Polyneuropathy associated with monoclonal gammopathy

cause and consequence

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Polyneuropathy associated with monoclonal gammopathy, cause and consequence

Polyneuropathie geassocieerd met monoclonale gammopathie, oorzaak en gevolg

(met een samenvatting in het Nederlands)

Proefschrift

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voor Sebastiaan en Rosemarij

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General introduction

Although results from epidemiologic and pathologic studies increasingly support a relationship between monoclonal gammopathy and polyneuropathy,[1-7] the differentiation between polyneuropathy caused by monoclonal antibody reactivity and polyneuropathy with coincidental presence of monoclonal gammopathy remains difficult. The monoclonal gammopathy can be directly related with polyneuropathy if monoclonal antibody reactivity against peripheral nerves can be demonstrated. In addition, monoclonal gammopathy can occur in association with inflammatory diseases like chronic inflammatory demyelinating polyneuropathy (CIDP) or vasculitis and neuropathy.[8-10] It remains unclear whether the presence of monoclonal antibodies produced by monoclonal B- or plasma cells is the primary cause of the neuropathy or alternatively, whether the monoclonal antibodies are produced as a result of an immune response against exposed neural antigens or antigens from microorganisms resembling neural antigens. Differentiation between causal and coincidental presence of monoclonal antibodies is important because the treatment strategies in polyneuropathy associated with monoclonal gammopathy of undetermined significance (MGUS) and chronic idiopathic axonal polyneuropathy (CIAP) or CIDP differ.[11-14] If the monoclonal protein (M-protein) is coincidental, treatment of the polyneuropathy by targeting the B cell and thereby lowering the M-protein level is not indicated.

The relationship between polyneuropathy and monoclonal gammopathy with antibody activity to the myelin associated glycoprotein (MAG) is supported by substantial evidence from pathologic and passive transfer studies.[2,3,5,15-24] Polyneuropathy associated with IgM monoclonal anti-MAG antibodies is characterized by slow progression, symmetric sensory or sensorimotor symptoms, ataxia and demyelination.[25] Patients with IgM monoclonal gammopathy but no anti-MAG reactivity often present with similar clinical signs and symptoms and the response to immunologic treatment is comparable with patients with IgM monoclonal anti-MAG antibodies. The former patients probably have antibody reactivity against other components of peripheral nerves, but a similar immune-mediated patho genesis.[6,17,18,21,22,26-31] The relationship of IgG or IgA monoclonal gammopathy with polyneuropathy is less clear. In patients with MGUS, IgG M-protein is more frequently found than IgM M-protein. In prevalence studies of monoclonal gammopathy, IgG M-protein is found in 75%, IgM M-protein in 15% and IgA M-protein in 10% of the cases.[32] Most of these cases with MGUS are asymptomatic and do not have neuropathy. Polyneuropathy is more frequently associated with IgM M-protein. [16,32,33]

In this thesis, the cause, coincidence and consequence of monoclonal gammopathy and polyneuropathy is described. To further discriminate clinical entities, we analyzed the presence of T cells in sural nerve biopsies in primary demyelinating and primary axonal polyneuropathies associated with monoclonal gammopathy. We postulated that T cell infiltrates should be absent in polyneuropathy associated with IgM MGUS and anti-MAG antibodies (Chapter 2).

In addition, we wanted to establish whether anti-MAG, anti-sulfoglucoronyl paragloboside (SGPG), and anti-ganglioside (AGM1, GM1, GM2, GD1a, GD1b, GQ1b) antibodies could have a prognostic value in patients with polyneuropathy associated with IgM monoclonal gammopathy (Chapter 3).

Monoclonal gammopathy of undetermined significance (MGUS) is characterized by the asymptomatic presence of a monoclonal protein (M-protein) in serum and/or urine without an underlying hematologic malignancy or other cause and stability of the M-protein level during six months. Criteria for MGUS include a serum M-protein level less than 30 g/L, a bone marrow plasma cell infiltration of less than 10%, absence of lytic bone lesions and laboratory abnormalities, i.e. anemia, hypercalcemia or renal insufficiency.[35-37] The risk of malignant transformation is 1% per year for patients with IgG MGUS to develop multiple myeloma and 1.5% per year for patients with IgM MGUS to develop lymphoma or immunocytoma (Waldenström's macroglobulinemia) and the risk of malignant transformation persists even after 30 years of follow-up.[32,38,39] Recently, Kyle et al. reported M-protein level as the most important independent predictor for malignant transformation in both IgG and IgM MGUS after studying 1384 patients with long-term follow-up.[32,39] Most of the patients with polyneuropathy associated with monoclonal gammopathy have MGUS, but one third of the patients have an underlying hematologic malignancy at presentation.[21] The risk of malignant transformation in polyneuropathy associated with MGUS is unknown.

The following case report illustrates the diagnostic process and importance of hematologic follow-up in polyneuropathy and monoclonal gammopathy (Chapter 4):

A 47-year-old woman had been healthy till the age of 44 years when the first symptoms occurred. She noted stiffness of her feet and unsteadiness of gait. She had numbness and tingling in both hands and feet. In the following years the numbness spread over the legs and the arms, she noted muscle cramps in her legs and wasting of hand and calve muscles. Her disease history was unremarkable; she did not use any medication, alcohol consumption had been modest and she had never smoked. Her 75-year-old mother had developed malignant IgM monoclonal gammopathy (Waldentröm's macroglobulinemia) at the age of 70 years and complained of numbness, tingling and weakness of the

hands and feet and unsteadiness of gait. At neurologic examination our patient was unable to stand with her eyes closed. The interossei muscles, muscles of the feet and calves were wasted. She had symmetrical weakness of the finger extensor muscles (Medical Research Council [MRC] 4), the anterior tibial muscle (MRC 3), the gastrocnemius muscle (MRC 4), and the extensor hallucis muscle (MRC 0). All tendon reflexes were absent. Touch and pinprick sensation was impaired in her feet and hands in a glove and stocking like distribution. Vibration sense was absent to her knees and joint position sense was normal. In conclusion, a distal symmetric sensorimotor polyneuropathy. Because of the areflexia demyelination was suspected. The electrophysiologic examination showed multifocal demyelination in arm and leg nerves and axonal degeneration in leg nerves. Laboratory investigations showed no abnormalities besides a monoclonal protein (M-protein) IgM kappa in a concentration less than 1 g/l and anti-MAG antibodies. DNA analysis showed no duplication on chromosome 17 or mutation on chromosome 1 (consistent with hereditary motor and sensory neuropathy (HMSN) type I). Examination of a sural nerve biopsy revealed IgM deposits and widening of the myelin lamellae in the external layers. Hematologic evaluation according to the CBO criteria (http://www.cbo.nl, including examination by a hematologist, laboratory analysis and bone marrow examination did not reveal a hematologic malignancy. The patient was diagnosed with a demyelinating polyneuropathy according to the American Academy of Neurology (AAN) criteria associated with IgM MGUS with anti-MAG antibodies.[34]

After two years the patient had to stop working as a sausage seller in a department store because she could not stand without support. She noted wasting of her thighs, she could not climb the stairs and could not move her feet. At neurologic examination she was unable to stand without assistance. The muscles of her lower arms, the muscles of her feet, lower and upper legs were wasted. There was symmetrical weakness of the finger extensor and finger flexor muscles (MRC 4), the iliopsoas muscle and the hamstrings (MRC 4), the anterior tibial, the gastrocnemius and the extensor hallucis muscle (MRC 0). All tendon reflexes were absent. Touch and pinprick sensation was impaired in her legs and lower arms. Vibration sense was absent in her legs and fingers and joint position sense of the toes was absent. Laboratory analysis revealed an increase of the IgM M-protein level to 5 g/l. Histologic examination of bone marrow biopsy identified infiltration of monoclonal B cells of 20% supporting the diagnosis immunocytoma.

Progression of the polyneuropathy in patients with MGUS seems to be associated with hematologic malignancies, as in the described case.[25] We wondered what the frequency of hematologic malignancies

was and whether we could identify patients with an increased risk of malignant transformation. To assess the frequency of hematologic malignancies in polyneuropathy associated with monoclonal gammopathy at diagnosis and during follow-up we performed a cohort analysis and a prospective follow-up study (Chapter 4).

The observation that the mother of our patient was diagnosed with Waldenström's macroglobulinemia suggests an unidentified genetic risk factor for monoclonal gammopathy and malignant transformation.[40,41] In hematologic malignancies genetic aberrations are involved in the development and progression of disease. For example in IgG MGUS and in multiple myeloma, chromosome 14 translocations and deletion of chromosome 13 have been identified.[42,43] Also in Non-Hodgkin's lymphoma chromosome 14 translocations and numerical chromosomal aberrations have been found.[44] To investigate whether chromosomal abnormalities could explain malignant transformation in patients with polyneuropathy and MGUS we performed cytogenetic analysis. The monoclonal B cell (or plasma cell) population producing the M-protein from the bone marrow of affected patients was identified and isolated. Cytogenetic aberrations in these B cells were identified with different techniques: 1. translocations or deletions of chromosomes (interphase fluorescent in situ hybridization, interphase FISH); 2. gain or loss of genes (comparative genomic hybridization based DNA array, CGH array, and multiple ligation-dependent probe amplification, MLPA) (Chapter 5).

The immunoglobulin is composed of heavy and light chains both consisting of a variable and a constant part. The variable part is responsible for the antigen binding. Immunoglobulin genes encoding the variable part (V, D, J) assemble during B cell development (somatic recombination). In individual B cells, a functional immunoglobulin gene (germline sequence) encoding the antigen binding part of the immunoglobulin molecule (antibody) is created in this way. When a B cell encounters an antigen, random replacement of nucleotides in this gene encoding the antigen binding part is initiated (antigen driven somatic hypermutation). These mutations can result in increased affinity of the immunoglobulin for antigen. As a consequence, B cells expressing such mutated immunoglobulin molecules are more efficiently activated by antigen, the proliferative capacity of these B cell increases, leading to selection of B cells expressing immunoglobulins with higher affinity. In hematologic malignancies with associated autoimmune disorders such as chronic lymphatic leukemia (CLL) restricted immunoglobulin genes are used.[45-47] We determined the immunoglobulin gene usage by monoclonal B cells and the presence of somatic mutations in patients with polyneuropathy associated with IgM MGUS (Chapter 6).

Aims of the study

Polyneuropathy associated with MGUS is heterogeneous. The spectrum is formed by demyelinating polyneuropathy with IgM MGUS and anti-MAG antibodies on one side and by chronic idiopathic axonal polyneuropathy with coincidental IgG MGUS on the other side. In between are various syndromes where the monoclonal gammopathy may be part of a clinical syndrome as the POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, M-protein and skin symptoms). Perhaps even more important, patients with polyneuropathy and MGUS have an increased risk of malignant transformation. Considering these aspects the aims of our study were:

1. To further delineate various syndromes of polyneuropathy associated with monoclonal gammopathy using clinical, pathologic and immunologic methods (Chapter 2).

2. To identify prognostic factors for these syndromes using clinical and immunologic methods (Chapter 3).

3. To determine the frequency of hematologic malignancies at diagnosis and the occurrence of malignant transformation during follow-up (Chapter 4).

4. To determine genetic risk factors for development of hematologic malignancies by identification of cytogenetic aberrations (Chapter 5).

5. To study immunoglobulin gene usage and the presence of somatic mutations in monoclonal B cells from patients with polyneuropathy associated with IgM monoclonal gammopathy (Chapter 6).

In Table 1 the nomenclature and characteristics of the hematologic diseases associated with polyneuropathy and monoclonal gammopathy are presented.[35,135-137]

Table 1 Monoclonal gammopathy type and associated hematologic disease

type	hematologic disease	important characteristics
IgM	Monoclonal gammopathy undetermined significance (MGUS)	- M-protein level < 30 g/l - Bone marrow plasma cell infiltration < 10% - Bone marrow B cell infiltration < 25% - No hepatospenomegaly, lymphadenopathy
	Immunocytoma Lymphoplasmacytic lymphoma (LPL)	- Bone marrow mature B- cell infiltration > 25%
	Waldenström's macroglobulinemia	- M-protein level > 30 g/l - Bone marrow mature B- cell infiltration > 25% (LPL)
	Chronic lymphocytic leukemia (CLL)	- Peripheral blood lymphocytosis > 10.000/ul - Lymphocyte infiltration in bone marrow > 30%
	Non Hodgkin's lymphoma (NHL)	- Lymphadenopathy +/- hepatosplenomegaly - Bone marrow B cell infiltration
IgG or IgA	MGUS	- Bone marrow plasma cell infiltration < 10% - No lytic bone lesions - No anemia, hypercalcemia or renal insufficiency
	Multiple myeloma (MM)	- Monoclonal plasma cell infiltration > 10%
	Osteosclerotic myeloma Plasmacytoma	- Localized plasma cell infiltration, sclerotic bone lesion
	POEMS	- Polyneuropathy: axonal/demyelinating on EMG - Organomegaly: echography/CT-abdomen - Endocrinopathy: TSH, LH/FSH, cortisol, glucose - M-protein - Skin symptoms - Localized plasma cell infiltration
	Associated light chain (AL) amyloidosis	- Amyloid (light chain) depositions in bone marrow, organs and skin - Autonomic failure - Rapidly progressive disease course
	Non Hodgkin's lymphoma (NHL)	- Lymphadenopathy, +/- hepatosplenomegaly - Bone marrow infiltration

Chapter 2

T cell infiltration in polyneuropathy associated with monoclonal gammopathy

adapted from

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T cell infiltration in axonal and demyelinating polyneuropathy associated with monoclonal gammopathy

Summary

The clinical presentation of polyneuropathy associated with monoclonal gammopathy is heterogeneous. As T cells in sural nerve biopsy specimens may represent a marker of inflammation, we analyzed whether the presence of sural nerve T cells in patients with axonal or demyelinating polyneuropathy associated with monoclonal gammopathy may help to define a specific clinical entity. Using immunohistostochemical analysis we investigated the number and distribution of sural nerve T cells in 25 patients with demyelinating polyneuropathy associated with monoclonal gammopathy (18 IgM, including 14 with antibodies to the myelin associated glycoprotein (MAG), 7 IgG), and in 23 patients with axonal polyneuropathy associated with monoclonal gammopathy (12 IgM, 11 IgG), and compared them with sural nerves T cells in 23 patients with chronic inflammatory demyelinating polyneuropathy (CIDP), 15 patients with chronic idiopathic axonal polyneuropathy (CIAP), and 10 normal controls. Six patients with demyelinating polyneuropathy associated with monoclonal gammopathy had increased T cell densities compared with CIAP patients and normal controls. No differences were found in distribution or phenotype of the T cells. T cell densities in patients with IgM monoclonal gammopathy were significantly lower than in patients with IgG monoclonal gammopathy or with CIDP. Increased sural nerve T cells were significantly associated with a subset of patients who had a more progressive disease course, more pronounced weakness, a monoclonal gammopathy of the IgG isotype, and a hematologic malignancy. Seven patients with axonal polyneuropathy associated with monoclonal gammopathy had increased T cell densities and a progressive disease course. Four of these patients were treated with prednisone with a good response, suggesting that vasculitis plays a role in the pathogenesis.

Introduction

Polyneuropathy associated with monoclonal gammopathy is widely recognized. [17,21,22,48] Both the type of polyneuropathy and the associated monoclonal gammopathies are heterogeneous, but several distinct syndromes have been identified.[17,21,22,48] For polyneuropathy and IgM monoclonal gammopathy with antibody activity to the myelin associated glycoprotein (MAG), there is substantial evidence from pathologic and passive transfer studies that the neuropathy is caused by autoreactivity of the monoclonal immunoglobulins.[2,3,5,15-24] However, in many patients antibody activity of the monoclonal gammopathy cannot be identified. These patients may have a similar type of polyneuropathy and response to immunologic treatment as patients with detectable serum antibodies, implicating an immune-mediated pathogenesis.[6,17,18,21,22,26-31]

The study of polyneuropathy associated with monoclonal gammopathy has been further confounded by its relation to chronic inflammatory demyelinating polyneuropathy (CIDP). The electromyographic features of CIDP may also occur in polyneuropathy associated with monoclonal gammopathy and the cerebrospinal fluid protein is usually elevated in both conditions.[49-52] Monoclonal gammopathies may occur non-specifically in many chronic inflammatory or infectious diseases, and approximately 1% of normal adults have a monoclonal gammopathy, so that in some cases, the monoclonal gammopathy may be coincidental and unrelated to the neurologic disease.[53] In CIDP the extent of involvement of cellular or humoral immune processes in the damage to the peripheral nervous system has not been established.[54,55] Evidence that supports a pathogenic role of T cells in CIDP is the presence of T cell infiltrates in sural nerve biopsy specimens of CIDP patients.[56-58] A role for T cells in polyneuropathy associated with monoclonal gammopathy has been suggested by the association with HLA class II, increased serum interleukin-2 receptor levels and activated CD4+ and CD8+ cells in blood of some patients.[59-61]

The association of monoclonal gammopathy and axonal polyneuropathy is less clear and the pathogenic role of antibodies against sulfatide, chondroitin sulfate and gangliosides is unknown.[62,63]A substantial number of patients with axonal polyneuropathy and monoclonal gammopathy had similar characteristics as patients with chronic idiopathic axonal polyneuropathy (CIAP), suggesting that monoclonal gammopathy may be a coincidental finding.[14] However, a subset of patients with axonal polyneuropathy and monoclonal gammopathy has a more progressive disease course and worse disability than patients with CIAP.[14]

An immunologic mechanism and a role for T cells in the pathogenesis of axonal polyneuropathy has been suggested by the

finding of T cell clones in peripheral blood in idiopathic sensory axonal polyneuropathy;[64] and the detection of perivascular T cells in sural nerve biopsies in cryoglobulinemia, which most frequently appears as a monoclonal protein.[65]

In the present study, we analyzed whether the presence of T cells in sural nerve biopsy specimens of patients with axonal or demyelinating polyneuropathy associated with monoclonal gammopathy, may help to further discriminate and define clinical entities.

Patients and methods

We studied the sural nerve biopsies of 48 patients between November 1989 and January 1998 who had polyneuropathy associated with a monoclonal gammopathy and electrophysiologic features compatible with demyelination or axonal degeneration (see below). The clinical and laboratory features of these patients are listed in table 1A and 1B. In all patients other causes of neuropathy including cryoglobulinemia, hepatitis C infection and primary amyloidosis were excluded.[25] Anti-MAG antibodies were measured using the Western blot system and anti-sulfatide antibodies by enzyme-linked immunoassay (ELISA), as previously described.[28,66]

Of the patients with a demyelinating polyneuropathy (table 1A) 18 patients had IgM monoclonal gammopathy, 14 of these had anti-MAG antibodies. In five patients with IgM monoclonal gammopathy with anti-MAG antibodies (1, 4, 7, 11, 12) elevated anti-sulfatide antibody titers were found and in one patient with IgM monoclonal gammopathy without anti-MAG antibodies (18). Seven patients had a monoclonal gammopathy of the IgG isotype. Twenty patients had a monoclonal gammopathy of undetermined significance (MGUS), one patient had non Hodgkin's lymphoma (16), two patients multiple myeloma (20, 25), one patient Castleman's disease (21) and one patient POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, M-protein and skin changes, 22). None of the patients received treatment before the nerve biopsy was taken. Treatment during follow-up consisted of intermittent cyclophosphamide and prednisone (18 patients),[14] prednisone (2 patients), dexamethasone (1 patient)[67] or intravenous immunoglobulins (IvIg: 2 patients).

Of the patients with axonal polyneuropathy (table 1B) 12 patients had IgM monoclonal gammopathy. In four of these patients (4, 7, 9, 12) elevated anti-sulfatide antibody titers were found. Eleven patients had a monoclonal gammopathy of the IgG isotype. Eighteen patients had MGUS, three patients had non Hodgkin's lymphoma (1, 8, 12), one patient Waldenström's macroglobulinemia (11) and one patient multiple myeloma (21). None of the patients received treatment before the nerve biopsy was taken. Treatment during follow-up consisted of intermittent cyclophosphamide and prednisone, or

monotherapy with prednisone. A positive treatment response was defined as improvement of motor and sensory sumscores and/or disability on the Rankin disability score during the year following treatment.[25] The course of the polyneuropathy was determined as either progressive (deterioration of the neuropathy leading to decline of motor and sensory sumscores of more than one point or disability on the Rankin disability score over months) or slowly progressive (deterioration over years). None of the patients had a relapsing remitting disease course. As normal controls we studied 10 sural nerves from autopsy cases without neuropathy.[58] As disease controls we used 23 sural nerves from patients with CIDP, who fulfilled established criteria for CIDP,[34] and 15 sural nerves from patients with a non-inflammatory chronic idiopathic axonal polyneuropathy (CIAP). The latter polyneuropathy had a slowly progressive course, and during a 5-year follow-up no cause was found.[68,69]

Electrophysiology
Electrophysiologic studies included nerve conduction and concentric needle examination using standardized techniques, and identified a predominantly axonal or demyelinating neuropathy according to conventional criteria, as previously described.[25,34,52,70,71] Briefly, conduction studies on both sides with surface electrodes included: motor nerve conduction and F waves in the median, ulnar, peroneal nerves and tibial nerves; antidromic sensory conduction on distal stimulation in the median, ulnar, radial and sural nerves; and presence or absence of spontaneous muscle fiber activity on concentric needle electromyography in the first dorsal interosseus and anterior tibial muscle were assessed. A demyelinating polyneuropathy was diagnosed when features of demyelination applied to the AAN criteria.[34]

Immunohistochemistry
Immunohistochemical staining was performed on 6 µm thick transverse acetone-fixed frozen sections.[58] To increase the sample size for each patient and control, two total sural nerve sections were cut from different tissue blocks for each staining procedure. The distance between two sections of the same biopsy was at least 500 µm. To examine infiltration of T cells, consecutive sections were incubated with the following primary antibodies for cell typing diluted in phosphate-buffered saline with 5% horse- or goat serum: rabbit-anti-CD3 (Pan-T cells, 1:200; DAKO, Carpinteria, CA), mouse-anti-CD4 (helper/inducer T cells, 1:400; DAKO), mouse-anti-CD8 (cytotoxic/suppresser T cells, 1:400; DAKO), mouse-anti-CD20 (B cells, 1:200; DAKO) and mouse-anti-CD68 (macrophages, 1:1000; DAKO). Labeling was visualized by the ABC-method (Vector Labs., Burlingame, CA). Color was developed using diaminobenzidine with cobalt and nickel intensification, and

sections were counterstained with nuclear fast red. Omitting the primary antibody did not produce any staining. As CD4 can also be expressed by macrophages,[72] CD4+ cells were examined but not quantified. Furthermore, infiltration of macrophages and B cells was examined, using mouse-anti-CD68 (Macrophages, 1:1000; Dako) and mouse-anti-CD20 (B cells, 1:200; Dako).

Quantification of T cells

The cellular infiltrates were quantified by light microscopy (40x objective) by the same investigator (M.E.) in blind fashion.[58] Nerve areas were measured using a digitizing tablet and image analysis software (Jandell, Erkrath, Germany). Densities (equals number of T cells/mm^2) of both CD3+ and CD8+ cells were measured in the total sural nerve areas, endoneurial areas and epineurial areas. For each sural nerve the mean of the measurements from the two sections from the different tissue blocks were used for further analysis. T cells were classified as lying perivascular or scattered throughout the endoneurial or epineurial space. A perivascular T cell was defined as lying within a distance of not more than one T cell diameter (10 µm) from a blood vessel or a perivascular infiltrate. We calculated the percentages of endoneurial T cells ((endoneurial T cells/total T cells) x 100%), of epineurial T cells ((epineurial T cells/total T cells) x 100%), of perivascular T cells ((perivascular T cells/total T cells) x 100%) and of CD8+ T cells ((CD8+ T cells/total T cells) x 100%). In patients with polyneuropathy associated with monoclonal gammopathy, we considered the T cell density to be increased if the T cell density exceeded those of normal controls.[58] The densities of myelinated nerve fibers were obtained from the histograms routinely made for diagnostic histopathologic reports. IgM and complement deposition was identified by immunofluorescence staining of the sural nerve biopsy.

Of the sural nerve biopsies with increased T cell densities, hematoxylin and eosin sections were examined to identify signs diagnostic of necrotizing vasculitis (inflammation of the vessel wall and necrosis of the vessel wall) and suggestive of vasculitis (mural or perivascular inflammation without tissue necrosis, or (previous) necrosis of the arteriole wall without inflammation).[10]

Statistical analyses

Statistical analyses were performed using the Mann-Whitney U test for comparison of T cell densities between patients and controls, and to compare T cell densities between subgroups with different clinical or electrophysiologic features. The Pearson Chi square-test was used for comparison of clinical symptoms between patients with increased and normal T cell densities. Values of $p<0.05$ were considered to be significant.

Results

A) Demyelinating polyneuropathy associated with monoclonal gammopathy

Sural nerve T cells

T cells were found in all sural nerves of patients and controls (figure 1A and 2A). Six patients with polyneuropathy associated with monoclonal gammopathy had increased T cell densities (higher than normal and CIAP controls, > 27/mm^2). Occasionally, a CD20+ B cell was present in large perivascular T cell infiltrates. Macrophages were found in all biopsies in the endoneurium and epineurium, both diffusely distributed and perivascular. Quantification of macrophages was difficult due to confluence of cells.[56]

In patients with polyneuropathy associated with IgM monoclonal gammopathy, T cell densities ranged from 5 to 57/mm^2 (median 11) and from 12 to 26/mm^2 (median 22), and were significantly lower than in patients with CIDP ($p<0.05$) (figure 2A). An increased T cell density was found only in one patient with IgM monoclonal gammopathy with anti-MAG antibodies who had a unusual clinical presentation for anti-MAG polyneuropathy: he developed profound proximal and distal weakness and sensory loss over months. In patients with an IgG monoclonal gammopathy, T cell densities ranged from 16 to 43/mm^2 (median 35). T cell densities were significantly higher in patients with an IgG monoclonal gammopathy than in normal controls ($p<0.01$) or in patients with an IgM monoclonal gammopathy ($p<0.01$) (figure 2A). Five of the 7 patients with an IgG monoclonal gammopathy had increased T cell densities (figure 2A).

The localization of T cells was in both the endoneurium and epineurium in all sural nerve biopsy specimens (figure 3A). In the majority of all sural nerves, the number of epineurial T cells was higher than the number of endoneurial T cells ($p<0.01$). The percentages of endoneurial and epineurial T cells in all subgroups of patients and controls were similar. In the total sural nerve area, a majority of T cells was localized around blood vessels (figure 3A). The percentage of perivascular T cells was similar in all subgroups of patients and controls. In all sural nerve biopsy specimens CD8$^+$ T cells were present in the endoneurium and epineurium (figure 3A).

Table 1A Clinical characteristics and T cell densities in demyelinating polyneuropathy

pat	age (y)	sural nerve SNAP/CV (mV, m/s)	clinical features		disease course	treatment response	CSF protein g/l	till biopsy (mo)	deposition	total T cells /mm2
IgM	*MAG +*									
1	54	no	sm	d+p	progr	+	0.75	12	IgM	57*
2	61	no	sm	d	slow	-	1.02	144	-	19
3	62	no	sm	d	slow	+	0.47	24	IgM	19
4	62	no	sm	d	slow	pred +	1.2	24	nd	18
5	65	no	sm	d	slow	-	nd	168	IgM,C	17
6	66	nd	s	d	slow	nt	0.71	24	IgM,C	15
7	54	9.1/53	s	d	slow	dexa +	nd	24	IgM,C	13
8	62	0.8/35	sm	d	slow	nt	nd	21	IgM,C	9
9	69	3/41	s	d	slow	-	1.32	12	-	8
10	64	no	sm	d	slow	+	nd	24	-	7
11	45	no	s	d	slow	+	0.85	36	IgM	7
12	70	no	s	d	slow	+	nd	20	IgM,C	6
13	52	3.8/41	s	d	slow	+	nd	30	IgM	5
14	52	4.1/40	s	d	slow	+	nd	30	IgM	5
IgM	*MAG -*									
15	73	no	s	d	progr	-	0.92	21	-	26
16	61	no	sm	d+p	slow	+	nd	5	-	24
17	69	nd	m	d+p	progr	+	0.55	8	nd	20
18	67	6/44	s	d	slow	+	0.55	60	-	12
IgG										
19	71	no	s	d	progr	+	nd	13	-	43*
20	53	10/37	m	d+p	progr	-	1.04	13	-	38*
21	63	1.3/28	m	d	progr	pred +	1.99	11	-	37*
22	61	nd	s	d+p	progr	-	1.46	8	-	35*
23	77	nd	m	d+p	progr	IVIg -	0.90	7	-	32*
24	49	no	m	d	progr	-	nd	14	-	26
25	44	no	sm	d	slow	IVIg -	nd	23	-	16

IgM/IgG = polyneuropathy associated with IgM/IgG monoclonal gammopathy; pat = patient; age = age at the time of the sural nerve biopsy; y = years; SNAP = sensory nerve action potential amplitude of the sural nerve (mV), CV = conduction velocity of the sural nerve (m/s), no = not obtainable, nd = not done; m = weakness, s = sensory loss; d = only distally affected, d+ p = distally and proximally affected; progr = progression over a period of months, slow = progression over a period of years; treatment response = response to treatment with endoxan/prednisone (see text) or as otherwise stated, + = good response, - = no response (see text), pred = prednisone only, dexa = dexamethasone, nt = no treatment; mo = months; deposition = IgM and complement factor (C) deposition on myelin sheaths, - = no deposition; * = increased T cells/mm^2

Characteristics of patients with increased T cell densities

A comparison of patients with and without increased T cell densities is shown in table 2. In all 6 patients with densities > 27/mm^2, deterioration occurred over months ($p<0.0001$). An increased T cell density was also associated with an IgG monoclonal gammopathy ($p<0.01$), predominantly motor ($p<0.05$) and proximal symptoms ($p<0.05$). Three (patients 20, 21, 22) of the six patients with increased T cells had a hematologic malignancy compared with two (patients 16, 25) of the 19 patients with T cell densities in the normal range ($p<0.05$) (table 1). No significant difference was found in the localization of T cells in the sural nerve. No significant difference was found in electrophysiologic features between patients with and without increased T cell density. All patients had electrophysiologic features compatible with demyelination, including increased F-wave latencies, decreased conduction velocity, conduction block, increased temporal dispersion or increased distal motor latencies. Myelinated nerve fiber densities were decreased in all biopsies, but no relationship with subgroups could be established.

Table 2A Characteristics of patients with and without increased T cell densities

	T cell density		
	increased	**normal**	**p- value**
	n=6 (%)	n=19 (%)	
Disease course			
Progressive	6 (100)	2 (11)	< 0.0001
Slowly progressive	0 (0)	17 (89)	
Symptoms			
Sensory	2 (33)	10 (53)	< 0.05
Sensorimotor	1 (17)	8 (42)	
Motor	3 (50)	1 (5)	
Distal	2 (33)	17 (89)	< 0.05
Proximal	4 (67)	2 (11)	
Monoclonal gammopathy			
IgG	5 (83)	2 (11)	< 0.01
IgM	1 (17)	17 (89)	
MGUS	3 (50)	17 (89)	< 0.05
Malignancy	3 (50)	2 (11)	
Treatment response			
-	2 (40)	4 (25)	n.s.
+	3 (60)	12 (75)	
CSF protein			
> 1 g/l	3 (60)	3 (33)	n.s.
< 1 g/l	2 (40)	6 (67)	
F-wave latencies			
Increased	5 (83)	16 (94)	n.s.
Normal	1 (17)	1 (6)	

increased = T cells/ mm^2 > 27, Normal = T cells/mm^2 < or = 27/mm^2; progressive = progression over months, slowly progressive = progression over years; sensory = predominantly sensory, sensorimotor = motor and sensory, ,motor = predominantly motor signs; distal = only distal affected, proximal = distal and proximal affected ; MGUS = monoclonal gammopathy of unknown significance, malignancy = hematologic malignancy; treatment response = response to treatment with endoxan/prednison (see text) or as otherwise stated, + = good response, - = no response; CSF protein = protein content of cerebro spinal fluid; n.s. = non significant

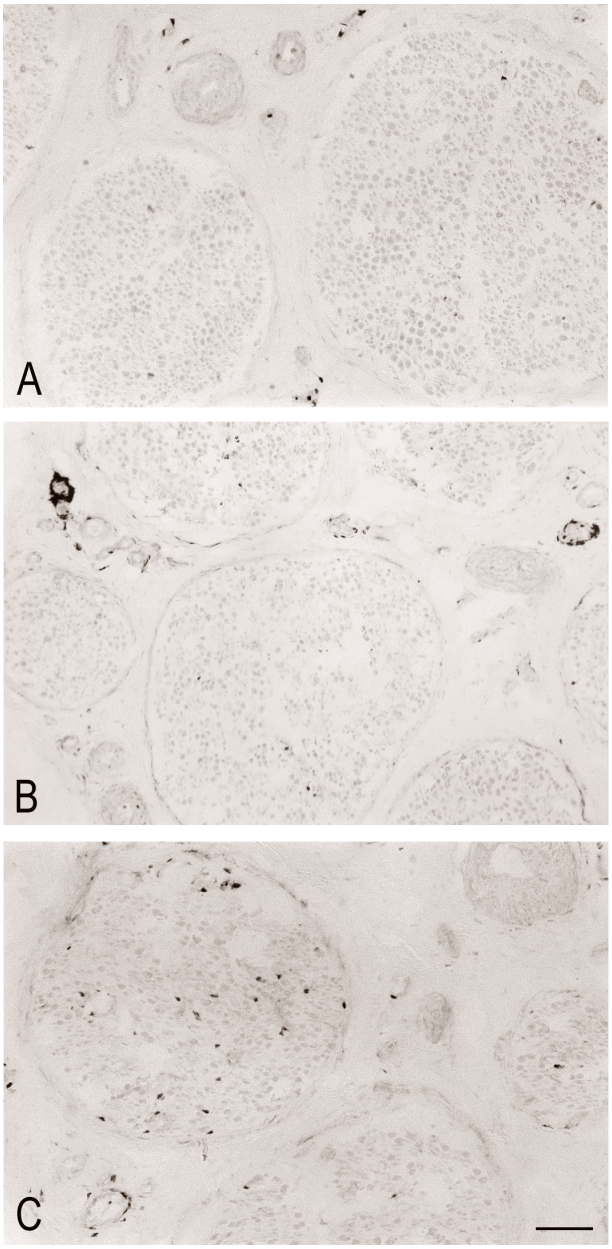

Figure 1A. CD3 staining of transverse sections of the sural nerve from a patient with (A) demyelinating polyneuropathy associated with IgM monoclonal gammopathy with anti-MAG antibodies with perivascular T cells (15/mm^2), (B) demyelinating polyneuropathy associated with IgG monoclonal gammopathy with perivascular T cells (35/mm^2), (C) demyelinating polyneuropathy associated with IgG monoclonal gammopathy with endoneurial T cells (37/mm^2) (bar=100um).

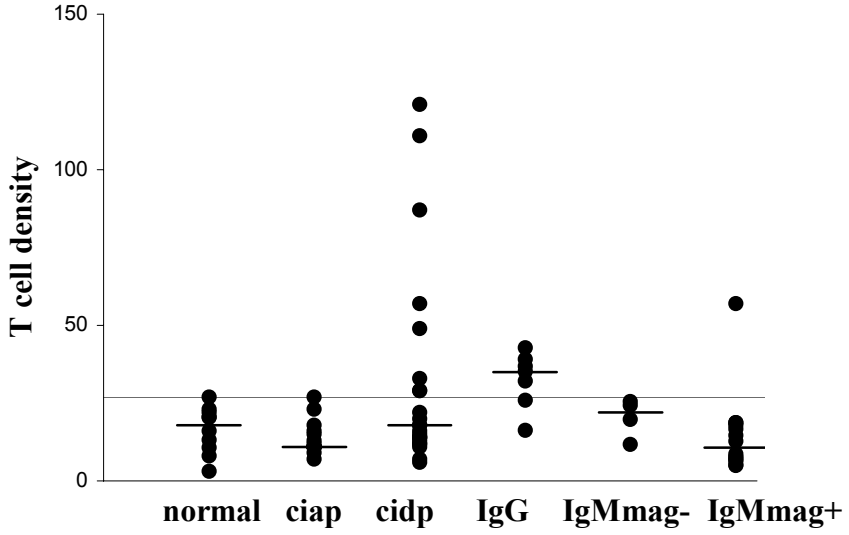

Figure 2A. T cell numbers/mm^2 in the sural nerve. normal = autopsy controls; ciap = chronic idiopathic axonal polyneuropathy; cidp = chronic inflammatory demyelinating polyneuropathy; IgG = polyneuropathy associated with IgG monoclonal gammopathy; IgMMAG+ = polyneuropathy associated with IgM monoclonal gammopathy with anti-MAG antibodies and IgMMAG- = polyneuropathy associated with IgM monoclonal gammopathy without anti-MAG antibodies. The short horizontal line represents median. Long horizontal line represents the upper limit for normal controls.

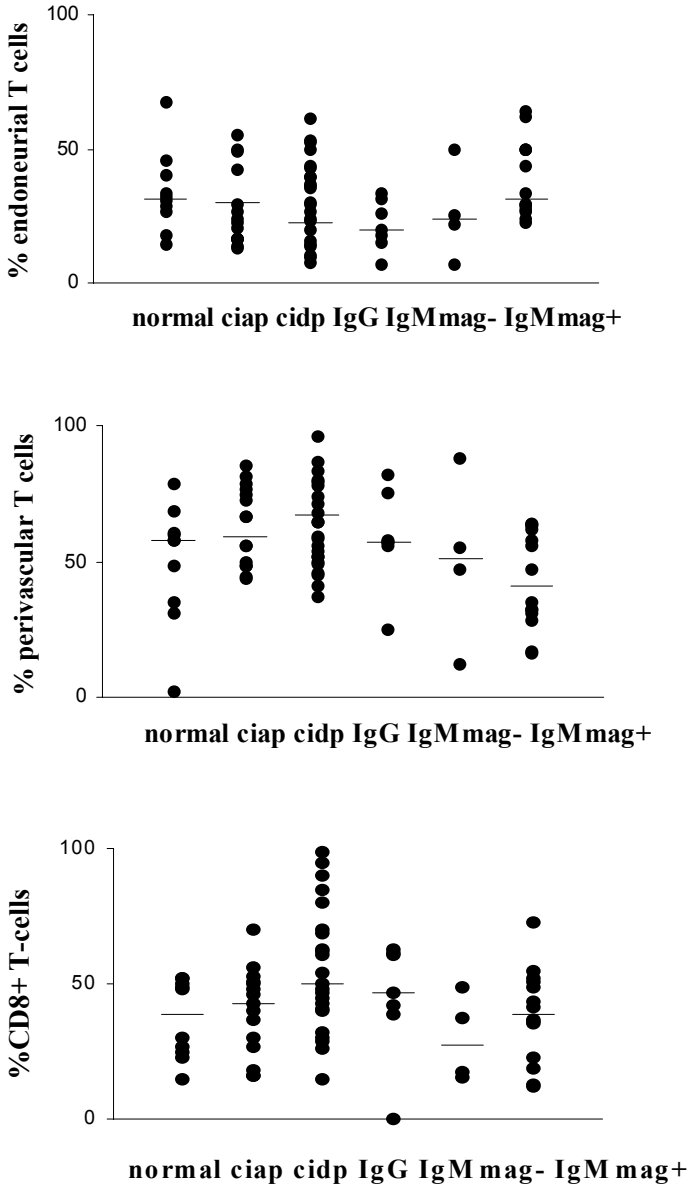

Figure 3A. Percentage of T cells in the endoneurium, perivascular and percentage of CD8+ T cells in the total sural nerve. The short horizontal line represents median.

B) axonal polyneuropathy associated with monoclonal gammopathy

Sural nerve T cells

T cells were found in all sural nerves of patients and controls (figure 1Ba and 2B). Occasionally, a CD20+ B cell was present in large perivascular T cell infiltrates. Macrophages were found in all biopsies in the endoneurium and epineurium. T cell densities ranged between 7 and 27/mm^2 (median 11) in patients with CIAP. All patients with CIAP had slowly progressive sensory axonal polyneuropathy. T cell densities ranged between 3 and 27/mm^2 (median 18) in normal controls. The sural nerves of seven patients with polyneuropathy and monoclonal gammopathy had increased T cells. Of the sural nerve biopsies with increased T cells, one biopsy had evidence of previous necrosis of the vessel wall with intimal proliferation and perivascular infiltration (Patient 1). One biopsy fulfilled the diagnostic criteria of necrotizing vasculitis (Patient 2). Patients 3, 13 and 14 had perivascular infiltration suggestive of vasculitis (figure 1B), of whom patient 3 had Sjögren's disease. Patients 4 and 15 had no signs of vasculitis. T cells were localized mostly perivascularly in the endoneurium and epineurium in all biopsies. The percentage of epineurial and perivascular T cells was higher in biopsies with increased T cells ($p<0.05$, $p>0.05$ when patients with definite vasculitis or hematologic disease were excluded, figure 3B). In all sural nerve biopsies, CD8$^+$ T cells were present in the endoneurium and epineurium (figure 3B).

T cell densities ranged between 4 and 183/mm^2 (median 11, table 1) in patients with axonal polyneuropathy and IgM monoclonal gammopathy. Four patients with a progressive disease course had increased T cell densities (two with symmetric and two with asymmetric features, three with pain). Two patients had IgM deposition in the sural nerve biopsy (patients 8 and 9), one of these (9) and three other patients had anti-sulfatide antibodies (patients 4, 7, 12). T cell densities ranged between 1 and 75/mm^2 (median 17, table 1) in patients with axonal polyneuropathy and IgG monoclonal gammopathy. Three patients with a progressive disease course had increased T cell densities (two with asymmetric and one with symmetric features with pain (table 1). All four patients with axonal polyneuropathy and increased T cells who were treated with prednisone responded ($p<0.05$). Of the five patients with axonal polyneuropathy and normal T cell densities who were treated with cyclophosphamide and prednison, only one patient with non-Hodgkin's lymphoma responded (patient 12). Four other patients had hematologic malignancies (patient 1 and 8 non-Hodgkin's lymphoma, patient 11 Waldenström's macroglobulinemia, and patient 21 multiple myeloma).

Table 1B Clinical characteristics and T cell densities in axonal polyneuropathy

pat	age	clinical features	disease course	pattern clin	pattern NCS	treatment response	till Biopsy (mo)	density	total T cells /mm²
IgM									
1	65	sm pain d	progr	S	S	nt	24	1610	183*
2	17	sm pain d	progr	As		p+	3	5864	130*
3	63	sm pain d	progr	As		p+	60	23	75*
4	76	s d	progr	S	S	nt	76	3533	46*
5	61	s pain d	slow	As	S	cp-	12	3583	21
6	43	sm pain d	slow	As	S	nt	36	3584	13
7	69	pain d	slow	S		nt	48	3178	9
8	65	sm d+p	slow	As	S	cp-	24	3306	8
9	64	sm d	slow	S		nt	18	2879	7
10	67	sm d	progr	As		nt	5	3389	7
11	76	s d	slow	S		nt	24	2686	5
12	66	sm pain d	slow	S	S	cp+	20	2454	4
IgG									
13	65	s pain d	progr	S	S	p+	19	3636	75*
14	73	sm d	progr	As	S	nt	15	3774	53*
15	39	sm pain d	progr	As	S	p+	1	3162	38*
16	45	pain d	slow	S		nt	5	2414	26
17	57	sm d	progr	S		cp-	3	3080	18
18	73	pain d	slow	As		nt	12	2736	17
19	67	sm d	slow	S		nt	25	2453	17
20	66	sm d	slow	S	S	nt	36	3463	14
21	68	sm d	slow	S		cp-	12	2577	11
22	71	s pain d	slow	S		nt	30	3438	6
23	72	pain d	slow	S	S	nt	10	2946	1
CIAP									
26-40		s d	slow	S	S	nt			< 27

IgM/IgG = polyneuropathy associated with IgM/IgG monoclonal gammopathy; CIAP = chronic idiopathic axonal polyneuropathy; pat = patient; age = age at the time of the sural nerve biopsy; m = weakness, s = sensory loss; pain = painfull paresthesia, progr = progression over a period of months, slow = progression over a period of years; clin = clinical pattern of neuropathy, NCS = nerve conduction studies, S = symmetrical sensory and motor neuropathy, As = asymmetrical sensory and motor neuropathy; treatment response + = improvement of symptoms, - = no response (see text), p = prednisone only, cp = cyclophosphamide and prednisone, nt = no treatment; antibodies = anti-MAG, anti-sulfatide, anti-ganglioside (AGM1, GM1, GM2, GD1a, GD1b, GQ1b); mo = months; density = myelinated nerve fibers/mm^2 (normal 6000/mm^2); total T cells/mm^2 = total number of T cells/mm^2, *=increased T cells/mm^2; Patient 1 had NHL and signs of recovered vasculitis, Patient 2 had signs of definite vasculitis in the sural nerve biopsy, Patient 3 had Sjogren's disease

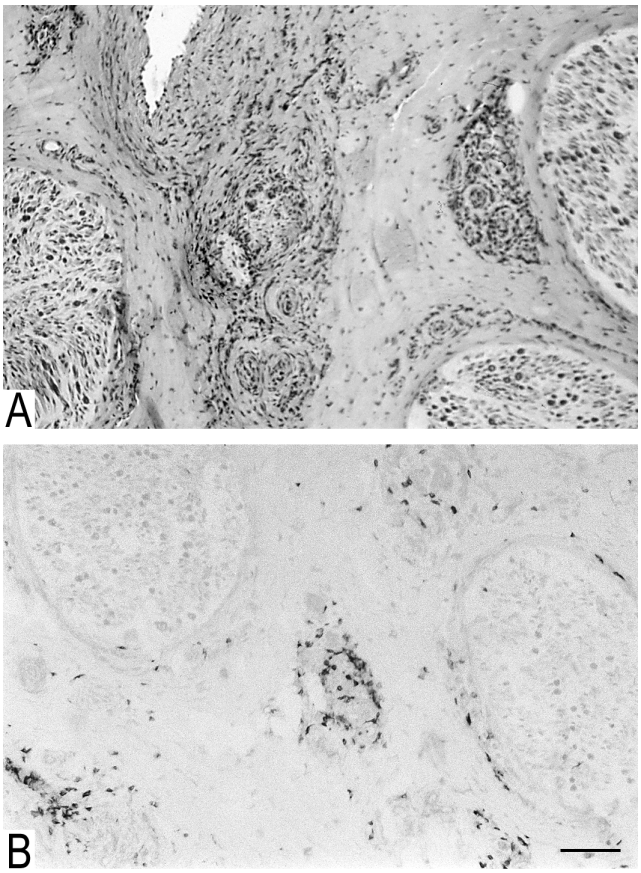

Figure 1B. Hematoxylin eosin staining (A) of transverse section of the sural nerve from a patient with axonal polyneuropathy associated with IgG monoclonal gammopathy showing perivascular infiltration around small epineurial arterioles and CD3 staining (B) showing perivascular T cells (76/mm^2), bar=100um.

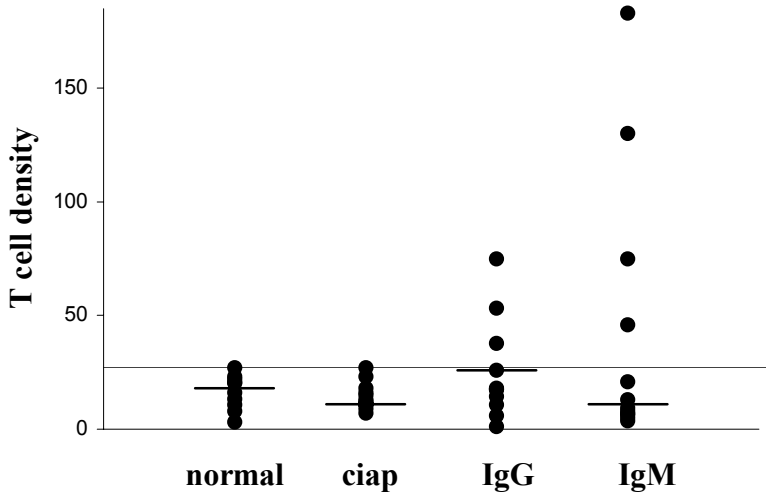

Figure 2B. T cell numbers/mm^2 in the sural nerve. normal = autopsy controls; ciap = chronic idiopathic axonal polyneuropathy; IgG = axonal polyneuropathy and IgG monoclonal gammopathy; IgM = axonal polyneuropathy and IgM monoclonal gammopathy. The short horizontal line represents median. Long horizontal line represents the upper limit for CIAP patients and normal controls.

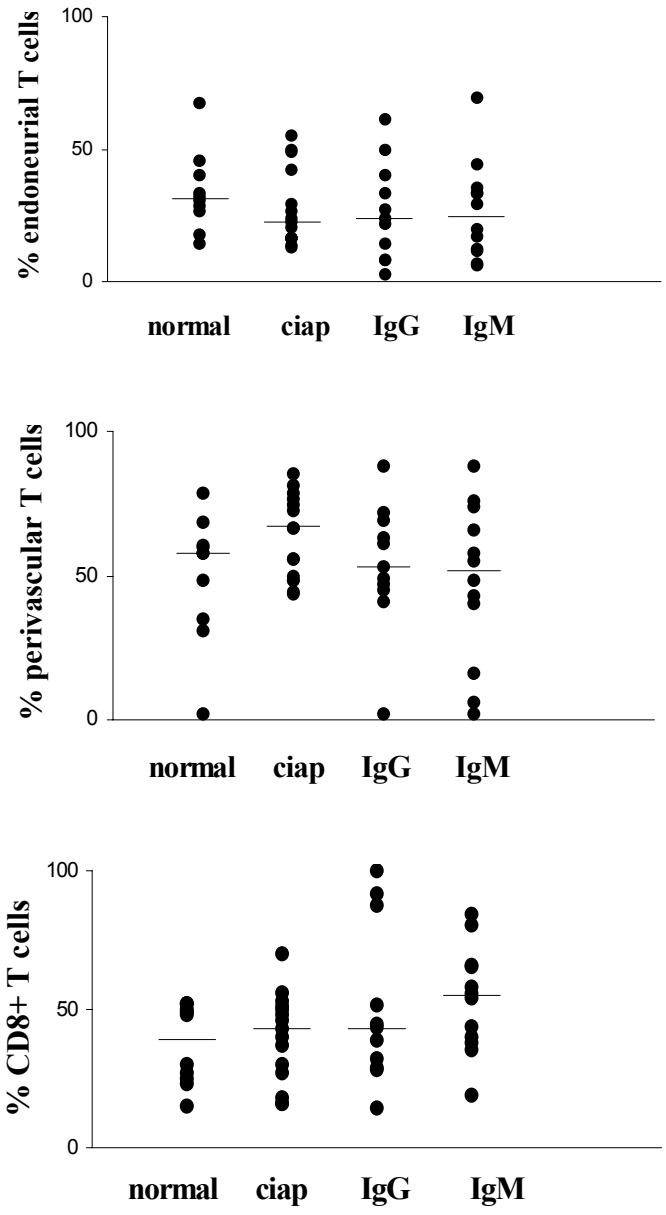

Figure 3B. Percentage of T cells in the endoneurium, perivascular and percentage of CD8+ T cells in the total sural nerve. The short horizontal line represents median.

Discussion

In the present study, the number and distribution of T cells in sural nerve biopsy specimens of patients with a demyelinating polyneuropathy associated with a monoclonal gammopathy were compared with those from CIDP, CIAP and normal controls using immunohistochemical techniques. Six of the 25 patients (24%) with a monoclonal gammopathy had a higher T cell density than normal and CIAP controls. Increased T cell density was associated with a more progressive disease course, predominantly motor and proximal symptoms, a monoclonal gammopathy of the IgG isotype and a hematologic malignancy. There was no specific distribution of T cells concerning endoneurial, epineurial or perivascular localization in the sural nerves of any of the subgroups of patients.

The association of motor and proximal symptoms with increased T cell density in a distal sensory nerve is probably the result of a generalized higher degree of inflammation in the peripheral nervous system in patients with these symptoms, but we cannot exclude the presence of focal T cell infiltration in between the sections in the patients without increased T cell density.

The role of T cells in anti-MAG polyneuropathy remains controversial. Substantial evidence for a pathogenic role of autoantibodies are provided by pathologic studies of sural nerve biopsies of patients with neuropathy and anti-MAG IgM monoclonal gammopathy showing demyelination associated with IgM deposits and complement on the affected myelin sheaths.[17,18,21,22] Passive transfer of patients' serum into cat nerve induces demyelination, and systemic administration of anti-MAG antibodies in chicken results in neuropathy and demyelination with characteristic separation of the myelin lamellae at the minor dense line, similar to that seen in human disease.[3,5,17-23] Furthermore, low titers of IgM anti-MAG antibodies are found in normal individuals, and lymphocytes from new-born umbilical cord blood can be induced to secrete antibodies of this specificity by Epstein-Barr virus.[73] These B cells belong to the CD5+ subpopulation, which has been implicated in the secretion of IgM antibodies with autoreactivity and in the development of IgM monoclonal gammopathies and chronic lymphocytic leukemia in both humans and mice. These B cells normally become anergic in early development, but in patients with monoclonal gammopathy they may be transformed spontaneously, by chemical mutation or viral infection, or as a consequence of chronic antigenic stimulation.[73] On the other hand, sequencing of some monoclonal anti-MAG antibodies from patients with polyneuropathy, has indicated that they are somatically mutated.[74,75] Furthermore, B cells producing anti-MAG antibodies were shown to be stimulated by activated T cells in vitro[59,73] and an association with HLA class II also suggests a role of T cells.[76] These findings suggest that the anti-MAG antibodies may be

produced by antigen-driven, T cell-dependent B cells. The results from our study, however, do not support a direct role for T cells in the demyelinating pathology of anti-MAG polyneuropathy as the T cell density of all but one patient with anti-MAG antibodies was in the same range as normal and CIAP controls, and IgM deposits sometimes associated with complement were found in 11 patients. One patient with an IgM monoclonal gammopathy had an increased T cell density. This patient had highly elevated anti-MAG antibody titers and direct immunofluorescence studies showed deposits of IgM in the sural nerve. The clinical presentation of the neuropathy, however, was compatible with CIDP: a predominantly motor, distal and proximal neuropathy, which developed over months. Patients with anti-MAG antibodies usually have a predominantly sensory, distal neuropathy which is slowly progressive over years, as was seen in patients 2-14 with anti-MAG antibodies in our study. Valldeoriola *et al*. described a similar patient with a predominantly motor polyneuropathy compatible with CIDP, who developed anti-MAG antibodies.[77] Monoclonal gammopathies with anti-MAG activity have also been reported to develop in Charcot-Marie-Tooth disease, which indicates that anti-MAG antibodies may be superimposed on other types of neuropathy, where they may contribute to the neurologic dysfunction.[78]

In a previous study by Solders *et al.* T cell infiltration was found in 13 of 36 patients (36%) with polyneuropathy associated with monoclonal gammopathy: in 7 of 19 IgM (including 2 out 7 with anti-MAG antibodies) and 6 of 16 IgG monoclonal gammopathies.[79] In this study, however, the number of T cells was not compared with normal or non-inflammatory controls. The present study and our previous study on sural nerve T cells in CIDP, shows that it is important to compare the number of T cells with controls as T cells were also present in all normal nerves and non-inflammatory controls.[58] We previously found that CIDP patients with an increased number of T cells had a significantly higher maximal Rankin scale score and CSF protein level as well as a tendency toward a faster disease course and worse outcome, indicating that an increased number of sural nerve T cells is associated with a more severe and active disease course.[58] Similar results were found in the present study, as patients with polyneuropathy associated with monoclonal gammopathy and increased sural nerve T cells had a more progressive disease course and more pronounced weakness. It is not known whether these T cells have a pathogenic role in the nerve or are attracted non-specifically to the nerve in more severe peripheral nerve damage.

Previous studies have found differences in the clinical presentation, antibody activity and response to treatment between patients with an IgM or IgG/IgA monoclonal gammopathy.[6,16,25,33,80,81] We found a significant difference in sural nerve T cells between patients

with an IgM and IgG monoclonal gammopathy, which provides further evidence that neuropathies associated with IgM monoclonal gammopathy are different than IgG monoclonal gammopathy. Comparing the clinical features between patients with 'idiopathic CIDP' and 'CIDP associated with monoclonal gammopathy of undetermined significance' showed that patients with an 'idiopathic CIDP' had a more progressive disease course and more pronounced weakness.[49,50,52] A more progressive disease course and prominent weakness were significantly associated with increased T cells in our study. It is possible that patients with increased T cells in our study have CIDP and that the monoclonal gammopathy occurs non-specifically, as reported in other chronic inflammatory or infectious diseases, or coincidentally as approximately 1% of healthy adults have a monoclonal gammopathy.[53,82]

Increased T cells were associated with a subset of patients with monoclonal gammopathy who had clinical features compatible with CIDP: a more progressive disease course and more pronounced weakness. Increased sural nerve T cells were found significantly more often in patients with a monoclonal gammopathy of the IgG isotype, which was frequently associated with a hematologic malignancy.

In this study we identified two groups of axonal polyneuropathy and monoclonal gammopathy: (1) axonal polyneuropathy with increased T cell densities in the sural nerve biopsies, all of which were progressive and (2) axonal polyneuropathy without increased T cell densities in the sural nerve biopsies, most of which were slowly progressive.

In patients with a progressive painful axonal polyneuropathy (deterioration of the neuropathy leading to decline of motor and sensory sumscores of more than one point and/or disability on the Rankin disability score over months) vasculitic polyneuropathy is the most likely explanation for the elevated T cell density although necrosis of the vessel wall was missing as diagnostic criterion in five of these seven patients.[10] The vasculitic signs in these sural nerve biopsies resembled the findings in cryoglobulinemic vasculitic neuropathy,[65] that is, perivascular inflammation, intimal proliferation and focal or multifocal fiber degeneration, but cryoglobulinemia was excluded in all patients.

In axonal polyneuropathy and monoclonal gammopathy without increased T cell densities an antibody-mediated pathogenesis is possible as two patients had IgM deposition in the sural nerve biopsy and one of these patients had anti-sulfatide antibodies.[62,63] In these patients with axonal polyneuropathy and a slowly progressive course, a sural nerve biopsy is non-contributive and should not be performed.

Although only nine patiens with axonal polyneuropathy associated with monoclonal gammopathy were treated with various

treatment strategies, patients with increased T cell densities seem to respond to treatment with prednisone. In patients with progressive axonal polyneuropathy and monoclonal gammopathy, a sural nerve biopsy is useful to identify a possible vasculitic neuropathy or increased T cell density as treatment response and prognosis may differ.

Chapter 3

Antibody reactivity in polyneuropathy associated with monoclonal gammopathy

adapted from

'*Eurelings M, Moons KG, Notermans NC, Sasker LD, De Jager AE, Wintzen AR, Wokke JH, Van den Berg LH. Neuropathy and IgM M-proteins: prognostic value of antibodies to MAG, SGPG, and sulfatide. Neurology 2001:56(2);228-33'*
and
'Eurelings M, Ang CW, Notermans NC, Van Doorn PA, Jacobs BC, Van den Berg LH. Anti-ganglioside antibodies in polyneuropathy associated with monoclonal gammopathy. Neurology 2001:57(10);1909-12'

Anti-MAG, anti-SGPG and anti-sulfatide in polyneuropathy associated with IgM monoclonal gammopathy

Summary

In polyneuropathy associated with IgM monoclonal gammopathy, antibodies to myelin-associated glycoprotein (MAG), sulfolucuronyl paragloboside (SGPG) and sulfatide have been associated with specific clinical and electrophysiologic features. However, it is not known whether the results of antibody tests provide additional information for the individual patient (and the neurologist) of future neurologic deficit or outcome. Therefore we studied the contribution of potential prognostic factors to the prediction of outcome of neuropathy associated with IgM monoclonal gammopathy. In accordance with the chronology in which prognostic factors are available in clinical practice, the association between prognostic factors and outcome was evaluated by univariate and multivariate logistic regression analysis in 65 patients with polyneuropathy and IgM monoclonal gammopathy. In univariate analysis, initial symptoms, IgM light chain type, electrophysiologic and pathologic studies, sural nerve IgM deposition, and anti-MAG or anti-SGPG antibodies were significantly associated with outcome. However, in multivariate analysis only initial symptoms and electrophysiologic studies are independent prognostic factors: initial sensory symptoms of the feet is prognostic for a slowly progressive disease course and less disability at four years, and demyelination with electrophysiologic study is prognostic for development of weakness and symptoms of upper extremities at four years. Addition of anti-MAG or anti-SGPG antibody tests did not yield any additional prediction of outcome. These results indicate that in clinical practice antibody tests in polyneuropathy associated with IgM monoclonal gammopathy do not have a prognostic value in terms of future neurologic deficit or outcome.

Introduction

Antibodies to the myelin-associated-glycoprotein (MAG) are detected in 50 to 60% of the patients with neuropathy and IgM monoclonal gammopathy. Most patients have a slowly progressive, sensory or sensorimotor, demyelinating polyneuropathy.[6,17,21,22,28,48,83,84] A causal relation between anti-MAG antibodies and the neuropathy is supported, because pathologic studies of sural nerve biopsies of patients with neuropathy and anti-MAG IgM monoclonal gammopathy show demyelination associated with IgM deposits on the affected myelin sheaths.[17,18,21,22,48] Passive transfer of patients' serum into cat nerve induces demyelination, and systemic administration of anti-MAG antibodies in chicken causes neuropathy and demyelination with characteristic separation of the myelin lamellae at the minor dense line, similar to that seen in human disease.[3,5,19,20,23] Anti-MAG antibodies cross-react with several other components of peripheral nerve myelin, including the glycolipids sulfoglucuronyl paragloboside (SGPG), sulfoglucuronyl lactosaminyl paragloboside (SGLPG) and sulfatide.[85-89] Anti-SGPG antibodies have also been described in patients with axonal neuropathy or motor neuron disease,[90,91] while anti-sulfatide antibodies are also associated with predominantly sensory polyneuropathy.[6,63,66,71,92]

Although previous studies have found an association between elevated anti-MAG, anti-SGPG or anti-sulfatide antibodies and specific clinical, electrophysiologic or pathologic characteristics,[6,29,33,71,81] it is not known whether the results of antibody tests provide additional information for the individual patient (and the neurologist) in terms of future neurologic deficit or outcome. In the present study, we determined the prognostic value of anti-MAG, anti-SGPG and anti-sulfatide antibody tests in patients with polyneuropathy associated with IgM monoclonal gammopathy.

Patients and methods

Patients

The study population comprised 65 patients with polyneuropathy associated with IgM monoclonal gammopathy, diagnosed by neuromuscular specialists at the Departments of Neurology of the University Hospitals Utrecht, Groningen and Leiden, The Netherlands, between 1988 and 1997. At the first visit of each patient to the hospital a clinical and laboratory work-up was done to exclude other causes of neuropathy. Medical history was obtained and physical examination and routine laboratory analysis, immunoelectrophoresis, immunofixation and electrophysiologic studies were carried out in all patients.[68] If a monoclonal gammopathy was found, hematologic investigations, including bone marrow analysis, to identify the type of monoclonal gammopathy and antibody tests followed. Furthermore, in

36 patients CSF examination, and in 42 patients a sural nerve biopsy was done.

Outcome variables
The following outcomes were investigated: (1) disease course which was distinguished as either 'progressive' (= deterioration over weeks or months) or 'slowly progressive' (= deterioration over more than one year); development within four years after onset of the first symptoms of (2) weakness, (3) sensory or motor symptoms or signs in the arms, (4) disability as determined by the modified Rankin disability scale after four years [93] (0= asymptomatic; 1= non-disabling symptoms not interfering with lifestyle; 2= minor disability leading to some restriction of lifestyle but not interfering with patients' capacity to look after themselves; 3= moderate disability with symptoms significantly interfering with lifestyle or preventing totally independent existence; 4= moderately severe disability with symptoms preventing independent existence, although patients do not need constant attention day and night; 5= severely disabled, totally dependent, requiring constant attention day and night).

Potential prognostic variables
Variables which could act as predictors of prognosis of the neuropathy were identified. These variables were obtained by reviewing the charts of the 65 patients with polyneuropathy associated with IgM monoclonal gammopathy. The potential prognostic variables were subdivided in two categories according to the chronology in clinical practice: I. variables which became available at or after the first visit to the neurologist, i.e. age at onset, sex, initial symptoms, IgM M-protein light chain specificity and electrophysiologic features; and II. variables which became available at subsequent visits, i.e. type of monoclonal gammopathy (monoclonal gammopathy of undetermined significance (MGUS), cryoglobulinemia or hematologic malignancy), CSF protein concentration, sural nerve pathologic features, and anti-MAG/SGPG/sulfatide tests results. Electrophysiologic studies included nerve conduction and concentric needle examination using standardized techniques, and identified a predominantly axonal or demyelinating neuropathy according to conventional criteria, as previously described.[14,34,70,71,94] Sural nerve biopsies were evaluated by light and direct immunofluorescence microscopy for the degree of demyelination, axonal degeneration, and deposits of IgM.

Antibody studies
Anti-MAG antibodies were measured using the Western blot system, as previously described.[28] Central nervous system myelin proteins obtained from human brain at autopsy were separated by

polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) and transferred to nitro-cellulose blots. Patients' serum antibody binding to the MAG band was tested at the initial dilution of 1:500 and titrated by serial two-fold dilution until negative, using peroxidase-conjugated rabbit immunoglobulin to human IgM. The antibody titer is given as the highest serum dilution at which antibody binding to the MAG band is detected.[95,96]

Antibody binding to SGPG in peripheral nerve myelin was determined by high-performance thin-layer chromatography (HPTLC). Bovine peripheral nervous system glycolipids were prepared from cauda equina by extraction with tetrahydofuran.[97] The glycolipid fraction (2 □g) was separated by HPTLC on aluminium-backed silica gel 60 HPTLC plates (Merck, Germany).[62] Patients' serum reactivity and the antibody titer were determined by immunostaining as described above for anti-MAG antibodies starting with a serum dilution of 1:100.

Anti-sulfatide antibodies were measured by enzyme-linked immunosorbent assay (ELISA), as previously described.[66] Briefly, wells of ELISA plates were coated with 200 ng of sulfatide (Sigma) in methanol, or bovine serum albumin (BSA) as control, saturated with BSA, and incubated with patients' serum beginning with a dilution of 1:200, followed by peroxidase-conjugated rabbit anti-human IgM. Reaction products were visualized with O-phenylene-diamine as substrate and read spectrophotometrically at 492 nm in a multiscan reader (Bio-Rad). The titer is given as the highest serum dilution at which the optical density in the antigen-coated wells was greater than 0.05 units above the control BSA-coated microwells. Serum IgM antibody titers were considered elevated when higher than the highest titer of 304 control patients with other neurologic or immunologic diseases and 50 normal controls as previously described;[66] i.e. 2000 for anti-MAG antibodies, 500 for anti-SGPG antibodies and 6400 for anti-sulfatide antibodies.

Statistical analysis

Associations between potential prognostic variables, including anti-MAG/SGPG/sulfatide antibody tests, and each outcome parameter were determined with univariate logistic regression analysis. Odds ratios with 95% confidential intervals were calculated. Variables that were associated ($p < 0.1$) were evaluated by multivariate logistic regression analysis to determine their independent contribution to the prediction of outcome. Models were constructed in accordance with the chronology in which variables became available in clinical practice. We first included univariate significant variables obtained from the first visit to the neurologist (category I: first symptoms, electrophysiologic findings and IgM light chain). Secondly, the model was extended with variables obtained from the following visits (category II: type of

monoclonal gammopathy, CSF protein concentration, pathologic features and anti-MAG/SGPG/sulfatide titers), to evaluate their added value in prediction of outcome. Reliability (goodness of fit) was quantified using the Hosmer&Lemeshow test.[98]

Results

The clinical, laboratory, electrophysiologic and pathologic features for all patients are summarized in table 1. Eighteen patients had a hematologic malignancy, i.e. non-Hodgkin's lymphoma (7), macroglobulinemia (9), multiple myeloma (1) and chronic leukemic lymphoma (1). One patient had a cryoglobulinemia. The CSF protein concentration was elevated (>0.50 g/l) in 27 out of 36 patients. Evidence for demyelination in a sural nerve biopsy was found in 34 out of 42 patients. Deposition of IgM was found in 19 out of 33 patients. Twenty-eight patients had ataxia. Twenty-four of the 36 patients who were treated had a favorable response to treatment (improvement of 1 or more points on the Rankin scale).[14] Treatment included different immunosuppressive therapies: cyclophosphamide and prednisone, plasmapheresis and intravenous immunoglobulin. Only patients with a progressive disease course received immunomodulating treatment within four years after onset of the polyneuropathy. In those patients the worst outcome was measured before treatment. Consequently, treatment did not interfere with the outcome variables. The duration of follow-up from the first symptoms ranged from four to 23 years (mean 6.7 years, SD 3.7). Elevated anti-MAG, anti-SGPG or anti-sulfatide antibody titers were found in 45 (69%) of the 65 patients (figure). Thirty-six patients developed a slowly progressive, sensorimotor, demyelinating polyneuropathy (11 MAG/SGPG/sulfatide+, 17 MAG/SGPG+, 2 SGPG/sulfatide+, 1 SGPG+, 5 no antibodies). Seven patients developed a slowly progressive, sensory, demyelinating polyneuropathy (2 MAG/SGPG/sulfatide+, 1 MAG/SGPG+, 1 SGPG/sulfatide+, 3 no antibodies). Two patients developed a slowly progressive, motor, demyelinating polyneuropathy (1 SGPG/sulfatide+, 1 no antibodies). Nine patients developed a slowly progressive, sensory, axonal polyneuropathy (3 sulfatide+, 6 no antibodies). Five patients developed a slowly progressive, sensorimotor, axonal polyneuropathy (3 SGPG/sulfatide+, 2 no antibodies). Six patients developed a progressive, predominantly motor, demyelinating polyneuropathy (1 SGPG+, 2 sulfatide+, 3 no antibodies).

In the univariate analysis of potential prognostic factors, a slowly progressive disease course was associated with sensory symptoms of feet as initial symptoms, kappa light chain of the IgM monoclonal gammopathy, deposition of IgM in the sural nerve biopsy as well as elevated antibodies to MAG or SGPG (table 2). Development of weakness within four years was associated with evidence for

demyelination on electrophysiologic and pathologic examination, sural nerve IgM deposition, as well as elevated antibodies to MAG or SGPG. Development of hand symptoms within four years was associated with evidence for demyelination on electrophysiologic examination. A less severe disability at four years (modified Rankin disability scale $\leq$ 2) was associated with sensory symptoms of feet as initial symptoms. Furthermore, ataxia was associated with a modified Rankin disability scale > 2 ($p<0.01$), demyelinating electrophysiologic features ($p<0.05$) and elevated anti-MAG antibodies ($p<0.05$). Tremor was associated with malignant monoclonal gammopathy; four of the six patients with tremor had an underlying hematologic malignancy (66%), compared to 14 of the 59 patients without tremor (24%) ($p<0.05$). Elevated anti-sulfatide antibodies were not associated with any of the outcome variables.

In multivariate analysis, a slowly progressive disease course was significantly associated with initial sensory symptoms of feet (table 3). Development of weakness and development of hand symptoms within four years were significantly associated with demyelinating electrophysiologic features. A less severe disability at four years was associated with initial sensory symptoms of feet. Addition of anti-MAG or anti-SGPG antibody tests to these models did not yield any additional prediction of outcome, neither did other laboratory evaluation such as CSF examination, sural nerve pathology or IgM deposition.

Table 1 Potential prognostic variables of 65 patients with polyneuropathy associated with IgM monoclonal gammopathy

	All, n=65
I	
Age at onset (mean in yrs (range))	62 (41-90)
Sex (male)	50 (77)
Initial symptoms	
sensory symptoms feet	55 (85)
sensory symptoms hands	1 (2)
sensory symptoms feet +hands	1 (2)
weakness feet	6 (9)
sensory + weakness feet + hands	2 (3)
IgM light chain type (k)	48 (74)
EMG (demyelinating)	51 (79)
II	
Monoclonal gammopathy (malignant)	18 (28)
CSF protein (>0.5g/l)	27 (75)
Sural nerve pathology (demyelinating)	34 (81)
Deposition in sural nerve (IgM)	19 (58)
Anti-MAG +	31 (48)
Anti-SGPG +	40 (62)
Anti-sulfatide +	25 (38)

I: prognostic variables available after the first visit to the neurologist; II: prognostic variables available after subsequent visits; yrs = years; EMG = electrophysiologic features; CSF protein = increased protein content of cerebrospinal fluid (>0.5 g/l); MAG = myelin-associated glycorotein; SGPG = sulfoglucuronyl paragloboside; in number and percentage in brackets, unless stated otherwise

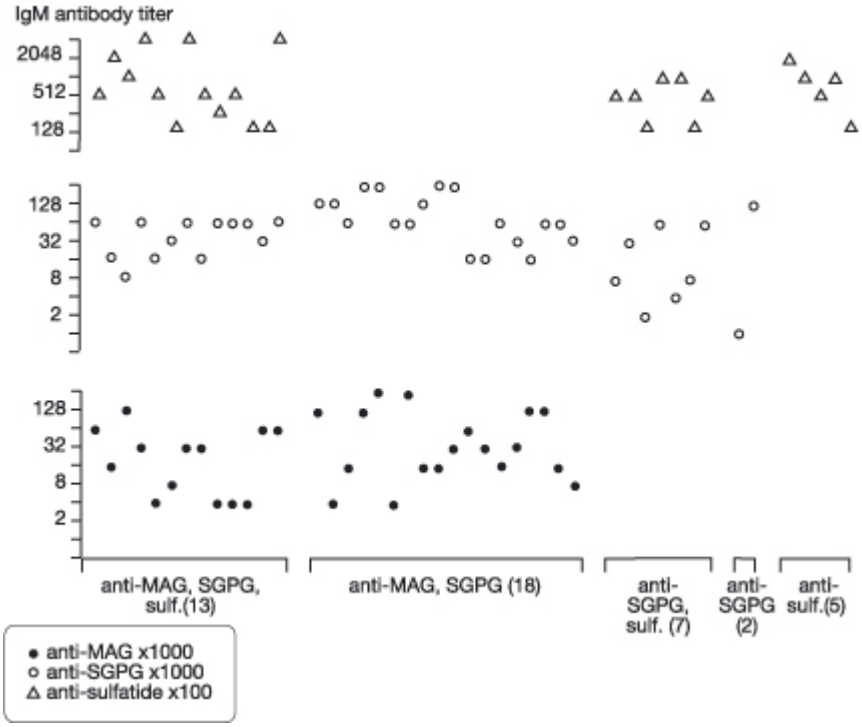

Figure. IgM anti-myelin-associated-glycoprotein (MAG), anti-sulfoglucuronyl paragloboside (SGPG) and anti-sulfatide antibody titers for each patient are presented on the vertical axis. Subgroups of patients subdivided by antibody specificities are presented on the horizontal axis. Anti-MAG, SGPG, sulf. (13*)* = 13 patients with antibodies against MAG, SGPG and sulfatide; anti-MAG, SGPG (18) = 18 patients with antibodies against MAG and SGPG; anti-SGPG, sulf. (7) = 7 patients with antibodies against SGPG and sulfatide; anti-SGPG (2) = 2 patients with antibodies against SGPG; anti-sulf. (5) = 5 patients with antibodies against sulfatide.

Table 2 Univariate analysis of the prognostic variables for each outcome

	disease course			**weakness 4y**		
	progressive n=6	slow n=59	OR (95%CI)	no n=21	yes n=44	OR (95%CI)
I						
Age at onset (mean in yrs)	68	62	n.s.	63	62	n.s.
Sex male	76	83	n.s.	75	81	n.s.
Initial symptoms sens sympt feet	0	93	165 (7.8-3510)**	95	80	n.s.
Light chain type kappa	33	78	7.1 (1.2-43.1)*	86	68	n.s.
EMG demyelinating	100	76	n.s.	48	93	15 (3.5-64.2)**
II						
Maligne gammopathy	34	27	n.s.	33	25	n.s.
CSF protein >0.5g/l	26	20	n.s.	71	76	n.s.
Pathology						
Demyelinating	100	78	n.s.	36	97	53 (5.1-545)**
IgM deposition in sural nerve	0	58	15 (0.8-317)*	22	71	8.5(1.4-51.5)**
Anti-MAG +	0	53	6.6 (1.1-58.6)*	29	57	3.3 (1.1-10.1)*
Anti-SGPG +	17	66	9.7 (1.1-89.1)*	43	70	3.2 (1.1-9.4)*
Anti-sulfatide +	33	39	n.s.	38	39	n.s.

	hand symptoms 4y			disability 4y		
	no	yes	OR	2	>2	OR
	n=42	n=23	(95%CI)	n=36	n=29	(95%CI)
I						
Age at onset (mean in yrs)	62	64	n.s.	60	66	n.s.
Sex male	83	65	n.s.	83	70	n.s.
Initial symptoms sens sympt feet	90	74	n.s.	94	72	0.2 (0-0.8)*
Light chain type kappa	79	65	n.s.	75	72	n.s.
EMG demyelinating	69	96	9.9 (1.2-81.2)*	69	90	n.s.
II						
Malignant gammopathy	31	22	n.s.	25	31	n.s.
CSF protein >0.5g/l	61	89	n.s.	71	79	n.s.
Pathology						
Demyelinating	71	94	n.s.	76	86	n.s.
IgM deposition in sural nerve	50	65	n.s.	64	52	n.s.
Anti-MAG +	43	56	n.s.	50	45	n.s.
Anti-SGPG +	57	70	n.s.	64	59	n.s.
Anti-sulfatide +	40	35	n.s.	36	41	n.s.

slow = slowly progressive disease course, deteriorating over > 1 year, progressive = progressive disease course, deteriorating over > 1 year; weakness 4y = development of weakness within 4 years; hand symptoms 4y = development of hand symptoms within 4 years; disability 4y = modified Rankin disability score after 4 years; I = prognostic variables available after the first visit to the neurologist; II = prognostic variables available after subsequent visits; yrs = years; sens sympt feet = sensory symptoms of the feet; EMG = electrophysiologic features; CSF protein = increased protein content of cerebrospinal fluid (>0.5g/l); * = $p < 0.05$, ** = $p < 0.001$; values are in % or OR (95% CI).

Table 3 Multivariate analysis of each outcome

	disease course	**Weakness 4y**	**hand symptoms 4y**	**disability 4y**
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
I				
Initial symptoms sens sympt feet	165 (7.8-3510)**	n.s.	n.s.	0.2 (0-0.9)*
+ EMG demyelinating	n.s.	15 (3.3-65)**	9.1 (1.1-76)*	n.s.
II				
+ Anti-MAG	n.s.	n.s.	n.s.	n.s.
+ Anti-SGPG	n.s.	n.s.	n.s.	n.s.

disease course = slowly progressive disease course, deteriorating over > 1 year; weakness 4y = development of weakness within 4 years; hand symptoms 4y = development of hand symptoms within 4 years; disability 4y = modified Rankin disability score >2 after four years; I = prognostic variables available after the first visit to the neurologist; II = prognostic variables available after subsequent visits; sens sympt feet = initial sensory symptoms of the feet; EMG = electrophysiologic features; MAG = myelin-associated glycorotein; SGPG = sulfoglucuronyl paragloboside; * = $p < 0.05$, *** = $p < 0.001$; n.s. = not significant.

Discussion

In the current study we analyzed whether the results of anti-MAG, anti-SGPG and anti-sulfatide antibody tests may have implications for future neurologic deficit or outcome of neuropathy associated with IgM monoclonal gammopathy. The prognostic value of elevated antibody titers was studied in a model, which simulated clinical practice. Multivariate analysis of the potential prognostic factors that were significantly associated with outcome in the univariate analysis, showed that initial sensory symptoms of feet and evidence for demyelination on electrophysiologic examination were independent prognostic factors for neuropathy associated with IgM monoclonal gammopathy. Sensory symptoms of the feet as the initial symptom of the neuropathy is prognostic for a slowly progressive disease course and less disability, and evidence for demyelination on electrophysiologic examination for development of weakness and symptoms of upper extremities at four years. Addition of anti-MAG or anti-SGPG antibody tests to these models did not yield any additional prediction of outcome. These findings imply that anti-MAG, anti-SGPG and anti-sulfatide tests are of limited value for the patient as it does not change management or prognosis of polyneuropathy associated with IgM monoclonal gammopathy.

We chose to use the outcome variables at disease duration of four years, which is the longest follow-up period for all 65 patients. Analysis of the outcome variables at a later stage of the disease shows similar results but concerns fewer patients. Although disease progression is slow in most patients with polyneuropathy associated with IgM monoclonal gammopathy, many patients, with or without antibodies, eventually develop severe disability, which is associated significantly with the presence of ataxia in our study. Immunosuppressive treatment is frequently given to patients with severe disability, usually in a later stage of the disease. Patients in our study were treated with various (combinations of) immunologic therapies, which makes a comparison of the response to treatment in polyneuropathy associated with IgM monoclonal gammopathy with and without antibodies difficult. However, our results and the results of previous studies do not show a difference in response to treatment between patients with and without antibodies.[2,14,31,99-101] From these studies it seems unlikely that antibody tests can identify a subgroup of patients that respond better to immunologic treatment, although this needs to be confirmed in a large prospective therapeutic trial.

In the current study we have chosen to detect anti-MAG or anti-SGPG antibodies by Western blot or TLC as these methods minimize binding to impurities in the antigen preparations, which may have major effects on the results of ELISA assays and the clinical correlation.[102] Similar to previous reports, anti-SGPG antibodies were

less specific for a slowly progressive, predominantly sensory, demyelinating polyneuropathy, than anti-MAG antibodies: three patients had axonal neuropathy and two patients had pure motor neuropathy.[28,29,91] This confirms the idea that anti-SGPG antibodies have affinity for epitopes in both myelin and axon of peripheral nerves and may cause nerve damage by different pathogenic mechanisms, leading to a different clinical presentation.[103]

Antibody tests may be useful for defining subgroups of patients for pathogenic studies or therapeutic trials of polyneuropathy associated with monoclonal gammopathy. However, in clinical practice elevated anti-MAG, anti-SGPG or anti-sulfatide antibody titers do not change management or prognosis in terms of future neurologic deficit or outcome of neuropathy associated with IgM monoclonal gammopathy.

Anti-ganglioside antibodies in polyneuropathy associated with monoclonal gammopathy

Anti-ganglioside antibodies in polyneuropathy associated with monoclonal gammopathy

Summary

Antibody reactivity to GA1, GM1, GM2, GD1a, GD1b and GQ1b gangliosides was measured in 87 patients with polyneuropathy associated with monoclonal gammopathy (60 IgM, 25 IgG, 2 IgA) and 42 control patients with monoclonal gammopathy without polyneuropathy (21 IgM, 21 IgG). Of these 87 patients 30 % had anti-MAG antibodies and 15 % had anti-ganglioside antibodies. Anti-ganglioside antibodies were significantly associated with demyelinating neuropathy and with IgM monoclonal gammopathy. Anti-GD1b and anti-GQ1b antibodies were significantly associated with predominantly sensory atactic neuropathy.

Introduction

Polyclonal antibodies to various gangliosides have been associated with specific types of polyneuropathy, such as anti-GM1 antibodies in multifocal motor neuropathy and Guillain Barré syndrome (GBS), and anti-GQ1b antibodies in Miller-Fisher syndrome (MFS). Anti-ganglioside antibodies have also been described in patients with polyneuropathy associated with monoclonal gammopathy,[17,22] but owing to the small number of patients in each study, an association of anti-ganglioside antibodies with a specific type of polyneuropathy is not clear (table 1). Such an association may be of diagnostic value and may support a role for anti-ganglioside antibodies in the pathogenesis of polyneuropathy associated with monoclonal gammopathy.

In this study we measured antibody reactivity to several gangliosides in 87 clinically well characterized patients with polyneuropathy associated with monoclonal gammopathy and compared this with that in patients with monoclonal gammopathy without neuropathy.

Table 1 Monoclonal anti-ganglioside antibodies in polyneuropathy

study	age	neuropathy	EMG		MAG	AGM1	GM1	GD1b	GQ1b	GD1a	GM2
Bollensen 1989	70	m	slow	D	IgM k	n.d.	-	-	-	n.d.	+	-
Oga 1998	75	m	relaps	D	IgM k	-	-	-	-	-	+	+
Nobile 1994	n.a.	m	slow	D	IgM l	-	-	-	+	-	-	-
Ellie 1997	n.a.	m	progr	D	IgM	+	-	+	+	-	-	-
Ilyas 1988b	60	m	slow	D	IgM l	**-**	+	+	+	n.d.	+	-
Carpo 1996	66	m	progr	D	IgM l	n.d.	+	+	+	n.d.	+	-
Carpo 1996	63	m	progr	D	IgM l	n.d.	-	+	+	n.d.	+	+
Kusunoki 1989	53	m	progr	D	IgM k	n.d.	-	+	+	-	-	+
Sadiq 1990	71	m	progr	D	IgM k	**-**	+	+	+	n.d.	n.d.	+
Sadiq 1990	51	m	slow	D	IgM l	**-**	+	+	-	n.d.	n.d.	+
Sadiq 1990	75	sm	progr	nd	IgM l	**-**	+	+	+	n.d.	n.d.	+
Sadiq 1990	77	sm	slow	D	IgM l	**+**	+	+	+	n.d.	n.d.	+
Sadiq 1990	72	sm*	slow	n.a.	IgM l	**-**	+	+	+	n.d.	n.d.	+
Younes 1992	50	sm	slow	A	IgM k	n.d.	-	-	+	n.d.	+	n.d.
Dalakas 1996	42	sm	n.a.	D	IgM	-	n.d.	n.d.	+	n.d.	n.d.	n.d.
Ilyas 1988b	66	sm	progr	D	IgM k	**+**	-	+	-	n.d.	-	+
Ilyas 1988a	n.a.	sm	n.a.	n.a.	IgM	n.d.	n.d.	+	n.d.	n.d.	+	+
Ilyas 1988a	n.a.	sm	n.a.	n.a.	IgM	n.d.	n.d.	+	n.d.	n.d.	+	+
Carpo 1998	55	sm	progr	D	IgM	-	-	+	+	+	-	-
Carpo 1998	74	sm a*	progr	D	IgM k	-	-	-	+	+	-	+
Hitoshi	79	s a	slow	A	IgM	n.d.	+	+	+	-	-	-

study	age	neuropathy		EMG		MAG	AGM1	GM1	GD1b	GQ1b	GD1a	GM2
Ilyas 1985	40	s a	slow	D	IgM k	-	n.d.	-	+	n.d.	-	n.d.
Arai 1992	53	s a	progr	D	IgM k	-	n.d.	-	+	n.d.	+	-
Daune 1992	67	s a	slow	A	IgM l	n.d.	n.d.	-	+	+	-	+
Yuki 1992	44	s a	relaps	A	IgM k	-	n.d.	-	+	+	-	-
Willison 1993#	55	s a *	slow	D	IgM l	n.d.	-	-	+	+	+	-
Herron 1994#	51	s a *	slow	D	IgM	n.d.	-	-	+	+	+	+
Oka 1996	n.a.	s a	n.a.	A	IgM	n.d.	n.d.	n.d.	+	+	+	n.d.
Tagawa 1997	n.a.	s a	n.a.	D	IgM	n.d.	n.d.	-	+	+	n.d.	n.d.
Tagawa 1997	n.a.	s a	n.a.	D	IgM	n.d.	n.d.	-	+	+	n.d.	n.d.
Tagawa 1997	n.a.	s a	n.a.	D	IgM	n.d.	n.d.	-	+	+	n.d.	n.d.
Ponsford 2000	47	s a	relaps	A	IgM l	-	-	-	+	+	-	-
Ponsford 2000	54	s a	relaps	D	IgM l	-	-	-	+	+	-	-
Brindel 1994	60	s*	relaps	D	IgM l	n.d.	-	-	+	+	+	-
Obi 1992	77	s	slow	A	IgM k	n.d.	-	-	+	+	+	-
Ilyas 1988b	71	s	slow	D	IgM k	+	-	+	-	n.d.	-	+

age = age at onset of the polyneuropathy in years; neuropathy = important clinical symptoms and disease course, m = predominantly motor signs s = predominantly sensory signs, sm = motor and sensory signs, a = ataxia, * = cranial nerve paresis and ophthalmoplegia, progr = progression over months, slow = progression over years, relaps = relapsing remitting; EMG = electrophysiologic analysis, D = demyelinating, A = axonal; M-protein = monoclonal protein; MAG = myelin-associated antibodies; sulf = anti-sulfatide antibodies; n.a. = not available; + = present, - = absent, n.d. = not done; Bollensen, 1989;[104] Oga, 1998;[105] Nobile-Orazio, 1994;[6] Ellie, 1997;[106] Ilyas, 1988b; Carpo, 1996;[107] Kusunoki, 1989;[108] Sadiq, 1990;[109] Younes, 1992; Farrer, 1996;[110] Ilyas, 1988a; [111] Carpo, 1998;[112] Hitoshi, 1994;[113] Ilyas, 1985;[114] Arai, 1992;[115] Daune, 1992;[116] Yuki, 1992;[117] Willison, 1993;[118] Herron, 1994;[119] # = described by Jacobs et al., 1997 as 2 patients with the CANOMAD syndrome,with IgM monoclonal antibodies to GQ1b, GD1b, GD1a, GM2 with a disialosyl group and NeuAc(a2-8)NeuAc(a2-3)Gal epitope; Oka, 1996;[120] Tagawa, 1997;[121] Ponsford, 2000; [122] Brindel, 1994;[123] Obi, 1992;[124]

Patients and methods

Patients

We studied 87 patients with polyneuropathy associated with monoclonal gammopathy. A diagnostic clinical and laboratory work-up was performed to exclude other causes for neuropathy.[68] Clinical, laboratory and electrophysiologic findings for all patients are summarized in table 2. The monoclonal gammopathy was IgM in 60 patients, IgG in 25 patients, IgA in two patients. Most patients had monoclonal gammopathy of unknown significance (77%), 12 patients had non-Hodgkin's lymphoma (20%) and two patients had Waldenström's macroglubulinemia (3%). Disease course was categorized as either 'progressive' (= deterioration over weeks or months) or 'slowly progressive' (= deterioration over more than one year). Sensory ataxia was defined by disturbance of gait or limb movements, which intensified when the eyes were closed. Electrophysiologic studies consisting of nerve conduction and concentric needle examination using standardized techniques identified a predominantly axonal or demyelinating neuropathy according to conventional criteria.[14] As controls, we used sera of 42 patients with monoclonal gammopathy (21 IgG, 21 IgM) without signs or symptoms of neuropathy. These serum samples were obtained from patients visiting the hematologic outpatient department**.**

Antibody studies

Anti-myelin-associated glycoprotein (MAG) antibodies were measured using Western blot, as previously described.[28] Patients' serum binding to MAG was tested at an initial dilution of 1:1000. The antibody titer is given as the highest serum dilution at which antibody binding to the MAG band is detected. Antibody reactivity against asialo-GM1 (GA1), GM1, GM2, GD1a, GD1b and GQ1b was measured by enzyme-linked immunosorbent assay (ELISA), as previously described.[66,125] Cut off values were determined by taking the mean plus three standard deviations of a population of 50 healthy control subjects. Antibody titers were considered elevated when >100. Antibody reactivity was confirmed with thin-layer chromatography as described before.[126] Antibody reactivity of the monoclonal gammopathy was further evaluated using kappa- or lambda chain specific peroxidase conjugated antibodies (DAKO, Glostrup, Denmark).

Statistical analysis

Differences in clinical and laboratory features between patients with and without anti-ganglioside antibodies, and between patients with and without polyneuropathy were analyzed with the Fisher's exact test (for categorical variables) and the Mann Whitney-U test (for continuous variables); $p < 0.05$ was considered significant.

Results

Thirteen of the 87 patients (15%) had elevated titers of antibodies to the gangliosides GA1, GM1, GM2, GD1a, GD1b or GQ1b (figure). Anti-MAG antibodies were elevated in 26 patients (30%). Two patients had anti-ganglioside and anti-MAG antibodies. Of the 60 patients with an IgM monoclonal gammopathy 60 % had anti-MAG or anti-ganglioside antibodies. Features that were found significantly more frequently in patients with neuropathy and anti-ganglioside antibodies were a monoclonal gammopathy of the IgM isotype, symptoms of both legs and arms and evidence of demyelination on electrophysiologic examination (table 2).

The clinical and laboratory features of he patients with anti-ganlioside antibodies are presented in table 3. Only one of these patients had an IgG monoclonal gammopathy. Six of the seven patients with anti-GQ1b antibodies and four of the five patients with anti-GD1b antibodies had a predominantly sensory ataxic neuropathy ($p<0.05$).

Patients with polyneuropathy associated with a monoclonal gammopathy and anti-ganglioside antibodies have been previously described in the literature (table 1). In accordance with the findings from our own study, most of these patients had a demyelinating polyneuropathy associated with an IgM monoclonal gammopathy.

Table 2 Clinical features of 87 patients with polyneuropathy associated with monoclonal gammopathy

	All	**IgM**	**Anti-MAG+**	**Anti-gang+**
	87 (100%)	60 (69%)	26 (30%)	13 (15%)
Age at onset (y mean range)	61 (35-90)	62 (42-90)	60 (44-73)	62 (44-76)
Sex (male)	64 (74)	46 (77)	19 (73)	12 (92)
Symptoms				
motor	12 (14)	7 (12)	1 (4)	4 (31)
sensorimotor	28 (32)	21 (35)	12 (46)	2 (15)
sensory	47 (54)	32 (53)	13 (50)	7 (54)
Ataxia	34 (39)	26 (43)	14 (54)	7 (54)
Location of symptoms				
distal only	73 (84)	51 (85)	24 (92)	9 (69)
distal and proximal	14 (16)	9 (15)	2 (8)	4 (31)
legs only	72 (83)	48 (80)	21 (81)	8 (61)
legs and arms	15 (17)	12 (20)	5 (19)	5 (39)*
Disease course				
slowly progressive	60 (69)	48 (80)**	24 (92)**	6 (46)
progressive	27 (31)	12 (20)	2 (8)	7 (54)
Treatment response	35 (66)	25 (74)	6 (43)	8 (80)
EMG				
Demyelinating	54 (62)	45 (75)***	24 (92)***	12 (92)*
Axonal	33 (38)	15 (25)	2 (8)	1 (8)
IgM M-protein	60 (69%)		26 (100)***	12 (92)*
IgG/A M-protein	27 (31%)		0 (0)	1 (8)
MGUS	64 (74)	46 (77)	23 (89)	11 (85)
Malignancy	23 (26)	14 (23)	3 (11)	2 (15)

y = years; treatment response = improvement or stabilization one year after treatment (53 patients treated); EMG = electrophysiologic features; MGUS = monoclonal gammopathy of unknown significance; malignancy = hematologic malignancy; Anti-MAG+ = anti-myelin-associated glycoprotein antibodies; anti-gang = anti-ganglioside antibodies; * = significant difference between patients with IgM and IgG, with and without anti-MAG or with and without anti-ganglioside antibodies with $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; values are number and % unless stated otherwise

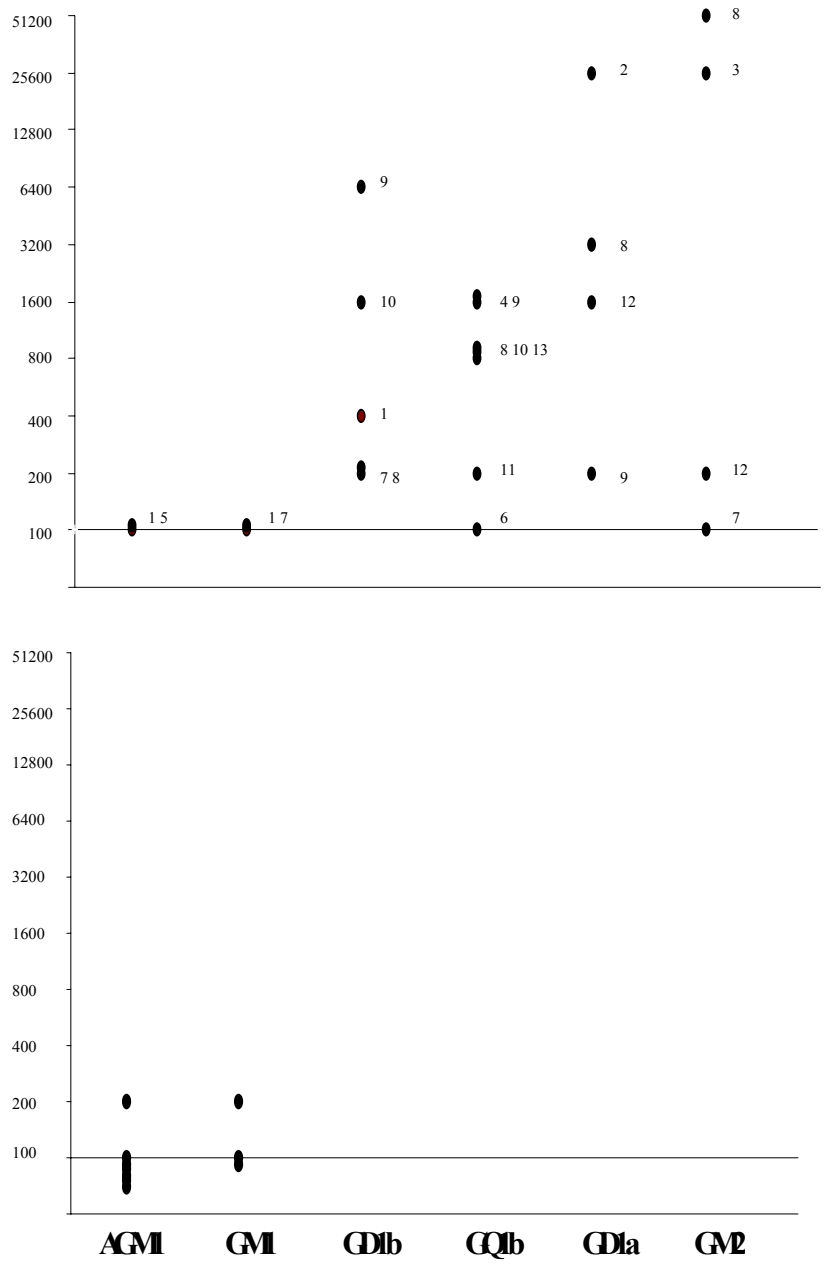

Figure. Anti-ganglioside antibody titers of patients with polyneuropathy associated with monoclonal gammopathy and control patients with monoclonal gammopathy without neuropathy. The numbers represent patients with anti-ganglioside antibodies shown in table 3. Eight control patients with monoclonal gammopathy without neuropathy had anti-ganglioside reactivity.

Table 3 Characteristics of patients with anti-ganglioside antibodies

age	neuropathy		treatment	EMG	M-prot	MAG	AGM1	GM1	GD1b	GQ1b	GD1a	GM2
61	m	slow	IvIg +	D	IgMl	-	100	100	400	-	-	-
45	m	progr	dexa +	D	IgMk	-	-	-	-	-	25600	-
69	m	progr	IvIg -, c/p +	D	IgMl	-	-	-	-	-	-	25600
76	m	progr	c/p +	D	IgMk	-	-	-	-	1600	-	-
69	sm	slow	n.t.	D	IgMk	-	100	-	-	-	-	-
53	sm a	slow	n.t.	D	IgMk	pos	-	-	-	100	-	-
73	s a	slow	c/p +	D	IgMk	pos	-	100	200	-	-	100
67	s a	slow	c/p +	D	IgMl	-	-	-	200	800	3200	51200
50	s a*	progr	IvIg -, c/p +	D	IgMk	-	-	-	6400	1600	200	
44	s a	progr	IvIg -, c/p -	D	IgMl	-	-	-	1600	800	-	-
73	s a	progr	c/p -	D	IgMl	-	-	-	-	200	-	-
57	s	slow	n.t.	D	IgMk	-	-	-	-	-	1600	200
57	s a	slow	plasma +	A	IgGk	-	-	-	-	800	-	-

age = age at onset of the polyneuropathy; yrs = years; neuropathy = important clinical symptoms and disease course, m = predominantly motor signs, s = predominantly sensory signs, sm = motor and sensory signs, a = ataxia,* = ophthalmoplegia, progr = progression over months, slow = progression over years; treatment = response to treatment with c/p (cyclophosphamide and prednisone), dexa (dexamethasone), IvIg (intravenous immunoglobulin) or plasmaph (plasmapheresis), + = positive response (stabilization or improvement following treatment), - = no response; EMG = electrophysiologic analysis, D = demyelinating, A = axonal; M-prot = monoclonal protein; pos = present, - = absent

Discussion

In the current study, we found elevated anti-ganglioside antibody titers in 15% of patients with polyneuropathy associated with monoclonal gammopathy. Anti-ganglioside reactivity was significantly associated with a demyelinating polyneuropathy and an IgM monoclonal gammopathy. These findings support a role for IgM anti-ganglioside antibodies in the pathogenesis of demyelinating polyneuropathy associated with monoclonal gammopathy.

We found that the presence of anti-GQ1b or anti-GD1b antibodies was associated with ataxic polyneuropathy. In three patients, the antibodies to GQ1b cross-reacted with GD1b, which indicates binding to disialosyl groups shared by GQ1b and GD1b. The association of anti-dialosyl antibodies, chronic ataxic polyneuropathy, ophthalmoplegia and cold agglutinins has been described as the CANOMAD syndrome (chronic ataxic neuropathy, ophthalmoplegia, M-protein, agglutination and anti-disialosyl antibodies).[127] In our study, one patient with IgM anti-GQ1b antibodies and a sensory ataxic neuropathy had ophthalmoplegia. High titers of polyclonal anti-GQ1b antibodies of the IgG isotype have been reported in 80 to 100% of patients with Miller Fisher syndrome (MFS) as well as in some patients with GBS and ophthalmoplegia.[112] Also, one patient with polyneuropathy associated with IgG monoclonal gammopathy had anti-GQ1b antibodies. This is the first reported patient with monoclonal IgG anti-GQ1b antibodies and a chronic ataxic polyneuropathy.

Polyclonal anti-GM1 antibodies have been found in 30-80% of patients with multifocal motor neuropathy and pure motor GBS. In patients with polyneuropathy associated with monoclonal gammopathy, this association of anti-GM1 antibodies with motor neuropathy was not observed. In the literature, seven patients with a motor neuropathy but also seven patients with a sensorimotor neuropathy and two patients with a sensory neuropathy were reported to have anti-GM1 reactivity (table 1). In our study, which included 12 patients with predominantly motor neuropathy, anti-GM1 antibodies were found in only one patient with a motor neuropathy and also in one patient with sensory neuropathy.

The highest titer of anti-GD1a antibodies was found in one of our patients with motor neuropathy. The presence of monoclonal anti-GD1a antibodies has been associated with motor neuropathy in previous studies (table 1). In addition, high-titer polyclonal GD1a antibodies have been reported in GBS with predominantly motor symptoms.[128] However, we found low anti-GD1a antibody titers in three patients with sensory polyneuropathy. Two of these patients also had anti-GM2 antibodies. Antibodies to the GD1a and GM2 gangliosides that share a GalNac(b1-4)Gal-NeuAc(a2-3)b1 epitope have also been described in two patients with a sensorimotor polyneuropathy.[129]

Larger and experimental studies may establish whether fine specificities or titers of antibodies to gangliosides determine the variety in the clinical presentation of the polyneuropathy in patients with monoclonal gammopathy.

Chapter 4

Risk factors for hematologic malignancy in polyneuropathy associated with monoclonal gammopathy

adapted from

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and
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Risk factors for hematologic malignancy in polyneuropathy associated with monoclonal gammopathy

Summary
Polyneuropathy associated with monoclonal gammopathy of undetermined significance (MGUS) is a well-known disease entity. Of the patients with monoclonal gammopathy without neuropathy 25 % develop a hematologic malignancy during long-term follow-up. Whether the frequency of hematologic malignancy is similar in patients with polyneuropathy associated with monoclonal gammopathy and whether hematologic screening is necessary in these patients is unknown. To determine the frequency of and risk factors for a hematologic malignancy, we investigated 104 patients with polyneuropathy and monoclonal gammopathy. Potential diagnostic variables were obtained from medical history, physical and neurologic examination, and laboratory analysis. The associations between potential diagnostic variables and outcome, hematologic malignancy, were evaluated by univariate and multivariate logistic regression analysis. Among our patients, 23 had a hematologic malignancy (eight patients multiple myeloma, ten patients low-grade lymphoma, three patients plasmacytoma, one patient Castleman's disease and one patient POEMS syndrome). Weight loss, progression of the neuropathy and an M-protein level > 1 g/l were independent risk factors for malignancy. Extensive screening is indicated in patients with these features.

Introduction

The relationship between polyneuropathy and monoclonal gammopathy is well established by epidemiologic and pathologic studies and the identification of IgM anti-myelin-associated-glycoprotein (anti-MAG) antibodies.[17,21,22,48,130] The neuropathy has an onset over 50 years of age and most patients are men.[21] The polyneuropathy is characterized by both sensory and motor signs.[25] The majority of patients with polyneuropathy associated with monoclonal gammopathy of unknown significance (MGUS) have an IgM monoclonal protein (M-protein).[16,33] In contrast, an IgG M-protein is most frequently found in patients with monoclonal gammopathy of undetermined significance (MGUS) without neuropathy.[131]

The presence of an M-protein in serum, urine, or both, without an underlying hematologic malignancy characterizes MGUS. Criteria for MGUS include a serum M-protein level less than 30 g/L; a bone marrow plasma cell infiltration of less than 10%; absence of lytic bone lesions, anemia or other laboratory or clinical abnormalities; and stability of the M-protein level.[132] After a median follow-up of 22 years approximately 25% of patients with MGUS develop a hematologic malignancy, mostly multiple myeloma and non-Hodgkin's lymphoma.[131] These malignancies have a great variation in presenting symptoms such as fatigue, back pain, weight loss and infections.[35,133] However, in 10-30% of patients the diagnosis is made during routine investigations.[134]

The frequency of a hematologic malignancy in polyneuropathy associated with monoclonal gammopathy is unknown. Because patients with polyneuropathy associated with monoclonal gammopathy often have a low M-protein level (< 1 g/L) and a rise in M-protein level indicating malignant transformation is usually not found,[25] it is questionable whether a full hematologic screening, including bone marrow examination and skeletal x-ray, should be routinely performed.[132]

In this study we recorded clinical and laboratory features in all patients with polyneuropathy associated with monoclonal gammopathy who were referred to our clinic from January 1987 to September 1999 and analyzed which features were associated with hematologic malignancy.

Patients and methods

Patients

From January 1987 to September 1999, 104 patients with polyneuropathy associated with monoclonal gammopathy were identified at the Departments of Neuromuscular Diseases of the University Medical Center Utrecht. They were part of a patient population of 1100 patients who were screened for causes of neuropathy. All patients were directly referred by general practitioners

or neurologists to our department. The initial work-up (clinical and laboratory) included medical history, physical examination, routine laboratory analysis, electrophysiologic studies, and a survey for a monoclonal protein (M-protein) in serum and/or urine (immunofixation of serum and 10 x concentrated urine). Other causes for neuropathy were excluded by standardized examination.[68]In patients who had autonomic features or in whom the polyneuropathy was progressive or painful, a sural nerve biopsy was performed (67 patients). In all sural nerve biopsies, Congo red staining was performed to exclude amyloidosis. In all patients with IgM monoclonal gammopathy, anti-MAG antibodies were measured using the Western blot system.[28]

If a monoclonal gammopathy was found, screening for the existence of an underlying hematologic malignancy was performed. This included (apart from physical examination and determination of blood chemical values) skeletal x-ray, x-ray of the lungs, sonography of the abdomen (in case of an IgM M-protein), and bone marrow investigation in 81 patients. Bone marrow aspirates and biopsies were obtained with a Jamshidi needle from the crista iliaca posterior. Additional investigations included immunophenotyping with plasma cell (CD38, antibodies against heavy and light chains), B cell (CD19, CD20) and T cell (CD3, CD4, CD8) specific monoclonal antibodies. Aspirates were stained with May-Grunwald giemsa. Bone marrow biopsies were embedded in paraffin, stained with hematoxylin-eosin and viewed by a pathologist. Congo red staining was performed for the detection of amyloid deposits. Kyle's[132]definition of MGUS was used. Criteria for multiple myeloma and plasmacytoma were defined according to Kyle and Greipp [135] and multiple myeloma patients were classified according to Durie and Salmon.[136] Indolent myeloma and smoldering myeloma fulfilling the criteria of Stage I myeloma were classified as stage 1 myeloma. Non-Hodgkin's lymphoma was defined according to the REAL classification.[137] The diagnosis of lymphoma was based on bone marrow biopsy, which included immunophenotyping with B cell and T cell specific monoclonal antibodies, by authorized pathologists.

None of the patients received treatment before the diagnosis of a hematologic malignancy or MGUS, after the hematologic evaluation including bone marrow examination. Treatment during follow-up consisted of intermittent cyclophosphamide and prednisone,[14] dexamethasone,[67] and melphalan. Treatment response was evaluated using motor and sensory symptoms and a positive response was defined as improvement or stabilization of symptoms during the year following treatment.[14]

Determination of follow-up

Duration of follow-up was defined by the time between the first symptoms of the neuropathy and the diagnosis of a hematologic

malignancy or the end of the study. Because an association of the M-protein and the neuropathy is found and a pathologic relation is well recognized, especially in patients with IgM monoclonal gammopathy,[17,21,22,48,130] we assumed that the M-protein was present since the onset of the neuropathy. In addition, the mean duration between the first screening for causes of neuropathy, including determination of M-protein and the hematologic screening, to detect hematologic malignancies was calculated.

Potential diagnostic variables

Potential diagnostic variables present at initial screening that could be predictive for an underlying hematologic malignancy, were identified by reviewing the charts of the 104 patients with polyneuropathy associated with monoclonal gammopathy. These were: age; sex; fatigue; unexplained bone pain; infections; unexplained fever, night sweats, unexplained weight loss > 5 kg/6 months; coagulopathy, defined by bleeding or thrombosis; metabolic disturbances, like polydipsia, polyuria and edema; and progression of the polyneuropathy, defined by deterioration of the neuropathy leading to disability.[93] Obtained laboratory features included: M-protein isotype, light chain type; anti-myelin-associated-glycoprotein (MAG) antibodies, measured using the Western blot system;[28] M-protein level, significant rise of M-protein level (> 25% if M-protein level = 5 g/L, doubling and minimal 3 g/L if M-protein level < 5 g/L), measured at least at two different time points; anemia (hemoglobin level < 7.4 mmol/L [< 12 g/L] in woman and < 8.6 mmol/L [< 14 g/L] in man); leukocytopenia or leukocytosis (<4x10^9/L or >10x10^9/L); renal insufficiency (plasma creatinine >120 micromol/L [> 0.014 mg/L]); hypercalcemia (calcium >2.8 mmol/L [0.11 g/L]); liver function abnormalities (serum alkaline phophatase >130 U/L, gamma glutamyl transferase >70 U/L, transaminase >50 U/L, or lactate dehydrogenates >620 U/L); and the type of neuropathy, either axonal or demyelinating. Electrophysiologic studies included nerve conduction and concentric needle examination using standardized techniques, and identified a predominantly axonal or demyelinating neuropathy according to conventional criteria, as previously described.[14,34,70,71,94]

Statistical analysis

The actuarial survival probability curve, as well as the cumulative risk of a hematologic malignancy was calculated using the Kaplan-Meier method. The associations between each potential diagnostic variable and outcome, i.e. hematologic malignancy, were determined with univariate logistic regression analysis. Odds ratios (OR) with 95% confidential intervals (95 % CI) were calculated. Variables that were associated ($p < 0.1$) were evaluated by multivariate logistic-regression

analyses to determine their independent contribution to the prediction of outcome. Models were constructed in accordance with the chronology in which the variables became available in clinical practice. Accordingly, we first included univariate significant variables obtained from medical history. Second, the model was extended with laboratory features, to evaluate their added value in prediction of outcome. Reliability (goodness of fit) of the models was quantified using the Hosmer-Lemeshow test.[98]

Results

Characteristics of all patients are summarized in table 1 and 2. Bone marrow examination was performed in 81 patients (79%). A hematologic malignancy was found in the 23 patients (22%) described in table 3, with multiple myeloma (eight patients), low-grade lymphoma (ten patients), plasmacytoma (two patients), multiple localization of plasmacytoma (one patient), POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, M-protein, and skin changes, one patient) and Castleman's disease (one patient). Six patients had multiple myeloma stage I and two patients had stage III multiple myeloma.[136] All eight patients with multiple myeloma had symptomatic myeloma: two patients had bone lesions causing bone pain, one patient had recurrent infections and diffuse bone pain, and five patients had symptomatic anemia with no other cause for anemia. Sixteen patients were diagnosed after bone marrow biopsy, six patients (patients 5, 8, 14, 19, 20, 21) had bone lesions found with radiographs of the spine, five patients (patients 12, 14, 21, 22, 23) had lymph node abnormalities found with computed tomography (CT) scan of the chest and abdomen. Of ten patients with low-grade lymphoma, eight patients had IgM monoclonal gammopathy and two patients had IgG monoclonal gammopathy. In one patient (patient 14) the diagnosis lymphoma was based on lymph node biopsy. This patient had pathologic mediastinal lymph nodes and a thoracic spine lesion without abnormalities of the bone marrow biopsy. Sixteen patients (15%) were diagnosed at the initial hematologic screening following the neurologic screening and seven patients (8%) were diagnosed during follow-up. Three of these seven patients (patients 5, 6, 8) had developed multiple myeloma (two patients stage III and one patient stage I), three patients had developed low-grade lymphoma (patients 9, 10, 16), and one patient had developed plasmocytoma (patient 20).

The actuarial probability of a hematologic malignancy at 5 and 10 years after the first neurologic screening was 30% and 42%. The duration between neurologic screening and full hematologic screening ranged from weeks to 11 years (median 1 year, mean 3 years). The median time between first symptoms of the neuropathy and neurologic screening was 1 year and between first symptoms of the neuropathy

and hematologic screening was 3 years (range, weeks to 20 years). The 20-year duration of neuropathy symptoms before neurologic screening occurred in two patients with a very slowly progressive axonal polyneuropathy. One of these patients developed multiple myeloma.

Nineteen of the 23 patients with a hematologic malignancy were treated. The indication for treatment was determined by both the progression of the polyneuropathy and the features of the hematologic malignancy. Thirteen patients had a good response. Of the six patients with stage I multiple myeloma, four patients received treatment with high-dose melphalan followed by stem cell rescue. Three of these patients achieved partial remission and the polyneuropathy improved; one patient died from treatment-related complications. Two patients had refractory myeloma and the polyneuropathy improved, one patient treated with cyclophosphamide and prednisone and one patient with melphalan. Two patients with multiple myeloma stage II were treated with melphalan and achieved partial remission, and the polyneuropathy improved. Eight patients with low-grade lymphoma were treated with cyclophosphamide and prednisone, and seven achieved partial remission. In all these patients the polyneuropathy improved. One patient with plasmocytoma was treated with cyclophosphamide and prednisone with good response. One patient with Castleman's disease was treated with prednisone, and both the Castleman's disease and the polyneuropathy responded. One patient with POEMS syndrome was treated with melphalan without response. Most patients had an IgM M-protein (53%). Patients with both IgM and IgG M-proteins were included in this group (4 patients) for analysis. Patients with IgG (37%) or IgA M-protein (5%), one patient with IgG and IgA M-proteins, and one patient with IgM, IgG and IgA M-proteins were included in the IgG/IgA group. The patient with triclonal gammopathy had multiple myeloma, weight loss, fatigue, and anemia (8 mmol/L, 13 g/L) and a progressive demyelinating polyneuropathy with severe weakness and sensory loss of the legs. One patient (No.19) had monoclonal lambda light chain only. This patient had no evidence of amyloidosis in the sural nerve biopsy, bone marrow biopsy, or biopsy of the sacral spine lesion. The patient died of pneumonia 2 weeks after diagnosis of plasmocytoma. At autopsy no evidence for amyloidosis was found. In this patient (patient 19) and two other patients (patients 20, 21) with plasmocytoma POEMS syndrome was excluded with endocrinologic blood tests and sonography of the abdomen. None of the patients had an M-protein level > 30 g/L. Ten patients had mild leukocytosis ($>10x10^9$/L, $<20x10^9$/L) and two patients had leukopenia ($<4x10^9$/L). Three patients had urine monoclonal protein < 1g/L (24-hour specimen). Four patients had kidney abnormalities. Three patients had only a minor elevation of creatinine level (130 mmol/L = 0.015 mg/L),

and one patient had creatinine level of 767 mmol/l (0.09 mg/L). None of the patients had hypercalcemia.

Comparison of patients with MGUS and hematologic malignancy
The odds ratios of the significant associations of the diagnostic variables with malignancy determined with univariate analysis are presented in table 4.

In multivariate analysis, weight loss, progression of the polyneuropathy and M-protein level >1g/L were the only independent variables significantly associated with hematologic malignancy (table 5).

Survival of patients with polyneuropathy and an underlying hematologic malignancy was 6.9 years, which was significantly shorter ($p<0.001$) than that of patients with polyneuropathy associated with MGUS (9.6 years). Of the 23 patients with an underlying hematologic malignancy, two patients had biclonal gammopathy (one patient IgM/IgG, one patient IgG kappa and lambda) and one patient had triclonal gammopathy (13%) compared to nine patients with biclonal gammopathy of 81 patients without a malignancy (three patients IgM/IgG, one patient IgG/IgA, four patients IgM kappa and lambda, one patient IgG kappa and lambda, 11%). Biclonal or triclonal gammopathy was not significantly associated with an underlying hematologic malignancy.

Table 1 Characteristics of 104 patients with polyneuropathy associated with monoclonal gammopathy

Symptoms	**%**
Mean age at onset (range)	59 (35-76)
Sex (male)	71
Fatigue	34
Bone pain	14
Infections	4
Night sweats/unexplained fever	5
Weight loss	22
Coagulopathy	4
Metabolic disturbance	7
Progression of polyneuropathy	45
MGUS	78
Malignancy	22
Treatment	48
Response	76

MGUS = monoclonal gammopathy of undetermined significance; malignancy = hematologic malignancy; treatment response = improvement or stabilization of symptoms during the year following treatment

Table 2 Laboratory analysis of 104 patients with polyneuropathy associated with monoclonal gammopathy

Laboratory analysis	**%**
Monoclonal gammopathy	
IgM	53
IgG	38
IgA	3
IgM/IgG	4
IgM/IgG/IgA	1
IgG/IgA	1
Light chain only	1
Light chain type	
kappa	64
lambda	25
kappa and lambda	11
Anti-MAG antibodies	47
Monoclonal protein level	
<1 g/l	55
1-10 g/l	27
>10 g/l	18
Increasing monoclonal protein level	7
Urine monoclonal proteins	13
Anemia	21
Liver function abnormalities	19
Kidney function abnormalities	5
Demyelinating findings on EMG	59
Axonal findings on EMG	41

Anti-MAG antibodies = anti-myelin-associated- glycoprotein antibodies; EMG = electrophysiologic features; anemia = hemoglobin level <7.4 mmol/L (< 12 g/L) in women, <8.6 mmol/L (< 14 g/L) in men

Table 3 Characteristics of patients with polyneuropathy and an underlying hematologic malignancy

Pat	M-protein		Hb	Infilt	Clinical symptoms	Course	Diagnosis	Duration
	type	g/l	g/l	%				mo
1	IgGl	<1	11	30	fatigue, metabolic	progr	MM I	12
2	IgAl+	7-11	13	20	fatigue, weight	progr	MM I	1
3	IgAl	8-13	11	20	fatigue, weight	progr	MM I	6
4	IgGl	19	12	15	fatigue, weight	progr	MM I	6
5	IgGk	20	-	30	bone	progr	MM III	48
6	IgGk	18-23	13	35	fatigue, bone, inf, weight	progr	MM I	72
7	IgGk	30	11	30		progr	MM I	6
8	IgGk	14-27	13	25	fatigue, bone, inf, fever, weight	progr	MM III	48
9	IgGk	<1	15	20	fatigue, weight	slow	NHL IV	36
10	IgMl	9	13	25		slow	NHL IV	97
11	IgGk+	11	15	20		slow	NHL IV	12
12	IgMk	<1-3	14	20		progr	NHL IV	6
13	IgMk	6-8	14	25	fatigue, weight, metabolic	progr	NHL IV	11
14	IgGk/l	8	12	n	fatigue, inf, weight	progr	NHL IV	24
15	IgMk	8	15	50	fatigue	progr	NHL IV	24
16	IgMk	6-11	14	20	fatigue, fever	progr	NHL IV	60
17	IgMk	10-18	-	20	fatigue, bone, weight	progr	NHL IV	11
18	IgMk	12	14	40	fatigue, fever, weight	progr	NHL IV*	12
19	l	<1	15	n	fatigue, bone, infections, fever, weight, coag	progr	plasma	2
20	IgAl	<1	13	n	bone, weight	progr	plasma	138
21	IgGk	6-8	15	5	fatigue, weight, metabolic	progr	plasma**	20
22	IgGl	3	9	5	fatigue, weight	progr	POEMS	12
23	IgGk	<1	15	5	fatigue, bone, weight	progr	Castle***	6

+ also IgGk/IgMl, Hb = hemoglobin level; infilt = percentage plasma cell or lymphocyte infiltration in bone marrow biopsy, n = normal; metabolic = metabolic disturbances; weight = weight loss; bone = bone pain; inf = recurrent infections; fever = night sweats or unexplained fever; coag = coagulopathy; progr = progressive deterioration of the polyneuropathy in weeks or months, slow = deterioration in more than one year; MM I, III = multiple myeloma stage I, III; NHL IV = non-Hodgkin's lymhoma stage IV (all cases low grade); plasma = plasmacytoma; * = amyloidosis found at autopsy; ** multiple localizations of plasmacytoma; *** histological diagnosis; duration mo = time in months between the first screening for causes of neuropathy and hematologic screening

Table 4 Univariate analysis of the diagnostic variables for hematologic malignancy

	MGUS n=81	**malignancy** n=23	Odds Ratio (95%CI)
Symptoms			
Fatigue	22	74	9.9 (3.4-29)
Bone pain	10	30	4.0 (1.2-13)
Infections	1	13	12 (1.2-122)
Metabolic disturbance	4	17	5.5 (1.1-27)
Night sweats/unexplained fever	1	17	16.8 (1.8-159)
Weight loss	10	65	17.1 (5.5-53)
Progression of polyneuropathy	33	87	13.3 (3.6-49)
Laboratory analysis			
Monoclonal gammopathy: IgG/IgA	37	64	3.0 (1.1-7.9)
Anti-MAG antibodies	31	9	0.2 (0.1-1.0)
Monoclonal protein level > 1g/L	35	83	9.0 (2.8-29)
Increasing monoclonal protein level	4	19	6.1 (1.3-30)
Anemia	16	42	3.9 (1.3-12)

MGUS = monoclonal gammopathy of undetermined significance; malignancy = hematologic malignancy; anti-MAG antibodies = anti-myelin-associated-glycoprotein antibodies; anemia = hemoglobin level <7.4 mmol/L (<12 g/L) in women, <8.6 mmol/L (<14 g/L) in men

Table 5 Multivariate analysis of outcome

	hematologic malignancy OR (95%CI)
Symptoms	
Weight loss	28.6 (3.2-257)
Progression of the polyneuropathy	8.3 (1.8-39)
Laboratory analysis	
Monoclonal protein level >1 g/L	34.1 (4.1-294)

Discussion

In our study we found a high frequency of an underlying hematologic malignancy in polyneuropathy associated with monoclonal gammopathy. Twenty-three patients out of a series of 104 patients were found to have a hematologic disease, according to standard classification criteria,[135,137] namely, multiple myeloma, non-Hodgkin's lymphoma, plasmacytoma, Castleman's disease, and POEMS syndrome. The majority of patients had symptoms related to the underlying malignancy.

In all patients, the polyneuropathy was the presenting symptom and reason for consultation. During initial neurologic screening, no indications were found of a malignancy. In all patients the serum M-protein was below 30 g/L which is in agreement with MGUS according to the criteria of Kyle.[132] As it is recommended that extensive screening, including bone marrow biopsy, should not be performed when the M-protein level is below 10 g/l,[138,139] an underlying hematologic malignancy would have been missed in 15 patients. Six patients even had M-protein levels < 1 g/L and these patients did not have a different presentation of their malignancy. More hematologic malignancies may be found in patients without neuropathy and low M-protein levels with full hematologic screening.[132]

Recently, Ponsford et al. reported a 6% incidence of hematologic malignancy in patients with polyneuropathy associated with monoclonal gammopathy.[122] The high frequency of hematologic malignancies in our study can partly be explained by selection bias. Patients with polyneuropathy associated with monoclonal gammopathy and an atypical disease course (e.q., a more progressive course) will be sent to our tertiary clinic earlier than will other patients. Furthermore, all patients presented to us as polyneuropathy associated with MGUS after hematologic evaluation (in other hospitals) were included in our study. Of these patients, 16 were found to have a hematologic malignancy at the initial hematologic work-up in our hospital. Seven patients developed a hematologic malignancy during follow-up, which is comparable to the reported incidence.[122] Comparison of the incidence and prevalence of hematologic malignancy in monoclonal gammopathy with and without neuropathy is difficult, however, as the M-protein in polyneuropathy associated with monoclonal gammopathy will be detected earlier because of the symptoms leading to neurologic screening. This problem can be partly solved by starting follow-up at the first neurologic screening. With this method the actuarial probability of hematologic malignancy was 30% and 42% at 5 and 10 years, respectively, which is higher than the reported probability of hematologic malignancy among patients with MGUS without neuropathy,[35,140,141] and the observation in our own hematology department (9% and 21% at 5 and 10 years, respectively).

The assumption that the M-protein was present from the onset of the neuropathy may be incorrect in some patients. Although the relation between M-protein and polyneuropathy is supported by several studies, the evidence for this relation is much stronger for IgM M-protein than for IgG M-protein.[17,21,22,48,130] Some patients with axonal polyneuropathy may have an idiopathic polyneuropathy and the coincidental presence of IgG M-protein, as both IgG monoclonal gammopathy and axonal polyneuropathy are more frequent in the older population.[53,142,143] Furthermore, the finding of IgG kappa M-protein in one patient (No. 9) with low-grade lymphoma could have been coincidental, as IgG M-protein is frequently found in the elderly and polyneuropathy can occur in association with low-grade lymphoma.[144]

The neurologic criteria for performing a bone marrow biopsy were autonomic features, a progressive or painful polyneuropathy, or both. In addition, bone marrow biopsies were performed based on hematologic criteria. Initially, a bone marrow biopsy was not performed in some patients as this was not part of the standardized hematologic evaluation at the start of the study. As the study progressed, however, a bone marrow biopsy was performed at least once in all patients, irrespective of hematologic or neurologic parameters.

Several risk factors for development of a hematologic malignancy among patients with MGUS have been reported. These include M-protein level,[141,145] increase of M-protein level during follow-up, light-chain proteinuria, age >70 years,[138] kappa light chain,[145] and IgA isotype.[140] We found that progression of the polyneuropathy, unexplained weight loss, and M-protein level >1 g/L were independent predictors for an underlying hematologic malignancy. We also found an association with other symptoms like fatigue, recurrent infections, bone pain, metabolic disturbances, or blood count abnormalities, but there were no independent risk factors. In polyneuropathy associated with monoclonal gammopathy, the M-protein level with a low risk of hematologic malignancy is lower (< 1 g/L) than levels reported in monoclonal gammopathy without neuropathy (< 15 g/L).[138]

Although we found no difference in treatment response of neuropathy symptoms between patients with polyneuropathy associated with benign and malignant monoclonal gammopathy,[99,146,147] early detection of patients with a hematologic malignancy is important, as careful investigations and follow-up may prevent serious complications and allows adequate timing of treatment. In this study the neuropathy symptoms improved in 13 of the 19 patients treated with the accepted therapy for the underlying malignancy. So, in patients with polyneuropathy associated with monoclonal gammopathy with a progressive disease course, unexplained weight loss or M-protein level >1 g/L, extensive hematologic work-up including skeletal

and chest radiographs, sonography of the abdomen (in case of an IgM M-protein), and bone marrow investigation is justified. In patients with a slowly progressive polyneuropathy without risk factors and a negative initial work-up for malignancy, annual neurologic and hematologic evaluation including measurement of M-protein is sufficient. Bone marrow examination should depend on clinical suspicion of malignant transformation (fatigue, bone pain, recurrent infections, metabolic disturbances, unexplained fever, night sweats, weight loss, progression of the neuropathy, anemia) or a steep rise in the M-protein. The possibility of amyloidosis should be suspected in every patient who has an M-protein with progressive sensorimotor peripheral neuropathy, particularly if autonomic involvement or organ involvement is present. In these patients Congo red staining of an abdominal fat aspirate or rectal biopsy should be done, even when a sural nerve biopsy and a bone marrow biopsy are negative.[148]

In conclusion, polyneuropathy associated with monoclonal gammopathy is frequently associated with an underlying hematologic malignancy. Independent risk factors for malignancy were rapid deterioration of the neuropathy, unexplained weight loss, or a level of M-protein >1 g/L. In patients with polyneuropathy associated with monoclonal gammopathy, especially with these characteristics, thorough hematologic screening and accurate follow-up should be performed.

Malignant transformation in polyneuropathy associated with monoclonal gammopathy

Malignant transformation in polyneuropathy associated with monoclonal gammopathy

Summary
To assess the frequency of hematologic malignancies at diagnosis and to determine the incidence and predictors of malignant transformation during follow-up in patients with polyneuropathy associated with monoclonal gammopathy we evaluated potential predictors of malignant transformation from medical history, hematologic, neurologic, and laboratory examination performed each six months by univariate and multivariate Cox' proportional hazard analysis. Of 193 patients with polyneuropathy associated with monoclonal gammopathy 17 patients had a hematologic malignancy at diagnosis. The incidence rate of malignant transformation in 176 patients without a malignancy at diagnosis was 2.7/100 patient years. Weight loss, progression of the polyneuropathy, unexplained fever or night sweats and M-protein level were independent predictors. Since hematologic malignancies occur frequently in polyneuropathy associated with monoclonal gammopathy we suggest that all patients should be screened at diagnosis and subsequently during follow-up if malignant transformation is suspected.

Introduction

Monoclonal gammopathy of undetermined significance (MGUS) occurs in 0.1-3% of the normal population and prevalence rises with age.[53] MGUS is characterized by the presence of a monoclonal protein (M-protein) in serum and/or urine without an underlying hematologic malignancy. Criteria for MGUS include a serum M-protein level less than 30 g/L, a bone marrow plasma cell infiltration of less than 10%, and absence of lytic bone lesions, anemia, hypercalcemia or renal insufficiency, and stability of the M-protein level.[35-37] Persons with MGUS are asymptomatic and have no signs of hematologic malignancy, including fatigue, weight loss, bone pain or susceptibility to infections. However, 1% of the patients with MGUS develop a hematologic malignancy per year, and the risk of malignant transformation persists even after 30 years of follow-up.[38] In IgG MGUS malignant transformation to a plasma cell malignancy has been reported to be 1% per year,[32] and in IgM MGUS malignant transformation to a malignant lymphoid disorder like lymphoma or immunocytoma occurs in 1.5% of patients per year.[39]

The relation between polyneuropathy and MGUS is supported by epidemiologic, pathologic and passive transfer studies.[1,2,4,5,19] In a retrospective cohort analysis we found that 22% of patients with polyneuropathy associated with MGUS developed a hematologic malignancy during long-term follow-up of median 6 years.[149] This suggests a higher frequency of hematologic malignancies in MGUS associated with polyneuropathy than in MGUS without polyneuropathy.

Therefore, we prospectively studied 193 non-selected patients with polyneuropathy associated with monoclonal gammopathy with a mean follow-up duration of three years, and assessed the frequency of underlying hematologic malignancies at diagnosis and the incidence of malignant transformation during follow-up. In addition, we analyzed which factors predicted malignant transformation in 104 patients without a hematologic malignancy at diagnosis in whom bone marrow examination was performed at the beginning and the end of follow-up.

Patients and methods

Patients

From January 1995 to August 2004, 193 patients with polyneuropathy associated with MGUS were identified at the Department of Neuromuscular Diseases of the University Medical Center Utrecht. These patients were referred to our clinic from all over the country for diagnosis and screening for causes. Before inclusion none of the patients was treated with chemotherapeutics. All patients with polyneuropathy and M-protein without other causes of the neuropathy were included after signed informed consent.[68] In all patients the initial work-up included: (1) medical history, (2) neurologic examination, (3)

routine laboratory analysis, (4) electrophysiologic studies, including nerve conduction and concentric needle examination using standardized techniques, identifying a predominantly axonal or demyelinating neuropathy according to the criteria of the American Academy of Neurology (AAN),[25,34,52,52] (5) a survey for M-protein by immunofixation of serum and 10 x concentrated urine, (6) antibody reactivity[28,71] (7) physical examination by a hematologist (8) skeletal X-ray, (9) X-ray of the lungs, (10) sonography or CT-scan of the abdomen (on indication) and (11) bone marrow investigation. Bone marrow aspirates and biopsies were obtained from the crista iliaca posterior and viewed by a pathologist. Additional investigations included immunophenotyping with specific plasma cell, B cell, and T cell monoclonal antibodies. Congo red staining was performed for the detection of amyloid deposits. In patients suspected of amyloidosis, i.e. painful axonal polyneuropathy or autonomic function disorder without amyloid deposits in bone marrow, Congo red staining of a rectal biopsy or a sural nerve biopsy was performed. During follow-up patients were examined each six months including: (1) medical history, (2) physical examination, (3) neurologic examination, (4) routine laboratory analysis and (5) determination of the level of M-protein. Duration of follow-up was defined by the time between the first hematologic screening and the diagnosis of an endpoint or the end of the study. We obtained the following variables i.e. fatigue, defined by difficulty to perform normal daily activities[14]; unexplained bone pain, defined as consistant localized pain other than joint or muscle pain; infections; B-symptoms, defined by unexplained fever, night sweats, unexplained weight loss > 5 kg/6 months; and progression of the polyneuropathy, defined by deterioration of the neuropathy leading to disability (graded with decrease of the Rankin disability score of one point) or decrease of sensory function or strength of one point in six months.[14,25] Strength was measured with MRC grading system in six muscles of both arms (deltoid, biceps and triceps brachii, finger extensors, finger flexors and first dorsal interosseus) and both legs (iliopsoas, quadriceps femoris, hamstrings, anterior tibial, gastrocnemius and peroneal) leading to a maximum score of 120. Sensory functions, both touch and pin prick sense were graded as normal = 4, abnormal distal to wrist/ankle = 3, distal half forearm/leg = 2, distal to elbow/knee = 1, distal to axilla/groin = 0. Vibration sense studied with tuning fork perception (128 Hz) was graded as present on middle finger/hallux = 4, ulnar styloid/medial malleolus = 3, elbow/knee = 2, clavicula/crista iliaca = 1, no perception = 0. Joint position sense was graded as present of middle finger/hallux = 2, diminished = 1, absent = 0. Summation of all sensory modalities could lead to a maximum score of 56. We obtained the following laboratory variables, i.e. M-protein isotype; M-protein level (g/L), significant rise of M-protein level (> 25% if M-protein level

≥ 5 g/L with an absolute rise of >5g/l, minimal 5 g/L if M-protein level < 5 g/L),[150] measured at least at two different time points; and anemia (hemoglobin level < 7.4 mmol/L = < 12 g/L in women and < 8.6 mmol/L = < 14 g/L in men).

Outcome
The main outcome of the study was malignant transformation to a hematologic malignancy i.e. multiple myeloma, plasmacytoma, amyloidosis, immunocytoma, Non-Hodgkin's lymphoma, Castleman's disease, and POEMS (polyneuropathy, organomegaly, endocrinopathy, M-protein and skin symptoms). Criteria for multiple myeloma and plasmacytoma were defined according to Kyle and Greipp[135] and multiple myeloma patients were classified according to Salmon and Durie.[136] Indolent myeloma and smouldering myeloma fulfilling the criteria of Stage I myeloma were classified as Stage 1 myeloma. Immunocytoma was defined based on WHO criteria, i.e. infiltration of the bone marrow of >25% by mature B cells. Non-Hodgkin lymphoma was defined according to the REAL classification.[137] MGUS was defined according to Kyle.[35]

Statistical analysis
The incidence of malignant transformation during follow-up was assessed in all patients without a hematologic malignancy at diagnosis. The analyses of predictive factors for malignant transformation during follow-up were performed on patients without a hematologic malignancy at the initial hematologic screening at base line (at diagnosis), with complete data on important predictive factors and bone marrow examination at the beginning and the end of follow-up. The association between each predictor and malignant transformation was first assessed by univariate Cox' proportional hazards analysis, with the hazard ratio (HR) and 95% confidence interval (95% CI) as measure of association. Variables that were univariately associated with hematologic malignancies at follow-up ($p < 0.1$) were then included in a multivariate Cox' proportional hazards analyses model to evaluate their independent contribution in the prediction of malignancies. First we included variables that can be obtained by history taking and physical examination, thereafter we included laboratory variables. Model reduction was performed by excluding variables that were not significantly related to hematologic malignancies (HR with $p<0.05$) from the overall model by the step-down method. The relationship between malignancy (at baseline or at follow-up) and mortality was studied using a statistical model relating a determinant whose status changes over time to survival type (censored) outcome data, i.e. Cox regression with time-varying covariates. The results were expressed as hazard ratios (and 95%

confidence intervals). The relationship between survival and type of monoclonal gammopathy were analyzed using Kaplan-Meier survival analysis.

Results

Base line and neurologic characteristics

For this study 193 patients underwent neurologic and hematologic screening. Most patients were men (69%) with a mean age of 60 years (table 1). Thirty-four patients had monoclonal IgM anti-MAG antibodies (29% of the patients with IgM monoclonal gammopathy or IgM/IgG biclonal gammopathy, table 2). Twenty-three patients had a slowly progressive demyelinating polyneuropathy associated with monoclonal IgM anti-MAG antibodies with ataxia or sensorimotor symptoms and signs. Eleven patients with anti-MAG antibodies had a more progressive disease course. Of the 83 patients with IgM monoclonal gammopathy (or biclonal gammopathy with IgM) without anti-MAG antibody reactivity 64% had a demyelinating polyneuropathy with predominance of sensory symptoms and signs. Of the 66 patients with IgG monoclonal gammopathy 73% had axonal polyneuropathy with predominance of sensory symptoms and signs. Nine patients had IgA monoclonal gammopathy with axonal sensory polyneuropathy.

Frequency of underlying hematologic malignancies

Of the 193 patients 17 (9 %) had a hematologic malignancy at first screening (three multiple myeloma (two stage I and one stage III multiple myeloma), three plasmacytoma, two amyloidosis, four immunocytoma, one Non-Hodgkin's lymphoma and multiple myeloma, one Castleman's disease, three POEMS). Characteristics and laboratory findings of patients are summarized in table 1 and 2.

Incidence of malignant transformation during follow-up

The incidence of malignant transformation during follow-up was assessed in 176 patients with polyneuropathy associated with MGUS in whom no hematologic malignancy was found after extensive hematologic screening at baseline. Bone marrow examination was performed in 139 patients (79%). After a mean follow-up of three years, 17 patients (incidence rate 2.7 per 100 persons years) developed a hematologic malignancy (four multiple myeloma (three stage I and one stage III multiple myeloma.), one plasmacytoma, ten immunocytoma, one Non-Hodgkin's lymphoma, and one POEMS). Five patients with polyneuropathy associated with IgM anti-MAG antibodies and a progressive disease course had malignant transformation (four immunocytoma, one Non-Hodgkin's lymphoma).

Predictive factors for malignant transformation

The analyses of predictive factors for malignant transformation during follow-up were restricted to 104 patients without a hematologic malignancy at diagnosis and complete data on important predictive factors including bone marrow examination at the beginning and end of follow-up (88 with MGUS and 16 with a hematologic malignancy at the end of follow-up). Of the 104 patients included in the analyses of potential predictors more patients had a progressive polyneuropathy and a hematologic malignancy than in the 72 patients who were not included in these analyses. Of these 72 patients 45 patients were lost to follow-up. Other characteristics did not differ. The hazard ratios for the associations between the potential predictive factors and hematologic malignancy determined with univariate analysis are presented in table 3. In multivariate analysis unexplained weight loss, progression of the polyneuropathy, unexplained fever or night sweats, and M-protein level were independent prognostic variables significantly associated with malignant transformation (table 4). Among patients who had or who developed a hematologic malignancy three patients died (pneumonia) compared to five patients of those without a malignancy (breast cancer, kidney cancer, pulmonary embolism after aortic bypass surgery, pneumonia and cardiac disease, hazard ratio for mortality 8.3, 95% CI: 2.1 –33.7). We did not find a difference in survival of patients with IgG/IgA or IgM type of monoclonal gammopathy.

Table 1 Clinical characteristics of patients with polyneuropathy associated with monoclonal gammopathy

	At diagnosis		During follow-up	
	MGUS	**malignancy**	**MGUS**	**malignancy**
	n=176	n= 17	n=159	n=17
Age at onset, mean (SD), years	60 (10)	57 (10)	60 (10)	56 (10)
Follow-up, mean (SD), months			38 (32)	55 (39)
Sex, male	68	75	69	61
Fatigue	21	50 *	18	44 *
Bone pain	6	13	5	17
Infections	1	6	1	6
Night sweats/unexplained fever	2	13	1	11 *
Weight loss	10	44 **	8	24 *
Kidney dysfunction	3	6	3	11
Progression of polyneuropathy			44	89 ***
Autonomic function disorder	7	17	7	0

In percentage unless stated otherwise; MGUS = monoclonal gammopathy of undetermined significance; Malignancy = hematologic malignancy; Kidney dysfunction = serum creatinine level > 20 mg/L; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

Table 2 Laboratory characteristics of patients with polyneuropathy associated with monoclonal gammopathy

	At diagnosis		During follow-up	
	MGUS	**malignancy**	**MGUS**	**malignancy**
	n=176	n= 17	n=159	n=17
IgM	56	13	55	61
IgG	30	53	32	17
IgA	4	13	3	17
Biclonal gammopathy	10	20	11	6
Kappa	63	38	63	61
Lambda	28	38	27	39
Kappa and Lambda	10	25	11	0
Monoclonal protein level mean (SD), g/L	3 (6)	7 (9) *	3 (5)	10 (9) ***
Increasing monoclonal protein level			6	41 ***
Anemia	22	50	20	50
Demyelinating EMG	56	69	54	72
Monoclonal population in bone marrow	48	64	39	100 ***
Plasma cell, mean (SD), %	3 (3)	3 (4)	2 (3)	6 (7)
B cell, mean (SD), %	8 (6)	9 (8)	7 (4)	15 (8) *

In percentage unless stated otherwise; MGUS = monoclonal gammopathy of undetermined significance; Malignancy = hematologic malignancy; IgM, IgG, IgA = IgM, IgG, IgA monoclonal gammopathy; Kappa, Lambda = kappa, lambda light chain, Kappa and Lambda = biclonal gammopathy kappa and lambda; Anemia = hemoglobin level < 12 g/L (<7.4 mmol/L) in women, < 14 g/L (<8.6 mmol/L) in men; Demyelinating EMG = demylinating electrophysiologic features according to the criteria of the American Academy of Neurology;[14] Monoclonal population in bone marrow = monoclonal B or plasma cell population in bone marrow aspiration; Plasma cell, B cell = plasma cell, B cell infiltration in bone marrow biopsy; * = $p < 0.05$, *** = $p < 0.001$

Table 3 Univariate analysis of the potential predictive factors for malignant transformation

	MGUS n=88	**malignancy** n=16	HR (95%CI)
Age at onset, mean (SD), y	59 (10)	56 (9)	1.0 (1.0-1.0)
Fatigue	20	40	2.9 (1.1-8.0)*
Bone pain	6	13	3.6 (0.9-14)*
Infections	1	0	0.7 (0.1-5.9)
Night sweats/unexplained fever	0	7	10.9 (2.0-60)***
Weight loss	11	20	74.1 (8-684)***
Progression of polyneuropathy	51	94	4.8 (0.8-36)*
IgG/IgA monoclonal gammopathy	38	21	2.0 (0.4-11)
Monoclonal protein level, mean (SD), g/L	2 (5)	11 (9)	1.1 (1.0-1.2)**
Increasing monoclonal protein level	7	43	2.9 (1.0-8.3)**
Anemia	14	50	121 (0.1-106267)
Anti-MAG antibodies	28	46	1.2 (0.4-3.9)

In percentages, unless otherwise specified, with hazard ratios (95% CI); MGUS = monoclonal gammopathy of undetermined significance; Malignancy = hematologic malignancy; Anemia = hemoglobin level < 12 g/L (<7.4 mmol/L) in women, < 14 g/L (<8.6 mmol/L) in men; Anti-MAG antibodies = anti-myelin-associated-glycoprotein antibodies; * = p < 0.1, ** = p<0.05, ***= p< 0.01

Table 4 Multivariate analysis of clinical, and laboratory predictors of malignant transformation

	model 1 clinical determinants HR (95%CI)	**model 2 laboratory determinants** HR (95%CI)	**model 3 clinical and laboratory determinants,** HR (95%CI)
Weight loss	226 (12-4381)		226 (12-4381)
Progression of the polyneuropathy	118 (3.0-4638)		118 (3.0-4638)
Unexplained fever or night sweats	27 (1.8-403)		27 (1.8-403)
Monoclonal protein level (per 1 g/L)		1.1 (1.0-1.2)	1.0 (0.9-1.1)

Values are hazard ratios (95% CI)

Discussion

In this prospective study of 193 patients with polyneuropathy associated with monoclonal gammopathy 34 patients were diagnosed with a hematologic malignancy at initial screening or during follow-up (18%). At initial screening 9% of the patients with polyneuropathy associated with monoclonal gammopathy had an underlying hematologic malignancy. During three years follow-up in 17 of the 176 remaining patients with polyneuropathy associated with MGUS malignant transformation occurred. The incidence of malignant transformation in polyneuropathy associated with MGUS (2.7/100 patient years, 95% CI 1.52/100-4.22/100 patient years) appears to be higher than in MGUS without polyneuropathy (1/100 patient years, 95% CI 0.85/100-1.24/100 patient years, $p<0.05$).[32] Unexplained weight loss, progression of the polyneuropathy, unexplained fever or night sweats and M-protein level were independent predictors of malignant transformation.

This study confirms the findings of our previous retrospective cohort study in which 22% of the patients with polyneuropathy associated with MGUS developed a hematologic malignancy during long-term follow-up.[149] The high frequency of hematologic malignancies could have been due to selection bias, because patients with polyneuropathy associated with MGUS and a progressive disease course may be referred earlier to a tertiary clinic than patients with slow progression of the disease. In addition, the high frequency of underlying hematologic malignancies could have been due to referral bias, since patients with a very slowly progressive polyneuropathy followed in a non-university hospital might not be screened for the presence of an M-protein and once they have a progressive polyneuropathy these patients are referred to an university hospital where the malignant monoclonal gammopathy is found. However, a high number of patients without a hematologic malignancy at initial screening developed a hematologic malignancy during prospective follow-up. To determine the incidence of and predictors for malignant transformation during follow-up, patients with polyneuropathy associated with MGUS were screened at regular intervals. The group of patients in the follow-up study for analyses of predictive factors included more patients with a progressive polyneuropathy and more patients with a hematologic malignancy than the group of patients not included. Patients without a progressive disease course were frequently lost to follow-up. This leads to under representation of patients with chronic polyneuropathy in the analysis, and may affect the association of progression of the polyneuropathy and malignant transformation.

Several prognostic factors for malignant transformation for patients with MGUS without polyneuropathy have been reported in previous retrospective studies, i.e. M-protein level,[141,145,145] increase of

M-protein level during follow-up, light-chain proteinuria, age >70 years,[138] kappa light chain,[145,145] and IgA isotype.[140] In the past two years, two large studies reported M-protein level as the most important independent predictor for malignant transformation in both IgG and IgM MGUS.[32,39,39] Patients with polyneuropathy associated with MGUS in this prospective multivariate study were comparable to patients with MGUS in these studies with respect to age, M-protein level and bone marrow infiltration and we confirmed the findings in MGUS without polyneuropathy. In addition to reported findings, we identified weight loss, unexplained fever or night sweats and progression of the polyneuropathy as predictive factors for malignant transformation. This is important since specialists in neurology, hematology and internal medicine should be aware of the underlying risk of a hematologic malignancy in patients with polyneuropathy associated with monoclonal gammopathy. Unexpectedly, the incidence of malignant transformation in patients with a demyelinating polyneuropathy associated with IgM anti-MAG antibodies is similar to the high incidence found in the total group of patients with polyneuropathy associated with MGUS. Also in the patients with anti-MAG antibodies, who normally have a slowly progressive disease course, progression of the polyneuropathy was associated with malignant transformation.[6,136]

Frequent bone marrow examination enables early detection of patients with a hematologic malignancy and may prevent serious complications by adequate timing of treatment. In this and other studies the neuropathy symptoms improved with the acknowledged therapy of the underlying malignancy.[14,14,17,21,21,151]

Chapter 5

Cytogenetic aberrations in polyneuropathy associated with IgM monoclonal gammopathy identified with FISH, MLPA and CGH-array

adapted from

'Eurelings M, Lokhorst HM, Notermans NC, Krijtenburg PJ, van Kessel B, Beleveld MJ, Bloem A, Wokke JHJ, Poot M, Buijs A. Acquired cytogenetic aberrations in polyneuropathy associated with IgM monoclonal gammopathy. Submitted'

Cytogenetic aberrations in polyneuropathy associated with IgM monoclonal gammopathy

Summary

The relation of polyneuropathy and IgM monoclonal gammopathy of undetermined significance (MGUS) is well supported. MGUS is characterised by the presence of a monoclonal protein (M-protein) without an underlying haematological malignancy. In patients with MGUS and polyneuropathy malignant transformation occurs more frequent than in patients with MGUS without polyneuropathy. The knowledge of cytogenetic aberrations in IgM MGUS is limited.

To determine the occurrence and nature of cytogenetic aberrations we applied interphase fluorescence in situ hybridisation (FISH), multiplex ligation-dependent probe amplification (MLPA) assay and genome-wide array-based comparative genomic hybridisation (CGH) in 22 patients with polyneuropathy associated with IgM monoclonal gammopathy, including 12 patients with MGUS and 10 patients with lymphoplasmocytic lymphoma (LPL). No cytogenetic aberrations were found in 12 patients with IgM MGUS. Of the 10 patients with LPL four patients had 10-20% and one patient had 30% B cells with IgH translocations; one patient had additional loss of 14qter; one patient had amplification of 6p and loss of 6q. Finding of 14q32 translocation makes early identification of patients with malignant transformation possible and thereby the initiation of early treatment and frequent follow-up.

Introduction

Monoclonal antibody activity to antigens in peripheral nerves has been implicated as a pathogenic mechanism in polyneuropathy associated with monoclonal gammopathy of undetermined significance (MGUS).[22] The pathogenic relation of polyneuropathy with these monoclonal antibodies is best supported in polyneuropathy associated with monoclonal IgM antibodies by pathologic and passive transfer studies.[5,17]

MGUS is characterised by the presence of a monoclonal protein (M-protein) in serum and/or urine without an underlying haematological malignancy.[132] The origin of the M-protein secreting cells in MGUS can be both plasma cells and B cells producing monoclonal IgG, IgA or IgM. Criteria for MGUS include a serum M-protein level less than 30 g/l, a bone marrow plasma cell infiltration of less than 10%, absence of lytic bone lesions, anaemia or other laboratory or clinical abnormalities and stability of the M-protein level.[132] Additional criteria for IgM MGUS include absence of constitutional symptoms, hepatosplenomegaly, or lymphadenopathy. The most important prognostic factor for malignant transformation in patients with MGUS is the M-protein level.[32] One percent of the patients with IgG MGUS develops a multiple myeloma per year.[32]} Translocations of the immunoglobulin heavy-chain gene (IgH), deletions of chromosome 13q14, and numerical abnormalities have been identified in these patients.[42,43,152-158,158-161] IgH translocations and numerical chromosomal aberrations have also been found in B cell malignancies.[44,162-167] The knowledge of cytogenetic aberrations and malignant transformation in IgM MGUS is limited.[168,169] Malignant transformation occurs more frequently in patients with MGUS and polyneuropathy than in patients with MGUS without polyneuropathy.[149,170] Whether cytogenetic aberrations occur more frequently and result in increased incidence rate of malignant transformation in patients with IgM MGUS and polyneuropathy is unknown. Finding of genetic aberrations predicting development of haematological malignancies is important to define a subset of patients at risk of malignant transformation. This may provide means to initiate frequent follow-up and early treatment of the patients at risk and less frequent follow-up of the patients not at risk.

To determine the occurrence and nature of cytogenetic aberrations in polyneuropathy associated with IgM monoclonal gammopathy and if possible aberrations in IgM MGUS predicting malignant transformation we analysed the presence of cytogenetic aberrations in monoclonal B cells in 22 patients diagnosed with polyneuropathy associated with IgM monoclonal gammopathy (12 MGUS and 10 lymphoplasmocytic lymphoma, LPL) with interphase fluorescence in situ hybridisation (FISH), multiplex ligation-dependent

probe amplification (MLPA) assay and genome-wide array-based comparative genomic hybridisation (CGH).

Patients and Methods

Patients

Patients were selected from a group of patients with polyneuropathy and MGUS referred to the University Medical Centre in Utrecht for diagnosis and treatment of their polyneuropathy. In these patients all other causes were excluded and the disease course, i.e. progression or pain, justified extensive examination and treatment.[68] All patients underwent a comprehensive evaluation including disease history, physical examination, routine laboratory analysis, immunoelectrophoresis, immunofixation, haematological examination, including bone marrow examination. Seven patients were newly diagnosed and 15 patients were analysed during follow-up. Of these 15 patients 10 patients had been treated previously with cyclophosphamide and prednisone, or fludarabine. Lymphoplasmocytic lymphoma (LPL) was diagnosed by a pathologist and defined based on WHO criteria, i.e. infiltration of the bone marrow of >25% by mature B cells in the bone marrow biopsy. MGUS was defined according to Kyle.[35] Electrophysiological studies consisting of nerve conduction and concentric needle examination using standardised techniques identified an axonal or demyelinating neuropathy according to conventional criteria.[14]

Antibody studies

Anti-myelin-associated glycoprotein (MAG) antibodies were measured using Western blot, as previously described.[28] Patients' serum binding to MAG was tested at an initial dilution of 1:1000. The antibody titre is given as the highest serum dilution at which antibody binding to the MAG band is detected. Antibody reactivity against asialo-GM1 (GA1), GM1, GM2, GD1a, GD1b and GQ1b was measured by enzyme-linked immunosorbent assay (ELISA).[66,125,149] Antibody reactivity was confirmed with thin-layer chromatography.[126]Antibody reactivity of the monoclonal gammopathy was further evaluated using kappa- or lambda chain specific peroxidase conjugated antibodies (DAKO, Glostrup, Denmark).

Bone marrow cells

Bone marrow cells were obtained from 22 patients after informed consent. Mononuclear cells are obtained by standard ficoll-hypaque density centrifugation. Flow cytometric analysis was used to identify B cells in the bone marrow samples. Infiltration of monoclonal B cells in the bone marrow samples was determined by analyzing the distribution

of kappa and lambda light chains (kappa/lambda ratio) of surface bound immunoglobulin (sIg) on CD 19^+ B cells. Mononuclear cells were purified using magnetic cell sorting (MACS) with anti-CD19 for monoclonal B cells and anti-CD138 for plasma cells. Cytospin slides were prepared from purified B- or plasma cells, dried overnight for direct use or sealed and stored at -70° C. Purified B- or plasma cells were stored as snap frozen cell pellets (0.5-1 x 10^6 cells) at -80° C.

Preparation of gDNA
Genomic DNA (gDNA) was extracted from 1 x 10^6 purified B cells according to manufacturer's instructions (QIAgen QIAamp DNA Blood Mini kit, QIAgen Inc., Chatsworth, USA). The DNA was eluted with 100ul buffer AE and stored at -4° C.

Interphase FISH
Interphase FISH was applied in 22 patients. For FISH studies the air dried cytospin slides of all 22 patients were fixed in icecold 10% acetic acid in ethanol and washed with PBS. The slides and probes were prepared and used according to manufacturer's instructions. The slides were mounted with antifade medium (Vectashield, Vector, Burlingame, CA) containing DAPI. Microscopic analysis of the samples was performed using CytoVision Applied Imaging software. To identify IgH translocations on chromosome 14q32 we used a break-apart strategy, using a red-labelled probe for the IgH constant regions and a green labelled probe for the IgH variable region (LSI IgH Dual Color, Vysis, Downers Grove, Il, USA). Break-apart of probe signals was defined by a distance of more than 3 signals widths between 2 differently labelled probe signals.[171] To identify partner chromosomes we used a fusion strategy, using a red labelled probe for the cyclin D1 locus on chromosome 11q13 and a green labelled probe for the IgH locus on chromosome 14q32 for t(11;14); using a red labelled probe for the BCL2 locus on chromosome 18 and a green labelled probe for the IgH locus on chromosome 14q32 for t(14;18) (LSI IgH/CCNDI, LSI IgH/BCL2, Vysis, Downers Grove, Il, USA). Fusion of probe signal was defined as 2 probe signals making contact. A cytospin was defined abnormal if the percentage of B cells with abnormal signal exceeded the mean percentage background level plus 3 SD found in cytospins of normal controls (>7%, for practical reasons we considered >10% abnormal). The mean percentage background was determined in five normal controls, and at least 1000 B cells were scored for each set of probes.

MLPA
The SALSA P006 human chromosomal aberration test kit 2 (MRC-Holland, Amsterdam, the Netherlands) was used to screen for copy

number changes of oncogenes in 18 of the 22 patients. The probe mix included in this MLPA kit contains probes for 41 different target sequences. For the test 50-500ng DNA was analysed according to manufacturer's recommendations (www.mlpaholland.com). The fragments were analysed on ABI 3100 Genetic Analyzer (Applied Biosystems) using Genescan-ROX 500 size standards (Applied Biosystems). Fragment analysis was performed using Genotyper softwere. The relative peak areas for each probe were calculated as fractions of the total sum of peak areas in a certain sample. Subsequently, the relative copy number for each probe was calculated by dividing the fraction of each peak by the average peak fractions of the corresponding probe in control samples.

Array-based CGH

In 8 of the 22 patients array-CGH was performed. For array-CGH 1 microgram of sonicated genomic DNA from one of these 8 patients (Tester) and 1 microgram of sonicated genomic DNA from a pool of 10 healthy male individuals (Reference) were labelled using the BioPrime DNA Labelling System (Invitrogen, Carlsbad, CA) with Cy3-dUTP and Cy5-dUTP (Amersham Biosciences, Little Chalfont, UK), respectively. These labelled DNA hybridised to the array-slides using a GeneTAC Hybstation (Genomic Solutions, Ann Harbor, MI).[172] The array has been created by spotting in triplicate DOP-PCR products of 3,343 BAC DNA probes, which have been chromosomally localised using FISH, and cover the entire human genome with a spacing of approximately 1 Mb.[172] After hybridisation the slides were scanned and imaged on a ScanArray Express HT (Perkin Elmer, Wellesley, MA) using the ScanArray Express software (version 2.1). Data were analysed with the GenePix Pro 5.0 software package (Axon Instruments, Union City, CA) followed by LOWESS normalisation using the Acuity 3.1 software package (Axon Instruments, Union City, CA). After analysis the averages of all triplicates were calculated. Clones with a standard deviation above 0.3 were excluded. The fluorescence ratios of all autosomes were used to calculate a 95% confidence interval. All BACs showing fluorescence intensity ratios outside the 95% confidence interval were considered to report segmental aneuploidy (i.e. an aberration).

Statistical analysis

Differences in cytogenetic aberrations between patients with MGUS and LPL, were analysed with the Fisher's exact test; $p < 0.05$ was considered significant.

Results

The clinical and laboratory characteristics of the 22 patients with polyneuropathy associated with IgM monoclonal gammopathy are shown in table 1. The median age at onset of the polyneuropathy was 57 years. Twelve patients had MGUS (55%), 10 patients had LPL (45 %) at the time of the bone marrow examination. Seven patients were newly diagnosed (3 MGUS, 4 LPL). Fifteen patients were analysed during follow-up (8 MGUS, 7 LPL). Ten patients had been treated previously with cyclophosphamide and prednison, or fludarabine. Five of the 15 patients analysed during follow-up had developed LPL after first being diagnosed as MGUS and three of these five patients who had developed LPL had been treated with cyclophosphamide and prednison seven years before. The median follow-up duration was eight years. Fourteen patients (64%) had elevated titres of antibodies to anti-MAG, gangliosides (GA1, GM1, GM2, GD1a, GD1b, GQ1b) or sulfatide (table 1). The B cell percentage in the bone marrow ranged from 3-90% (median 9%, table 2). Plasma cell percentage was < 1%. In all patients with polyneuropathy associated with IgM monoclonal gammopathy a monoclonal B cell population was identified in the bone marrow. After MACS with CD19 > 95% purified B cells were obtained for cytogenetic analysis.

Interphase FISH

Interphase FISH with 14q32 probe identified IgH translocations in 10-20% of the purified B cells in four of the 22 patients and in 30% of the purified B cells in one patient with a large clonal involvement of the bone marrow (patient 8, table 2). In one patient (patient 14) loss of the variable region of the IgH locus (deletion of green signal with the LSI IgH Dual Colour) was found in 50% of the B cells. All patients with IgH aberrations had LPL. The representative image of patient 19 is shown in figure 1. No IgH translocation partners were identified with dual colour probes of t(11;14) involving cyclin D1 or t(14;18) involving BCL2.

MLPA and Array-based CGH

MLPA was performed in 18 patients and array-based CGH was performed in eight patients. In one of the 18 patients cytogenetic aberrations were found with both MLPA and CGH array. In the purified bone marrow B cells of patient 22 using MLPA five loci on the short arm of chromosome 6 were amplified (n=3); four loci on chromosome 6 band p21.3, i.e. KIAA0170-D01, IER3-D02, TNF-D01, BAK1-D01, and one locus on chromosome 6 band p12, i.e. VEGF-D02 (figure 2a). Interphase FISH confirmed triplication of centromere 6 with deletion of 6q. In the same patient array CGH revealed amplification (n=3) of the short arm of chromosome 6 and deletion of the long arm (n=1) (figure

2b). Therefore, these data indicate a gain of the short arm and loss of the long arm of chromosome 6 in this patient.

In patient 14 with loss of the variable region of the IgH locus array with interphase FISH (figure 3a), array-based CGH revealed loss of all loci of 14qter (figure 3b). These data can be explained by splitting of the variable part of the IgH locus, most likely due to non-reciprocal translocation of IgH locus on 14q32 resulting in derivative chromosome 14 with loss of partner derivative (loss of 14qter).

All patients with cytogenetic aberrations, IgH translocations or other, were patients with LPL (p=0.01).

Table 1 Characteristics of patients with polyneuropathy associated with IgM monoclonal gammopathy

pat	age	fup	neuropathy		EMG	antibody	M-prot	g/l	type	treatment
1	65	10	progr	sm	A	-	IgMk	8	LPL	* 1995
2	64	8	progr	sm	D	MAG, sulfatide	IgMk	16	LPL	* 1997
3	69	3	slow	s	A	-	IgMk	<1	MGUS	-
4	45	11	progr	smm	D	GM2, GD1a, GD1b	IgMk/l	<1	MGUS	1999
5	64	8	slow	s	A	sulfatide	IgMk	<1	MGUS	-
6	64	4	progr	s	D	-	IgMk	<1	MGUS	-
7	66	4	progr	s	D	-	IgMk	7	LPL	-
8	55	1	slow	s	n.d.	GQ1b	IgMk	<1	LPL	-
9	74	4	slow	s	A	-	IgMl	23	LPL	-
10	56	2	progr	sm	A	-	IgMk	<1	MGUS	-
11	45	12	progr	sm	D	GD1a, sulfatide	IgMk	16	MGUS	** 2002
12	53	15	slow	s	A	-	IgMk	7	MGUS	-
13	54	9	progr	sm	D	MAG	IgMk	<1	MGUS	** 2000
14	59	5	progr	sm	D	MAG	IgMk	15	LPL	-
15	57	1	progr	sm	D	n.d.	IgMk	<1	MGUS	-
16	60	9	progr	sm	D	MAG	IgMk	12	LPL	* 1998
17	67	3	progr	sm	D	GM1, GM2	IgMk	5	MGUS	-
18	50	8	progr	s	D	GD1a,GD1b, GQ1b	IgMk	<1	MGUS	* 1999
19	50	12	progr	sm	D	MAG	IgMk/l	<1	LPL	* 2000
20	52	18	progr	sm	D	MAG	IgMl	9	LPL	* 1997
21	57	10	progr	s	D	GM1, GM2, GD1a	IgMk	11	LPL	-
22	52	11	progr	sm	D	MAG	IgMk	9	MGUS	* 1999

Bone marrow examination performed in 2004; fup = follow-up in years; progr = progressive, slow = slowly progressive; s = sensory, sm = sensorimotor; n.d. = not done; M-prot = M-protein; type = hematologic disease, LPL = lymphoplasmocytic lymphoma; last treatment with * cyclophosphamide and prednison, or ** fludarabin

Table 2 cytogenetic aberrations in patients with polyneuropathy associated with IgM monoclonal gammopathy

pat	% B cells *	monoclonal population	kappa/ lambda	FISH 14q32	MLPA	CGH array
1	9	B, IgM k	, 11:1	n.a.d.	n.a.d.	n.d.
2	11	B, IgM k	, 90:1	14q32 break, 11%	n.a.d.	n.a.d
3	7	B, IgM k	, 3:1	n.a.d.	n.a.d.	n.d.
4	12	B, IgM k	, 2:1	n.a.d.	n.a.d.	n.a.d
5	3	B, IgM k	, 2:1	n.a.d.	n.a.d.	n.d.
6	7	B, IgM k	, 2:1	n.a.d.	n.d.	n.d.
7	8	B, IgM k	, 9:1	n.a.d.	n.a.d.	n.a.d
8	68	B, IgM k	, 16:1	14q32 break, 30%	n.a.d.	n.d.
9	14	B, IgM l	, 9:1	14q32 break, 18%	n.a.d.	n.a.d
10	8	B, IgM k	, 11:1	n.a.d.	n.a.d.	n.a.d
11	3	B, IgM k	, 3:1	n.a.d.	n.a.d.	n.d.
12	7	B, IgM k	, 3:1	n.a.d.	n.a.d.	n.d.
13	9	B, IgM k	, 2:1	n.a.d.	n.a.d.	n.d.
14	90	B, IgM k	, 99:1	del 14q32, 50%	n.a.d.	del 14 q ter
15	5	B, IgM k	, 3:1	n.a.d.	n.d.	n.d.
16	10	B, IgM k	, 11:1	14q32 break, 17%	n.a.d.	n.d.
17	6	B, IgM k	, 2:1	n.a.d.	n.d.	n.d.
18	9	B, IgM k	, 2:1	n.a.d.	n.a.d.	n.d.
19	50	B, IgM k	, 67:1	14q32 break, 12%	n.a.d.	n.a.d
20	11	B, IgM l	, 6:1	n.a.d.	n.a.d.	n.d.
21	8	B, IgM k	, 15:1	n.a.d.	3 x 6p	3 x 6p/1x 6q
22	7	B, IgM k	, 3:1	n.a.d.	n.d.	n.d.

* in aspirate; after MACS >95% B cells for further analyses; n.a.d. = no aberrations detected; n.d. = not done 14q32 break, % B cells with slit signal of 14q32, no translocation with chromosome 11 or 18 was identified; del 14q32, % of B cells with absent variable part of IgH locus

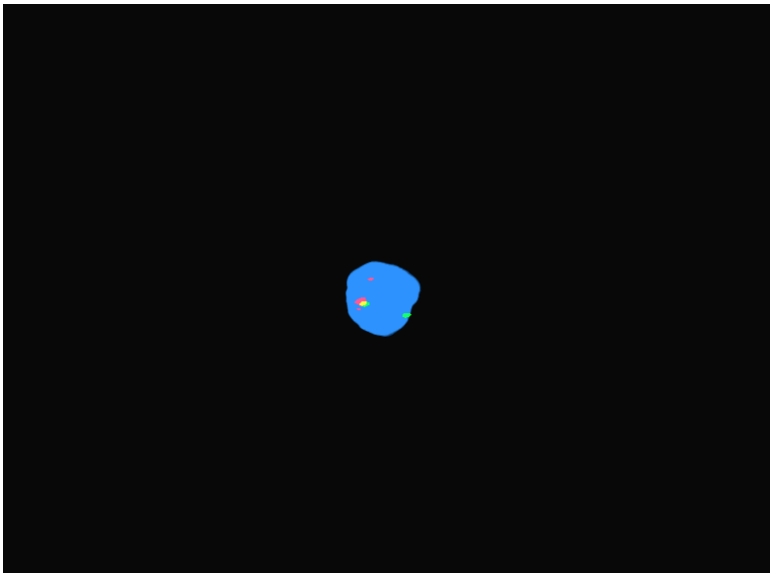


Figure 1. Interphase FISH with 14q32 probe identified IgH translocation in patient 19.

Figure 3a. Interphase FISH with 14q32 probe identified deletion of variable part of IgH locus (green signal) in patient 14.

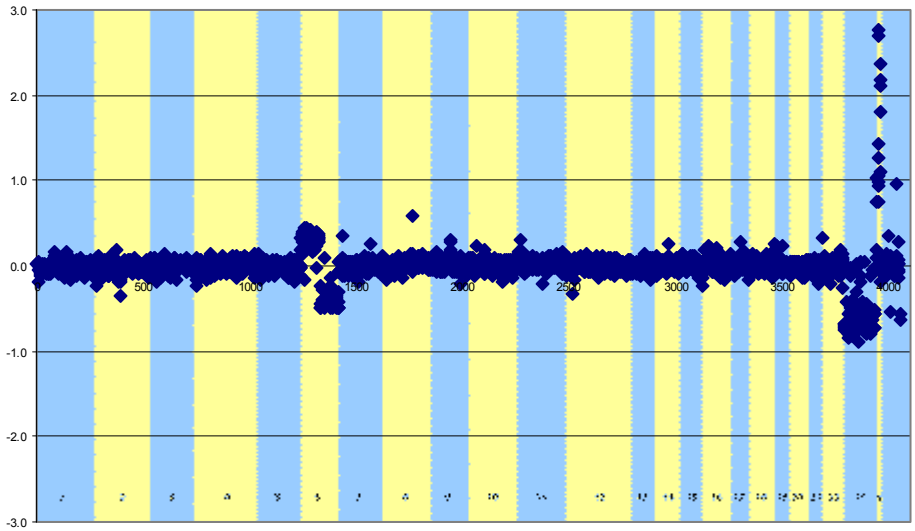


Figure 2b. Array-based CGH result of patient 22 with a gain of 6p (n=3) and loss of 6q (n=1). The abscissa shows the ^{2}Log of the fluorescence intensity ratio's for all BACs of our patient vs. the reference sample (a pool of DNAs from 50 female subjects); the ordinate shows BACs arranged according to chromosomal position.

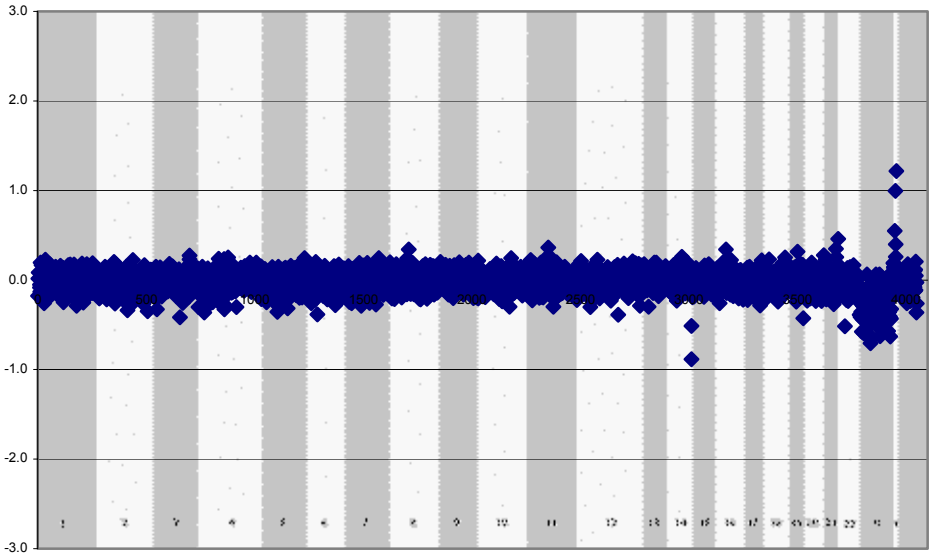


Figure 3b. Array-based CGH result of patient 14 with a deletion of the 14qter region. The abscissa shows the ^{2}Log of the fluorescence intensity ratio's for all BACs of our patient vs. the reference sample (a pool of DNAs from 50 female subjects); the ordinate shows BACs arranged according to chromosomal position (for further details see text).

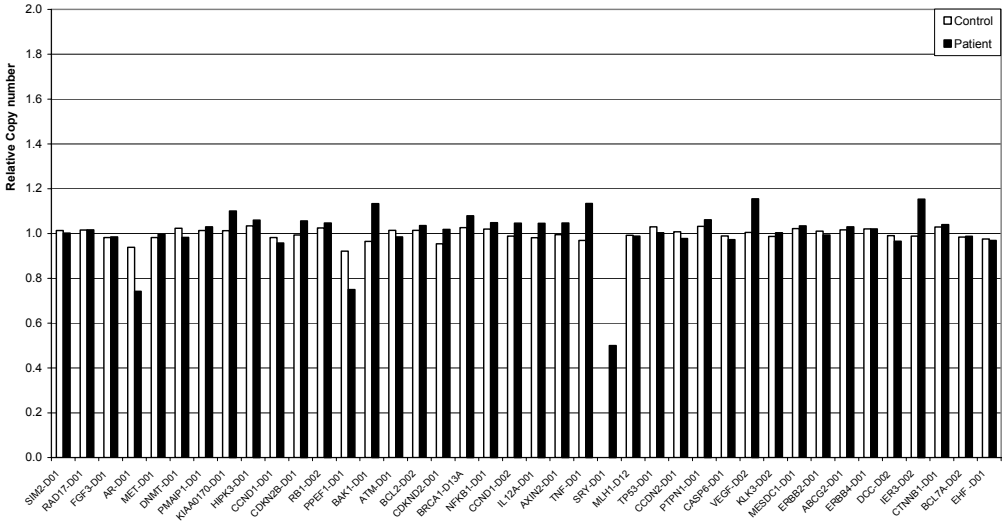


Figure 2a. MLPA result of patient 22 with a gain of 6p (n=3). The vertical axis shows the copy number of the tested probes. Four loci on chromosome 6 band p21.3, i.e. KIAA0170-D01, IER3-D02, TNF-D01, BAK1-D01 , and one locus on chromosome 6 band p12, i.e. VEGF-D02 were increased.

Discussion

Cytogenetic aberrations were analysed with molecular cytogenetic techniques in 22 patients with polyneuropathy associated with IgM monoclonal gammopathy, i.e.12 patients with MGUS and 10 patients with lymphoplasmocytic lymphoma (LPL). Cytogenetic aberrations, predominantly IgH translocations were identified in seven of these patients. Remarkably, all seven patients had LPL according to the WHO definition. None of the patients with IgM MGUS had genetic aberrations. Four of the seven patients with genetic aberrations were previously diagnosed with MGUS and had developed LPL during follow-up. The finding of cytogenetic aberrations supports the difference between IgM MGUS and LPL. However, it remains unclear whether the patients with IgM MGUS without cytogenetic aberrations remain MGUS or whether these patients can still get these aberrations after long-term follow-up (median follow-up of 8 years) and will develop LPL in the future.

Structural aberrations of the IgH locus were found in six patients, including in five patients IgH translocations involving 11-36% of the B cells analysed with interphase FISH using a 14q32 break-apart probe and loss of 14qter, as detected by array-based CGH and interphase FISH in one patient. Previous series of 31 and 22 patients with Waldenstrom's macroglobulinemia report very few patients with IgH translocations (3-14%).[167,171] Our patients with LPL differ from these series of patients since none of our patients had M-protein levels > 30 g/l. In non-secretory lymphoplasmocytic lymphoma (LPL) IgH translocations, i.e. t(9;14)(9p13;q32) resulting in PAX-5 up-regulation, have been identified in 50% of the patients.[173] These results suggest that a 14q32 IgH translocation is an oncogenic event in malignant transformation in IgM monoclonal gammopathy. By FISH we excluded t(11;14) and t(14;18) identified in Non-Hodgkin's lymphoma.[44] Further research is necessary to identify partner chromosomes, such as t(9;14) identified in non-secretory LPL, and t(4;14), t(6;14), and t(14;20) identified in multiple myeloma.

In contrast to the frequent occurrence of IgH translocations in IgG MGUS in almost half of the patients, IgH translocations were absent in our patients with IgM MGUS.[159] Neither did we identify deletions of 13q14 RB-1 frequently detected in IgG MGUS using MLPA or array-based CGH. MLPA could only be performed in 18 of the 22 patients and array-based CGH only in 8 of the 22 patients. The differences in cytogenetic aberrations between IgM and IgG MGUS could be due to the fact that IgM MGUS and IgG MGUS result from a different type of B cell. The incidence rate of malignant transformation in IgM MGUS (to B cell malignancies i.e. lymphoma or LPL) is higher than in IgG MGUS (to multiple myeloma or plasmacytoma). In IgM monoclonal gammopathy and LPL monoclonal autoimmune antibodies

are frequently found.[174] Continuous auto antigen stimulation of the IgH locus might result in aberrations in the IgH region of the monoclonal antibody producing B cell. However, in our study of patients with polyneuropathy associated with IgM monoclonal gammopathy we did not find IgH translocations in IgM MGUS and the frequency of IgH translocations in LPL was comparable to the frequency reported in patients with IgG monoclonal gammopathy.

Of the patients treated with cyclophosphamide three patients had 14q32 IgH translocations. Of these three patients two patients were first diagnosed as IgM MGUS and had developed LPL during follow-up seven years after treatment. Cytogenetic aberrations in treatment related secondary haematological malignancies, i.e. acute myeloblastic leukaemia (t-AML) and myelodysplastic syndrome (t-MDS) after treatment with alkylating chemotherapeutics like cyclophosphamide have been found. However, the cytogenetic aberrations reported were 11q23 mutation, 21q22 mutation, t(8;21), t(9;11), del(5), del(7), del(13) and no translocations of 14q32.[175-177]

In one patient with LPL deletion of 14qter was detected with array-based CGH and interphase FISH. Interphase FISH with LSI IgH Dual Color 14q32 break-apart probe showed loss of the green signal of the variable region, suggesting an unbalanced translocation, resulting in derivative chromosome 14. This event can result in inappropriate expression of near-by oncogenes in B cells due to the proximity of the strong IgH enhancer from the derivative chromosome 14. This may explain the very large clonal B cell infiltration of the bone marrow (90%) in this patient.

In one patient we identified gain (n=3) of the long arm and loss of the short arm of chromosome 6 using MLPA and array-based CGH. MLPA provides a possibility to detect numerical changes, gain or losses, in malignant cells.[178] The probe mix included in the MLPA kit we used contains probes for 41 different target sequences. Among these are many genes that are often deleted or amplified in various tumours. Deletions of chromosome 6 band q21 have been reported to occur frequently in Waldenstrom's macroglobulinemia,[171]but also in chronic lymphatic leukaemia (CLL), lymphoma and leukaemia and LPL. Probably tumour supressor genes at this locus are inactivated during malignant transformation or tumour progression.

In this study we demonstrate that in patients with polyneuropathy associated with IgM monoclonal gammopathy cytogenetic aberrations, mainly recurrent 14q32 translocations, are present in a majority of patients with polyneuropathy associated with IgM monoclonal gammopathy with lymphoplasmocytic lymphoma (LPL) but not in patients with polyneuropathy associated with IgM MGUS. With the results of this study it is not yet possible to identify the patients at risk of malignant transformation. However finding of 14q32

translocation makes early identification of patients with malignant transformation possible and thereby the initiation of early treatment and frequent follow-up. To investigate whether such IgH translocations are a risk factor for malignant transformation in IgM MGUS a longitudinal follow-up study is needed to evaluate how disease progresses in time.

Chapter 6

Immunoglobulin genes in polyneuropathy associated with IgM monoclonal gammopathy

adapted from

'Eurelings M, Notermans NC, Lokhorst HM, van Kessel B, Jacobs BC, Wokke JHJ, Sahota S, Bloem A. Immunoglobulin gene analysis in polyneuropathy associated with IgM MGUS. Submitted'

Immunoglobulin gene analysis in polyneuropathy associated with IgM monoclonal gammopathy

Summary

Autoantibody activity of the M-protein against antigens in peripheral nerve has been implicated as pathogenic mechanism in polyneuropathy associated with monoclonal gammopathy of undetermined significance (MGUS). Autoantibody recognition of antigen will depend on molecular motifs displayed by immunoglobulin variable (V) regions, encoded by V genes, which can reveal evidence of selection in clonal origins. Previous studies have been incomplete, based on few cases in each study. Furthermore, these early studies mined a map of the human immunoglobulin variable loci at a time when the available germline repertoire was not fully characterized. These studies suggest in contrast to other anticarbohydrate activity, that antibody response against gangliosides or myelin-associated glycoprotein (MAG) is not dominated by a restricted set of immunoglobulin variable genes, although somatic mutations appear common. We investigated V_HDJ_H and V_LJ_L gene use by clonal analysis, correlating them with antibody reactivity and the clinical symptoms in 21 patients diagnosed with polyneuropathy associated with IgM monoclonal gammopathy. In all patients a monoclonal B cell population was found in the bone marrow. Seventeen patients used V_H3 genes, two patients used V_H1 genes and two patients used V_H4 genes. In our cohort of patients immunoglobulin V genes associated with bacterial responses appear over-represented with a preferential utilization of the V_H3-23 gene. Vh3-23 was not preferentially associated with D or J_H gene segments or with V_LJ_L. Notably, all immunoglobulin V genes revealed extensive somatic mutation. Polyneuropathy associated with IgM anti-neural antibodies may be caused by an immune response to bacterial polysaccharides which recruit somatically mutated igM autoreactive B cells.

Introduction

Autoantibody activity of the M-protein against antigens in peripheral nerve has been implicated as pathogenic mechanism in polyneuropathy associated with monoclonal gammopathy of undetermined significance (MGUS).[22] The relation of IgM monoclonal gammopathy and demyelinating polyneuropathy is supported by the presence of IgM deposits on myelin sheaths,[19,179-181] and widening of the outer myelin lamina in sural nerve biopsies.[182] In half of the patients antibodies to the myelin-associated glycoprotein (MAG) are found,[6] and transfer of the IgM anti-MAG antibodies can induce demyelinating neuropathy in cat and chicken.[5] In addition, anti-ganglioside antibodies are found in 20% of the patients with polyneuropathy associated with IgM monoclonal gammopathy,[149] and human monoclonal anti-ganglioside antibodies produced by a hybridoma cell line interfere with neuromuscular transmission in a mouse model.[183] Patients with polyneuropathy associated with IgM monoclonal gammopathy lacking anti-MAG or anti-ganglioside activity probably have autoantibody activity against different peripheral nerve antigens such as sulfatide, and chondroitin sulfate.[62,63]

Autoantibodies like anti-DNA antibodies and rheumatoid factor often share idiotypic determinants resulting from shared immunoglobulin variable genes.[184] The structural basis of autoantibodies directed against neural antigens has been studied by research groups in early 1990. Different techniques and approaches were employed to address the question whether the different autoantibody responses involved in polyneuropathy could be attributed to a restricted set of immunoglobulin genes and whether these genes were somatically mutated. The findings showed that, in contrast to other anticarbohydrate reactivity like those against galactan, 3-Fl and the blood group I/i antigen, antibody reactivity against MAG or gangliosides (GM1, GA1, GD1b) was not dominated by a restricted set of immunoglobulin genes and furthermore that the antibodies exhibited somatic mutations.[74,75,185-190] These studies however, were limited by few cases and use of hybridoma approaches and the interpretations were hampered by the limited knowledge of V_H germ-line gene repertoire and of polymorphic alleles. Now, complete maps of the human immunoglobulin V gene loci are available. For the V_H locus on chromosome 14q32, 51 germline genes are potentially functional. The V_H genes are clustered in seven families based on sequence homology (V_H1 - V_H7). At the functional repertoire level however, there is a biased representation of individual V_H gene use. From the two largest families (V_H3 (n = 22) and V_H4 (n = 11)) 10-12 genes are used in 70 - 80% of the peripheral B cell repertoire. This biased representation is already evident during the earliest stages of B cell development and persists until the mature B cell stage. Among the most frequently used

germline V_H genes are V_H 3-23, V_H 3-30, V_H 4-59. These V_H genes are over-represented in certain B cell malignancies and are often found in autoimmune disorders. Interestingly, these V_H genes are also associated with responses to bacterial polysaccharides. This has raised the question whether immune responses driven by bacterial antigens could result in subsequent pathologic states.

In polyneuropathy caused by IgM monoclonal autoantibodies a limited number of patients haven been studied so far for their immunoglobulin V gene usage. [74,75,185-190] In this study we investigated V_H and V_L gene usage and the mutational status in monoclonal surface IgM B cells from 21 patients with polyneuropathy associated with IgM monoclonal gammopathy and identify an apparent restriction at the level of V_H gene family usage, and a preferential over-utilization of the V_H 3-23 gene. All cases revealed mutations in the immunoglobulin variable region genes. These data extend previously reported observations and contribute to the understanding of the pathogenesis of polyneuropathy associated with IgM monoclonal gammopathy.

Patients and Methods

Patients

We studied 21 patients diagnosed with polyneuropathy associated with IgM monoclonal gammopathy in whom all other causes were excluded.[68]. These patients were derived from a group of patients with polyneuropathy and MGUS referred to the University Medical Center in Utrecht for diagnosis and treatment. All patients underwent a comprehensive evaluation including history, physical examination, routine laboratory analysis, immunoelectrophoresis, immunofixation, hematological evaluation, including bone marrow examination. Each six months patients were examined by the same neurologist including a Rankin disability score. Disease course was categorized as either 'progressive' (= deterioration of the motor, sensory or disability scores of one or more points in less than 12 months) or 'slowly progressive' (= deterioration over more than one year). Ataxia was defined by disturbance of gait or limb movements, which intensified when the eyes were closed.[191] Electrophysiologic studies consisting of nerve conduction and concentric needle examination using standardized techniques identified an axonal or demyelinating neuropathy according to conventional criteria.[14]

Antibody studies

Anti-MAG antibodies were measured using Western blot, as previously described.[28] Patients' serum binding to MAG was tested at an initial dilution of 1:1000. The antibody titer is given as the highest serum dilution at which antibody binding to the MAG band is detected. Antibody reactivity against asialo-GM1 (GA1), GM1, GM2, GD1a, GD1b and GQ1b was measured by enzyme-linked immunosorbent assay (ELISA).[66,125,149] Antibody reactivity was confirmed with thin-layer chromatography.[126]Antibody reactivity of the monoclonal gammopathy was further evaluated using kappa- or lambda chain specific peroxidase conjugated antibodies (DAKO, Glostrup, Denmark).

Bone marrow cells

Bone marrow cells were obtained from 21 patients after informed consent. Mononuclear cells were obtained by standard ficoll-hypaque density centrifugation. Flow cytometric analysis was used to identify B cells in the bone marrow samples. Infiltration of monoclonal B cells in the bone marrow samples was determined by analyzing the distribution of kappa and lambda light chains (kappa/lambda ratio) of surface bound immunoglobulin (sIg) on CD 19^+ B cells. All patients had kappa/lambda ratio's <0.3 or >3.0 indicative for the presence of a monoclonal B cell population. B cell purification was performed using magnetic cell sorting (MACS) based on CD 19 expression. Purified B

cells (>95% $CD19^+$) were stored as snap frozen cell pellets (100.000-1.000.000 cells) at -80° C.

Extraction of RNA
RNA was extracted from 100.000-1.000.000 purified mononuclear cells using the RNAbee reagent following the manufacturer's instructions. The RNA pellet was resuspended in RNAse-free H_2O (10-30ul) and stored at -20° C until required.

Preparation of cDNA
cDNA was generated using 3.0 µl RNA, AMV reverse transcriptase (Promega) and a hexaprimer following the manufacturer's instructions. cDNA was stored at -20° C.

PCR analysis
For PCR amplification of V genes, we used a specific 5' primer corresponding to one of the seven human variable heavy chain family leaders (V_H family 1-6, V_H7 is amplified by the V_H1 primer) and a 3' constant region (C_H) primer corresponding to mu (Cµ) heavy chains matching the tumor immunoglobulin isotype.[192] The reaction volume was 50ul containing 140 ng of each primer and Taq polymerase used according to the manufacturer's instructions (Promega). PCR cycle conditions were 1 cycle of [94° C for 2', 60° C for 2', 72° C for 1.5'], 30 cycles of [94° C for 1', 60° C for 1', 72° C for 1.2']. We runned out the PCR products on a 2% agarose gel with ethidium bromide (5 µl/100ml gel volume) and a phichi174RF DNA/HaeIII fragments (0.5µg/µl) for sizing and electrophoresis at 100Volt for 45'. If the V gene product (~360-480bp) was contaminated we cutted out the predicted size of V gene product and elute DNA from agarose slice using manufacturer's protocols for DNA recovery (Quiagen). This DNA was used for cloning.

Cloning
Within the purified B cell population, the predominant cDNA is derived from tumor cells. By cloning PCR DNA and analyzing multiple colonies, the predominant sequence with a common CDR sequence represents the tumor V gene.[193] Cloning of the PCR product was done using the pGEM-T Easy Vector System II (Promega) with E.coli JM109 following the manufacturer's instructions. We picked 12-20 white colonies representing inserts. Plasmids were prepared from 1.5 ml culture broth using manufacturer's instructions for column based purification (Qiagen). Plasmid preparation was eluted in 50 µl sterile H_2O. We confirmed cloned PCR DNA insert by V_H PCR as described in the PCR analysis using 1 µl purified plasmid. Plasmids with verified inserts were sequenced (bidirectional).

DNA sequence analysis

For DNA sequence analysis the ABI Prism 3100 Genetic analyzer was used with the following protocol. Sequencing reactions were set up: Big Dye Terminator version 3.1 (BDT, 1 µl), 3 µl 5x buffer, sequence primer 3.2 pmol/ul (1 µl), ddH_2O (7 µl), eluted PCR DNA (3 µl). PCR amplification was 25 cycles of [96° C for 10'', 50° C for 5'', 60° C for 2']. Sequencing products were purified and the DNA was resuspended in Hi-DI: formamide for fractionation in a sequencing gel. Sequences of 12-20 plasmids of each patient were compared and the sequence which occurred in most plasmids (at least >2) was identified as the clonal sequence of the variable gene of the monoclonal B cell.

Mutational analysis

V gene sequences were analysed using BioEdit Version 5.0.9 and aligned to a web directory: http://www.ncbi.nlm.nih.gov/igblast. The web alignments also defined each functional V gene by FR and CDR domains. For analysis we first identified each germline V(D)J gene element utilized in monoclonal B cell V gene arrangement. The percentage homology to the germline V_H or V_L gene segment was determined from the beginning of FR1 to the end of FR3. For the determination of somatic mutation frequencies, nucleotide differences relative to the corresponding germline genes were considered. The sequence was translated into an amino acid sequence by using http://www.ncbi.nlm.nih.gov/igblast. The number of observed replacement mutations was noted. The ratio of replacement to silent mutations (R:S) was calculated for CDR and FR domains.

Results

The characteristics of the 21 patients with polyneuropathy associated with IgM monoclonal gammopathy are shown in table 1. The median age at onset of the polyneuropathy was 59 years. The median follow-up duration was 9 years. Ten patients had MGUS (48%), ten patients had developed lymphoplasmocytic lymphoma (LPL, 48%), and one patient had developed NHL (5%) at the time of the bone marrow examination. Thirteen (62%) had elevated titers of antibodies to anti-MAG, gangliosides (GA1, GM1, GM2, GD1a, GD1b, GQ1b) or sulfatide (table 1). All patients had infiltrating monoclonal B cells; the percentages of the B cells in the bone marrow samples ranged from 3-90% (median 6%, table 2). From these samples B cells were purified by MACS sorting based on CD19 expression. After MACS with CD19 preparations with > 95% $CD19^+$ B cells were obtained for cDNA preparation and cloning.

Immunoglobulin V gene expression

Multiple clones displaying an identical CDR3 sequence (at least >2) were identified in all 21 patient's samples analyzed and established the presence of a clonal infiltration of B cells. Of these 17 utilized V_H3 family genes, 2 cases V_H1 and 2 cases V_H4 genes in the overrepresented monoclonal B cells (table 2). Identified D and J_H genes are also shown in table 2 as well as the identified V_L and J_L of the light chain. Notably, a predominant usage of V_H3-23 in nine of the 17 patients expressing V_H3 genes (53%) was found (table 2). Analysis of expressed D and J_H as well as V_L and J_L gene segments did not reveal a predominant use of a particular immunoglobulin gene.

IgV gene mutation status

Each of the identified tumor-derived V genes displayed somatic mutations, as evident in deviation in homology to germline genes. For V_H differences in homology ranged from 83% - 97%. Mutations yielded replacement [R] and silent [S] changes in amino acids. The median R:S ratio in CDR was 4 range 0/1 - 12/0), in FR was 1.5 (1/4 – 9/1). The homology of utilized V_L genes to the germline gene sequence was 86% - 98%.

In the tumor-derived B cell clones utilizing V_H3-23 no common mutations in the V_H FR1, 2 and 3 or in the V_H CDR1 and 2 were found (data not shown). The V_H CDR3 length was similar, but without sequence similarities (see table 3).

Table 1 Characteristics of patients with polyneuropathy associated with IgM monoclonal gammopathy

pat	age	M-prot	g/l	type	fup	neuropathy		EMG	treatment	antibody
1	64	IgMk	<1	MGUS	8	slow	s	A	-	sulfatide
2	64	IgMk	16	LPL	8	progr	sm	D	* 1997	MAG, sulfatide
3	67	IgMk	5	MGUS	3	progr	sm	D	-	GM1, GM2
4	57	IgMk	11	LPL	10	progr	s	D	-	GM1,GM2,GD1a
5	69	IgMk	<1	MGUS	3	slow	s	A	-	-
6	64	IgMk	<1	MGUS	4	progr	s	D	-	-
7	66	IgMk	7	LPL	4	progr	s	D	-	-
8	55	IgMk	<1	LPL	1	slow	s	n.d.	-	GQ1b
9	45	IgMk	16	MGUS	12	progr	sm	D	** 2002	GD1a, sulfatide
10	54	IgMk	<1	MGUS	9	progr	sm	D	** 2000	MAG
11	60	IgMk	12	LPL	9	progr	sm	D	* 1998	-
12	50	IgMk	<1	MGUS	8	progr	s	D	* 1999	GD1a,GD1b,GQ1b
13	56	IgMk	8	NHL	22	progr	sm	D	* 1997	MAG, sulfatide
14	52	IgMl	9	LPL	18	progr	sm	D	* 1997	MAG
15	50	IgMk/l	<1	LPL	12	slow	sm	D	* 2000	MAG
16	65	IgMk	8	LPL	10	progr	sm	A	* 1995	-
17	56	IgMk	<1	MGUS	2	progr	sm	A	-	-
18	53	IgMk	7	MGUS	15	slow	s	A	-	-
19	59	IgMk	15	LPL	5	progr	sm	D	-	MAG
20	45	IgMk/l	<1	MGUS	11	progr	sm	D	* 1999	GM2, GD1a, GD1b
21	74	IgMl	23	LPL	4	progr	s	A	-	-

Bone marrow examination performed in 2004; M-prot = M-protein; type = hematologic disease, LPL = lymphoplasmocytic lymphoma; fup = follow-up in years; progr = progressive, slow = slowly progressive; s = sensory, sm = sensorimotor; n.d. = not done; last treatment with * cyclophosphamide and prednison, or ** fludarabin.

Table 2 analysis of immunoglobulin genes in polyneuropathy associated with monoclonal gammopathy

pat	% B cells	monoclonal population	germline VH gene	% =	R/S CDR	R/S FR	D	JH	germline VL gene	% =	JL
1	3	IgM k	Vh1-2	92	3/2	7/7	D1-26	JH4	VK L11	87	JK4
2	11	IgM k	Vh1-3	89	9/0	11/5	D3-16	JH1	VK L2	96	JK1
3	6	IgM k	Vh3-15	91	4/3	5/8	no	JH4	VK A30	93	JK4
4	8	IgM k	Vh3-15	90	4/3	6/9	D7-27	JH4a	VK B3	93	
5	7	IgM k	**Vh3-23**	90	8/2	7/4	D5-24	JH3b	VK L8	92	JK1
6	7	IgM k	**Vh3-23**	92	5/2	6/4	no	JH3b	VK L5	96	JK4
7	8	IgM k	**Vh3-23**	92	8/1	4/1	D4-17	JH4b		92	
8	68	IgM k	**Vh3-23**	85	8/2	10/4	D5-24	JH4a	VK A27	94	JK2
9	3	IgM k	**Vh3-23**	92	8/1	4/4	D4-17	JH6	VK O2	92	JK4
10	9	IgM k	**Vh3-23**	87	10/1	9/5	D7-27	JH4b	VK A20	87	JK4
11	10	IgM k	**Vh3-23**	90	8/1	5/2	no	JH3b	VK L14	95	
12	9	IgM k	**Vh3-23**	90	8/2	7/7	D3-10	JH4	VK L8	90	
13	15	IgM k	**Vh3-23**	91	8/2	3/5	no	JH5	VK L8	89	
14	11	IgM l	Vh3-30	93	5/1	5/3	D6-19	JH3b	VL2-23	90	JL3
15	50	IgM k	Vh3-48	93	6/1	4/4	no	JH5	VK L5	91	JK4
16	9	IgM k	Vh3-72	91	6/4	3/2	no	JH4b	VK A30	94	JK4
17	8	IgM k	Vh3-72	97	0/1	2/3	D2-2	JH3		97	
18	7	IgM k	Vh3-73	90	8/0	3/1	D5-24	JH5	VK B3	95	JK1
19	90	IgM k	Vh3-73	86	12/0	6/5	no	JH6	VK O2	98	JK2
20	12	IgM k	Vh4-59	96	2/1	5/1	no	JH3b	VK A30	93	JK1
21	14	IgM l	Vh4-59	95	3/2	1/4	no	JH5	VL2-11	94	JL7

% B cells after MACS > 95 %, % = % homology with closest germline gene

Table 3 Aminoacid sequence of CDR3 of Vh3-23

pat	antibody reactivity	germline gene	CDR3 length	amino acid sequence
5	-	VH3-23	9	R N G R D G Y I S
6	-	VH3-23	9	L L L P N G R W G
7	-	VH3-23	10	T T E I Q W X L X G
8	GQ1b	VH3-23	9	R N G R D G Y I S
9	GD1a, sulfatide	VH3-23	10	T T V T K K F L F T
10	MAG	VH3-23	10	R P E V T N V G G P
11	-	VH3-23	9	L P Q T R S D D A
12	GD1a, GD1b, GQ1b	VH3-23	9	G G G S G S H F W
13	MAG, sulfatide	VH3-23	9	H T W R Q S I G V

Discussion

We analyzed the expression of immunoglobulin variable regions genes in bone marrow derived surface IgM (sIgM) monoclonal B cells from 21 patients with polyneuropathy associated with IgM monoclonal gammopathy. This is the largest single group of patients that has been studied. So far, in the literature 14 patients have been analysed by several research groups.[74,75,185-190] Our results confirm and extend the findings published previously by showing that the immunoglobulin variable region gene of most patients carried mutations. Replacement/silent mutations in framework and variable regions show a distribution pattern that is comparable to that found in antigen selected memory B cells.[192]

V_H gene family usage in normal peripheral sIgM B cells resembles germline complexity with the largest V_H gene family (V_H3) found most often. The 22 functional gene segments of V_H3 are used by approximately 50% of functional sIgM positive B cells.[193,194] In our cohort of patients most (17/21, 81%) express V_H3 gene family encoded immunoglobulin with the V_H3-23 gene being used in 43% of the patients (9/21). This observation suggests a disease associated prominence. It parallels other findings associating V_H3-23 in autoimmune responses against DNA, rheumatoid factor, I-antigens, myosin and is also identified in Graves' disease, ankylating spondylitis and Wegener's granulomatosis, either in germline or somatic mutated configuration.[195-199] It is unclear at present whether autoreactivity is based on recognition of single or multiple, different antigen determinants. Since in this and in other studies no preference or specific D, J_H or specific CDR3 configurations are found, the former explanation seems unlikely.

Our findings show a consistent feature of somatic mutations in immunoglobulin V genes. These results agree with previously published data.[74,75,185-190] The percentages are comparable to those described for normal antigen-selected memory B cells.[200,201] While it is not readily feasible to ascribe a role for a conventional antigen in selecting mutations, a role for antigen is implicated as there is a mechanistic requirement for signals via surface immunoglobulin to initiate mutational activity. Although, human memory B cells may be generated in response to T cell dependent antigens in conventional germinal centers, ectopic events can also occur.[200] The splenic marginal zone is an unique B cell compartment that contains B cells capable of responding to blood borne T cell independent antigens.[202] These B cells carry somatic mutations that can be raised after immunization with T-independent polysaccharides.[203] Interestingly, nearly all V_H genes in our patient group (18/21) are associated with antibody responses to capsular polysaccharides of H. Influenza type b, Staphylococcus A., Streptococcus pneumoniae and Neisseiria meningitides (V_H1-3, V_H3-15,

V_H3-23, V_H3-30, V_H3-48, V_H3-73 and V_H3-74, V_H4-59).[204-207] In addition, if available information of patients with polyneuropathy associated with IgM anti-MAG antibodies reported in earlier studies (8 patients) is included in this analysis, 26 from 29 patients use V_H genes associated with polysaccharide responses.[74,75,185-188] Protective IgM antibodies to encapsulated bacteria all carry somatic mutated immunoglobulin genes.[208] Collectively, these data suggest that polyneuropathy associated with IgM anti-neural antibodies may be caused by an immune response to bacterial polysaccharides, which recruit somatically mutated auto reactive B cells.

B cell malignancies are considered to arise from normal lymphocytes at different stages of B cell development. Analysis of the V_H genes of the monoclonal immunoglobulin molecule is a useful approach to trace the stage of neoplastic transformation. The analysis presented in this study show that the monoclonal B cells from the majority of patients with polyneuropathy with an associated IgM M-protein contain mutated V_H genes in a pattern that suggests a role for antigen. The presence and involvement of the V_H genes found in our patients in early life, autoimmune and polysaccharide responses suggest a mantle zone origin and clearly differs from V_H gene distributions found in malignancies derived from post-germinal center B cells, like multiple myeloma. [208,209, 210]

In summary, monoclonal B cells from patients with polyneuropathy associated with IgM monoclonal gammopathy display a limited set of V_H genes that are also employed in polysaccharide responses. The large majority of patients showed somatic mutations in their immunoglobulin variable region genes that are likely the result of antigenic stimulation. We hypothesize that the autoantibodies in patients with polyneuropathy arise during bacterial antigen driven B cell responses. Induction of continuous B cell proliferation either by bacterial polysaccharides or autoantigen is a risk factor for malignant transformation resulting in B cell malignancy secreting autoreactive monoclonal antibodies.

Chapter 7

General discussion: the work-up of a patient with polyneuropathy and monoclonal gammopathy

adapted from

'Eurelings M, Franssen H, Notermans NC. The work-up of a patient with polyneuropathy and monoclonal gammopathy. In preparation'

General discussion

The general discussion of this thesis concerns three questions: 1) What is the first event in the pathogenesis of polyneuropathy associated with monoclonal gammopathy? 2) What can explain the increased incidence of malignant transformation in patients with MGUS and polyneuropathy? 3) What are the guidelines for additional investigations and follow-up in a patient with polyneuropathy and a monoclonal gammopathy?

The discussion concerning questions 1) and 2) is concentrated on polyneuropathy associated with IgM monoclonal gammopathy, since the relationship between IgM monoclonal gammopathy and polyneuropathy is best supported.[17,22]

What is the first event in the pathogenesis of polyneuropathy associated with monoclonal gammopathy?
Theoretically there are two possibilities. A) A clonal B cell population, developed by mistake during B cell development, coincidentally produces monoclonal antibodies with reactivity against peripheral nerve autoantigens. B) Infectious agents, like bacteria, share epitopes with peripheral nerve antigens, and in this way induce a cross reactive immune response against these self antigens. Chronic stimulation of these B cells by antigens leads to an expansion of a single B cell clone. This phenomenon has been demonstrated in B cell malignancies Helicobacter pylori (H. pylori) in mucosa associated lymphoid tissue (MALT) lymphoma, hepatitis C in chronic lymphatic leukemia (CLL) and Epstein-Barr virus (EBV) in Burkitt's lymphoma.[211-214] In addition, the peripheral nerve autoantigens can chronically stimulate the B cell to proliferate and expand. In all patients with monoclonal IgM antibodies the immunoglobulin V genes used for encoding nucleotide sequences of the antigen binding site were associated with responses against encapsulated bacteria. This finding supports the possibility that during immune responses against bacterial antigens IgM antibodies are generated that cross-react against nerve antigens, like myelin-associated glycoprotein (MAG) or gangliosides, eventually leading to neuropathy (possibility B, Chapter 6). To gain more insight in this intriguing mechanism we plan to study the anti-bacterial specificity of the autoreactive monoclonal IgM antibodies in our cohort of patients.[127] Another line of research could be serial analysis of the mutational status of immunoglobulin genes, antibody affinity, effect of the monoclonal antibodies in muscle-nerve preparations and the progression of polyneuropathy.[215] Hereby, the relation of ongoing mutations, antibody affinity and progression of the polyneuropathy can be analyzed.

What can be the explanation of the increased incidence of malignant transformation in patients with MGUS and polyneuropathy?

In patients with polyneuropathy and MGUS the incidence of malignant transformation is increased compared to patients with MGUS without polyneuropathy (Chapter 4).[149,170] One explanation could be the prevalence of cytogenetic risk factors for malignant transformation in patients with polyneuropathy associated with MGUS. Using a cytogenetic approach we did not identify a high prevalence of one cytogenetic aberration in patients with polyneuropathy and IgM monoclonal gammopathy. However, we identified IgH translocations, also found in MGUS, multiple myeloma and B cell malignancies in half of the patients (Chapter 5). Another explanation for the relation of progression of the polyneuropathy and malignant transformation could be the chronic stimulation of the (pre-malignant) monoclonal B cells by autoantigen. The proliferative capacity and expanding clonal size would increase the risk for additional genetic aberrations and eventually for a full malignant transformation.[212] We found a high incidence of intra-clonal heterogeneity within the clonal B cell populations of patients with polyneuropathy associated with IgM monoclonal gammopathy. This suggests that the monoclonal B cells are still subject to stimuli, possibly autoantigens. This mechanism of malignant transformation is also identified in MALT lymphoma where continuous presence of viral or bacterial antigens of H. pylori (or Borrelia Burgdorferi, Campylobacter jejuni) eventually leads to clonal B cell expansion.[213] Whether intra-clonal heterogeneity leads to higher affinity IgM autoantibodies and thereby to progression of the polyneuropathy is going to be addressed.

What are the guidelines for additional investigations and follow-up in a patient with polyneuropathy and a monoclonal gammopathy?

For a clinical neurologist, the most important question is how to evaluate a patient with polyneuropathy and a monoclonal gammopathy. In every standard protocol for the work-up of a patient with polyneuropathy a search for monoclonal gammopathy is advocated (http://www.cbo.nl).[216-218] Once a monoclonal gammopathy is found it is important to have guidelines for additional investigations and follow-up. Is an intensive search for an underlying hematologic malignancy indicated in every patient with polyneuropathy and monoclonal gammopathy, even if the monoclonal gammopathy level is low? Is there evidence in an individual patient for a relationship between the polyneuropathy and the monoclonal gammopathy? Is there a difference between patients with IgM MGUS and patients with IgG or IgA MGUS?

In patients with polyneuropathy and monoclonal gammopathy the disease course indicates the diagnostic process and the additional investigations (Chapter 2, Chapter 4). A progressive disease course is defined as a deterioration of the neuropathy leading to decline of motor and sensory sum scores or disability on the Rankin disability score over months (< 12 months). A slowly progressive or non-progressive disease course is defined as a deterioration of the neuropathy over years.

Progressive course (see flow chart)

Progressive polyneuropathy is an independent predictor of malignant transformation in these patients (Chapter 4).[170] Therefore, in patients with a progressive polyneuropathy associated with monoclonal gammopathy, extensive hematologic screening, including laboratory analyses and bone marrow examination (both immunophenotyping and pathologic examination), is justified. A hematologic malignancy is found in 10-30% of the patients with polyneuropathy associated with monoclonal gammopathy.[21,170]

Both in patients with a hematologic malignancy as in patients in whom a hematologic malignancy has been excluded, EMG examination should be performed. Demyelination, fulfilling the electrophysiologic criteria[34] is in favor of the diagnosis of polyneuropathy associated with IgM MGUS or CIDP. Progressive axonal asymmetric painful polyneuropathy can be caused by vasculitis in association with cryoglobulinemia, non-systemic vasculitis, or amyloidosis.[10,65] If cryoglobulines are absent a nerve/muscle biopsy must be done to identify vasculitis or amyloid depositions (Chapter 2).[219-221] In patients suspected of amyloidosis with a rapidly progressive disease course, painful neuropathy and autonomic failure without amyloid deposits in

bone marrow or nerve biopsy, Congo red staining of a rectal or skin biopsy should be performed.

Non-progressive course (see flow chart)
When the disease course of the polyneuropathy in patients with MGUS is not progressive the level and isotype of the M-protein and the antibody reactivity indicate the further process. In case of an M-protein level >10 g/l or clinical risk factors for a hematologic malignancy (anemia, bone pain, weight loss, night sweats) screening is indicated accordingly (Chapter 4).[32,39] If the M-protein level is low and no clinical risk factors are present the chance to find a hematologic disease that influences the prognosis is very small.

When the disease course of the polyneuropathy is not progressive and the M-protein is of the IgM isotype, than antibody reactivity should be tested. Half of the patients with polyneuropathy associated with IgM monoclonal gammopathy have anti-MAG antibodies. These patients predominantly have a polyneuropathy that is characterized by slow progression, symmetric sensory or sensorimotor symptoms, ataxia and demyelination.[25] Of the patients with IgM monoclonal gammopathy without anti-MAG reactivity, 30% have monoclonal anti-ganglioside antibodies (Chapter 3).[149] Patients with monoclonal IgM anti-ganglioside (GM1, GM2, GD1a, GD1b and GQ1b) antibodies predominantly have a demyelinating polyneuropathy and ataxia. EMG examination is recommended to confirm demyelination. Patients with similar initial symptoms and electrophysiologic features have a similar prognosis independent of antibody reactivity (Chapter 3). T cell infiltrates are small in slowly progressive neuropathy associated with IgM monoclonal gammopathy in both patients with and without anti-MAG antibodies (Chapter 2) This suggests that the pathogenesis of the polyneuropathy in patients with IgM monoclonal gammopathy without anti-MAG or anti-ganglioside reactivity is similar and these patients most likely have antibody reactivity against other components of the nerve, although these antigens are not yet identified.[17,22] When no antibody reactivity is found, EMG examination is performed. At the onset of the disease the EMG examination shows demyelination characteristic of polyneuropathy associated with IgM MGUS, but after long term disease axonal degeneration may predominate.[8,52,222] In patients with polyneuropathy associated with monoclonal gammopathy and a non-progressive disease course nerve biopsy is not indicated (Chapter 2).

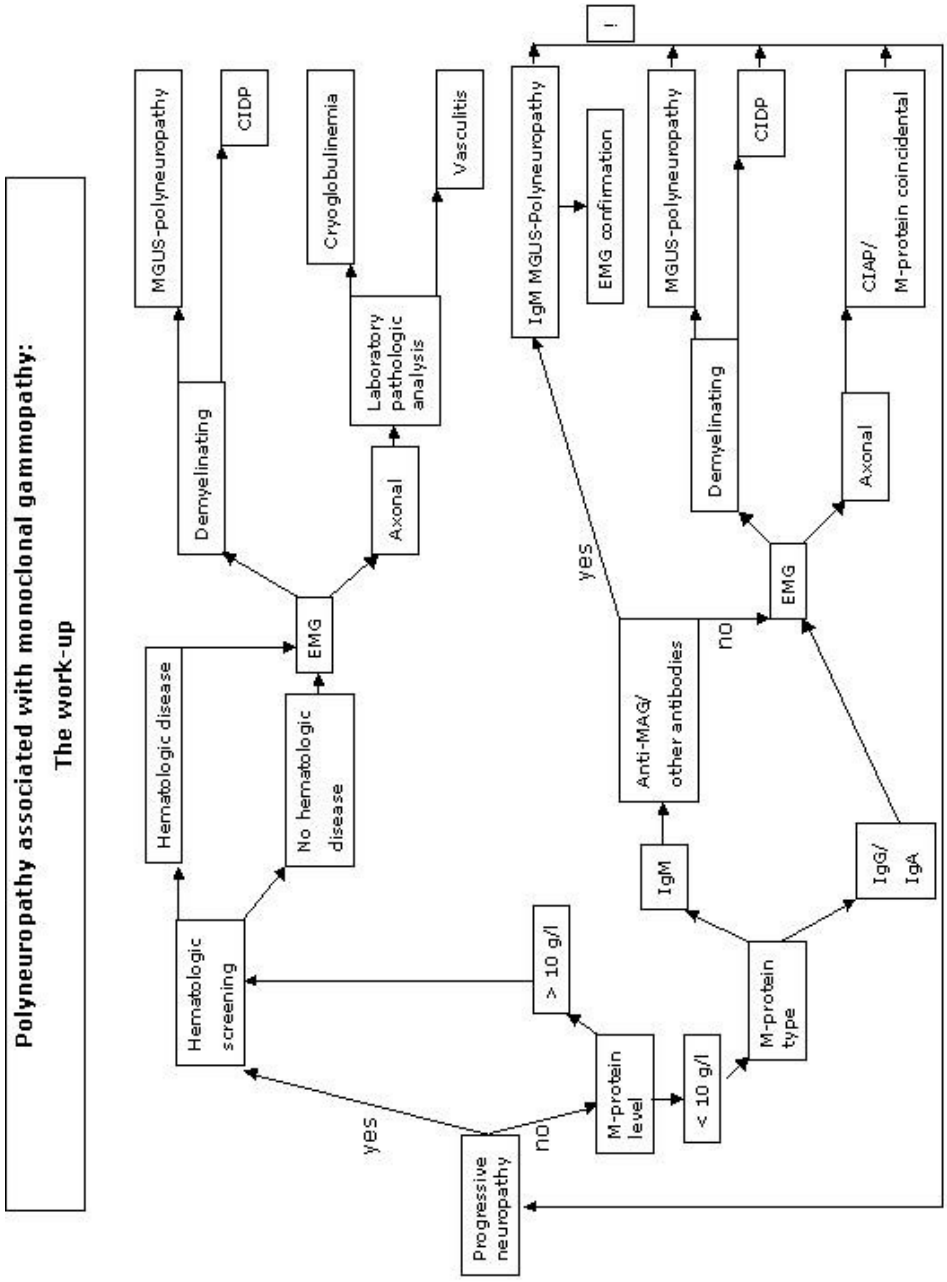
During follow-up hematologic screening, including bone marrow examination, should be repeated when the polyneuropathy is progressive, the M-protein level rises, or the patient has symptoms like weight loss, bone pain or night sweats (Chapter 4).[32,39,149,170]

When the disease course of the polyneuropathy is not progressive and the M-protein is of the IgG or IgA isotype EMG examination is performed. The relation of IgG or IgA monoclonal gammopathy with polyneuropathy is less evident. Most persons with MGUS have IgG monoclonal protein (75% IgG M-protein) but no polyneuropathy. In contrast, patients with neuropathy more frequently have IgM MGUS.[16,33] Interestingly, patients with a demyelinating polyneuropathy and IgG monoclonal gammopathy show clinical and electrophysiologic features of CIDP. Consequently, we and others favor a diagnosis of CIDP with IgG MGUS in these patients.

The significance of monoclonal gammopathy for the pathogenesis of primary axonal polyneuropathy is not clear. The chance that the monoclonal gammopathy is a coincidental finding in elderly patients with chronic axonal polyneuropathy is high since both the frequency of monoclonal gammopathy and the frequency of axonal idiopathic polyneuropathy rise with age.[25,53] The disease course of chronic idiopathic axonal polyneuropathy with and without monoclonal gammopathy does not significantly differ.[14,223]

In conclusion the work-up of patients with polyneuropathy associated with monoclonal gammopathy is complicated and awareness of possible hematologic malignancies in these patients is of great importance.

In the flow chart we propose a scheme for the work-up of a patient with polyneuropathy and monoclonal gammopathy. Yearly follow-up of all patients must include medical history taking with attention for clinical risk factors (fatigue, bone pain, weight loss, night sweats), physical examination, neurologic examination, routine laboratory analysis and determination of the M-protein level. A hematologic screening, including bone marrow examination should be performed if the patient has clinical risk factors or anemia, the M-protein level rises, or the polyneuropathy is progressive (!). Bone marrow examination enables early detection of malignant transformation and timely installment of appropriate treatment to prevent serious complications.


Polyneuropathy associated with monoclonal gammopathy:
The work-up
Progressive neuropathy
yes
Hematologic screening
Hematologic disease
No hematologic disease
EMG
Demyelinating
Axonal
MGUS-polyneuropathy
CIDP
Laboratory pathologic analysis
Cryoglobulinemia
Vasculitis
no
M-protein level
> 10 g/l
< 10 g/l
M-protein type
IgM
IgG/ IgA
Anti-MAG/ other antibodies
yes
IgM MGUS-Polyneuropathy
EMG confirmation
no
EMG
Demyelinating
Axonal
MGUS-polyneuropathy
CIDP
CIAP/ M-protein coincidental
!

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Summary

Polyneuropathy associated with monoclonal gammopathy is a peripheral nerve disease. In patients with polyneuropathy associated with monoclonal gammopathy serum monoclonal antibodies are present. The relation between these monoclonal antibodies and polyneuropathy is best supported for polyneuropathy associated with IgM monoclonal anti-myelin associated glycoprotein (anti-MAG) antibodies. These anti-MAG antibodies are reactive against peripheral nerve autoantigen, thereby causing an autoimmune mediated polyneuropathy. Polyneuropathy associated with monoclonal IgM anti-MAG antibodies usually is a slowly progressive, symmetrical, sensory or sensorimotor polyneuropathy with ataxia.

The studies of the cause and consequence of polyneuropathy associated with monoclonal gammopathy presented in this thesis are now summarized.

To further discriminate clinical entities, we analyzed the presence of T cells in sural nerve biopsies in primary demyelinating and primary axonal polyneuropathies associated with monoclonal gammopathy. Using immunohistochemical analysis we investigated the number and distribution of sural nerve T cells. T cells were predominantly localized perivascular and perineurial. T cell infiltration was compared with normal controls and patients with chronic idiopathic axonal polyneuropathy (CIAP), a non-inflammatory neuropathy. Increased T cell densities compared with CIAP patients and normal controls were found in: 1) patients with progressive demyelinating polyneuropathy associated with IgG monoclonal gammopathy, with T cell densities (T cells/mm^2) comparable to densities in patients with chronic inflammatory demyelinating polyneuropathy (CIDP); 2) patients with progressive axonal polyneuropathy associated with monoclonal gammopathy. The pathologic features in the biopsies of these patients resembled those found in vasculitic neuropathy. T cell densities in patients with IgM monoclonal gammopathy were significantly lower than in patients with IgG monoclonal gammopathy. In the nerve biopsies of patients with polyneuropathy associated with monoclonal IgM anti-MAG antibodies very low T cell densities were found.

In polyneuropathy associated with IgM monoclonal gammopathy, antibodies to myelin-associated glycoprotein (MAG), sulfoglucuronyl paragloboside (SGPG) and sulfatide have been associated with specific clinical and electrophysiologic features. However, it is not known whether the results of antibody tests provide additional information for the individual patient (and the neurologist) on future neurologic deficit

or outcome. Therefore, we studied the potential prognostic value of clinical, electrophysiologic, pathologic and antibody determination (Chapter 3). In univariate analysis, initial symptoms, IgM light chain type, electrophysiologic and pathologic studies, sural nerve IgM deposition, and anti-MAG or anti-SGPG antibodies were significantly associated with outcome. However, in multivariate analysis only initial symptoms and electrophysiologic studies are independent prognostic factors: initial sensory symptoms of the feet is associated with a slowly progressive disease course and less disability at four years. Demyelination on electrophysiologic examination is prognostic for development of weakness and symptoms of upper extremities at four years.

Half of the patients with polyneuropathy associated with IgM monoclonal gammopathy have anti-MAG antibodies. Since patients with IgM monoclonal gammopathy without anti-MAG antibodies have similar symptoms and comparable response to treatment, it is most likely that these patients have antibody reactivity against other components of the nerve. We found monoclonal anti-ganglioside antibodies in half of the patients with polyneuropathy associated with IgM monoclonal gammopathy without anti-MAG antibodies (Chapter 3). Monoclonal IgM anti-GQ1b antibodies were associated with ataxia.

Monoclonal gammopathy is of undetermined significance (MGUS) as long as no hematologic malignancy, like multiple myeloma or lymphoma is found. In patients with polyneuropathy associated with monoclonal gammopathy the frequency of hematologic malignancy is high (22%). A follow-up study of patients with polyneuropathy associated with MGUS showed that the incidence of malignant transformation is higher (2.7/100 patient years, 95% CI 1.52-4.22) than in patient with MGUS without polyneuropathy (1/100 patient years, 95% CI 0.85-1.24). Progression of the polyneuropathy, weight loss, unexplained fever or night sweats and M-protein level were independent predictors (Chapter 4).

To find an explanation for the increased frequency of hematologic malignancies in polyneuropathy associated with monoclonal gammopathy we studied the occurrence of genetic aberrations (Chapter 5). We isolated B cells from bone marrow samples of patients with polyneuropathy associated with IgM monoclonal gammopathy. In these B cells we studied the occurrence and nature of cytogenetic aberrations by using interphase fluorescence in situ hybridisation (FISH), multiplex ligation-dependent probe amplification (MLPA) assay and genome-wide array-based comparative genomic hybridisation (CGH). We identified different genetic aberrations in patients with polyneuropathy associated with IgM monoclonal gammopathy. Half of

the patients had chromosome 14 translocations, which are also frequently identified in multiple myeloma and B cell malignancies.

The immunoglobulin is composed of heavy and light chains both consisting of a variable and a constant part. The variable part is responsible for the antigen binding. Immunoglobulin genes encoding the variable part (V, D, J) by assembling lead to a functional antigen binding site of the immunoglobulin (antibody). In autoimmune diseases certain immunoglobulin genes are preferentially used. In addition to previous studies of immunoglobulin usage in patients with polyneuropathy associated with anti-MAG antibodies, we determined the immunoglobulin usage in patients with polyneuropathy associated with IgM monoclonal gammopathy (Chapter 6). Therefore, we isolated the B cell of bone marrow samples of patients with polyneuropathy associated with IgM monoclonal gammopathy. Of these B cells we isolated the DNA. Using constant and variable part specific primers DNA was amplified. The PCR product (copies of the immunoglobulin gene) was cloned after transfection to bacteria. In this way each bacterial clone contains the DNA sequence of one immunoglobulin gene. After comparison of the predominant sequence with known immunoglobulin gene sequences the used immunoglobulin gene is identified and base pare differences are identified as mutations. We found a preferential use of immunoglobulin genes associated with immune responses to encapsulated bacteria (preferentially V_H3-23) with mutations in the variable part of the immunoglobulin gene suggesting an antigen driven immune response.

In patients with polyneuropathy and monoclonal gammopathy, after excluding other causes, the disease course leads the diagnostic process and the additional investigations (Chapter 7, flow chart). In patients with a progressive polyneuropathy associated with monoclonal gammopathy, extensive hematologic screening is indicated to exclude a hematologic malignancy. Both in patients with a hematologic malignancy and in patients in whom a hematologic malignancy has been excluded, EMG examination should be performed to differentiate between demyelinating and axonal polyneuropathy. In patients with progressive axonal polyneuropathy, cryoglobulinemia needs to be excluded and muscle/nerve biopsy is performed to exclude vasculitis. In patients with progressive demyelinating polyneuropathy CIDP needs to be considered. When the disease course of the polyneuropathy is not progressive antibody reactivity is determined in patients with IgM M-protein. EMG examination is performed to identify a demyelinating or an axonal neuropathy. In patients with slowly progressive axonal polyneuropathy the monoclonal gammopathy is probably a coincidental finding. After exclusion of other causes these patients probably have

chronic idiopathic axonal polyneuropathy (CIAP). In conclusion, the work-up of patients with polyneuropathy associated with monoclonal gammopathy is complicated and awareness of possible hematologic malignancies in these patients is of great importance. We advise a yearly follow-up of all patients with polyneuropathy associated with monoclonal gammopathy, including M-protein level determination. A hematologic screening, including bone marrow examination should be performed if the patient has clinical risk factors of malignant transformation (weight loss, night sweats, bone pain, anemia, rise of M-protein level) or a progressive polyneuropathy.

Samenvatting

Polyneuropathie geassocieerd met monoclonale gammopathie is een aandoening van perifere zenuwen. Bij polyneuropathie geassocieerd met monoclonale gammopathie wordt in het bloed een monoclonale antistof gevonden. De relatie tussen monoclonale antistoffen en polyneuropathie is het best bewezen voor polyneuropathie geassocieerd met monoclonale IgM anti-myeline geassocieerde glycoproteine (anti-MAG) antistoffen. De anti-MAG antistoffen zijn gericht tegen antigenen in het eigen zenuwweefsel. Hierdoor ontstaat een autoimmuun gemedieerde neuropathie. Polyneuropathie geassocieerd met IgM monoclonale anti-MAG antistoffen is meestal een langzaam progressieve, symmetrische sensorische of sensorimotorische polyneuropathie met ataxie.

Het onderzoek naar de oorzaken en gevolgen van polyneuropathie geassocieerd met monoclonale gammopathie beschreven in dit proefschrift worden hier samengevat.

Om verschillende entiteiten te onderscheiden binnen polyneuropathie geassocieerd met monoclonale gammopathie hebben we T cel infiltratie in zenuwbiopten geanalyseerd (hoofdstuk 2). Met immuunhistochemische technieken hebben we de dichtheid en verdeling van T cellen in zenuw biopten bestudeerd. In alle biopten werd T cel infiltratie gevonden. Deze T cellen waren vooral gelokaliseerd rondom de vaatjes in het perineurium. De T cel infiltratie werd vergeleken met normale controles en met patiënten met chronische idiopathische axonale polyneuropathie (CIAP) waarbij in het ziekteproces inflammatie geen rol speelt. De T cel infiltratie was, in vergelijking met deze controles verhoogd in twee groepen: 1) patiënten met progressieve demyeliniserende polyneuropathie geassocieerd met IgG monoclonale gammopathie en de T cel densiteit (T cellen /mm^2) in deze biopten was in dezelfde range als in patiënten met chronische inflammatoire demyeliniserende polyneuropathie (CIDP); 2) patiënten met progressieve axonale polyneuropathie geassocieerd met monoclonale gammopathie. De afwijkingen in de zenuw biopten van patiënten met progressieve axonale polyneuropathie met T cel infiltratie leken op de afwijkingen welke gevonden worden bij non-systemische vasculitis neuropathie. Pijn stond bij deze patiënten op de voorgrond en zij reageerden goed op behandeling met prednison. T cel infiltratie in zenuwbiopten van patiënten met IgM monoclonale gammopathie was significant lager dan in biopten van patiënten met IgG monoclonale gammopathie. Bij patiënten met polyneuropathie geassocieerd met monoclonale IgM anti-MAG antistoffen werd nauwelijks T cel infiltratie gevonden.

In polyneuropathie geassocieerd met IgM monoclonale gammopathie zijn de antistoffen tegen MAG en sulfatide geassocieerd met bepaalde klinische en electrofysiologische kenmerken. Omdat het onbekend is of de bepaling van de antistoffen een toegevoegde waarde heeft bij het bepalen van de prognose hebben wij de onafhankelijke voorspellende waarde van klinische, electrofysiologische, pathologische en antistofbepalingen onderzocht (hoofdstuk3). In univariate analyse waren de eerste symptomen, de electrofysiologische kenmerken (axonaal of demyeliniserend), depositie van IgM in het zenuwbiopt en anti-MAG antistoffen geassocieerd met de prognose, maar in multivariate analyse waren alleen de eerste symptomen en electrofysiologische kenmerken onafhankelijke voorspellers voor de prognose. Gevoelstoornissen aan de voeten als eerste symptoom was voorspellend voor een langzaam progressief beloop. Demyelinisatie voorspelt het ontwikkelen van krachtsverlies en symptomen aan de armen.

Bij patiënten met polyneuropathie geassocieerd met IgM MGUS wordt in ongeveer 50% van de patiënten antistoffen tegen MAG gevonden. Aangezien de klinische verschijnselen en de reactie op immuunmodulerende therapie bij patiënten met polyneuropathie geassocieerd met IgM monoclonale gammopathie met en zonder anti-MAG antilichamen gelijk is spelen bij de patiënten zonder anti-MAG antistoffen waarschijnlijk antistoffen tegen andere antigenen een rol. Wij vonden bij de helft van de patiënten met IgM MGUS zonder anti-MAG antistoffen monoclonale antistoffen tegen gangliosiden. Monoclonale IgM anti-GQ1b antistoffen waren geassocieerd met sensorische ataxie (hoofdstuk 3).

De monoclonale gammopathie is van onbekende betekenis, "monoclonal gammopathy of unknown significance" (MGUS) zolang geen onderliggende hematologische maligniteit wordt gevonden zoals multipel myeloom of lymfoom. Bij patiënten met polyneuropathie geassocieerd met monoclonale gammopathie is de prevalentie van hematologische maligniteiten hoog (22%). Uit follow-up onderzoek bij patiënten met polyneuropathie geassocieerd met MGUS bleek dat de kans op maligne transformatie van MGUS groter is bij patiënten met polyneuropathie en MGUS (2.7/100 patiënt jaren, 95% CI 1.52/100-4.22/100 patiënt jaren), dan bij patiënten met MGUS zonder polyneuropathie (1/100 patiënt jaren, 95% CI 0.85/100-1.24/100 patiënt jaren). Onafhankelijke voorspellers voor maligne transformatie waren progressie van de polyneuropathie, gewichtsverlies, B-symptomen en de hoogte van het monoclonale eiwit gehalte in het bloed (hoofdstuk 4).

Om een verklaring te vinden voor de verhoogde prevalentie van hematologische maligniteiten bij polyneuropathie geassocieerd met monoclonale gammopathie hebben we gekeken naar het voorkomen van genetische aberraties (hoofdstuk 5). Wij hebben uit beenmergaspiraten van patiënten met polyneuropathie geassocieerd met IgM monoclonale gammopathie B-cellen geïsoleerd. In deze B-cellen hebben wij met behulp van interfase fluorescence in situ hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA) assay en genome-wide array-based comparative genomic hybridization (CGH) gezocht naar genetische afwijkingen welke bij B-cel maligniteiten voorkomen. Wij vonden verschillende genetische aberraties bij patiënten met polyneuropathie geassocieerd met IgM monoclonale gammopathie. Het meest frequent werden chromosoom 14 translocaties gevonden welke ook bij multipel myeloom en bij verschillende lymfomen voorkomen.

Het immunoglobuline is opgebouwd uit zware en lichte ketens, welke bestaan uit een variabel en een constant deel. Het variabele deel is verantwoordelijk voor de antigen binding. Immunoglobuline genen die het variabele deel coderen (V, D, J) vormen door aaneenschakeling een functionele antigen bindingsplaats van het immunoglobuline (antistof). Bij autoimmuunziekten worden bepaalde variabele immunoglobuline genen preferentieel gebruikt. In aansluiting op eerder onderzoek bij patiënten met polyneuropathie geassocieerd met anti-MAG antistoffen hebben wij het gebruik van immunoglobuline genen bij patiënten met polyneuropathie geassocieerd met IgM monoclonale gammopathie onderzocht (hoofdstuk 6). Wij hebben daarvoor B-cellen geïsoleerd uit beenmergaspiraten van patiënten met polyneuropathie geassocieerd met IgM monoclonale gammopathie. Uit deze B-cellen hebben wij DNA geïsoleerd. Met primers voor de variabele en constante delen van de immunoglobuline genen hebben wij het DNA geamplificeerd. Het PCR product (kopie van het immunoglobuline gen) hebben wij met behulp van een vector ingebracht in bacterieën en gekloneerd. Iedere bacterie bevat op deze manier de DNA sequentie van één immunoglobuline gen. De sequentie welke in het merendeel van de bacterie klonen wordt gevonden is de sequentie van het monoclonale immunoglobuline gen. Na vergelijking met bekende sequenties van immunoglobuline genen kan het gebruikte gen geïdentificeerd worden en enkele base paar grote afwijkingen van de sequentie worden geduid als mutaties. Wij vonden preferentieel gebruik van immunoglobuline genen betrokken bij de immuunrespons tegen bacteriële polysacchariden (vooral V_H3-23) en mutaties in de variabele delen van de immunoglobuline genen passend bij een antigeen gedreven immuunrespons.

Bij de diagnose en work-up van een patiënt met polyneuropathie geassocieerd met monoclonale gammopathie, waarbij andere oorzaken van polyneuropathie uitgesloten zijn, is het ziektebeloop bepalend voor de uitgebreidheid van de diagnostiek (hoofdstuk 7, flow chart). Bij patiënten met een snel progressief beloop is allereerst hematologisch onderzoek nodig om een hematologische maligniteit uit te sluiten. Vervolgens is, zowel bij patiënten met een hematologische maligniteit als bij patiënten met MGUS, EMG onderzoek nodig om te differentiëren tussen een demyeliniserende of axonale polyneuropathie. Bij een snel progressieve axonale polyneuropathie moet aanvullend cryoglobuline bepaald worden en indien dit negatief is, moet een spier/zenuwbiopt plaats vinden voor het aantonen van vasculitis. Bij een snel progressieve demyeliniserende polyneuropathie moet differentiaal diagnostisch gedacht worden aan CIDP. Bij patiënten met een langzaam progressief beloop worden bij patiënten met een IgM MGUS antistoffen bepaald. EMG onderzoek vindt plaats om een demyeliniserende en een axonale polyneuropathie te onderscheiden. Bij een langzaam progressieve axonale polyneuropathie is MGUS waarschijnlijk een toevalsbevinding. Indien andere oorzaken zijn uitgesloten is waarschijnlijk sprake van chronische idiopathische axonale polyneuropathie (CIAP). Al met al is de work-up van patiënten met polyneuropathie geassocieerd met monoclonale gammopathie ingewikkeld en is het vooral belangrijk om de mogelijkheid van een hematologische maligniteit niet uit het oog te verliezen. Wij adviseren patiënten jaarlijks te vervolgen en een M-proteine gehalte te bepalen. Verder adviseren wij om bij iedere patiënt met een klinische verdenking op maligne transformatie (gewichtsverlies, nachtzweten, botpijn, anemie, of stijging van het M-proteine gehalte in het bloed) of progressie van de polyneuropathie een hematologische screening inclusief beenmergonderzoek te doen.

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