syndrome or a combination of these), in whom no tumor was known at the time of testing and for whom no other satisfatory cause could be given. Patients with characteristic paraneoplastic neurological symptoms without other satisfactory explanation and with a known tumor at time of testing were scored as grade 3. All patients with either grade 2 or 3 were selected for this study. Also all sera showing anti-neuronal specific antibody reactivity on the indirect immunofluorescence (IIF) assay were selected for this study, regardless of grade of suspicion.
As controls (table 2 A), four groups were selected: sera of 52 patients with cancer with neurological complications (like brain metastasis or therapy-induced neurotoxicity) but no PNS, 98 cancer patients without neurological complications, 51 patients with other immune-mediated neurological disease (multiple sclerosis, myasthenia gravis) without cancer and 20 healthy controls.

Screening-assay
Sera of all 1600 patients were screened for the presence of anti-neuronal antibodies by the IIF as described previously[17,31] and in short below. Sera of patients with grade 2 or 3, and those positive on the first IIF assay were tested for anti-neuronal antibodies by IIF assay and by Western blotting on neuronal protein extracts.

Anti-neuronal antibody testing
As tissue-substrates for the IIF assay sections of human and rat cerebral and cerebellar cortex were used. As control tissue sections of liver, muscle and kidney were used. Human tissue was obtained at autopsy performed between 0-16 hours post mortem. Rat tissue was obtained immediately after decapitation. Tissues were snap frozen in chilled isopentane and stored between -80 ^{0}C and -120 ^{0}C. Sections of 5-7 µm were cut on a cryostate, air dried for 20 minutes at room temperature and fixed in cold acetone for 10 minutes. After rinsing sections one time in phosphate buffered saline pH 7.4 (PBS), sections were incubated for 20 minutes in PBS containing 5-10% of bovine serum albumin (BSA) in order to block non-specific binding. Sections were then incubated with patient serum in a 1:100 dilution of a 5% BSA solution in PBS for 1 hour at room temperature. After washing with PBS three times for five minutes, sections were incubated with secondary anti-human IgG(Fab_2) antibody (DAKO, Glostrup, Denmark) labeled with fluoresceinisothiocyanate (FITC) in a 1:40 dilution for 1 hour. After washing three times for five minutes sections were mounted with mounting fluid consisting of a 9% glycerol solution in PBS and evaluated under a fluorescent microscope. Positivity was scored when a clear fluorescence pattern was seen on neuronal tissue but not on control tissue. Titer of positive sera was determined by serial dilution of patient's serum on rat cerebellar sections using the IIF assay.

Table 1 Possible antibody mediated paraneoplastic neurological syndromes with incidence rates in The Netherlands calculated over a 5 years period (1990-1995)*

Antibody	Paraneoplastic neurological syndrome	Associated malignancy	Antigen location
Anti-Hu	Encephalomyelitis Sensory neuronopathy	Small cell lung cancer	Nuclei of all neurons
Anti-Yo	Cerebellar degeneration	Ovarian	Purkinje cell cytoplasma
Anti-PCA	Cerebellar degeneration	Hodgkin's lymphoma	Purkinje cell cytoplasma
Anti-Ri	Opsoclonus myoclonus	Breast cancer	All nuclei of CNS neurons
Anti-CAR	Visual loss	Small cell lung cancer	Retinal neurons
Anti-128kD	Stiff-man syndrome	Breast cancer	Synapses of CNS neurons
Anti-VGCC	Lambert-Eaton myasthenic syndrome	Small cell lung cancer	Voltage gated calcium channels

* Numbers are based on data from the Netherlands cancer registry 1989, Cancer incidence and survival 1955-1994 from the Eindhoven cancer registry.

Western blot

Western blotting was essentially performed following the method described by Towbin et al. and slightly modified as described below[31,32]. Protein samples of cerebral and cerebellar cortex, liver and muscle were prepared by homogenizing 0.5 - 1.0 grams of frozen tissue pieces in a bullet homogenizer and dissolving the powder in PBS. After centrifugation the supernatant was recovered and protein contents measured by an MBradford protein assay. Protein samples were boiled in 1:5 Laemli's buffer (0.0625 M Tris hydrochloric acid (pH 6.8), 2% sodium dodecyl sulfate, 0.001% bromophenol blue and 5% 2-mercaptoethanol for 5 minutes. Protein samples were then separated by electrophoresis on a 10% SDS-polyacrylamide gel. Gels were loaded with 20 µg of protein per well or 150 µg per whole gel. After being transferred by liquid electrophoresis to Hybond nitrocellulose membrane (Amersham, Buckinghamshire, United Kingdom), separate lanes were incubated with patient's serum in a 1:1000 dilution. After washing, lanes were incubated with alkaline phosphatase labeled anti-human IgG (Fab2) antibody (DAKO). To visualize reactive bands, lanes were then incubated with developing substrate (5-bromo-4-chloro-3-indolyl-phosphate and nitroblue-tetrazolium (Bio-Rad, Herculus, California) in developing buffer (100mM Tris/HCl, 100mM NaCl, 5 mM MgCl, pH 9.5) for 10-30 minutes. Molecular weight of reactive bands was estimated using a prestained protein sample (Bio-Rad) as reference. Reactivity was scored positive when distinct bands were visible in neuronal extracts and not seen on liver or muscle extracts.

Table 1 Possible antibody mediated paraneoplastic neurological syndromes with incidence rates in The Netherlands calculated over a 5 years period (1990-1995)* (continued)

Immunoblot	Total no. of associated tumors/year in The Netherlands	Total number of patients with PNS diagnosed/year	Total incidence rate of PNS per tumor/year(%)
38 to 40 kD	1300	7	0.53
34 and 62 kD	1275	1.2	0.09
negative	375	0.6	0.16
55 and 80 kD	8000	0.6	0.01
23 and 48 kD	2500	0	0
128 kD	8000	0	0
?, 50 kD	1300	n.k.	n.k.

* The Netherlands central bureau of statistics 1993: Vademecum of health statistics of The Netherlands

Control assays for systemic autoantibodies

In order to exclude false positive reactivity or cross-reactivity, all sera showing reactivity in one of the assays were tested for the presence of systemic autoantibodies (ANA, ANCA, anti-SSA, anti-SSB, RNP, Scl70, Sm, Jo1, IgM and IgA rheumatic factor, anti-mitochondrial, anti-parietal cell, anti-smooth muscle, anti-thyroglobulin and microsomal antibodies using standardized assays[31]. IIF assays were performed on all sera using various substrates as antigenic source including rat liver, culture preparations of Hep-2 cells, Crithidia luciliae (DNA), rat kidney (mitochondria and brush border antigen), thyroid (thyroglobulin and microsomal fraction), stomach (parietal cells and smooth muscle)[33-35]. Antibodies to Sm, nRNP and Scl 70 were determined by immuno-precipitation reaction in agar gel[36,37]. Presence of anti-Ro/SS-A and anti-La/SS-B antibodies were tested by an ELISA method using affinity purified antigens[38]. Rheumatoid factor (IgM and IgA) was determined by an ELISA[39]. Positive reference sera against ANA, ANCA, SS-A, SS-B, RNP, Scl70, Sm and mitochondria were tested on both IIF and Western blot assays in order to determine reactivity patterns of these antisera against neuronal sections and protein extracts.

Results

Selected patients

Of 1600 patients, 514 scoring grade 2 or grade 3 or showing positive reactivity in the first IIF assay were selected for further study. A summary of the clinical features of these 514

Table 2 A Overview of control groups

Controls	No.
Healthy individuals	20
Diseased controls:	
Multiple sclerosis	20
Myasthenia gravis	30
Primary Sjögren's syndrome	45
Systemic lupus erythematosus	10
Tumor controls:	
Small cell lung cancer	55
Ovarian cancer	33
Breast cancer	10
	Total 223

Table 2 B Tumor type in 514 selected patients (grade 2 or 3 or positive for anti-neuronal antibody)

Tumor diagnosis	No.
Small cell lung cancer	68
Non-small cell lung cancer	74
Breast cancer	94
Ovarian cancer	20
Cervical cancer	7
Prostate cancer	23
Hodgkin's lymphoma	9
Non-Hodgkin's lymphoma	16
Other tumors	64
No tumor found	139
	Total 514

Table 2 C Paraneoplastic syndrome in relation to presence of tumor

Neurological syndrome	Tumor +	Tumor -
Sensory neuronopathy	36	27
Sensori-motor neuropathy	84	28
Motor neuropathy	26	17
Cerebellar syndrome	85	39
Opsoclonus myoclonus syndrome	1	4
Organic brain syndrome	37	9
Brain stem syndrome	8	1
Otherwise or combinations of above	98	14
	Total 375	Total 139

patients is presented in tables 2 B and 2 C. At the onset of neurological symptoms no tumor was known in 138 patients and 68 had SCLC, 74 non-SCLC, 20 ovarian cancer, 94 breast cancer, 7 endometrial carcinoma, 16 non-Hodgkin lymphoma, 9 Hodgkin's disease, 23 prostate carcimoma and 64 patients had other primary tumors (table 2 B). Neurological symptoms consisted of 46 patients with organic brain syndrome, 63 with sensory polyneuropathy/neuronopathy, 112 with sensori-motor polyneuropathy, 43 with motor neuron disease, 124 with cerebellar syndrome, 5 with opsoclonus myoclonus syndrome, 9 with bulbar encephalitis and 112 with other neurological signs or a combination of two or more of the above syndromes (table 2 C).

Frequency of autoantibodies
By IIF, Western blot and other autoantibody assays, we found autoantibodies in 17.9% (287/1600) patients. A total of 117 patients had different non-neuronal specific autoantibodies (see below). In assays for organ specific autoantibodies we found positivity in 103 of the 735 sera of patients and controls tested in these assays.
Anti-neuronal antibodies were found in 67 (4.2%) patients. Forty-six (2.9%) patients had one of the well-known paraneoplastic antibodies, including 35 anti-Hu antibodies, 6 anti-Yo antibodies, 3 anti-Purkinje cell (anti-Tr) antibodies associated with Hodgkin's disease and 2 anti-Ri antibodies. Antibodies against the cancer associated retinopathy (CAR) protein and the 128 kD protein associated with stiff-man syndrome were not found. Clinical data of patients positive for anti-Hu, -Yo, -Tr and -Ri are not included this report.

Novel anti-neuronal antibodies
Antibodies against 5 different not previously identified neuronal antigens were found in 21 of 1600 (1.3%) patients (see below and table 3). An overview of different antibodies and clinical data is presented in table 4.

Anti 60-64 kD: In 4 patients, an antibody against a 60-64 kD neuronal protein complex on Western blot were detected. This antibody showed cytoplasmic staining of all neurons. The antigen was not detected in other tissues (see Patient and Methods) by IIF or Western blot (fig. 1, appendix). In three of these patients, two with sensory neuropathy and one with a cerebellar syndrome, SCLC was detected. In one patient with organic brain syndrome no tumor could be found (see also chapter 5). This antibody reactivity was not seen in any of the control sera.

Anti-55 kD: In 4 patients an antibody was detected that reacted with a 54-55 kD neuronal protein on Western blot. On IIF a neuronal nuclear staining pattern was seen. Weak reactivity was found in non-neuronal tissues (see Patients and Methods) in both IIF and Western blot (fig. 2, appendix). All 4 patients had a tumor of the genito-urinary system. Two patients had a sensory neuronopathy and cervical carcinoma. Two patients suffered from a cerebellar syndrome of which one patient had ovarian cancer and the other bladder carcinoma. The 55 kD antibody reactivity could not be detected in sera of the control groups.

Table 3 Overview of anti-neuronal antibodies identified in 1600 patients with suspected paraneoplastic neurological syndromes

Anti-neuronal antibody	No of positives (% of 1600)	Most frequently associated neurological syndrome	Most frequently associated tumor
Anti-Hu	35 (2.2)	Encephalomyelitis Sensory neuronopathy	Small cell lung cancer
Anti-Yo	6 (0.4)	Cerebellar syndrome	Ovarian cancer
Anti-Tr	3 (0.2)	Cerebellar syndrome	Hodgkin's disease
Anti-Ri	2 (0.1)	Opsoclonus myoclonus	Breast cancer
Anti-CAR	0 (0.0)	-	-
Anti-amphyphisin	0 (0.0)	-	-
Anti 60-64 kD	4 (0.3)	Cerebellar syndrome Sensory neuropathy Organic brain syndrome	Small cell lung cancer
Anti 55 kD	4 (0.3)	Cerebellar syndrome Sensory neuropathy	Cancer of genito-urinary tract
Anti 40-200 kD	3 (0.2)	Polyneuropathy	Lung cancer
Anti 37 kD	7 (0.4)	Heterogeneous	Heterogeneous
Anti 210 kD	3 (0.2)	Sensory neuropathy Cerebellar syndrome	Heterogeneous
Total	67 (4.2)		
Other neuronal autoantibodies	117 (7.3)	Heterogeneous	Heterogeneous
Systemic autoantibodies	103 (6.4)	Heterogeneous	Heterogeneous
Total	220 (13.8)		

Anti-40-200 kD: An antibody reactive with a pan-neuronal cytoplasmic antigen was detected in three patients. On Western blot these sera showed reactivity against an antigen which was homogeneously distributed over the range of 40-200 kD on electrophoresis with the strongest reactivity in the upper 200 kD molecular weight range (fig. 3, appendix). This antibody was not found in any of the control sera and did not react with other tissues (see Paterials and Methods). Two patients suffered from a polyneuropathy and the other showed signs compatible with limbic encephalitis. Two patients had adenocarcinoma of the lung and in one no tumor was found.

Anti-37 kD: In 7 patients, an antibody against a 37 kD neuronal protein was found on Western blot. The antigen migrated just below the Hu complex and showed one sharp small band that could clearly be distinguished from the Hu complex. The serum of the patients did

not react with the HuD recombinant protein. The antibody reacted with all neuronal nuclei on IIF. No reactivity was seen on other tissues (see Patients and Methods) on both IIF and Western blot (fig. 4, appendix). Three patients suffered from a polyneuropathy or sensory neuronopathy, one had a cerebellar syndrome. One patient had the clinical picture of bulbar encephalitis and one of limbic encephalitis. Three patients had lung cancer (one small-cell and two adenocarcinoma), one Hodgkin's disease and one patient had melanoma. In two patients no tumor was detected. Only in one of the sera from the control groups, this antibody was found in a patient with SCLC and without neurological signs.

Anti-210 kD: In three patients, high-titer antibodies (all > 1:6400) against a 200-210 kD neuronal protein were observed. On IIF, a neurofilamentous staining pattern was seen with these sera (fig 5, appendix). No reactivity was present on other tissues (see Patients and Methods). This antibody reactivity was seen in one patient of the control group who suffered from Miller-Fisher syndrome. Neurologically and oncologically, these three patients did not show common paraneoplastic characteristics. One patient had non-SCLC and suffered from sensory polyneuropathy and cerebellar ataxia. One patient had sensory neuronopathy without cancer. One patient had breast carcinoma, cerebellar ataxia and brain metastasis. This high-titered anti-neurofilamentous antibody was not found in any of the controls.

Other neuronal autoantibodies

In 117 patients a range of other autoantibodies was found. Low titered (<1:500) antibodies against neurofilaments NF-L, H and M of 70, 150 and 200 kD molecular weight, respectively, were seen in 58 (11%) patients (data not shown). No correlation was found between neurological and oncological data in this patient group.

In 21 patients antibodies were found against a 40 kD antigen which was detectable on both neuronal and muscle protein extracts but not in the other tissue extracts (see Patients and Methods). This antibody reactivity was also detected in 6 sera from controls. This patient group was heterogeneous without correlation between the antibody, neurological symptoms and tumor.

In 38 patients antibodies were identified against a 35-36 kD antigen, appearing just below the 37 kd and the 38-40 kD (Hu) antigen. This antigen was present in both the neuronal and the other tissue protein extracts. This antibody reactivity was seen in seven sera of the controls. No correlation was found between antibody, neurological symptoms or tumor.

Other autoantibodies

A variety of autoantibodies against other antigens was found in 103 (20%) of the 514 sera tested. Of these, 98 sera had anti-nuclear antibodies (ANF) in titers higher than 1:100 on Hep-2 cells. In 42 of these 98 sera, antibodies were found against SS-A, SS-B, RNP or Sm. Nine patients had antibodies against SS-A alone, seven patients had antibodies against both SS-A and SS-B. In 8 patients antibodies against SS-A and Sm or RNP were found and in 10 patients against Sm or RNP only. Eight patients had antibodies against SS-B and RNP. Except for 3 patients, none had a known diagnosis of SLE or Sjögren's syndrome. All patients presented with neurological symptoms. In 69 (67%) of these patients, a tumor was known at the time of presentation of neurological symptoms.

Table 4 Five different anti-neuronal antibodies found in 1600 patients suspected of PNS

No.	M/F	Age	Tumor	Neurological symptoms	IIF-reactivity	kD
1	M	65	SCLC	Sensory neuronopathy, cerebellar syndrome	Neuronal cytoplasmic	60-64
2	M	64	SCLC	Sensory neuronopathy	Neuronal cytoplasmic	60-64
3	F	80	SCLC	Sensory neuronopathy	Neuronal cytoplasmic	60-64
4	F	69	n.t.d.	Organic brain syndrome	Neuronal cytoplasmic	60-64
5	F	60	Cervical carcinoma	Sensory neuronopathy	Pan-neuronal cytoplasmic and nuclear	55
6	M	80	Bladder carcinoma	Cerebellar syndrome, bulbar encephalitis	Pan-neuronal cytoplasmic and nuclear	55
7	F	60	Ovarian cancer	Cerebellar syndrome	Pan-neuronal cytoplasmic and nuclear	55
8	F	69	Cervical and breast carcinoma	Sensory neuronopathy	Pan-neuronal cytoplasmic and nuclear	55
9	M	80	n.t.d	Polyneuropathy	Pan-neuronal cytoplasmic	40-200
10	M	66	Adenocarc.lung	Polyneuropathy	Pan-neuronal cytoplasmic	40-200
11	M	40	Adenocarc.lung	Limbic encephalitis	Pan-neuronal cytoplasmic	40-200
12	M	69	Adenocarc. lung	Polyneuropathy	Anti-neuronal nuclear	37
13	n.k.	n.k.	Adenocarc. lung	Polyneuropathy	Anti-neuronal nuclear	37
14	F	25	Hodgkin's disease	Cerebellar syndrome	Anti-neuronal nuclear	37
15	F	74	n.t.d.	Bulbar encephalitis	Anti-neuronal nuclear	37
16	F	62	n.t.d.	Limbic encephalitis	Anti-neuronal nuclear	37
17	F	37	Melanoma	Sensory neuronopathy	Anti-neuronal nuclear	37
18	M	73	SCLC	Cerebellar syndrome	Anti-neuronal nuclear	37
19	M	72	non-SCLC	Sensory neuronopathy, cerebellar syndrome	Neurofilamentous	210
20	F	61	Breast carcinoma	Cerebellar syndrome, metastasis	Neurofilamentous	210
21	F	89	n.t.d.	Sensory neuronopathy	Neurofilamentous	210

M: male; F: female; n.k.: not known; IIF: indirect immunofluorescence; SCLC: small cell lung cancer; n.t.d.: no tumor detected; kD: molecular weight in kilodalton.

Discussion

In this study, we found autoantibodies in 17.8 % of patients suspected of a PNS. Using appropriate controls and assays to exclude non-neuronal antibody reactivities, only 4.1 % of sera had anti-neuronal antibodies. Apart from the well-characterized paraneoplastic antibodies, five novel anti-neuronal antibodies were identified.

Previously, others have suggested the possibility that anti-neuronal antibodies might be associated with a number of neurological or psychiatric disorders. In one study, anti-neuronal antibodies were found against proteins of various molecular weight in approximately 30% of patients, but no significant association with clinical symptoms was found[40]. However, in this analysis the relationship between individual reactivity patterns of antibodies and patients was not studied, and there were no control assays on non-neuronal extracts. When sera from a random population are tested against a crude protein extract of neuronal or non-neuronal tissue on Western blots, many reactive bands can be found, especially when tested at low dilution[40,41]. The frequency of autoantibodies rises with age and even at young age antibodies against many autoantigens can be detected in serum of normal healthy controls[40,41]. Therefore, the finding of autoantibodies has to be carefully interpreted, if using a sensitive technique as Western blotting. In our study, only antibodies were considered positive with titers above 1:500 and when in both IIF assays and Western blotting a reactivity against non-neuronal antigens was excluded. Only the cloning or purification of corresponding antigens will lead to more direct detection of these antibodies.

Frequency of antibodies and incidence of PNS

In 67 (4.1%) of the patients examined, we found anti-neuronal antibodies, including 35 anti-Hu antibodies, 6 anti-Yo antibodies, 3 anti-Purkinje cell antibodies associated with Hodgkin's disease (anti-Tr) and 2 anti-Ri antibodies. Antibodies against the visual related protein and the 128 kD protein associated with stiff-man syndrome were not found. In 21 patients (1.3%) other, novel anti-neuronal antibodies were identified.

As we serve in The Netherlands as the only reference laboratory for determining paraneoplastic anti-neuronal antibodies, we presume that we receive material from virtually all patients suspected of PNS in The Netherlands. Approximately 2% had an immunologically confirmed diagnosis of PNS, in a population of 3000 patients with a clinical suspicion of PNS grade 1. The incidence of paraneoplastic neurological syndromes in The Netherlands thus does not reach above 0.1%. For each syndrome, the incidence can be calculated (table 1). For the Hu syndrome, the incidence would be 7 on a total of 1300 new SCLC cases per year or 0.53% of all new SCLC/year in The Netherlands. For anti-Yo positive PCD, the incidence would be 1.2 cases on a total of 1275 newly diagnosed ovarian cancers/year in The Netherlands yielding an incidence of less than 0.09%. For anti-Tr and anti-Ri, the incidences are 0.16% in Hodgkin's disease and 0.01% in breast cancer patients (table 1).

Novel antibodies

We identified 21 (1.3%) patients with other non-Hu, -Yo, -Ri, and -Tr anti-neuronal antibodies in a group of 1600 sera from patients with clinical suspicion of PNS. Five different types of antibodies were found that reacted specifically with a neuronal antigen in both IIF and Western blotting.

Many previous studies, mostly case-reports, indicate that other as yet not characterized antibodies can be identified in patients with PEM/PSN or other PNS. One study reported antibodies to Purkinje and dorsal root ganglion cells reactive with a 62 and 110 kD protein in 3 patients with sensory neuronopathy without cancer[28]. Another non-Yo antibody reacting with Purkinje cell cytoplasm has been described, identifying 65, 120 and 150 kD proteins in neurons and neuro-ectodermal tumor cells[24]. Recently Jaeckle et al. reported the cloning of a novel antigen called Br in a patient with PSN and lung alveolar carcinoma[42]. An antibody against 45, 59 and 135 kD proteins in a patient with PEM, uveitis and undifferentiated carcinoma was reported by Antoine et al.[28]. They demonstrated that different findings may be explained by the techniques used and showed that not each antigen is resistant to routine immunofluorescence or Western blot procedures. Methods of fixation can influence the antigenic epitopes to a large extent and thus interfere with antibody detection[28].

In control sera these antibodies could not be found[23,42,43]. However, no systematical study has previously been carried out to determine the actual existence or diagnostic value of these newly identified anti-neuronal antibodies in PNS.

Although the relation between individual antibody and tumor type is not as direct as in other antibody-associated PNS, we think that our results suggest that each antibody identifies a subgroup of patients with PNS. The number of identified patients harbouring these antibodies is small and the relation with neurological symptoms and tumor type may become more apparent when more patients are investigated.

The criteria we used met the guidelines for the detection of relevant PNS antibodies, that have recently been defined[44,45]. Nevertheless, patients with identical antibodies may have a variable neurological syndrome or more than one type of tumor. Different tumor types may express identical onco-neural antigens with corresponding anti-neuronal antibodies that may evoke different neurological syndromes. This phenomenon is not unique and is clearly apparent in the Hu-syndrome, in which a large variety of clinical presentations has been recognized and the Hu expression is not solely restricted to one tumor type[4,46].

Although the tumor type varies, for example in the group of patients with the 37 kD and 55 kD reactive antibodies, it may well be that all tumors in one group express the same reactive antigen. Further study needs to clarify whether antigens recognized by the identified antibodies are present in the corresponding tumors. Nevertheless, our data indicate that the identified antibodies relate to patients with neurological syndromes associated with cancer, as they were not observed in sera of any of the control groups, although we only tested 20 normal controls and 203 diseased controls.

Anti-60-64 kD: In patients with 60-64 kD antibody, three of the four had a diagnosis of SCLC and in one no tumor was found. All had a clinical picture of central nervous system involvement similar to symptoms of a anti-Hu associated paraneoplastic encephalomyelitis.

The anti-60-64 kD antibody may therefore be a new marker of PEM/PSN associated with SCLC.

Anti-55 kD: All patients harbouring serum reactivity against a 55 kD neuronal protein had cancers, that were derived from the Mullerian tract, which is ontogenetically closely related to the nervous system. This may indicate that an antigen expressed by the tumor induces an anti-55 kD immune response.

Anti-40-200 kD: Antibodies to the 40-200 kD antigen were found in two patients with adenocarcinoma of the lung and in one patient without tumor. The antigen detected by these sera is most likely not a protein, considering the behaviour of the antigen on Western blots and the immunoreactivity in IIF. The antigen may be of a lipid or mucoid structure or a large protein with positive degradation products on Western blots. Further study has to reveal the nature of this antigen.
Polyneuropathies as found in two of these patients are not characteristic for classical PNS, although they may occur as a paraneoplastic syndrome. The third patient had a clinical picture of a limbic encephalitis, which is a well-known PNS.

Anti-37 kD and 35-36 kD: In seven patients antibodies against a 37 kD neuronal protein were identified. Their sera did not react with the HuD recombinant protein indicating that the antigen is different from Hu. This group was clinically heterogeneous. Thus, although the data indicate that the antigen is solely expressed by neurons, little can be said as yet on the clinical relevance of this antibody.
The identification of antibodies against a 35-36 kD non-neuronal protein in 38 patients and controls underscores the importance of appropriate controls and the quality of techniques to detect and discriminate the antibodies. The Hu, the 37 kD antigen and the 35-36 kD antigen all comigrate closely on Western blots and are difficult to distinguish. Almost inevitably degradation products of proteolysis limit discrimination of these antigens. It is essential that electrophoresis gels are homogeneously polymerized and that during the preparation of protein extracts proteolysis is prevented by either proteolytic enzyme inhibitors or short processing times of the tissue materials. Parallel performing of IIF assays and eventually cloning of the antigens is necessary for unequivocal determination of antibody reactivities.

Anti-210 kD: Patients with sera recognizing the 210 kD neurofilamentous antigen had different neurological signs and various underlying tumors. Although their antibody titers were very high (all > 1:6400), it is difficult to draw conclusions on whether this antibody is clinically relevant. Preliminary studies using monoclonal anti-neurofilament antibodies show that the antigen does not comigrate with the neurofilament proteins NF-L, H or M (data not shown).

Other autoantibodies
In the present study, we found autoantibodies in 20% (103 of 514) of the patients evaluated. One remarkable finding is that a relatively high percentage had antibodies against SS-A or

SS-B antigens associated with Sjögren's syndrome or SLE. However, this percentage may be biased by selection of patients. Only 514 patients from a total of 1600 were tested in assays for a variety of autoantibodies. Patients with Sjögren's syndrome may suffer from neurological involvement in 10-25% of cases, and neurological symptoms may be the first manifestation of the disease[47]. In that way, the finding of SS-A or SS-B antibodies in patients presenting with neurological symptoms may indicate an underlying Sjögren's syndrome or SLE. Besides, the neurological complications of Sjögren's syndrome have been associated with anti-neuronal antibodies[47]. In our study, only three of the 514 patients had a diagnosis of Sjögren's syndrome.

Conclusions

We conclude that a number of novel anti-neuronal antibodies can be recognized in patients with PNS. Our study indicates that these antibodies identify hitherto unrecognized subsets of PNS. The anti-60-64 kD antibody may be a novel marker for a subgroup of patients with SCLC associated PEM/PSN. Anti-55 kD antibodies have been identified in PNS associated with urogenital malignancies. The abnormally reacting 40-200 kD antigen on Western blots may indicate that not all antigens involved in PNS are proteins, but that other antigenic structures may be involved as well. The relatively high percentage of systemic autoantibodies indicates that new neurological symptoms can well be the first presenting symptoms of a systemic connective tissue disorder.

Expression and cloning of novel "onco-neural" antigens may reveal the nature and function of these proteins and may enable more reliable detection of corresponding antibodies. Understanding the molecular mechanisms by which neurons and tumor cells co-express shared antigens may help to reveal the pathogenesis that underly paraneoplastic syndromes of the nervous system.

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7 Immunological characterization of a neuronal antibody (anti-Tr) associated with paraneoplastic cerebellar degeneration and Hodgkin's disease

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Abstract

The anti-neuronal antibodies found in some patients with paraneoplastic cerebellar degeneration (PCD) and Hodgkin's disease (HD) have not been sufficiently characterized to allow their appropriate identification among different laboratories. We studied an antibody (called anti-Tr) found in five patients with HD-associated PCD, that immunoreacted with the cytoplasm of Purkinje cells of human and rat cerebellum. The molecular layer of rat cerebellum showed a characteristic, dotted pattern suggestive of immunoreactivity of dendritic spines of Purkinje cells. This dotted pattern was not produced by anti-Yo, or five different non anti-Yo anti-Purkinje cell antibodies from patients with neurologic disorders other than HD-associated PCD. In the rat nervous system, anti-Tr immunoreactivity was also observed in multiple regions. Immunoblots of human Purkinje cells or mouse cerebellum were negative. Patients with cerebellar disorders without cancer (96), opsoclonus (14), recent infection with Epstein-Barr virus (16), HD without PCD (30), or PCD associated with other neoplasms (21) did not harbor anti-Tr antibodies.
We conclude that the anti-Tr antibodies defined in this study are specific for HD-associated PCD. Until the cloning of the Tr antigen(s), which will allow the unambiguous identification of these antibodies, the immunohistochemical staining pattern described in the rat cerebellum coupled with the absence of reactivity in the immunoblot may be used to identify anti-Tr antibodies.

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Introduction

Paraneoplastic cerebellar degeneration (PCD) is characterized by the subacute development over weeks or months, of a severe pancerebellar syndrome. Postmortem study shows extensive loss of Purkinje cells with relative preservation of other cerebellar neurons. Inflammatory infiltrates in the deep cerebellar nuclei and brainstem are also identified in some patients. PCD has mostly been reported in association with gynecologic tumors, breast and lung cancer, and Hodgkin's disease (HD)[1].

Except for the anti-Yo antibody, that is a marker of PCD associated with ovarian or breast cancer[2,3], no other antineuronal antibodies have been well characterized in patients with PCD[4,5]. A few patients with PCD and HD harbor anti-neuronal antibodies, different from anti-Yo[6-10]. However, in none of the previous studies were these antibodies analyzed extensively enough to allow their correct identification among different laboratories, to differentiate them from anti-Yo or other less well characterized antineuronal antibodies[11-15], and to demonstrate their specificity for PCD associated with HD.

In the present study, we evaluated the immunohistochemical and immunoblot characteristics of an antineuronal antibody (that we called anti-Tr), found in the serum and cerebrospinal fluid (CSF) of five patients with HD-associated PCD.

Methods

Patients

The most relevant clinical features of the five patients are summarized in table 1. Four men developed a progressive subacute pancerebellar syndrome that left them disabled after one to four months. The fifth patient was a 14 year-old girl who developed a cerebellar syndrome and bilateral retrobulbar optic neuropathy two weeks after an episode of fever and tonsillitis. The neurologic deficit completely resolved over the ensuing two months after treatment with corticosteroids, 1 g/day for 3 days followed by tapering doses during three months. All five patients had CSF pleocytosis and normal MRI scans of the head in the initial evaluation. HD appeared after a median of 5 months (range: 0-12 months) following the diagnosis of PCD. In the last visit, the HD was in remission in all patients. However, patients 1 to 4 were disabled from the cerebellar dysfunction and the MRI showed cerebellar atrophy. Patient 5 had a normal neurological examination but visual evoked potentials remained abnormal and there was a mild cerebellar atrophy in the MRI scan.

Serum samples

Serum was collected at the onset of PCD or diagnosis of the HD, and after the treatment. CSF samples were also obtained at the onset of PCD in patients 2 and 3. As controls, we studied serum from 21 patients with PCD (15 associated with lung cancer and 6 with other solid tumors), 14 with opsoclonus-myoclonus syndrome (8 paraneoplastic), 30 with newly diagnosed HD without neurologic disorders, 16 with recent Epstein-Barr virus (EBV)

infection, and 96 with different cerebellar disorders without cancer (8 with probable postinfectious cerebellitis). All sera were negative for anti-Hu, Yo, or Ri antibodies. The anti-Tr antibody reactivity of the five HD-PCD patients was compared with that of (1) anti-Yo antibodies; (2) five non anti-Yo anti-Purkinje cell antibodies from patients with different neurologic disorders (two with PCD and solid tumors[15], two with non-paraneoplastic cerebellar disorders and one with neuromyotonia); and (3) anti-glutamic acid decarboxylase (GAD) antibodies from a patient with stiff-man syndrome[16]. Samples were alliquoted and kept at -70°C until use.

Table 1 Clinical features of paraneoplastic cerebellar degeneration and Hodgkin's disease.

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age/Gender	72/Male	43/Male	29/Male	36/Male	14/Female
Previous febrile episode (time)	"flu-like" (2 weeks)	No	No	No	Tonsillitis (2 weeks)
Ataxia					
Truncal	Severe	Moderate	Moderate	Moderate	Severe
Limbs	Mild	Moderate	Moderate	Moderate	Mild
Nystagmus	Horizontal	Downbeat	Horizontal	Downbeat	Downbeat
Non cerebellar signs	Extensor plantar responses	No	No	No	Retrobulbar optic neuropathy
WBC/μl in CSF	20	17	102	50	12
Initial MRI	Normal	Normal	Normal	Normal	Normal
Time (months) to HD diagnosis	7	5	2	0	12
Type/stage HD	NS/I A	MC/II A	NS/III A	LD/II A	NS/II A
Treatment HD	Chemotherapy	Chemotherapy	Chemotherapy	Chemotherapy	Radiotherapy
Follow-up (months)	52	48	12	72	38
HD/PCD	NED/Stable	NED/Stable	NED/Stable	NED/Stable	NED/NED

NS: Nodular sclerosis; MC: Mixed cellularity; LD: Lymphocytic depletion; NED: No evidence of disease.
WBC/μl: White blood cells per microliter;

Tissue samples

Human brain samples were obtained at autopsy from patients without neurologic disorders, and HD lymph node samples from surgical pathology specimens. Wistar rats were killed by decapitation and the brain and other organs were snap frozen in isopentane chilled in liquid nitrogen. Some rats were anesthetized and perfused with saline and with 4% paraformaldehyde in phosphate buffered saline (PBS) subsequently. After perfusion, brains were removed and frozen after further fixation with 4% paraformaldehyde for 4 hours, and cryoprotection with 20% sucrose in PBS overnight. In some experiments, brains were removed and immersed in the same fixative for 24 hours. Samples were stored in PBS at 4°C until vibratome sections were obtained.

For immunoblot analysis, human Purkinje neurons were purified as previously described[17]. Fifteen BALB/c mice were killed by cervical dislocation and the cerebellum was isolated. The cortical gray matter was separated from the white matter, and homogenized in phospholysis buffer (1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 120 mM sodium deoxycholate in PBS).

Preparation of biotinylated IgG containing anti-Tr antibodies

IgG was purified from serum of normal blood donors and patient 2 using a protein G column. Anti-Tr reactivity was confirmed by immunohistochemistry only in the IgG fraction. The purified IgG was diluted in PBS and reacted with biotin N-hydroxysuccinimide ester (Vector Labs, Birlinghame, USA) in dimethyl sulfoxide (DMSO)(1mg/10mg of IgG) for 2 hours. The reaction was terminated by the addition of 10 mg glycine and the free biotin was removed by dialysis[2].

Immunohistochemistry

Frozen sections of unfixed normal human brain and rat tissues were fixed with acetone for 10 minutes, or 100% alcohol for 5 minutes at 4°C. After inhibition of endogenous peroxidase with 0.3% hydrogen peroxide in PBS for 10 minutes, sections were sequentially incubated with 10% normal goat serum for 20 minutes, patient's serum (screening dilution 1:500) for 3 hours at 37°C (tissue sections fixed with paraformaldehyde), or overnight at 4°C, biotinylated goat anti-human IgG, kappa or labda specific, for 30 minutes, and the avidin biotin immunoperoxidase complex (Vector Labs) for 30 minutes. The reaction was developed with 0.05% diaminobenzidine with 0.01% hydrogen peroxide in PBS with 0.5% Triton X-100. Dilution of antibodies was done in PBS, or PBS with 0.1% Triton X-100 in the experiments using paraformaldehyde fixed sections.

For a preliminary evaluation of the protein nature of the antigen recognized by anti-Tr antibodies, paraformaldehyde fixed frozen sections of rat cerebellum were pretreated, after suppression of the endogenous peroxidase, with 0.4% pepsin (Sigma, St. Louis, USA) in Titrisol buffer (Merck, Darmstadt, Germany) for 10 minutes at 37°C. To ascertain if anti-Tr antibodies recognized carbohydrate epitopes, sections of rat cerebellum were deglycosilated by incubation in 10 mM periodic acid in 50 mM sodium acetate (pH 4.5) for 1 hour at 25°C, washed in PBS, and incubated in 20 mM glycine in PBS for 1 hour[18]. After these pretreatments, immunohistochemistry was proceeded as described above.

To study if anti-Tr antibodies bound to samples of HD, frozen sections of pathological lymph nodes from 30 patients with HD were fixed in cold (4°C) acetone or 4% paraformaldehyde and sequentially incubated with 0.3% hydrogen peroxide, 10% normal human serum, biotinylated anti-Tr or control IgG (10 μg/ml) overnight at 4°C, and the Vectastain Elite ABC complex (Vector Labs). The substrate staining was developed with diaminobenzidine as described above. To evaluate the anti-Tr antibody binding to rat brain, serial sections of brain from perfused animals were cut at 5 μm in the vibratome. Free-floating sections were blocked with 3% normal goat serum and incubated at 4°C overnight with the serum diluted at 1:500 or biotinylated IgG (10 μg/ml). The sections were subsequently developed by the avidin-biotin immunoperoxidase technique described above. Serum and CSF anti-Tr antibody titers were defined as the reciprocal value of the highest dilution that showed positive staining on rat cerebellum. Titration of paired serum and CSF samples were done in the same experiment and repeated twice. To estimate the intrathecal synthesis of anti-Tr antibodies we used an antibody index defined by the formula: CSF titer x serum IgG / serum titer x CSF IgG. A value >1.0 suggested intrathecal synthesis of the antibody[19].

Immunoblotting

Protein extracts of human Purkinje cells (75 μg) or homogenates of mouse cerebellum (200 μg) were separated in a 10% SDS polyacrylamide gel electrophoresis, and transferred to nitrocellulose filters[20]. The nitrocellulose filters were placed in a blocking solution containing 5% Carnation evaporated milk (Carnation Company, CA, USA) overnight at 4°C, then incubated with patients' sera (1:500) diluted in TBST buffer (1% bovine serum albumin, 10 mM Tris-HCl (pH 7.4), 0.9% NaCl, and 0.5% Triton X-100) for 1 hour at room temperature. Strips were then washed in 0.1% Tween-20, and incubated with sheep anti-human IgG diluted 1:20,000 in 0.1% Tween-20 for 1 hour at room temperature. After washing, strips were incubated with the chemiluminescence ECL detection reagents (Amersham, Buckinghamshire, United Kingdom) according to manufacturer's instructions, exposed to Kodak X-OMAT-AR film (Sigma, St. Louis, USA) for 5, 10, and 20 minutes.

Results

Specificity of anti-Tr antibodies

The sera of the five patients with anti-Tr antibodies stained both human and rat brain. The staining was obtained with anti-human IgG, kappa, or lambda, but not IgM. The immunoreactivity observed with the biotinylated IgG anti-Tr antibodies from one of the patients was abolished when the tissue sections were preincubated with undiluted serum from the five patients with anti-Tr antibodies but not with normal human serum or serum containing high titers of anti-Yo antibodies. The titers of anti-Tr antibodies ranged from 1:8,000 to 1:80,000 (median: 1:20,000). Intrathecal synthesis was observed in the two

patients who had paired serum and CSF samples. The anti-Tr antibody titer dropped below 1:500 immediately after treatment of the patients for their HD.

Immunohistochemical staining pattern of anti-Tr antibody on human brain regions in comparison with anti-Yo antibodies

Anti-Tr antibodies reacted with the cytoplasm and proximal dendrites of Purkinje cells in human cerebellum. Unlike the immunoreactivity observed with anti-Yo antibodies, the basket and stellate neurons of the molecular layer were negative (data not shown). In rat cerebellum, the serum or biotinylated IgG anti-Tr antibodies strongly bound to the cytoplasm, with a coarse granular pattern, and dendrites of the Purkinje cells. Unlike anti-Yo immunoreactivity, basket and stellate neurons were negative but the molecular layer presented multiple positive small dots suggestive of immunoreactivity against dendritic spines of the Purkinje cells (Fig 1, appendix). The dotted pattern in the molecular cell layer was similar to that observed with anti-GAD antibodies that label GABA-ergic nerve terminals. However, unlike anti-GAD, anti-Tr antibodies did not label nerve terminals around the Purkinje cell bodies and the dots were smaller and confined to the molecular cell layer (Fig 1, appendix).

The dotted staining pattern was observed after fixation with acetone, absolute alcohol, or paraformaldehyde. This pattern disappeared at higher dilutions while the cytoplasm of Purkinje cells still remained positive. Anti-Tr immunoreactivity was abolished by treatment with pepsin but not with periodic acid or delay of fixation with paraformaldehyde up to 16 hours after death of the animal. The characteristic staining pattern was not seen with any of the control sera. Serum with anti-Yo, or non anti-Yo anti-Purkinje cell antibodies from patients with other neurologic disorders did not show the dotted staining pattern in the molecular layer of rat cerebellum (Fig 1, appendix).

In human brain sections, anti-Tr antibodies did not stain neurons of cerebral cortex, hippocampus, putamen, thalamus, mammillary bodies, lateral geniculate body, substantia nigra, dentate nucleus and dorsal root ganglia. Also no anti-Tr immunoreactivity was observed in the pathological lymph nodes from 30 patients with HD.

Immunohistochemical staining pattern of anti-Tr antibody in the rat brain

The anti-Tr antibody staining of the rat brain was evaluated in frozen and vibratome sections. In the rat, anti-Tr antibody staining was restricted to the nervous system. No staining was observed in liver, kidney, spleen, thymus, stomach, colon, adrenal cortex, and testis. Sensory neurons of the gasserian ganglia were faintly positive; a few showing strong reactivity. The satellite and Schwann cells in the gasserian ganglia were negative.

Identical results were obtained with whole serum or biotinylated IgG (table 2). In the cerebellum, the strongest staining was seen in the cytoplasm and proximal dendrites of Purkinje cells and in the molecular cell layer that showed the immunoreactive dots (Fig. 2B, appendix). A few Golgi cells of the granular cell layer were positive but neurons of the deep cerebellar nuclei, that are strongly positive with anti-Yo antibodies[21] were faintly positive (Fig. 3A, appendix). Many neuronal groups in the thalamus, diencephalon and brainstem were immunoreactive but only weakly positive. Neurons of the piriform and entorhinal

cortex (Fig. 2A, appendix), lateral and basal amygdala, and isolated neurons in the basal ganglia, hilus of the dentate gyrus and plexiform layers of the hippocampus (Fig. 3B, appendix) and neocortex were strongly positive. In the neocortex, positive neurons were randomly distributed in the molecular layer and layers II and III, whereas large neocortical projection neurons were negative. The dotted pattern of the molecular layer of the cerebellum was not observed in other areas of the brain. Leptomeninges, glial and ependymal cells, and choroid plexus were negative.

Immunoblot studies

Immunoblots of isolated Purkinje cells or mouse cerebellum homogenates under reducing and nonreducing conditions failed to identify a common band in the serum of the patients with anti-Tr antibodies.

Table 2 Distribution of anti-Tr immunoreactivity in the rat nervous system

Structure	Intensity	Comments
Neocortex	++	Isolated cells, probably interneurons (see text)
Entorhinal cortex	++	Neurons of layer II, isolated neurons in the other layers
Hippocampus/dentate gyrus	++	Isolated cells, probably interneurons (see text)
Striatum	++	Isolated large-sized neurons
Thalamus	+	Reticular thalamic nuclei
Habenula	++	
Substantia nigra	+	
Red nucleus	+	
Locus ceruleus	–	
Basis pontis	+	
Olivary nuclei	++	
Dentate nuclei	+	
Purkinje cells	+++	
Granular cells	-	
Gasserian ganglia	++	Isolated neurons
Non neuronal cells	-	

Discussion

PCD associated with HD presents clinical features different than that associated with ovarian or breast cancer and anti-Yo antibodies. In HD-associated PCD, men are more often affected than women. PCD may occur in the setting of prolonged HD remission, and often improves

spontaneously, or after treatment with corticosteroids or the underlying HD[10]. Our patients with anti-Tr antibodies, unlike those in the series of Hammack et al.[10] with anti-Purkinje cell antibodies, had PCD before the diagnosis of HD and in all but one the clinical course was similar to that seen in patients with anti-Yo antibodies. In patient 5, the remitting cerebellar syndrome was associated with an optic neuropathy. The normal MRI reasonably excluded the possibility of postinfectious encephalomyelitis. Optic neuropathy is not described in children with postinfectious cerebellar ataxia[22,23] but it has rarely been reported in patients with PCD[24] or paraneoplastic encephalomyelitis[25,26]. Unlike patients with PCD associated with gynecological cancer who almost always have anti-Yo antibodies[3,27,28], anti-neuronal antibodies have been reported only in a few patients with HD-associated PCD and never been well characterized[6-10]. As Trotter et al. were the first to indicate the presence of anti-Purkinje cell antibodies in the serum of a 21 year-old woman with PCD and HD[6], we elected to name the antibodies examined in the current study, anti-Tr antibodies.

Anti-Tr antibodies were not observed in PCD associated with solid tumors, in patients with HD without neurologic symptoms, or in patients with cerebellar disorders other than PCD. These data suggest that anti-Tr antibodies are in fact highly specific for HD-associated PCD.

Anti-Tr antibodies gave a similar pattern to that observed with anti-Yo or other anti-Purkinje cell antibodies in immunohistochemical studies on frozen human cerebellum. However, a more specific immunoreactivity was observed in rat cerebellum. Anti-Tr antibodies showed a characteristic dotted staining pattern of the molecular layer probably due to immunoreactivity with dendritic spines of the Purkinje cells. In a previous patient with HD-associated PCD, Greenlee et al.[8] found an antibody that, although not specifically mentioned by the authors, also produced a dotted pattern in the molecular layer[8]. Other anti-Purkinje cell antibodies, including anti-Yo, did not show this characteristic immunoreactivity in the molecular layer of the rat cerebellum. In addition, anti-Tr antibodies, unlike anti-Yo, did not bind to the cytoplasm of stellate and basket neurons of the molecular layer, and did react very weakly with the dentate nucleus of the rat cerebellum[21].

The abolition of the immunoreactivity by preincubation of the tissue sections with pepsin suggest the Tr antigen is a protein. Our failure to identify the neural antigens recognized by anti-Tr antibodies in immunoblots is in agreement with previous studies[10]. Unger et al.[9] studied the serum of a patient with anti-Purkinje cell antibodies and HD-associated PCD. After affinity chromatography, the serum reacted against two bands of 43 kDa and 18 kDa, and three weak bands of 74 kDa, 56 kDa and 30 kDa. However, this finding should be confirmed with other antibody positive patients to conclusively demonstrate that these bands are characteristic. In the current study, the negative results in immunoblots of purified Purkinje cell extracts suggest that anti-Tr antibodies may be directed against conformational epitopes that are destroyed during processing rather than that the negative results are due to a low concentration of the antigen in the sample.

Anti-Tr antibodies reacted with several neuronal types of the central and peripheral nervous system in the rat brain. Anti-Purkinje cell antibodies found in a previously reported patient with HD-associated PCD did not stain neurons outside the rat cerebellum[8]. The reasons for the discrepancy with our results are unclear, but could be due to the use of different

immunohistochemical procedures, antibody dilutions or, less likely, that the patients had different antibodies.

The distribution of the anti-Tr immunoreactivity in the rat brain does not provide any clue about the possible functional role of the recognized antigen(s). Anti-Tr binding neurons do not belong to a particular functional group of the nervous system. In the cortex and hippocampus, the location and morphology of anti-Tr positive neurons is suggestive of local circuit rather than projective neurons, a similar pattern observed with antibodies against some calcium-binding proteins[29]. The anti-Tr staining in the cerebellum and brainstem was similar to the distribution of P-type calcium channel detected by a polyclonal rabbit antibody[30], although the immunolabeling of the hippocampus was clearly different from that shown in the present study[31].

The origin of anti-Tr antibodies in HD-associated PCD is unknown. HD patients present with dysfunction of T-cell mediated immune responses that leads to increased risk for viral infections[32]. In some patients, the onset of PCD is preceded by mild symptoms of a viral-like infection suggesting that the cerebellar syndrome, or the presence of anti-neuronal antibodies, or both, could be the manifestation of postinfectious cerebellitis[23]. Antibodies against Purkinje cells were reported in a patient with cerebellitis following EBV infection[12]. In contrast, we were unable to find anti-Tr antibodies in six patients with post-infectious cerebellitis or in patients with recent EBV infection. Other findings that cast doubt on the possible infectious origin of the anti-Tr antibodies in our patients include the persistence of high titers of anti-Tr antibodies for many months until the HD was treated, and the decrease of the titer after treatment of the HD, suggesting that the neoplasm itself was the initial trigger of the antibody response. Our failure to detect anti-Tr immunoreactivity in HD tumor samples from patients without PCD does not rule out this possibility. Yo antigens are always present in the tumors of patients with PCD and anti-Yo antibodies[2] but in only 20% of tumors from patients without PCD[33]. Future evaluation of Tr antigen expression by the tumor of HD-associated PCD patients with anti-Tr antibodies will help to resolve this issue.

Conclusion

We conclude that anti-Tr antibodies defined as in this study are characteristic for HD-associated PCD. Anti-Tr antibodies may be easily distinguished from other anti-Purkinje cell antibodies observed in patients with different neurologic disorders in immunohistochemical studies of rat cerebellum. In addition, the distribution of the anti-Tr immunoreactivity in the rat brain may help in their identification. Until the cloning of the Tr antigen(s), which will allow the unambiguous diagnosis of anti-Tr antibodies, the immunohistochemical pattern described in this study coupled with the absence of reactivity in immunoblots of isolated Purkinje cells or mouse cerebellum homogenates should be used to identify the anti-Tr antibodies.

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8 Guidelines on the detection of paraneoplastic anti-neuronal specific antibodies: anti-Hu, anti-Yo and anti-Ri

Report from a workshop to a Symposium on Paraneoplastic Neurological Disease, held on 22-23 October 1994 in Rotterdam, The Netherlands.

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Introduction

Paraneoplastic diseases of the central nervous system (PNS) consist of a variety of neurological disorders including encephalomyelitis (limbic encephalitis, brainstem encephalitis and acute myelitis), sensory neuronopathy, subacute cerebellar degeneration, visual paraneoplastic syndrome, stiff-man syndrome and motor neuron disease[1-3].

For the clinician, it is often difficult to arrive at a reliable diagnosis of the neurological syndrome and to detect the underlying tumor. Neurological signs and symptoms, although often stereotypic, are not specific. In two thirds of cases the underlying tumor is not discovered until after the first neurological symptoms[4]. Furthermore the neoplasm may be small and difficult to detect[5-7].

PNS are frequently associated with serum autoantibodies reactive with neuronal antigens that are also present in the underlying tumor. These antibodies include **(1)** anti-Yo antibodies also called type 1 anti-Purkinje cell antibody (PCA-1), APCA-1 or Type I antibody, **(2)** anti-Hu, also called type 1 anti-neuronal nuclear antibody (ANNA-1) or Type IIa antibody and **(3)** anti-Ri, also called type II anti-neuronal nuclear antibody (ANNA-2) or Type IIb antibody (table 1). A clinical diagnosis of PNS is supported by the finding of these specific anti-neuronal antibodies. The detection of these antibodies should also lead to a focused search for specific underlying neoplasms[8-12].

A major problem in comparing results obtained by different groups of investigators has been that individual laboratories have employed different laboratory techniques for detection of these antibodies. Although identification of these antibodies has rested on immunohistochemistry and Western blot analysis, not all laboratories have used Western blot analysis to identify the molecular weight of detected antigens. Furthermore, material from a number of animal species has been used, methods of preparing substrates for Western blot are not always spelled out and concentrations of serum and end-point staining have not always been defined. In some cases, this has resulted in substantial differences in sensitivity and specificity of these techniques for antibody detection[12-17]. Some early publications and recent reports on several incompletely characterized novel anti-neuronal antibodies have complicated this situation and make it even more difficult for the clinician to interpret the diagnostic value of these antibodies [18-22].

Approach to consensus

In view of these uncertainties and the existing controversy between some research groups on the techniques for proper detection and on the terminology of anti-neuronal antibodies in paraneoplastic neurological disease, a workshop on this topic was organized during an international symposium on Paraneoplastic Neurological Disease, held on 22-23 October 1994 in Rotterdam, The Netherlands. The primary aim of this workshop was to develop a consensus report that contained generally accepted guidelines in order to make laboratory

testing of anti-neuronal antibody detection more uniform and easily interpretable and to clarify the terminology.
This paper reports on the work from 11 international research groups that formed a consensus group which discussed the terminology, methodology of detection and interpretation of paraneoplastic anti-neuronal antibodies.

Terminology

Since the initial description of a paraneoplastic anti-neuronal antibody by Wilkinson and Zeromski in 1965, different terminologies for paraneoplastic antibodies have been proposed by different groups working in this field[23]. It is important to realize that during a relatively short period of time both denominations and methods of detection have been refined along with changes in improvement of techniques and disclosure of antibody characteristics. A brief survey on the terminology will be presented first.
In 1982 an anti-Purkinje cell antibody was identified by Greenlee and later by Jaeckle et al. and Greenlee and Brashear in patients with paraneoplastic cerebellar degeneration and gynaecological malignancies[8,10,24,25]. Neither group designated a specific name but described the antibody as an anti-Purkinje cell antibody. Various names were designated later. The name anti-Yo was introduced in 1990 for those anti-Purkinje cell antibodies reacting with 34 and 62 kD proteins from isolated Purkinje cells on Western blot[26]. The abbreviations PCA or PCab were used by Lennon et al. in 1989 using immunohistochemical criteria and were modified to PCA-1 in 1994, based solely on immunohistochemical criteria[12,15,16]. The term APCA-1 was introduced in 1989[27].
The original description of possible anti-neuronal nuclear antibodies mainly emphasized the cytoplasmic staining by the antibody[23]. Graus et al. noted the nuclear reactivity and described reactivity with 37-40 kD molecular weight proteins on Western blotting[9,28]. In 1986, this antibody was designated anti-Hu (after the first patient in whom it was discovered[28]. The anti-Ri antibody was first described in 1988 using both immunohistochemical and Western blot characterization[29] and its name was designated in 1991[11]. The term ANNA was introduced by Lennon et al. in 1989 and was modified to ANNA-1 and ANNA-2 in 1994 based on immunohistochemistry alone. Others identified differences in ANNA reactivity as anti-Hu and anti-Ri by additional testing on Western blots[12,15,16].
The term Type I for anti-Purkinje cell antibodies and Type II for the anti-neuronal nuclear antibody were designated in 1983, later in 1991 and further specified as Type IIa and Type IIb in 1993, based on the combination of immunohistochemistry and Western blotting[25,30-32].
Although each of these investigators have made valuable contributions to current understanding of this area, it is clear that unequivocal definition and terminology is necessary in order to be able to compare results of different laboratories and to avoid confusion to the clinician. Obviously, the main point is not as much how these antibodies are being named as to formulate criteria on the proper methods of detection and classification of these antibodies.

Methods of detection

The nomenclatures with numerical designations like ANNA 1 and 2, PCA-1 or Type I, IIa and IIb (table 1) were originally given to those antibodies determined by immunohistochemical assays only[12,25,30-32]. The terms anti-Hu, -Ri and -Yo are based on determination by both immunohistochemistry and Western blotting[11,26,33]. Hence, strictly speaking, these terminologies cannot be considered to be interchangeable. Nevertheless, all nomenclatures do intend to refer to equivalent antibodies and should therefore preferably be regarded as synonymous, as suggested in the December 1994 issue of Neurology [15,16,34,35]. However, to do this identical methods and criteria should be used, which implies redefinition of the criteria in such a way that for proper detection of all three PNS-associated anti-neuronal antibodies both immunohistochemical and Western blot assays are required. The consensus group agrees that identification by immunohistochemistry alone, even when performed in experienced laboratories and according to previously given recommendations[15,16], cannot be considered as a sufficiently reliable test for the characterization of these antibodies. If on sole immunohistochemistry, anti-Purkinje cell cytoplasmic antibodies or anti-neuronal nuclear antibodies are detected, one should speak of anti-Purkinje cell antibody (APCA or PCA) or anti-neuronal nuclear antibody (ANNA), not further specified. In line with the recommendations of "Neurology" we consider the anti-Yo antibody synonymous with APCA-1 or PCA-1, anti-Hu synonymous with ANNA-1 and anti-Ri synonymous with ANNA-2, providing that both immunohistochemical and Western immunoblot assays are used.

A specimen yielding no reactivity on immunohistochemistry is interpreted as negative for anti-neuronal antibodies, although Western blot analysis may detect low titers of anti-Hu antibodies in sera of neurologically normal patients with small cell lung cancer using highly affinity-purified recombinant proteins.

Some general guidelines on the appropriate methods together with technical details will be given here in order to provide non-controversial and accurate criteria for the definition of these antibodies. We also expect that, by following these guidelines, identification of novel antibodies will become more easily comparable and interpretable.

Immunohistochemistry

The first screening for the presence of anti-neuronal antibodies in serum or CSF consists of an immunohistochemical assay. Immunohistochemical screening can also detect other atypical (non-Yo, non-Hu or non-Ri) anti-neuronal antibodies.

Patients' sera should be sent overnight and tested within 48 hours or can be stored at 4^0C for several weeks. For longer delays samples should preferably be stored at -80 ^{0}C until tested.

Human cerebellar and cerebral cortex are preferentially used as tissue substrates for immunohistochemistry. Occasionally, dorsal root ganglia, myenteric plexus, medulla, spinal cord and control tissues such as liver, muscle and kidney can be used for special interests, although on indirect immunohistochemistry some background staining may be found due to the presence of irrelevant IgG in systemic tissues. If obtaining human tissue is difficult,

alternatively mouse or rat tissue can be used for avidin biotin or peroxidase anti-peroxidase methods as these species express most human PNS antigens. However misleading results can be obtained when fluorescence staining is used in non-human tissue[36]. For novel antibody screening one should always employ human tissue and it may probably prove valuable to use non human tissue as well.

Criteria for positive immunohistochemical identification of antibodies are presented in table 1. It is possible to discriminate immunohistochemically between anti-Hu and anti-Ri by using peripheral neurons, for example myenteric plexus or dorsal root ganglion cells as substrate[15,37]. In contrast to the Hu antigen, the Ri antigen is not expressed in these cells.

Antibody titers should be determined by serial dilutional titration. In each series of tests, parallel control sections should include a section without use of primary serum, a section using a known positive serum and one with a known negative serum. The secondary antibody can be labeled with either fluorescein-isothiocyanate (FITC), horse-radish peroxidase (HRP), alkaline-phosphatase (AP), biotin or other enzymes to visualize bound antibodies. Either polyclonal or monoclonal secondary antibodies against total human Ig or IgG may be chosen. Titers of antibodies depend on many factors among which are the purity of the antigen, concentration of the secondary antibody, time of incubation, quality of tissues, and type of secondary antibody. Dilution and diluent of the secondary antibody is of great importance in this respect. The secondary antibody should be diluted in the same medium used as blocking agent. Initial assays from the early groups used the FITC-labeled secondary antibody [8-10,38] which is easy to work with and reliable. Other frequently used techniques are avidin-biotin peroxidase and streptavidin-peroxidase methods [39]. Different labeling techniques will often lead to difference in end-point staining. In general, immunoperoxidase and avidin-biotin methods give higher endpoint titers than indirect immunofluorescence. Therefore each laboratory should critically establish dilution and method of visualisation of secondary antibody, using appropriate positive and negative controls.

Ideally, a set of positive reference control sera are assembled by exchange of various sera between experienced laboratories.

End-point staining titers for positive identification of antibody should be determined using large series of controls and positive standards in order to prevent false positive detection. In most immunohistochemical assays using FITC labeled secondary end-point titers of > 1:500 can be regarded as positive, yielding high specificities, although in some patients lower titers can represent true positive reactivity [14].

With high serum IgG concentrations background staining can occasionally make the assay difficult to interpret. Isolation and direct labeling of serum IgG may eliminate background reactivity and may improve the quality of the assay[39].

Table 1 Criteria for identification of paraneoplastic anti-neuronal antibodies

Name of antibody	First description of antibody	Paraneoplastic Neurological Syndrome	Most frequently associated tumors
Anti-Hu ANNA-1 Type IIa	Graus et al. 1986[28] Lennon et al. 1989[12] Greenlee et al. 1991[32]	Encephalomyelitis, sensory neuronopathy, autonomic neuropathy	Small cell lung cancer, neuroblastoma; rarely non-small cell lung cancer, prostate cancer, seminoma
Anti-Yo PCA-1 Type I APCA-1	Furneaux et al. 1990[26] Lennon et al. 1994[15] Jaeckle et al. 1985[25] Greenlee et al. 1982[24]	Subacute cerebellar syndrome, dysarthria and nystagmus	Ovarian cancer, breast cancer, other gyneacological cancer
Anti-Ri ANNA-2 Type IIb	Luque et al. 1991[11] Lennonet al. 1994[15] Brashear al. 1994[30]	Opsoclonus, ataxia, nystagmus dizziness, dysarthria	Breast cancer (total number of patients described still small), small cell lung cancer (one case)

Table 1 Criteria for identification of paraneoplastic anti-neuronal antibodies (continued)

Immunohistochemical criteria for detection	Western blot criteria for detection	Comments
Strong staining of all CNS and PNS neuronal nuclei, sparing of nucleoli and weaker fine granular staining of cytoplasm. Negative on systemic tissues.	35-40 kD reactive bands on extracts of isolated CNS neurons or (optional) reactivity with recombinant protein (HuD, HuC, Hel-N1).	Anti-Hu: both immunohistochemistry and Western blot identification required. ANNA-1 or Type IIa: immunohistochemically identical to anti-Hu, initial description used no Western blot identification, but Western blot identification now required for diagnostic purposes. For Western blot identification proteins from isolated CNS neurons are sufficiently reliable when 37-40 kD protein complex is identified. Use of recombinant proteins is optional (licensed in USA).
Coarse granular staining of Purkinje cell cytoplasm and proximal axon and dendrites, negative nuclei. Negative on systemic tissues.	62 kD band and mostly 34 kD band on purified Purkinje cell extracts or (optional) reactivity with CDR 34 and CDR 62, p52/PCD17 recombinant proteins.	Anti-Yo: both immunohistochemical and Western blot required. APCA-1, PCA-1 or Type-I: initial description used no Western blot identification, but for diagnostic purposes now required. For Western blot confirmation proteins from isolated Purkinje cells reliable when 34 and 62 kD bands are identified. Use of recombinant proteins is optional (licensed in USA).
Strong staining of all CNS but not of PNS neuronal nuclei with sparing of nucleoli and weaker fine granular staining of cytoplasm. Negative on systemic tissues.	55 and 80 kD bands on neuronal proteins or (optional) reactivity with 55 kD recombinant protein Nova.	Anti-Ri: both immunohistochemical and Western blot identification required ANNA-2 or Type IIb: initial description used no Western blot identification, but now required for diagnostic purposes. For Western blot identification proteins from isolated CNS neurons are sufficiently reliable when 55 and 80 kD band are identified. Use of recombinant protein Nova is optional (licensed in USA).

Western blot
Essential and optional criteria for positive identification on Western blot are described in table 1. Since the availability of recombinant antigens (HuD, HelN-1/ple21, HuC, Ri (Nova), CDR34, CDR62 and p52/PCD17, CZF) it may be useful, though not obligatory, to use these complementary to neuronal extracts[40-46]. One should realize, especially in the United States, that the commercial diagnostic use of the recombinant proteins HuD, Yo (CDR62) and Ri(Nova) is licensed by Genica (Boston, Mass. USA).
When using neuronal proteins as antigenic substrate, an important factor for the diagnostic reliability of this technique is the nature and concentration of the protein extracts. Collected tissues should be processed as rapidly as possible (preferably within 6-8 hours, maximally 24 hrs) post mortem.
For anti-Hu detection on Western blots, a protein extract from isolated neurons or neuronal nuclei from normal human, rat or murine cerebral or cerebellar cortex is used[39]. If available, the recombinant HuD protein can also be used. For recognition of anti-Yo and other anti-Purkinje cell antibodies on Western blots, a protein extract of isolated human or murine Purkinje cells, separated on a sucrose/ficoll gradient, is used. Optionally the Yo-recombinant protein (CDR 62) or the p52 recombinant protein of Sakai can be used next to the Purkinje cell proteins [40,42].
For identification of anti-Ri antibodies, the same guidelines as used for anti-Hu antibodies are applicable. For the reliable detection of anti-Ri antibodies, Western blotting on neuronal proteins is required. The Ri recombinant protein may also be used for proper recognition of anti-Ri antibodies. However, immunohistochemistry and Western blot on neuronal proteins are sufficiently reliable for demonstrating the anti-Ri antibody.
As a control antigenic substrate, protein extracts from human and murine liver, kidney and or muscle can be used.

Diagnostic value

Although no large clinico-pathological studies are available to accurately establish sensitivity and specificity of these assays for detection of PNS-associated anti-neuronal antibodies, it is clear that their presence has high diagnostic value[4,11,14,25]. However, in a minority of patients with characteristic PNS and high antibody titers no tumor could be found despite extensive evaluation.[5,47]. One presumes that in these cases the tumor is either too small to be detected or may have spontaneously regressed [4,5]. On the other hand, failure to detect anti-Yo, anti-Hu or anti-Ri antibodies in patients with suspected paraneoplastic syndromes does not exclude a paraneoplastic neurological disease as it does not exclude an underlying malignancy.
If, following the guidelines above, anti-Yo (APCA-1), -Hu (ANNA-1) or -Ri (ANNA-2) antibody is identified, a sufficiently reliable diagnosis of PNS can be made in spite of the lack of proof of the presence of cancer.

Lastly, some cases with a clear clinical diagnosis of PNS are antibody-negative in the presence of cancer. Whether these patients harbour paraneoplastic antibodies that cannot be detected with presently available methods remains unclear.

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9 General Discussion

Definition

Paraneoplastic syndromes of the nervous system (PNS) are syndromes associated with cancer, without invasion of tumor cells into the nervous system. The incidence of PNS has been reported as high as 9% of new cases with cancer, but largely depends on the definition of PNS used to establish this incidence[1]. In its widest definition PNS include neurological syndromes secondary to metabolic changes, opportunistic infections or changes in haemostasis induced by cancer and an important group of cancer-associated syndromes without clear cause. For example metabolic encephalopathy can be induced by hypercalcaemia secondary to osseous metastasis or by hyponatriaemia secondary to inappropriate secretion of antidiuretic hormone. Mucinous products from cancer cells can evoke chronic intravascular coagulation producing occlusion of small vessels in the nervous system or fibrin deposits on mitral heart-valves leading to embolic occlusion of large cerebral arteries. A classic example of opportunistic infection is progressive multifocal leucoencephalopathy caused by selective invasion of the papova (JC and SV-40) virus into oligodendrocytes. Opportunistic infections can be the direct consequence of cancer or its treatment, but can also be secondary to diseases not associated with cancer, like diabetes mellitus, or acquired immunodeficiency syndrome or be the consequence of immunosuppressive therapy in patients with autoimmune disorders or organ transplants[2,3].

Therefore it would be more accurate to restrict the term paraneoplastic disorders of the nervous system for those syndromes which are uniquely associated with cancer. If defined in that way the first description of a true paraneoplastic syndrome was published in 1888 by Oppenheim[4]. Later, in 1938, the Dutchmen Brouwer and Biemond pointed at the possible relationship between the occurrence of cerebellar degeneration and carcinoma[5]. The association of encephalomyelitis with carcinoma was noted by Henson, Hoffman and Urich in 1965[6]. Abnormalities of the peripheral nervous system in cancer were first recognized by Denny-Brown in 1948[7] and later by Henson, Russell and Wilkinson in 1954[8]. From then on, it became clear that a number of specific paraneoplastic syndromes could be associated with cancer, each having its own characteristic clinical presentation, sometimes associated with certain types of cancer and specific neuropathological findings. A common and important feature of these syndromes is that at the beginning of neurological symptoms, patients usually have no apparent cancer.

Henson and Urich in 1982 were the first to give a definition of PNS: "A paraneoplastic syndrome is a degenerative disease of the nervous system that is associated with the presence of cancer but not caused by the direct effect of the tumor, its metastasis or therapy"[2].

By now we know that the "true" PNS can affect any portion of the nervous system from the cerebellar cortex through the brainstem and spinal cord to peripheral nerves, neuromuscular junction or muscle. Clinical manifestations can reflect pathologic damage to a single specific structure in the nervous system (e.g. degeneration of Purkinje cells in isolated paraneoplastic cerebellar degeneration (PCD)) or can reflect damage to multiple areas of the nervous system simultaneously, as in paraneoplastic encephalomyelitis (PEM). In other florid PNS,

for instance in some patients with paraneoplastic opsoclonus-myoclonus syndrome, no pathological damage is found at all.
Regarding the recent insights in clinical course and possible pathogenesis of the various forms of PNS the original definition of Henson and Urich does not fulfil its purpose sufficiently. Also it is better to distinguish "true" PNS, i.e. is possibly immune-mediated, from other "paraneoplastic" neurological complications of cancer as mentioned in table 1. A better definition would be: "A paraneoplastic syndrome is an inflammatory disease of the nervous system that occurs in the presence of a tumor, but is not caused by the direct effect of the tumor, its metastasis or therapy, and characterized by the finding of autoantibodies that react with a neuronal antigen, which similar antigen or antigenic epitope is also expressed on the underlying tumor."
In the future this definition might be simplified by stating that PNS are true autoimmune diseases caused by the presence of an ectopically expressed neuronal antigen on a tumor which induces an autoimmune response against the nervous system, resulting in neurological disease. This is until now only true for the PNS of the peripheral nervous system like the Lambert-Eaton myasthenic syndrome and myasthenia gravis, which can be regarded as PNS of the motor-endplate.

Incidence

Several studies have addressed the incidence of PNS. Results of these studies depend on the definition of the syndrome and on how strictly other causes of neurologic dysfunction are excluded. For example a paraneoplastic sensory neuronopathy occurs in less than 1% of patients with small cell lung cancer (SCLC), but a combined sensori-motor polyneuropathy occurs in almost 40% of patients with SCLC and is likely to be related to weight loss and nutritional status[3,9,10].
Another example on the different frequencies is found in patients with cerebellar degeneration. Croft and Wilkinson found only 2 of 319 patients with lung cancer suffering from cerebellar ataxia and only 3 of 1476 patients with any type of cancer[8]. Wessel et al. found 13 of 50 patients with lung cancer showing cerebellar signs when analysed by posturographic analysis[11] (table 1). These conflicting reports make it difficult to estimate the incidence of true paraneoplastic syndromes. In general it can be said that the clinically significant PNS (i.e. that can be diagnosed by the demonstration of anti-neuronal antibodies) occur in less than 1% of patients with cancer. In **chapter 6** we estimated the frequencies and incidence of PNS in The Netherlands. We found anti-neuronal specific antibodies in 67 (4.1%) of the 1600 examined patients, including 35 anti-Hu antibodies, 6 anti-Yo antibodies, 3 anti-Purkinje cell antibodies (anti-Tr) associated with Hodgkin's disease and 2 anti-Ri antibodies. Antibodies against the CAR-protein and the 128 kD protein associated with stiff-man syndrome were not found. In 21 patients (1.3%) other, novel anti-neuronal specific antibodies were identified.

In The Netherlands we represent the only reference laboratory for the detection of paraneoplastic anti-neuronal antibodies and we presume that we receive material from virtually all patients suspected of PNS. Given this assumption, in a population of 3000 patients with a clinical suspicion on PNS, approximately 2% had an immunologically confirmed diagnosis of PNS.
The overall incidence of PNS in patients with cancer in The Netherlands thus is not higher than 0.1%. For each syndrome separately the incidence can be calculated (table 1 and see **chapter 6**). For the Hu syndrome the incidence would be 7 on a total of 1300 new SCLC cases per year or 0.53% of all newly diagnosed SCLC patients in The Netherlands. Incidence of anti-Yo positive PCD would be 1.2 cases on a total of 1275 newly diagnosed ovarian cancers/year in The Netherlands yielding an incidence of less than 0.1%. For the anti-Tr and anti-Ri antibody associated PNS, incidence rates are 0.16% and 0.01% in Hodgkin's disease and breast cancer patients, respectively.

Importance

Although PNS are rare, their recognition is important for several reasons. First, in 80% of cases the neurological symptoms precede the diagnosis of the underlying cancer[12]. In these cases, diagnosis of PNS will direct the search for an underlying malignancy and may lead to early detection of the tumor. Second, since no effective treatment is known for most PNS, it is important to distinguish true PNS from other neurological complications of cancer, that might be treatable[13]. Third, the hypothesis of the cross-reactive anti-tumor immune response and the discovery of the antibodies and their antigens provide a unique model to study the interaction of cancer, immunological mechanisms and the nervous system[13]. The identification of novel proteins, playing key roles in the development and maintanance of neurons, may in future prove to benefit studies on regeneration of neurons and thus be applicable in therapeutic strategies[14-17].
The elucidation of the exact pathogenic mechanism may be more feasible in PNS than in for instance multiple sclerosis, because of the isolated and cloned antigens. Parallel mechanisms may play a role in the pathogenesis of multiple sclerosis and other immune mediated diseases, which occur more frequently. In this way studies on the pathogenesis of PNS may show its benefit in another way.
Direct use of the identification of novel anti-neuronal/anti-tumor specific antibodies may be found in the application of adoptive immune-therapy and immune imaging. Toxin or tracer labeled monoclonal antibodies or antigen specific cytotoxic T-cells may be examples of such applications. First studies using radioactively labeled anti-Hu monoclonal antibodies to detect early stages of SCLC or micrometastases are currently ongoing and first steps to study the cytotoxicity of Hu specific cytotoxic T-cells on SCLC are taken.

Table 1 Frequency of paraneoplastic syndromes in several studies

No. of patients with cancer examined	Type of cancer	Type of examination	Neurologic diagnosis	Percentage of patients with cancer and neurologic signs	Ref.
1465	Any	Clinical	Neuromyopathy	6.6	9
1465	Any	Clinical	Cerebellar ataxia	0.2	9
150	SCLC	Clinical, EMG	LEMS	2.0	18
150	SCLC	Clinical	Weakness	44.0	18
171	Any	Sensory, clinical	Sensory neuropathy	12.0	10
50	Lung	Postural testing	Cerebellar ataxia	26.0	11
100	Lung	Muscle biopsy	Neuromuscular	33.0	19
641	SCLC	Clinical, EMG	LEMS	0.3	20
3843	Lung	Clinical	Encephalomyelitis	0.36	2
3843	Lung	Clinical	Peripheral neuropathy	0.7	2
908	Ovary	Clinical	Cerebellar degeneration	0.1	21

EMG = electromyogram; LEMS = Lambert-Eaton myasthenic syndrome; SCLC = small-cell lung cancer
Source: J.B. Posner. Neurologic complications of cancer. Contemporary neurology series, vol. 45, p.355. F.A. Davis Company, Philadelphia, USA, 1995.

Pathogenesis

The pathogenesis of most PNS is unknown, but several mechanisms have been put forward. Oppenheim proposed that neurological complications of cancer might be caused by a toxic substance released by the tumor[4]. We now know that tumors indeed can secrete substances like cytokines or hormones that interfere with central nervous system (CNS) function[22]. Cachexia, fatigability, asthenia and general weakness can affect up to 50% of patients with cancer during some stage of their disease.

Another mechanism was proposed by Denny-Brown, when he probably described the first case of a pure paraneoplastic dorsal root ganglionitis. He suggested competition for a vital nutrient, in this case pantothenic acid[7]. Deprivation of pantothenic acid in swine gives rise to identical features as found in dorsal ganglionitis[7].

A third pathogenic mechanism may be an opportunistic viral infection involving the CNS. Progressive multifocal leucencephalopathy, which was originally classified as a paraneoplastic neurological disease, is such an example[13]. Opportunistic viral infections mainly play a role in patients immune-compromised by tumors such as malignant lymphoma or by chemotherapy. However, no viral particles have been isolated from affected brain regions of PNS patients.

A fourth pathogenic mechanism, discussed in more detail below, is that of PNS being an immune mediated disease. In the last 10 years, this hypothesis has gained support by the results of many studies demonstrating the involvement of both the humoral and cellular immune system. However, when thinking about the pathogenesis of PNS we have to realize that each separate syndrome might have a different pathogenesis.
For example, in PCD the antibodies react selectively with Purkinje cells possibly resulting in degeneration of these cells[23]. Interestingly hardly any lymphocytic infiltrates are found in the cerebellum of PCD[24-26].
On the other hand, in paraneoplastic encephalomyelitis/sensory neuronopathy (PEM/PSN) the antibody seems to react with all neurons and yet clinically and pathologically only distinct areas are affected. Dorsal root ganglia and the limbic region are most frequently involved and infiltrates with CD8+ lymphocytes are found[27].
The situation in anti-Ri paraneoplastic opsoclonus myoclonus and in anti-Yo positive paraneoplastic cerebellar degeneration is also different from the anti-Hu syndrome. The antigens (Ri and Yo) are only expressed by tumors (e.g. breast and ovarian carcinoma) of patients with PNS and not by tumors of patients without PNS. This is in contrast to the expression of the Hu antigen associated with PEM/PSN, which is expressed in virtually each SCLC.

Autoantibodies and anti-neuronal antibodies
The question can be raised whether the anti-neuronal antibodies are true autoantibodies when compared to the autoantibodies associated with other autoimmune diseases. Several aspects need to be addressed to answer this question.
In the normal individual there is evidence of autoimmunity in the B-cell repertoire. Normal donor serum often contains autoantibodies which increase in titer during infections, immunization and pregnancy[52]. In addition, activated or EBV-transformed B-cells as well as human lymphocyte hybridomas may also secrete autoantibodies in vitro[53].
In one group of autoimmune diseases (i.c. systemic autoimmune diseases) the autoantibodies bind to key intracellular proteins or to nucleic acids involved in the regulation of transcription and translation, DNA replication, cell division and RNA processing and the antibodies are usually non-organ specific[54]. Examples are systemic lupus, scleroderma, Sjögren's syndrome, mixed connective tissue disease and poly- and dermatomyositis[54]. However, in neither of these diseases a direct pathogenic role of the antibody has been proven. On the other side of the spectrum of autoimmune diseases are those diseases in which autoantibodies are directed against membrane proteins (e.g. TSH receptor) or cell specific products (e.g. thyroglobulin), like thyrotoxicosis and Hashimoto's thyroiditis. In this group of diseases evidence does exists that the antibodies, that are usually organ-specific, are directly involved in the pathogenetic mechanisms causing disease[55].
Whether PNS belong to the group of autoimmune diseases can be discussed. Paraneoplastic antibodies clearly react with "self" antigens. In this way the anti-neuronal antibodies should be regarded as true autoantibodies. From the side of the tumor, however, they may be regarded as effective anti-tumor antibodies raised to defend the body against the harming tumor cells.

One parallel between PNS and the known autoimmune diseases can be drawn. Compared to the nature of the antigens the PNS of the CNS clearly belong to the group of generalized autoimmune diseases. The paraneoplastic antibodies are all directed against intracellular antigens, which probably play key roles in cell development and maintanance. On the other hand, it can be reasoned that PNS of the peripheral nervous system, e.g. LEMS and myasthenia gravis, belong to the other end of the spectrum of autoimmune disease. LEMS and myasthenia gravis show parallel features with for instance thyrotoxicosis with respect to the nature of antigen (membrane bound voltage-gated calcium channel and acetyl-choline receptor versus membrane-bound TSH receptor). In these diseases there is more evidence on the pathogenic role of antibodies than in PNS of the CNS.
Major difference, however, is that autoantibodies against nuclear antigens, e.g. ANA such as anti-SS-A, anti-SS-B or anti-DNA can also be found in individuals without symptoms of disease (chapter 3). This is definitely not true for the PNS. PNS associated antibodies are solely found in patients with clinically definite PNS.
Another difference between PNS and other autoimmune diseases is that in PNS no HLA-class association has been found yet[56]. However, our finding of a high frequency of systemic autoantibodies in patients with PNS suggests that a genetic susceptibility to develop autoimmune phenomena may be a prerequisite to develop PNS (**chapter 3**).

Immune pathogenesis
There is circumstantial evidence that a humoral, and recently also a cellular autoimmune response, may be involved in the generation of PNS. However, it is uncertain whether autoantibodies or cellular cytotoxicity are pathogenic or represent an epiphenomenon of the disease process.
The idea that paraneoplastic neurological syndromes might have an autoimmune basis originates from neuropathologic findings of lymphocytic parenchymal infiltrates, without a causative infectious agent [6,7]. Wilkinson raised the question of a humoral immune pathogenesis when describing the first anti-brain antibodies in serum of patients with carcinomatous sensory neuronopathy [28].
Further support for an autoimmune pathogenesis was provided by the finding of a pathogenic antibody to voltage-gated calcium channels (VGCC) in paraneoplastic Lambert-Eaton myasthenic syndrome (LEMS)[20,29], and the demonstration of the serum and CSF autoantibodies in PNS patients that are reactive with antigens in both the underlying neoplasm and in the central nervous system neurons[30-33].
Evidence for an autoimmune genesis is convincing in LEMS[34]. Both active and passive immunisation experiments have demonstrated the role of anti-VGCC antibodies in the development of LEMS. For other PNS, the finding of specific antibodies only suggests a similar pathogenic role of the antibody. However, neither in vivo nor in vitro studies have been able to demonstrate a direct pathogenic role for anti-neuronal antibodies in other PNS[35-39]. One crucial difference between LEMS and PNS of the central nervous system seems the nature of the antigen against which the antibodies are directed, being a membrane-bound ion-channel in LEMS towards nuclear or intracytoplasmic proteins in PEM/PSN and PCD. Binding of anti-VGCC antibodies to the calcium channel has been shown to block the

influx of calcium[29], whereas little is known about the effect of the binding of anti-Hu or anti-Yo and other antibodies to their corresponding antigens. If binding of the antibodies to the paraneoplastic antigens does not influence their function, this might explain the failure of the passive and active immunization experiments in PNS. Furthermore, in contrast to LEMS, the in vivo accessibility of the Hu and Yo antigens for antibodies is clearly different (intracytoplasmic versus membrane bound) and has been disputed lately[40].

The paraneoplastic antibodies, if produced in response to antigenic stimulation by a tumor in the periphery, must first gain access to the CNS. Intraperitoneal injections of large quantities of rabbit IgG in rats with intact blood-brain barrier has resulted in retrograde axoplasmic transport to spinal cord, medullary motor neurons and Purkinje neurons[23]. However, quantitative analysis of anti-Hu and anti-Yo antibody in the cerebrospinal fluid of PNS patients has also shown evidence of intrathecal anti-Hu IgG synthesis. Once in the CSF, it appears that PNS antibodies can reach their target; intraventricularly injected IgG in rats or guinea pigs localizes within Purkinje cells. Pathologic examination of CNS tissues and tumors of patients with PEM has revealed IgG bound to nuclei of neurons and tumor cells[12]. However, immunohistochemical localization of anti-Hu IgG has shown only limited correlation with clinical symptoms and regions of major tissue injury[11,41-42]. Immunoglobulin depositions within neuronal tissues are primarily of the IgG_1 and IgG_3 subclasses. The finding of IgG deposition within neurons in a biopsy taken from a patient with PNS suggests that antibody gains intracellular entry prior to death[26]. However, the recent finding of anti-Hu IgG in post mortem specimens of HuD immunized mice and of patients with PEM/PSN has been disputed by data suggesting that the apparent uptake of anti-Hu IgG by neurons in vivo may be an artifact and possibly due to postmortem perivascular diffusion of anti-Hu IgG and tissue processing methods[40]. If this is true, these findings have wide implications on the thinking about the role of anti-Hu in the pathogenesis of PEM/PSN and support the notice that anti-Hu (and other paraneoplastic) antibodies are merely an epiphenomenon of an as yet unknown pathogenic process.

Anti-Hu antibodies in the pathogenesis of PEM/PSN

Several findings indicate that anti-Hu associated PEM/PSN may result from either a humoral or a cellular immune response against one or more of the Hu antigens. One study showed that the anti-Hu antibody produces lysis of rat cerebellar granular neurons in vitro, which could be enhanced by the addition of complement[37]. Others, however, were not able to reproduce these results[43]. Other findings supporting a pathogenic role for the anti-Hu antibodies include (a) the strong correlation between the presence of high titers of anti-Hu antibodies in CSF and serum and the development of PEM/PSN in patients with SCLC or neuroblastoma, (b) intrathecal synthesis and deposits of anti-Hu antibodies in the CNS[44], and (c) the highly restricted expression of Hu antigens by the nervous system and the tumor[43]. This restricted extraneuronal expression of Hu antigens suggests that the aberrant or ectopic expression of these proteins may trigger the immune mechanisms involved in PEM/PSN. The possibility of a mutation of the HuD gene from SCLC cell lines has been mentioned[45] as a cause for this trigger, but Sekido et al. and Dalmau et al. (unpublished data) were unable to confirm earlier reported mutations[45].

Attempts at modeling anti-Hu PEM/PSN in animals until now have included (a) passive transfer of anti-Hu serum and IgG, and (b) immunization with recombinant HuD[40]. Although animals developed high titers of anti-Hu antibodies with a similar pattern of epitope recognition, they did not develop clinical or pathological symptoms of PEM/PSN. These findings, along with the nature of the inflammatory infiltrates in the nervous system of patients with PEM/PSN, suggest that a cell-mediated cytotoxic response may be involved in the disorder[27,46]. This is supported by the correlation found between expression of HuD coexpressed with major histocompatibility complex (MHC) class I proteins on the tumor and development of anti-Hu associated PEM/PSN[47], indicating that T-cells are able to recognize the Hu antigen on the tumor.

Other studies (chapter 4) examined the IgG subclasses of the anti-Hu antibodies in serum, CSF and nervous system of PEM/PSN patients[48]. These studies revealed predominantly IgG1 and IgG3 subclasses of anti-Hu IgG with only weak complement activation in the affected areas of the CNS and no infiltration of natural killer (NK) cells, suggesting that complement mediated cytotoxicity and cytotoxicity mediated by NK cells do not play a major role in PEM/PSN. Severely damaged regions of the CNS contained infiltrates rich of EBM11+ cells (monocytes/macrophages) in the perivascular spaces[48]. The ability of IgG1 and IgG3 to bind Fc receptors may play a role in the recruitment of these cells.

The inflammatory infiltrates primarily consist of CD4+ T-cells and CD19+ B-cells in the perivascular spaces, while infiltrating lymphocytes are primarily CD8+ T-cells[16,27,48]. However, it remains to be established if these cytotoxic T-cells are Hu specific. Monocytes and macrophages have also been demonstrated within the interstitium[48]. Other authors have reported some antibody-secreting cells[27].

Taken together these data suggest that the anti-Hu associated PEM/PSN is a complex disorder in which both humoral and cell mediated mechanisms may play a role. The reactivity of IgG anti-neuronal antibodies to the cell-surface of neurons and in vitro studies using tissue cultures suggest that internalization of anti-Hu IgG is possible. Internalization of anti-Hu IgG may result in inhibition of the function of the Hu protein which, by homology with the Drosophila proteins Sex-lethal and Elav, appears to have a crucial role in establishing and maintaining the neuronal phenotype[16]. This inhibition may then result in irreversible cell damage and neuronal death. In addition, the analysis of the inflammatory infiltrates suggests a role of cell-mediated cytotoxicity in the pathogenesis of the disorder. Therefore, further studies on the pathogenesis of PEM/PSN and efforts to develop an animal model of PEM/PSN should also be directed towards a cellular immune response against HuD.

Anti-Yo antibodies in PCD and other PNS

As for the anti-Hu antibody, the pathogenic role for the anti-Yo antibodies in PCD has not been established. There is only circumstantial evidence like (a) a strict correlation between the presence of anti-Yo antibody and PCD[32], (b) the demonstration of uptake of antibodies by Purkinje cells[23], (c) the demonstration of intrathecal synthesis of antibodies, and (d) the restricted antigen expression by Purkinje cells and tumor cells[49]. The expression of Yo is even more restricted than Hu, since only ovarian cancer of patients with PCD and anti-Yo antibodies seem to express the antigen. This, however, has recently been disputed since the

the Yo-antigen seems also to be expressed intracytoplasmically in ovarian cancer cells of non-PCD patients [50].

Graus et al have reported negative results following passive transfer of anti-Yo IgG to guinea pigs[35]. Also intraventricular injection of anti-Yo IgG did not produce cell damage, although uptake of IgG by Purkinje cells could be demonstrated. Even injection of anti-Yo IgG together with complement failed to induce any damage[38,39,51]. Neither peripherally recruited mononuclear cells, nor lymphocytes recruited from the CSF from a PCD patient injected into the peritoneum or brain of SCID mice caused ataxia or Purkinje cell loss[38]. Mice immunized with recombinant Yo produced high titers of anti-Yo antibody during a 3 month period but no signs of disease[39].

Explanations for the failure to produce disease in animals are numerous. Differences between human and rodent Yo protein and immunological make up of the host have to be taken into account.

Still it can be reasoned that the anti-Yo antibody plays a pathogenic role in loss of Purkinje cells. The Yo antigen possesses a leucine-zipper motive, a DNA-binding sequence, that may influence the regulation of gene transcription. Binding of anti-Yo to the protein might influence the function of the protein and affect cell metabolism and survival.

In contrast to the findings in patients with PEM/PSN, the lack of finding of lymphocytic infiltrates in cerebellum of patients with PCD supports the hypothesis of direct antibody mediated cytotoxicity[24,26,49].

No studies are currently available providing data on possible pathogenic mechanisms in other PNS (e.g. anti-Ri associated opsoclonus, PCD in Hodgkin's disease).

Self tolerance

In recent years it has been postulated that self tolerance usually results from deletion and suppression of potentially reactive T-cell clones during intrathymic maturation. Acquired self tolerance may also result if the antigen fails to associate with MHC class II antigens or is expressed at such low levels that generation of self reactive T-cells does not occur. Furthermore excessive amounts of exogenous antigen may induce tolerance of B-cells and CD8+ T-suppressor cells. How the immune tolerance breaks down in PNS is largely unknown and may vary from disease to disease. In most autoimmune diseases, however, genetic factors next to enviromental influences play a role.

With regard to the antigens of the CNS, one theory is that neuron-specific proteins are not presented to the immune system during the establishment of immune tolerance. The implication is that the expression of neuron-specific proteins in tumor tissue (that expresses MHC antigens) may result in a profound immune reaction. This is an even more interesting model for PNS if we realize that SCLC normally does not express MHC abundantly but SCLC cells in patients whith anti-Hu antibodies harbours relatively high MHC expression. In this study, 17 of 20 tumors from seropositive patients expressed both Hu and MHC class I proteins, but only 4 of 30 tumors from seronegative patients expressed both proteins[47].

Another intriguing question remains whether during the ontogeny self-reactive T or B cells were produced against antigens that are normally restricted to an immune privileged site as the CNS. This could explain why aberant expression of the antigen on a tumor then would

induce such a fulminant immune respons. Recently it has been suggested that peripherally activated or memory T-cells randomly wander to and from the CNS through the blood-brain barrier. Only those cells recognizing their antigen will be activated and proliferate resulting in inflammation. This model would perfectly fit the hypothesis on the pathogenesis of PNS. PNS therefore provide a unique model to study neuro-immune inflammation also because some of the antigens are well characterized. It is therefore of great interest to focus future research on cellular cytotoxicity to see if the PNS specific cytotoxic cells exists. Extrapolation of findings to other immune-mediated neurological diseases like multiple sclerosis further warrants this research.

Clinical variation
A wide variety of clinical presentations can be encountered in the Hu-syndrome as illustrated in **chapter 4.** As yet it is unknown why only certain areas of the CNS are involved and others spared. It may be that the Hu antigen, although expressed by all neurons, only plays a functional role in the dorsal root ganglion and limbic area. Also differences in fine specificity or affinity to the Hu antigen of anti-Hu antibodies in individual patients may play a role in variability of clinical symptoms. There are no data on differences in affinity of anti-Hu IgG. The finding that anti-Hu sera obtained from patients with PEM/PSN, but with different neurological symptoms, react with all Hu antigens (HuD, HuC, Hel-N1) indicates that none of these proteins in particular can be causative of specific neurological symptoms. Also differences in IgG subclasses of anti-Hu antibodies have been shown not to play crucial role in determining differences in clinical course of PEM/PSN.

Diagnosis

PNS are found in patients with occult or active cancer or with cancer in remission after treatment. As in most PNS patients, the cancer is subclinical and undetected at presentation. In patients with undiagnosed cancer the diagnosis can easily be made when neurological symptoms present as one of the classical paraneoplastic syndromes. Still other possible neurological diseases should be excluded. A CT or MRI scan to exclude metastasis and CSF examination should be carried out. Rigorous evaluation for systemic cancer, especially when one of the anti-neuronal antibodies is found in serum or CSF, is necessary. These antibodies can direct the search for the underlying tumor.
In general several clinical features suggest a paraneoplastic origin of the neurological symptoms:

1. The onset of disease is usually subacute with development of signs in days to some weeks and occasionally months after which stabilization follows. Acute onset with a relapsing-remitting course is less likely to be paraneoplastic.
2. Most PNS cause severe neurological disability. Recently a more indolent, slowly progressive type of PNS has been described.

3. CSF examination reveals mostly mild lymphocytic pleocytosis, elevated protein concentration and oligoclonal bands.
4. Imaging procedures, like MRI and CT scan of the brain, usually reveal no abnormality. In PCD some cerebellar atrophy may be encountered.
5. Usually at the onset of the disease one area of the CNS is mainly affected, resulting in a typical clinical presentation.

As indicated throughout this study, detection of the various antibodies is of great help in making the diagnosis of PNS with a specificity of almost 100% and both prompts and directs the search for an underlying tumor. Specific actions that are recommended to be taken when a distinct antibody is found are summarized in table 2.

Table 2 Anti-neuronal antibodies and guidelines for tumor detection

Syndrome	Test for	Action if positive
PEM/PSN	Anti-Hu	Look for SCLC: chest X-ray, CT scan, frequent follow-up
Cerebellar	Anti-Yo	Look for gynaecological malignancy: abdominal echogram, laparoscopy, mammogram, adnex extirpation, frequent follow-up
	Anti-Hu	Look for SCLC
Opsoclonus myoclonus	Anti-Ri	Look for breast cancer: mammography. If negative look for SCLC
Visual loss	Anti-CAR	Look for SCLC
Stiff-man	Anti-128kD	Look for breast cancer If negative look for anti-GAD
Motor neuron disease	No tests available	Look for lymphoma or paraproteinemia

Diagnostic value of antibodies

Although many studies have established that anti-neuronal antibodies are highly specific for PNS and associated tumors, hardly any systematic study has been performed reporting the actual diagnostic value of these antibodies. One major problem (see chapter 1 or 2) in such a study is the gold standard to make a definite diagnosis of PNS in calculating the sensitivity and specificity for the diagnosis of PNS. In order to overcome this problem one can use a set of clinical definitions to provide reliable diagnostic criteria. Consequently one will find different values in different studies when different criteria are used or criteria are differently interpreted. It is therefore important to try to standardize methods of detection in order to make studies from different laboratories comparable. Also the population under study will influence the outcome of diagnostic sensitivity. The more rigourous the criteria used for making a clinical diagnosis of PNS in such a study, the higher sensitivity of antibody

detection will be. However, it seems feasable that even when it would be possible to perform such a study using the gold standard of post mortem confirmation of the diagnosis of PNS, sensitivity would still be relatively low. Probably not all anti-neuronal antibodies can be identified yet, using currently available and applied methods (see also chapters 5, 6 and 7). Also it is very well possible that not all PNS are associated with the presence of anti-neuronal antibodies.
The criteria for the detection of paraneoplastic anti-neuronal antibodies as diagnostic tool and the technical methods for laboratory assay are described in **chapter 8**. These criteria are the result of a consensus meeting held in 1994 in Rotterdam, from 11 international research groups which discussed the terminology, methodology and proper interpretation of paraneoplastic anti-neuronal antibodies.

Novel PNS antibodies

In spite of the specific association between anti-Hu antibodies, PEM/PSN and SCLC, 38-50% of patients whith a clinical picture of PEM/PSN do not harbour anti-Hu antibodies. Some studies, mostly case-reports, indicate that other as yet not characterized anti-neuronal antibodies can be found in some of the anti-Hu negative PEM/PSN patients. Others have not been able to detect other antibody reactivities in patients with PEM and SCLC. The different findings in these studies may be explained by the low frequency of antibodies against novel antigens, the heterogeneity of patients and the techniques used to detect the antibodies. It is probable that not all antigens can be detected by routine IIF or Western blot assays. Methods of fixation can largely influence the conformation of the antigens and thus interfere with antibody detection. It is therefore important to try to identify novel anti-neuronal antibody reactivities in PNS and to establish a correlation with the neurological and oncological symptoms.
In **chapters 5, 6 and 7** several novel anti-neuronal antibodies are described in relation to the neurological symptoms and the underlying tumor. Although the exact diagnostic value of some of these antibodies is not precisely known, as indicated in **chapter 5**, we think that the discovery of these novel antibodies and their antigens will help to identify new subgroups of PNS, and thus help to clarify the clinical pictures of patients with a yet undefined type of PNS.
In **chapter 6** we report four patients with PEM and SCLC in whom a novel anti-neuronal antibody against a 60-64kD neuronal antigen complex was detected by IIF and Western blot.
In **chapter 7** the anti-neuronal antibodies found in some patients with PCD and Hodgkin's disease (HD) are reported. The antibody, designated anti-Tr, found in five patients with HD-associated PCD, bound with the cytoplasm of Purkinje cells of human and rat cerebellum. The molecular layer of rat cerebellum showed a characteristic dotted pattern suggestive of reactivity with dendritic spines of Purkinje cells. This dotted pattern was not produced by anti-Yo, or five different anti-Purkinje cell antibodies from patients with neurologic disorders other than HD-associated PCD. They are sufficiently characterized to allow

appropriate identification in different laboratories and discriminate the anti-Tr from the anti-Yo reactivity. In this chapter we conclude that anti-Tr antibodies, as defined in this study, appear to be specific for HD-associated PCD. Until the Tr antigen(s) are cloned, which will allow the unambiguous identification of these antibodies, the IIF pattern in rat cerebellum in combination with the absence of reactivity in the immunoblot assay may be used to identify anti-Tr antibodies.

Treatment

In general, treatment of PNS has been unrewarding and most patients suffer from severe neurological disability. Most therapies tried forms of immune-modulation, like plasmapheresis, corticosteroids or intravenous immune-globulin, and were evaluated in the antibody-associated syndromes. With the exception of LEMS, in which plasmapheresis is clearly effective, and the paraneoplastic opsoclonus-myoclonus syndrome in children, in which removal of the tumor may be of benefit, results have been disappointing. One valid explanation for the failure of therapy is that rapid onset of disease has resulted in irreversible destruction of neurons at the time therapy is started. In most cases the interval between onset of neurological symptoms to the definite diagnosis of PNS and start of therapy is more than one month up until several years. However, when considering a therapeutic strategy, we have to bear in mind that the model of immune-mediated pathogenesis of the PNS has not been proven. The hypothesis that the antibodies are only an epiphenomenon of a different non-immune pathogenic mechanism leading to neuronal damage may be another explanation why immune-modulatory therapies are not effective in PNS.

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Summary

Patients with cancer develop neurological symptoms in the course of their disease in more than 40% of cases. The neurological symptoms may be caused by either metastatic spread of the tumor, by metabolic complications, by direct or indirect side-effects of anti-tumor therapy or by a paraneoplastic mechanism. The paraneoplastic syndromes of the nervous system (PNS) are those syndromes that are associated with cancer, but not caused by direct invasion of tumor cells in the nervous system and not caused by cancer therapy. Defined in this way, PNS occur in less than 1% of patients with cancer. The PNS consists of a number of characteristic clinical syndromes that can involve the peripheral or central nervous system. The PNS of the central nervous system form a separate group of syndromes that can clinically be divided in encephalomyelitis, sensory neuronopathy, subacute cerebellar degeneration, opsoclonus myoclonus syndrome, visual degeneration and stiff-man syndrome. These syndromes are all considered to be induced by an immune-mediated mechanism. The central hypothesis rests on the assumption that a neuronal antigen is expressed by the tumor which is usually of neuro-ectodermal origin. The antigen is expressed in such way that immune tolerance for the antigen is broken and a fulminant humoral and/or cellular immune response is induced against this tumor and particularly against the neuronal antigen. This misdirection of the immune reaction against the antigen in the nervous system then causes the neuronal damage and thus clinical symptoms. Following this hypothesis, each syndrome would be associated with a specific immune reaction, which can be demonstrated by the presence of a specific anti-neuronal autoantibody.

Another characteristic feature of these syndromes is that in more than two-third of cases the neurological symptoms precede the detection of the tumor.

Thus detection and characterization of specific anti-neuronal antibodies is important, since they can be used as diagnostic markers for the presence of a paraneoplastic syndrome. The specificity of the presence of these antibodies approaches 100% indicating that a reliable diagnosis of PNS can be made including the presence of a tumor. On the other hand, a negative assay does not rule out that the neurological symptoms have a paraneoplastic origin. It is therefore of major importance that these antibodies can be detected and identified in a reliable way in order to justify an extensive and sometimes invasive search for the underlying tumor.

In patients with PNS, the antibodies, or better the humoral and cellular immune response, seem to control the underlying tumor as compared to patients with similar tumors without these antibodies. In this way the autoimmune response may benefit the patient through impairment of the tumor growth. However, recent studies indicate that the overall survival of patients with PNS and cancer equals the survival of patients with a similar cancer but without PNS, primarily because PNS patients frequently die of the neurological complication.

The major focus of this study concerned the detection of the different anti-neuronal antibodies including the comparison and reliability of techniques, the diagnostic value of the antibodies, and the description of some newly discovered anti-neuronal antibodies.

In **chapter 1** a detailed overview is given of different PNS, with a focus on the immune-diagnosis of these syndromes.
Chapter 2 reports on a study, in which the diagnostic value of the presence of two of these antibodies (anti-Hu and anti-Yo) is determined, using the indirect immunofluorescence assay only. **Chapter 3** shows that PNS patients harbour not only anti-neuronal antibodies but in a large percentage other organ- and non-organ specific autoantibodies. This may indicate that patients with PNS have a genetic predisposition to develop autoimmune phenomena. This predisposition, together with the presence of a tumor may be a prerequisite to develop PNS.
A series of 29 patients with the most frequently appearing PNS, the encephalomyelitis/sensory neuronopathy, is described in **chapter 4**. The wide variety in clinical presentation is described in relation to the different IgG subclasses of anti-Hu antibodies.
In **chapters 5, 6 and 7** several novel anti-neuronal antibodies are described in relation to neurological syndromes and underlying tumor. Although the true diagnostic value of some of these antibodies is not precisely known, we think that the discovery of these novel antibodies and their associated antigens will help to identify new subgroups of PNS and thus clarify some clinical pictures of PNS.
Since the presence of systemic autoantibodies interferes with a reliable identification of anti-neuronal specific antibodies in an indirect immunofluorescence assay, the finding of systemic autoantibodies in more than 50% of PNS patients has pointed out that the indirect immunofluorescence assay alone is not a reliable method of detection of these antibodies.
Chapter 8 extensively describes the criteria for the detection of anti-neuronal antibodies as a diagnostic tool for the neurologist and gives the basic technical rules for the laboratory assay. This chapter is the result of an international consensus meeting held in 1994 in Rotterdam, with 11 research groups that discussed the terminology, methodology and interpretation of paraneoplastic anti-neuronal antibodies.
Another important aspect of the PNS is found in the nature of the antigen. Although this study does not focus on this aspect, the analysis of involved antigens has thus far shown that they may play key roles in cell-regulatory processes of neurons and in pathogenesis of tumors. The gene coding for the Hu antigen has been shown to be closely related to other onco-genes as c-myc and c-fos and is now called an onco-neural gene. These findings led to speculations that PNS antigens and antibodies may be used in the future for immune-imaging of malignancies and even for adaptive immuno-therapy. When applied in this way, the finding of specific antibodies and antigens in very rare, severe and often untreatable diseases, provides a unique model for cancer, and warrants further research.

Samenvatting

Meer dan 40% van de patiënten met kanker ontwikkelen neurologische symptomen in de loop van hun ziekte. Deze neurologische symptomen kunnen zowel worden veroorzaakt door tumor-metastasen, metabole complicaties, directe of indirecte bijwerkingen van de anti-tumor therapie, als door een paraneoplastisch mechanisme. Paraneoplastische syndromen van het zenuwstelsel zijn syndromen die zijn geassocieerd met kanker, maar niet worden veroorzaakt door invasie van tumorcellen in het zenuwstelsel of door tumortherapie.
Op deze manier gedefinieerde syndromen worden in minder dan 1% van alle kankerpatiënten gezien. Paraneoplastische syndromen vormen een groep karakteristieke klinische syndromen, waarbij zowel het perifere als het centrale zenuwstelsel betrokken kan zijn.
De paraneoplastische syndromen van het *centraal* zenuwstelsel vormen een aparte groep die klinisch kan worden onderverdeeld in encephalomyelitis, sensorische neuronopathie, subacute cerebellaire degeneratie, opsoclonus myoclonus syndroom, retina degeneratie en het "stiff-man" syndroom. De hypothese is dat al deze syndromen een immuungemedieerde pathogenese hebben. De basis voor deze hypothese wordt gevormd door de bevinding dat de tumor, meestal een ectodermale tumor, een neuronaal antigen tot expressie brengt, zodanig dat de immuuntolerantie voor dit antigen wordt doorbroken en een fulminante humorale en/of cellulaire afweerreactie wordt opgewekt tegen de tumor en in het bijzonder tegen het neuronale antigeen. Deze primair tegen de tumor gerichte afweerreactie kruisreageert met het zenuwstelsel, waardoor neuronale schade met klinische verschijnselen ontstaat. Volgens deze hypothese is elk paraneoplastisch syndroom geassocieerd met een specifieke immuunreaktie, die kan worden aangetoond door de aanwezigheid van karakteristieke anti-neuronale antilichamen.
Een ander kenmerk van deze syndromen is, dat in meer dan tweederde van de gevallen de neurologische symptomen voorafgaan aan de ontdekking van de tumor. Het is om die reden zo belangrijk de specifieke anti-neuronale antilichamen op te sporen en te karakteriseren aangezien ze kunnen dienen als diagnostische merker voor een paraneoplastisch syndroom en voor de aanwezigheid van een bepaald type tumor. De specificiteit van deze antilichamen is bijna 100%. Aan de andere kant sluit de afwezigheid van deze antilichamen een paraneoplastisch syndroom niet uit. Het is van groot belang dat deze antilichamen betrouwbaar kunnen worden geïdentificeerd, alvorens een uitgebreid en soms invasief onderzoek naar de aanwezigheid van een bepaalde tumor kan worden gedaan.
Uit vergelijking van patiënten met identieke tumoren, met en zonder anti-neuronale antilichamen, lijkt de immuunrespons een groeiremmend effect te hebben op de tumor. Als zodanig zou vanuit oncologisch gezichtsveld deze auto-immuunreactie voor de patiënt gunstig kunnen zijn. Recent onderzoek daarentegen laat zien dat de overleving van kankerpatiënten met of zonder paraneoplastisch syndroom niet verschilt, voornamelijk omdat patiënten met een paraneoplastisch syndroom doorgaans overlijden aan de neurologische complicaties.
Het in dit proefschrift beschreven onderzoek spitst zich toe op het aantonen van verschillende antilichamen, de hiervoor gebruikte technieken, de betrouwbaarheid van deze technieken en de voorspellende waarde van de aanwezigheid van deze antilichamen. Voorts worden enkele nieuw ontdekte anti-neuronale antilichamen beschreven.
In hoofdstuk 1 wordt een gedetailleerd overzicht gegeven van de verschillende paraneoplastische syndromen, gericht op de immunodiagnose. Hoofdstuk 2 beschrijft een van de eerste studies,

waarin de diagnostische waarde van twee anti-neuronale antilichamen (anti-Hu en anti-Yo) wordt aantgetoond. In hoofdstuk 3 wordt beschreven dat patiënten met een paraneoplastisch syndroom niet alleen anti-neuronale antilichamen maken, maar dat zij in een hoog percentage tevens andere auto-antilichamen hebben die niet gericht zijn tegen neuronale antigenen. Dit kan betekenen dat patiënten met een paraneoplastisch syndroom een genetische predispositie hebben voor het krijgen van auto-immuunziekten. Misschien is deze predispositie een voorwaarde voor het ontwikkelen van een paraneoplastisch syndroom bij aanwezigheid van een tumor.
In hoofdstuk 4 worden 29 patiënten beschreven met de meest bekende verschijningsvorm van de paraneoplastische syndromen, te weten de encephalomyelitis/sensorische neuronopathie. Onderzocht werd of de gevarieerde klinische presentatie van dit syndroom kon worden gecorreleerd met de verschillende IgG subklassen van de met encephalitis geassocieerde anti-Hu antilichamen.
In de hoofdstukken 5, 6 en 7 worden enkele nieuwe anti-neuronale antilichamen beschreven in relatie tot de klinisch neurologische symptomen en de onderliggende tumor. De diagnostische waarde van sommige van deze antilichamen is niet precies bekend. Echter, de verdere karakterisatie van deze nieuwe antilichamen en het antigeen waartegen zij gericht zijn, zal waarschijnlijk bijdragen tot de identificatie van enkele nieuwe subgroepen paraneoplastische syndromen. Op deze manier kan beter inzicht worden gekregen in het klinisch beeld van patiënten met een paraneoplastisch syndroom dat zich niet als een van de bekende, karakteristieke paraneoplastische syndromen presenteert.
Daar de aanwezigheid van andere auto-antilichamen interfereert met een betrouwbare bepaling, heeft het vinden van niet-neuronale auto-antilichamen in meer dan 50% van de paraneoplastische syndroom patiënten, duidelijk gemaakt dat het gebruik van alleen indirecte immunofluorescentie technieken niet altijd even betrouwbaar is voor het aantonen van anti-neuronale antilichamen. In hoofdstuk 8 worden uitgebreid de criteria beschreven waaraan het aantonen van anti-neuronale antilichamen als diagnostische test voor de neurologisch specialist dient te voldoen. Tevens worden de technische details van de laboratoriumbepalingen nader toegelicht. Hoofdstuk 8 is het resultaat van de consensus bijeenkomst gehouden in 1994 te Rotterdam, waar 11 internationale onderzoeksgroepen discussieerden over de terminologie, methodologie en interpretatie van anti-neuronale antilichamen bij paraneoplastische syndromen.
Een ander belangrijk aspect van paraneoplastische syndromen vormt de aard van het antigeen waartegen de antilichamen gericht zijn. Hoewel dit proefschrift zich hierop niet specifiek richt, is het vermeldenswaard dat analyse van de betrokken antigenen heeft aangetoond dat deze antigenen een sleutelrol spelen in de celregulatie van neuronen en van tumorcellen. Met name het Hu antigeen blijkt nauw verwant te zijn aan oncogenen als c-myc en c-fos en wordt derhalve ook wel een onconeuro gen genoemd. Deze bevindingen hebben tot de speculatieve verwachting geleid dat paraneoplastische antigenen en de daartegen gerichte antilichamen in de toekomst gebruikt kunnen worden voor "immuno-imaging" van maligniteiten en misschien zelfs een rol kunnen vervullen in immunotherapie. De aanwezigheid van specifieke antilichamen en hun antigenen bij zeldzame, vaak onbehandelbare aandoeningen, vormt een uniek model voor toekomstig kanker onderzoek. Onderzoek hiernaar is dus zeer de moeite waard.

List of Abbreviations

ADCC	antibody dependent cell-mediated cytotoxicity
ANA	anti-nuclear antibody
ANF	anti-nuclear factor
ANNA	anti-neuronal nuclear antibody
ANSA	anti-neuronal specific antibody
AP	alkaline phosphatase
APCA	anti-Purkinje cell antibodies
BSA	bovine serum albumin
CAR	cancer associated retinopathy
CDR	cerebellar degeneration related
CNS	central nervous system
CSF	cerebrospinal fluid
CT	computerized tomography
DNA	deoxyribonucleic acid
EEG	electroencephalogram
EMG	electromyogram
ENA	extractable nuclear antigen
FITC	fluorescein isothiocyanate
GABA	gamma-aminobutaric acid
GAD	glutamic acid decarboxylase
HD	Hodgkin's disease
Hep	human epithelial pharyngeoma
HRP	horseraddish peroxidase
Hu	antigen called after the first patient's initials
IIF	indirect immunofluoresence
Ig	immunoglobulin
kD	kilo dalton
LEMS	Lambert-Eaton myasthenic syndrome
Nd	antigen called after the first patient's initials
OMS	opsoclonus myoclonus syndrome
PBS	phosphate buffered saline
PCA	Purkinje cell antibody
PCab	Purkinje cell antibody
PCD	paraneoplastic cerebellar degeneration
PEM	paraneoplastic encephalomyelitis
PNS	paraneoplastic syndromes
PSN	paraneoplastic sensory neuronopathy
Ri	antigen called after the first patient's initials
RNA	ribonucleic acid
RNP	ribonuclear protein
SCLC	small cell lung cancer
SDS	sodiumdodecylsulfate
SLE	systemic lupus erythematosus
St	antigen called after the first patient's initials
Tr	antigen called after name of author who first reported the antibody
VGCC	voltage-gated calcium channel
WBC/μL	white blood cell count per microliter
Yo	antigen called after the first patient's initials

Curriculum Vitae

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1982	Graduated from Grammar School, Alphen aan de Rijn, The Netherlands.
1982-1991	M.D., Medical School, State University of Leiden, The Netherlands.
1983-1985	Research fellow, Dept. of Pathology, Leiden. Studies on immunoglobulin deposits in glomerular basal membrane in relation to heparan sulfate. Prof.dr. G.J. Fleuren.
1987	Research fellow, Dept. of Anaesthesiology, State University Medical Center Utah, U.S.A. Studies on respiratory depression and pain perception of Midazolan, Alfentanil and Fentanyl. Prof.dr. T.H. Stanley.
1988	Research fellow, Dept. of Neurology, State University Medical Center Utah, U.S.A. Studies on paraneoplastic disorders of the central nervous system. K.A. Jaeckle, M.D.
1989 and 1991-1992	Research fellow, Dept. of Neuro-oncology, Dr. Daniel den Hoed Cancer Center, Rotterdam, the Netherlands. Studies on paraneoplastic disorders of the central nervous system. KWO-NWO fellowship on paraneoplastic disease (1993-1996). Dr. Ch.J. Vecht. Participating in multi-center study on Cisplatinum induced polyneuropathies treated with Org 2766 during and after therapy. Dr. Ch.J. Vecht.
1992-	Resident in neurology (AGIKO), Dept of Neurology, University Hospital Rotterdam, Dijkzigt, The Netherlands. Prof. dr. F.G.A. van der Meché.

Social activities

* Playing the violin in various orchestra's
* Bicycles and cycling
* Participation in organisation committees of the Leiden Baroque Orchestra and the Zuid-Holland Symphonic Orchestra "Bellitoni".

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Submitted.

Dankwoord

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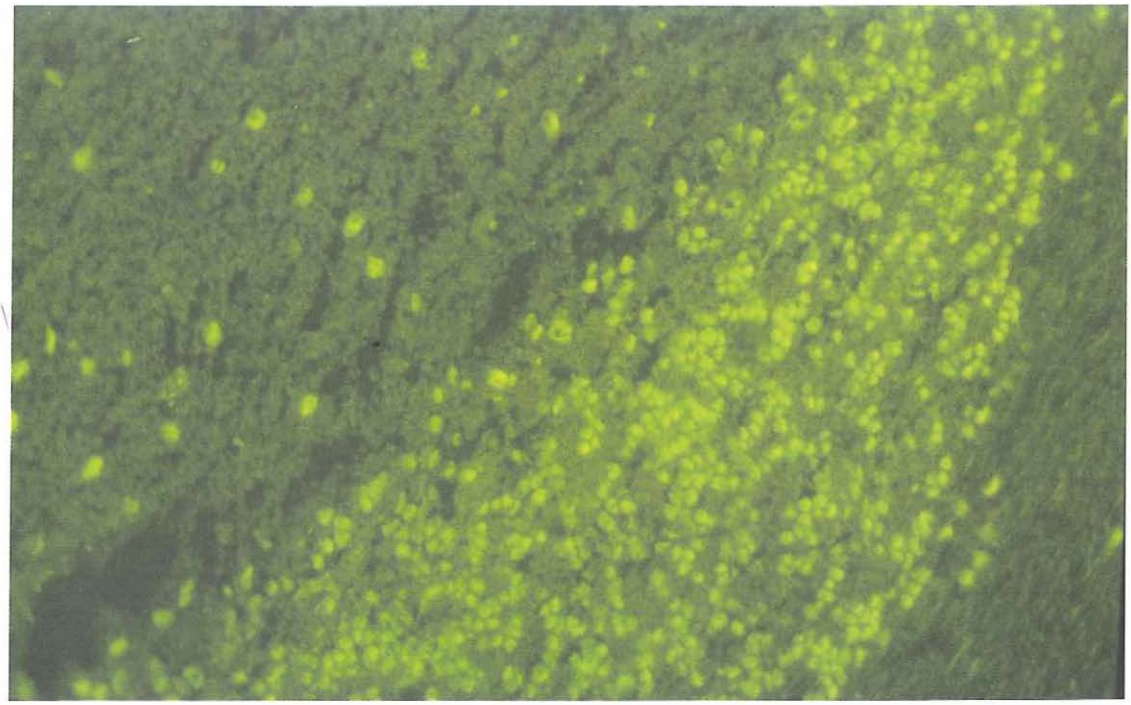
Bedankt

Appendix

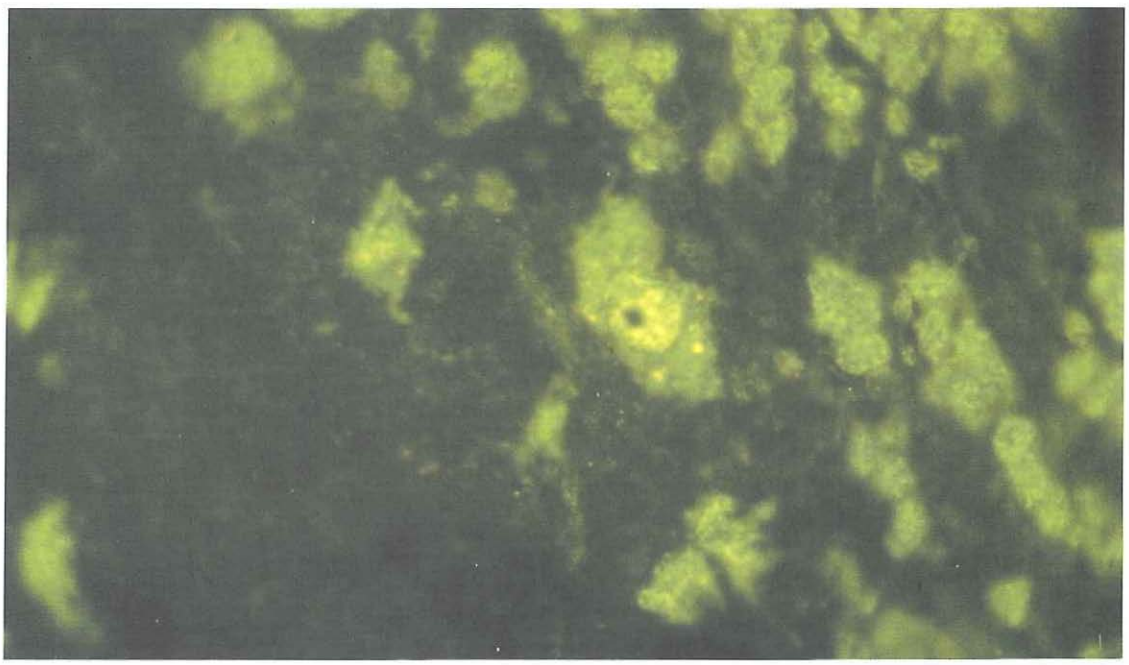
Figures

Chapter 2 Figure 1
Indirect immunofluorescence (dilution 1:100) of nuclei of granular and Purkinje cells with sparing of nuclei (100 x).

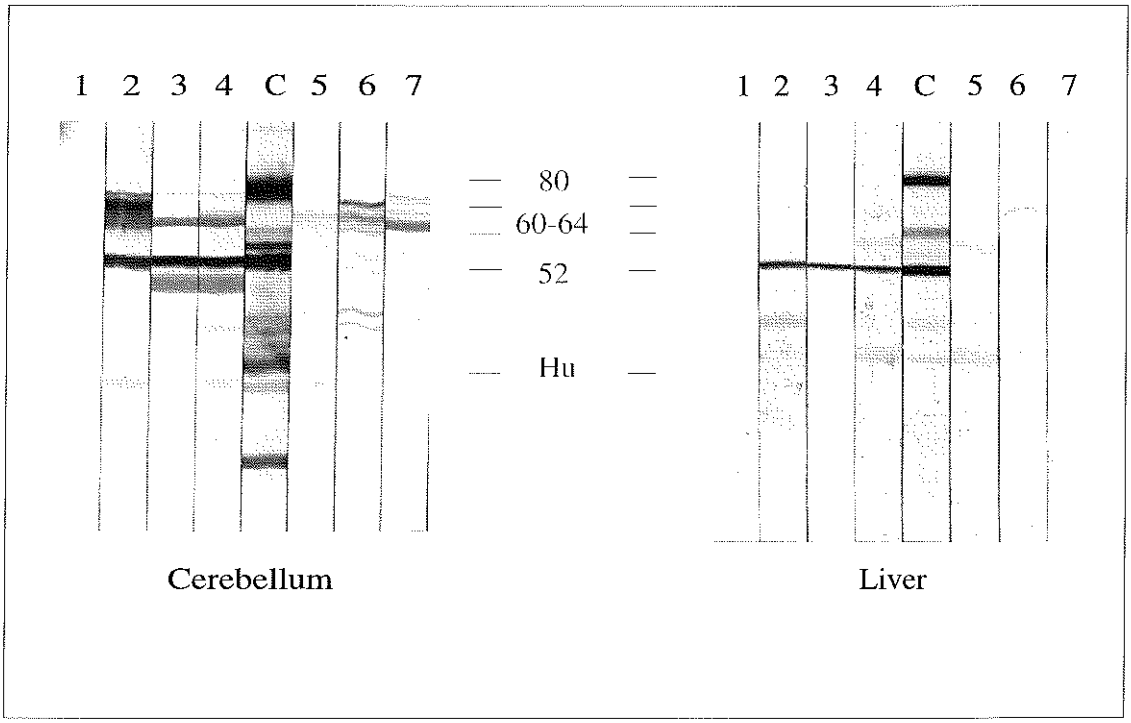
Chapter 2 Figure 2
Indirect immunofluorescence (dilution 1:100) of nuclei of granular and Purkinje cells with sparing of nuclei (400 x).



Chapter 2 Figure 1

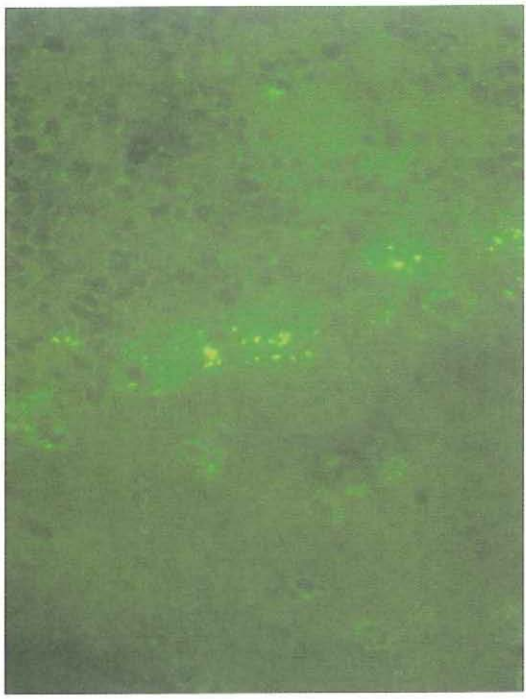


Chapter 2 Figure 2

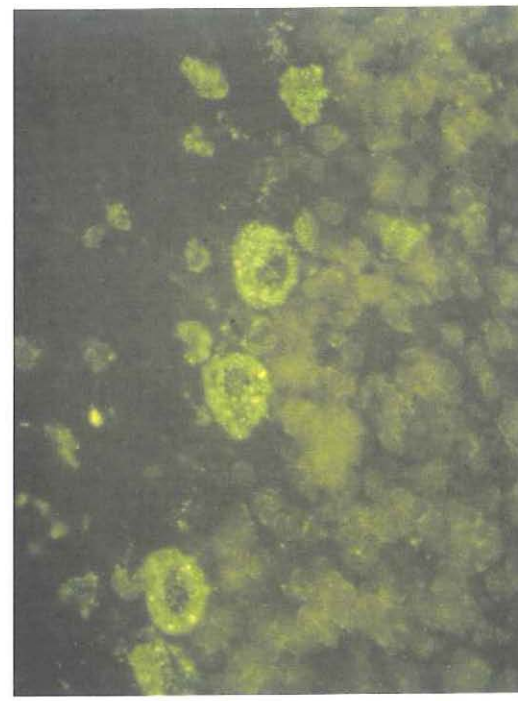


Chapter 5 Figure 1

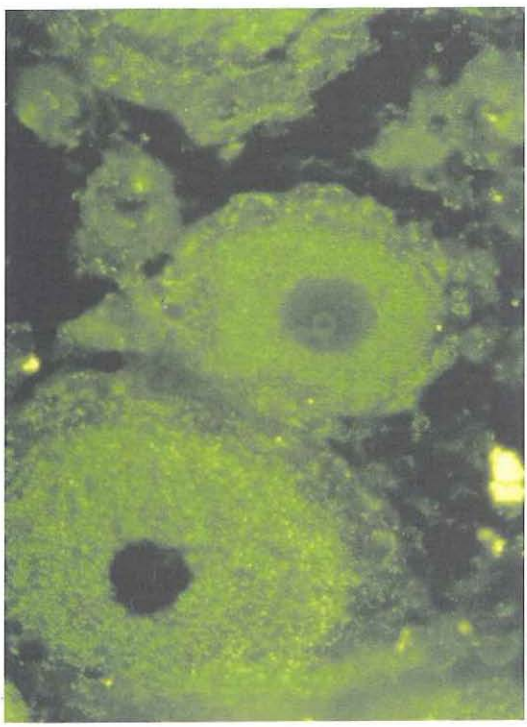
Chapter 5 Figure 1
Western immunoblot showing reactivity against rat cerebellar and liver protein extracts. Clearly visible is the 60-64 kD neuronal reactive protein complex in cerebellar extracts (lanes 2,3,4 and 7 incubated with serum of patient no. 1 to 4) not seen in liver extracts. Also visible is the 52 kD reactive band in lanes 2,3 and 4 in both cerebellar and liver extracts indicating the presence of a systemic auto-antibody (ANA). Lane 1 shows reactivity of normal healthy control serum. Lanes 5 and 6 are incubated with serum of patients with SCLC without neurological complications. Lanes marked C show reactivity of several sera known to have antibodies against neuronal and liver proteins and is used as control to indicate relative molecular weights (numbers in centre of figure).



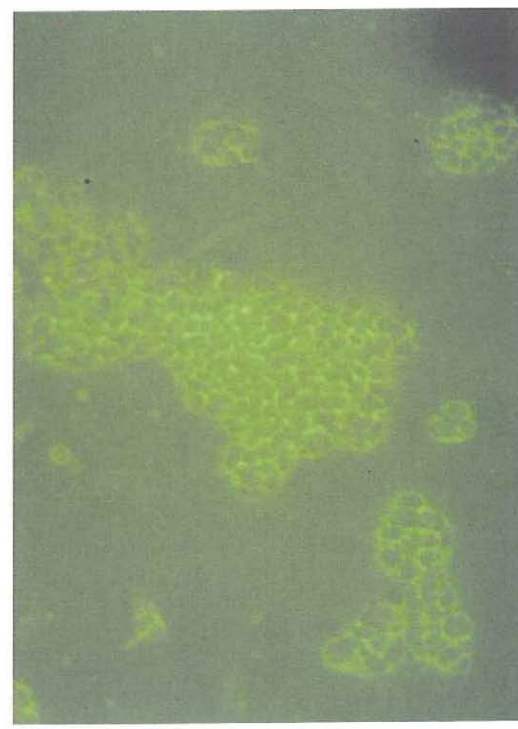
Chapter 5 Figure 2a



Chapter 5 Figure 2b



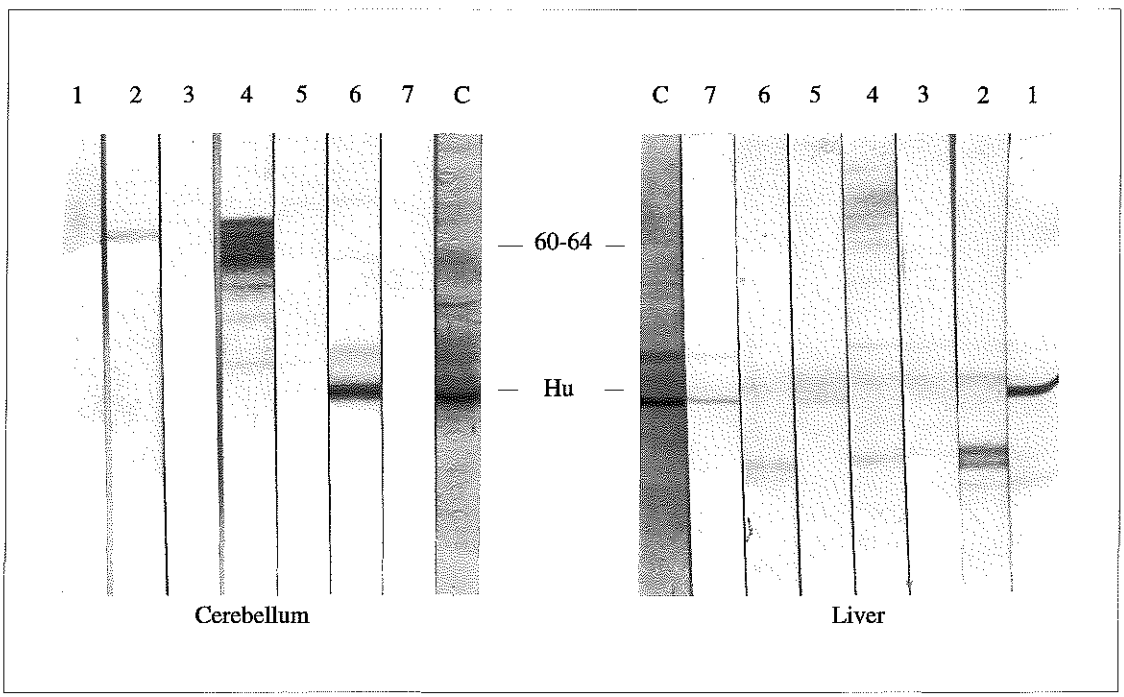
Chapter 5 Figure 2c



Chapter 5 Figure 2d

Chapter 5 Figure 2
Figure 2a and 2b: Indirect immunofluorescence with anti-St reactivity (patient 4, 1:500 dilution, 400 x magnification) showing cytoplasmic staining of Purkinje and granular layer cells compared to coarse granular staining of Purkinje cells as seen in figure 2b (anti-Yo positive patient, 1:500, 400 x magnification).
Figure 2c: The anti-St reactivity seen on human dorsal root ganglion cells showing clear speckled cytoplasmic staining (patient 4, 1:500 dilution, 400 x magnification).
Figure 2d: Cytoplasmic staining of a smal cell lung carcinoma cell line with the anti-St anti-serum of patient 4 (1:100 dilution, 100 x magnification).

Chapter 6 Figure 1a
Western immunoblot of rat cerebellar and liver protein extracts incubated with serum of normal healthy controls (lane 1, 2 and 3), serum of patient 4 (lane 4) and serum of a patient with SCLC without neurological symptoms (lane 5). Lane 6 and lane marked C are incubated with anti-Hu positive serum. Lane 7 is incubated with serum of a patient with sensory neuropathy without cancer. Clearly visible is the 60-64 kD protein complex not seen in liver protein extract or with control sera. Lane 1 shows reactivity against a liver protein not seen in neuronal extract with serum of normal healty control.

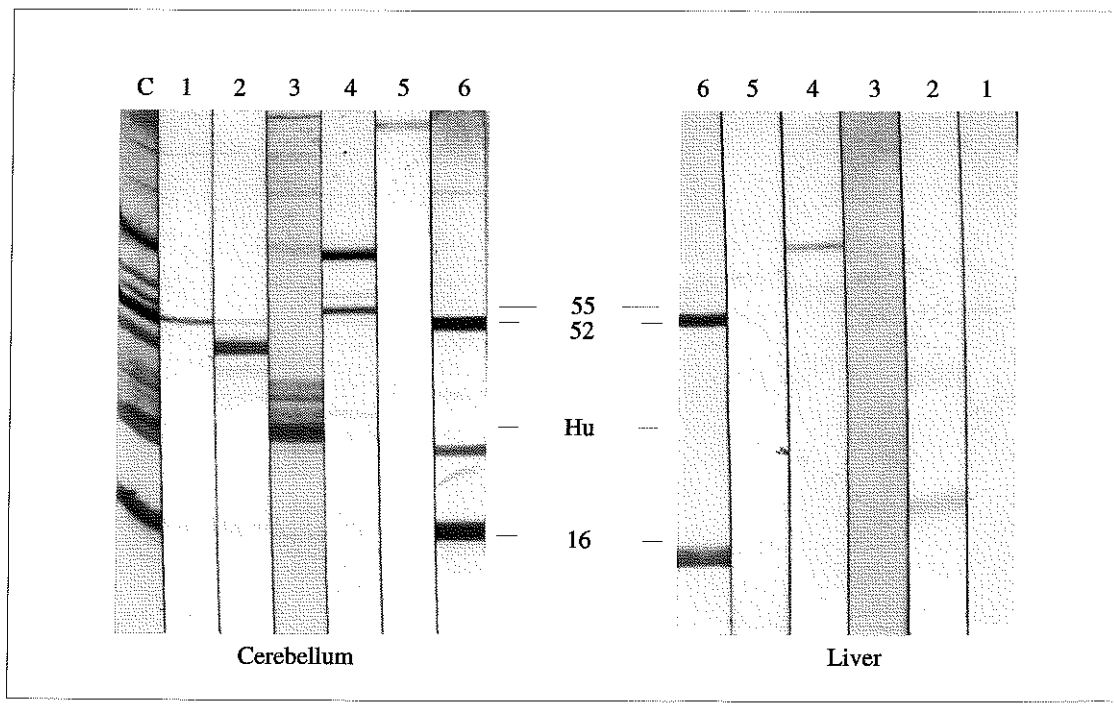


Chapter 6 Figure 1a

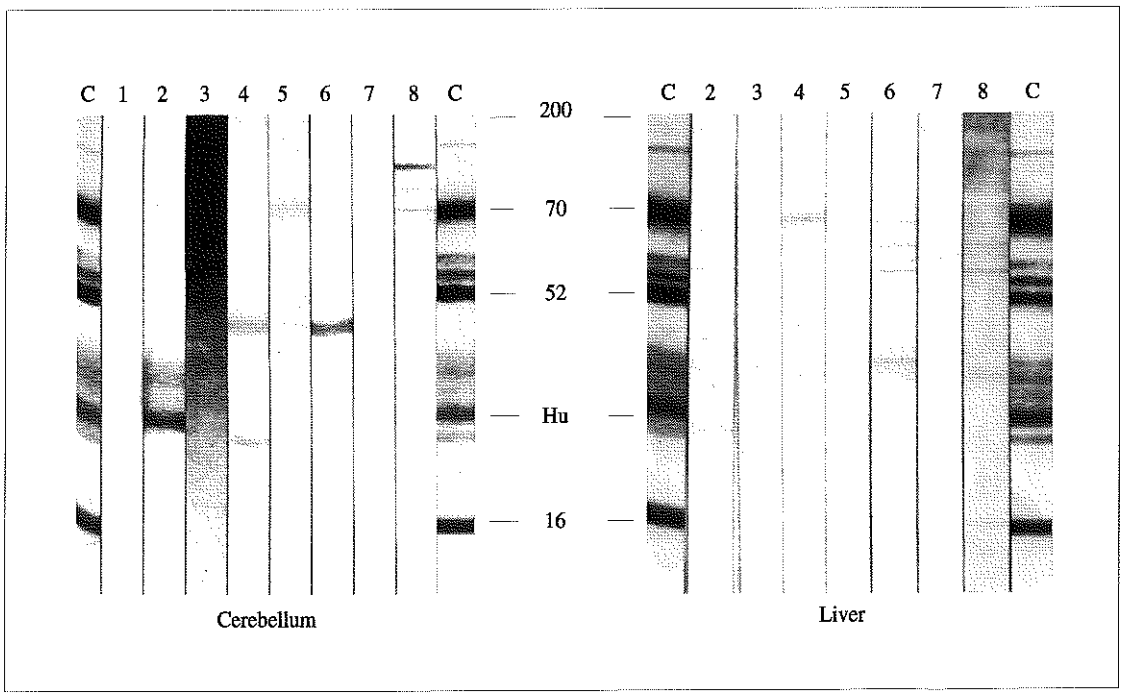
Chapter 6 Figure 2a
Western immunoblot of rat cerebellar and liver protein extracts showing a clear 55 kD reactive band in lane 4 incubated with serum of patient 6. This serum also showed reactivity against a 80 kD mitochondrial protein also seen in liver. Lane 3 incubated with anti-Hu positive serum. Lanes marked C are incubated with sera harbouring various anti-neuronal an liver antibodies and are used as marker to indicate relative molecular weights (numbers in center of text).

Chapter 6 Figure 3a
Western immunoblot of rat cerebellar and liver protein extracts showing reactivity against a 40-200 kD neuronal antigen when incubated with serum of patient 13. Lane 1 incubated with serum of normal healthy control. Lane 2 incubated with serum of anti-Hu positive patient. Lanes 4-8 incubated with different sera from control groups. Lanes marked C (all rat cerebellar extract) are incubated with several sera harbouring various anti-neuronal antibodies used as marker to indicate relative molecular weights (numbers in center of figure).

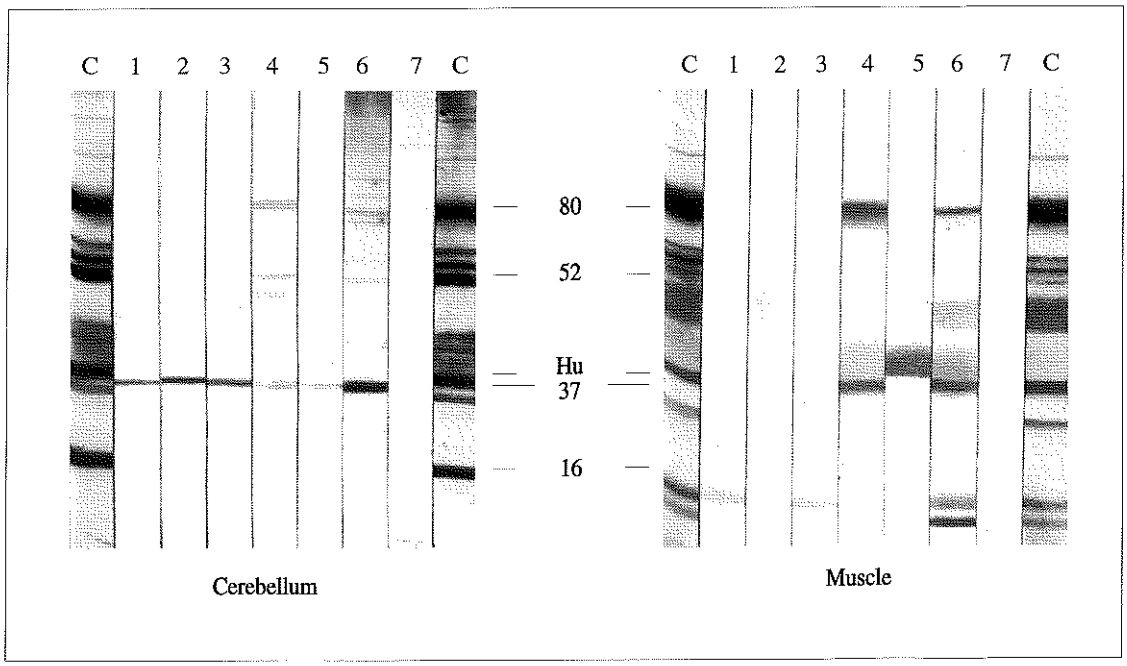
Chapter 6 Figure 4a
Western immunoblot of rat cerebellar and muscle protein extract. Lanes 1 to 6 all show anti-37 kD anti-neuronal reactivity not seen in control sera (lane 7) and only in 2 sera on muscle extract showing clearly weaker reactivity. Reactivity was not seen on liver extracts. Lanes marked C (all rat cerebellar extracts) are incubated with sera harbouring various anti-neuronal antibodies and used as marker to indicate relative molecular weights (numbers in center of figure).



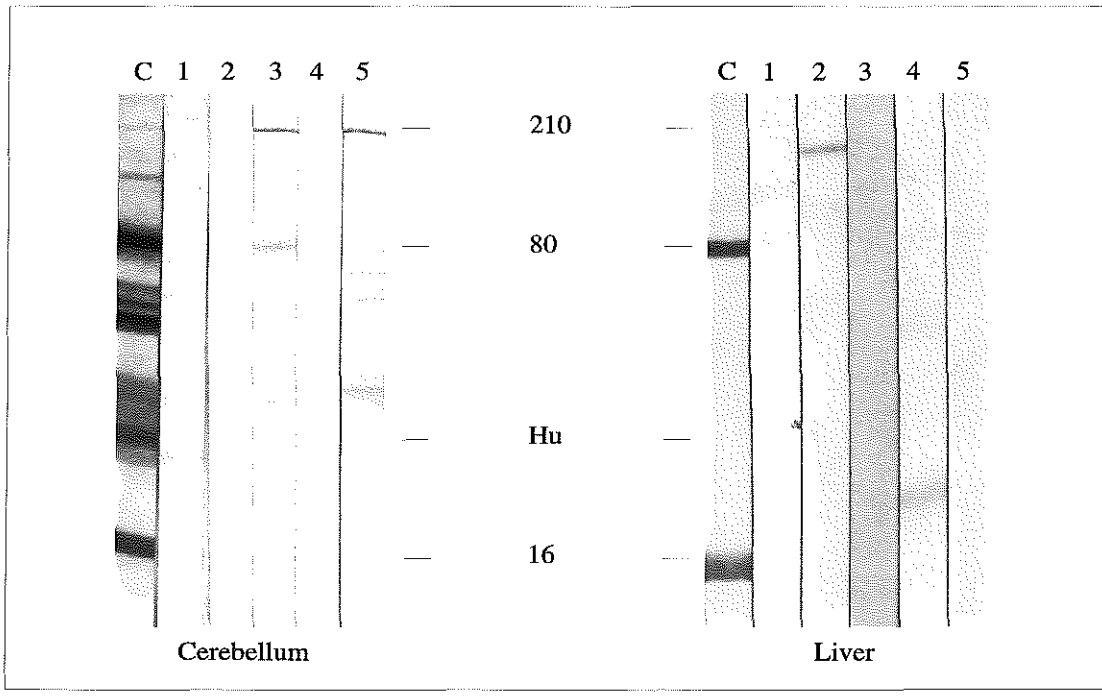
Chapter 6 Figure 2a



Chapter 6 Figure 3a



Chapter 6 Figure 4a



Chapter 6 Figure 5a

Chapter 6 Figure 5a
Western immunoblot of rat cerebellar and muscle protein extracts. Lanes 3 and 5 show anti-210 kD anti-neuronal specific reactivity not seen in control seral (lane 1,2 and 4). Lanes marked C (all rat cerebellar extracts) are incubated with several sera harbouring various anti-neuronal antibodies used as marker for relative molecular weights (numbers in center of figure).

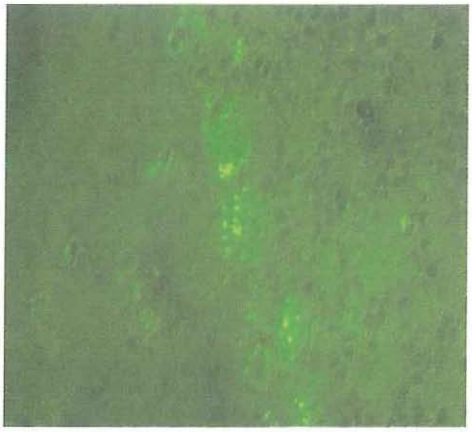
Chapter 6 Figure 1b
Indirect immunofluorescence on frozen rat cerebellar section (5um) with anti-60-64 kD reactivity (patient 4, 1:500 dilution, 400 x magnification) showing diffuse cytoplasmic staining of both Purkinje and granular layer cells.

Chapter 6 Figure 2b
Indirect immunofluorescence on frozen rat cerebellar section (5 um) with anti-55 kD reactivity (patient 5, 1,500 dilution, 400 x magnification) showing clear speckled nuclear staining of virtually all neurons.

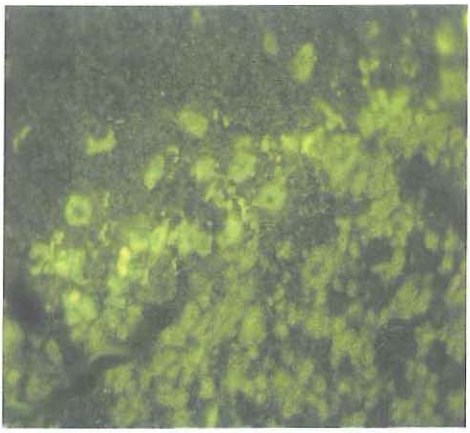
Chapter 6 Figure 3b
Indirect immunofluorescence on frozen rat cerebellar section (5um) with anti-40-200 kD reactivity (patient 12, 1:500 dilution, 400 x magnification) showing diffuse homogeneous staining of cerebellum.

Chapter 6 Figure 4b
Indirect immunofluorescence on frozen rat cerebellar section (5um) with anti-37 kD reactivity (patient 20, 1:500 dilution, 400 x magnification) showing bright nuclear staining of neurons not seen on systemic tissue.

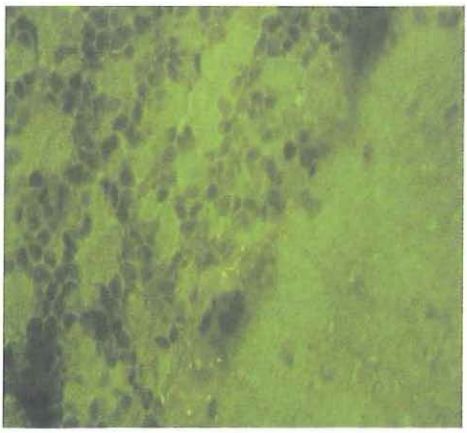
Chapter 6 Figure 5b
Indirect immunofluorescence on frozen rat cerebellar section (5um) with anti-210 kD reactivity (patient 61, 1:500 dilution, 400 x magnification) showing filamentous staining of astrocytes in molecular layer.



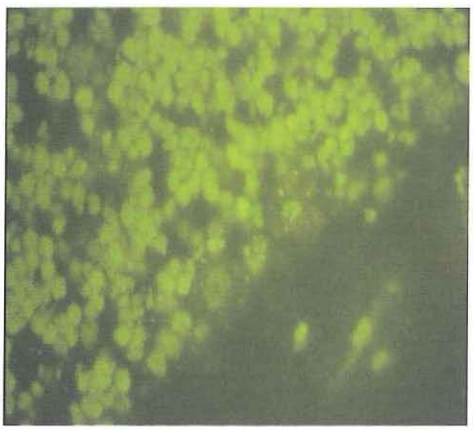
Chapter 6 Figure 1b



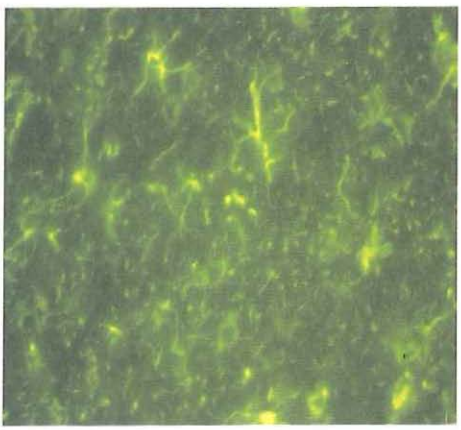
Chapter 6 Figure 2b



Chapter 6 Figure 3b



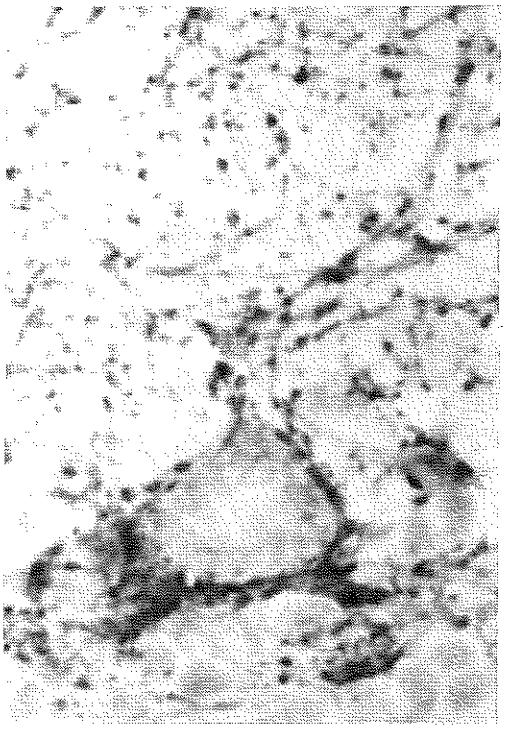
Chapter 6 Figure 4b



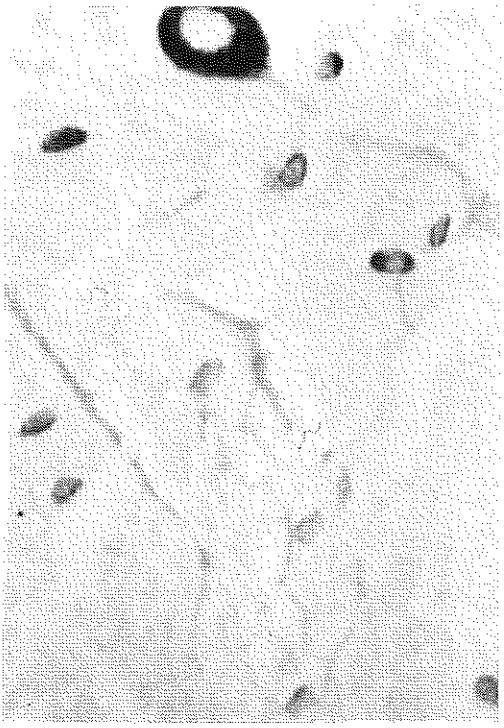
Chapter 6 Figure 5b

Chapter 7 Figure 1

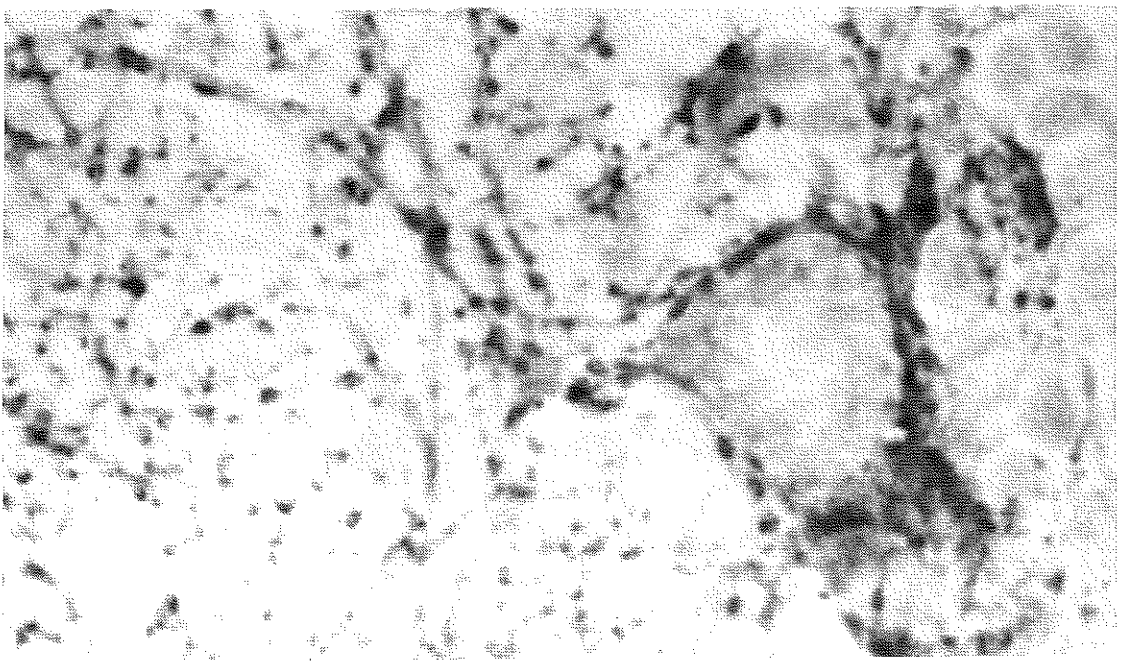
Anti-Tr (A), Anti-GAD (B), and anti-Yo (C) immunoreactivity in rat cerebellum. Both anti-Tr and anti-Yo antibodies immunoreact with the cytoplasm of Purkinje cells. Anti-Tr antibodies show a dotted staining in the molecular layer suggestive of immunoreactivity of dendritic spines of the Purkinje cells. Compare the dot pattern with that of anti-GAD antibodies (B) that label GABA-ergic nerve terminals. Basket and stellate neurons are anti-Tr negative but anti-Yo positive. A and B slightly counterstained with hematoxylin. Bar = 10 μm for panels A and B; bar = 15 μm for panel C.



Chapter 7 Figure 1a



Chapter 7 Figure 1c

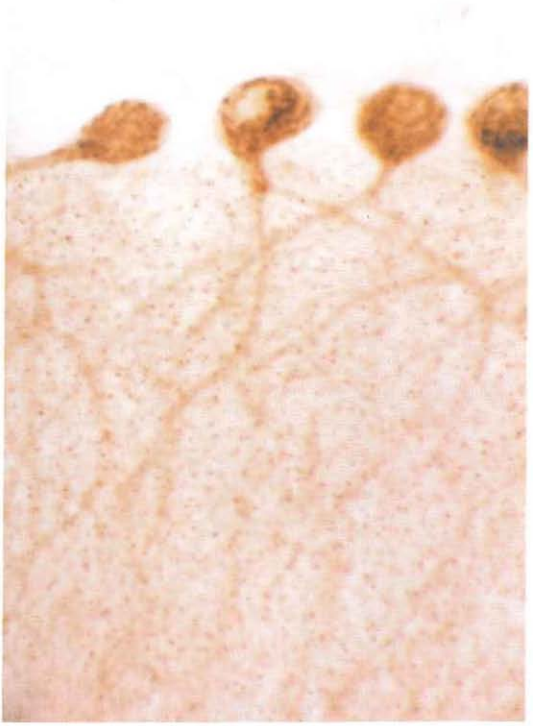


Chapter 7 Figure 1b

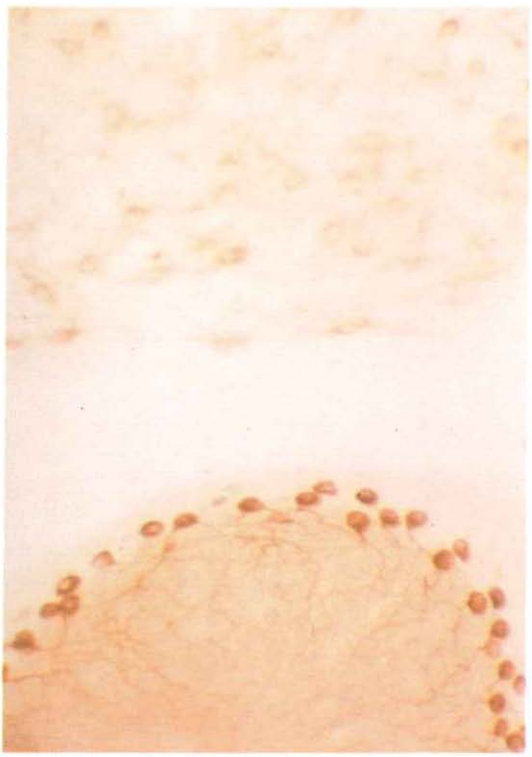
Chapter 7 Figures 2 and 3

Anti-Tr immunoreactivity in vibratome sections of rat cerebellum incubated with biotinylated anti-Tr IgG. Strong anti-Tr immunoreactivity is observed in Purkinje cells. The molecular layer shows the fine dotted pattern (2B). Neurons of the dentate nucleus are only weakly positive (3A). Anti-Tr immunoreactivity in vibratome sections of the rat entorhinal cortex (2A) and hippocampus (3B). Anti-Tr positive cells are present in layer II and scattered in the inner layers. Isolated Tr-immunoreactive non-pyramidal neurons are located in the stratum oriens, stratum pyramidale and stratum radiatum.

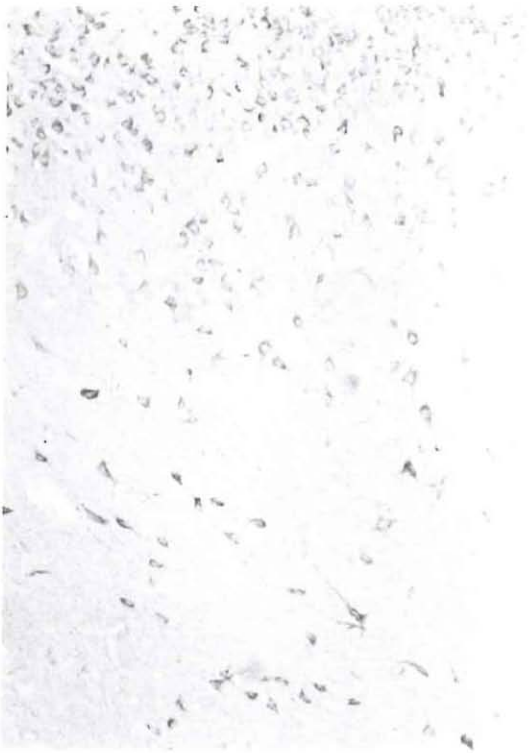
Chapter 7 Figure 2a



Chapter 7 Figure 2b



Chapter 7 Figure 3a



Chapter 7 Figure 3b