

A New Line Immunoassay for the Multiparametric Detection of Antiganglioside Autoantibodies in Patients with Autoimmune Peripheral Neuropathies

KARSTEN CONRAD,^a HAUKE SCHNEIDER,^b TJALF ZIEMSEN,^b THOMAS TALASKA,^c DIRK REINHOLD,^d RENE-LOUIS HUMBEL,^e AND DIRK ROGGENBUCK^f

^a*Institute of Immunology, Medical Faculty "Carl Gustav Carus," Technical University Dresden, Fetscherstrasse, Dresden, Germany*

^b*Department of Neurology, Technical University Dresden, Fetscherstrasse, Dresden, Germany*

^c*Regional Consiliary Center for Tick-borne Diseases, Georg-Schacht-Strasse, Brieskow-Finkenheerd, Germany*

^d*Institute of Immunology, Otto-von-Guericke-University Magdeburg, Leipziger Strasse Magdeburg, Germany*

^e*LLIP Laboratoire Luxembourgeois d'Immuno-Pathologie, Rue Romain Fandel, Esch-sur-Alzette, Luxembourg*

^f*GA Generic Assays GmbH, Ludwig-Erhard-Ring, Dahlewitz, Germany*

ABSTRACT: A novel line immunoassay for the multiparametric detection of 11 antiganglioside autoantibodies (GM1, GM2, GM3, GM4, GD1a, GD1b, GD2, GD3, GT1a, GT1b, and GQ1b) was evaluated by comparing the reactivities in sera of 77 patients with suspected or definite autoimmune peripheral neuropathies (PNP), 60 blood donors, and 30 systemic lupus erythematoses (SLE) patients. At least one antiganglioside autoantibody was detectable in 97.4% of the patients with neuropathies compared to 12.2% in the control group. A broad spectrum of reactivities with more than two antiganglioside autoantibodies including all tested gangliosides except GD2 and GT1b was found in nearly one-third of the patients with neuropathies whereas in the control group autoantibody profiles with more than two reactivities were observed in two SLE patients only. For the first time anti-GM4 IgG and IgM antibodies were shown in PNP including Guillain-Barré syndrome (GBS), Miller-Fisher

Address for correspondence: Dr. Karsten Conrad, Institute of Immunology, Medical Faculty "Carl Gustav Carus," Technical University Dresden, Fetscherstrasse 74, 01307 Dresden, Germany. Voice: +49351-458 6540; fax: +49351-458 6308.
k.conrad@mail.zih.tu-dresden.de

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syndrome (MFS), and multifocal motor neuropathy (MMN). Different autoantibody profiles detectable by this multiparametric assay may help to diagnose different entities within the growing spectrum of autoimmune PNP.

KEYWORDS: line immunoassay; ganglioside; autoantibodies; autoimmune peripheral neuropathies

INTRODUCTION

Peripheral neuropathies (PNP) are characterized by motoric, sensoric, or autonomic dysfunctions. The causes are manifold and may be hereditary or acquired. Detection of antiganglioside autoantibodies and their association with clinically defined subtypes implicate an autoimmune mechanism of peripheral and cranial nerve damage in some forms of acquired PNP.^{1,2} The prevalence of autoimmune neuropathies seems to be underestimated due to limitations of diagnostic tools in clinical practice.^{3,4} The prototype of an acute autoimmune-mediated PNP is the Guillain-Barré syndrome (GBS), which is not a defined entity but a group of conditions with diverse clinical manifestations, such as acute inflammatory demyelinating polyradiculoneuropathy (AIDP) or Miller-Fisher syndrome (MFS).⁵ As early as 1988 antiganglioside antibodies were demonstrated in patients suffering from GBS.⁶ Another condition with similar heterogeneity where antiganglioside antibodies can be found is the chronic inflammatory demyelinating polyneuropathy (CIDP).⁷ A clinically and electrophysiologically well-defined neuropathy syndrome with a slowly progressive multifocal muscle weakness and atrophy is the multifocal motor neuropathy (MMN).⁸ In addition to these disorders, other neuropathies may exist, which are caused by immune attacks against gangliosides on peripheral nerves.²

Certain gangliosides are components of the plasma membrane of Schwann cells, the myelin, and the axolemma. They represent membrane-sialylated glycolipids that bear autoantigenic epitopes confined to the carbohydrate moiety. The relative accumulation of various gangliosides at defined locations of the peripheral nerve system may account for an association of the clinical presentation with ganglioside autoantibody specificities.^{9,10} For better serological diagnostics and for a preferable differentiation of pathological entities, the determination of different antiganglioside autoantibodies of both IgG and IgM isotypes is suggested. Until recently no standardized tools for the determination of antiganglioside autoantibodies were available. Thin layer chromatography with subsequent immunodetection is proposed to be the "golden standard." This method, however, is not applicable for routine use. Enzyme immunoassays with highly purified gangliosides have been introduced in the routine diagnostics of PNP but the determination of antibody profiles with this method is not cost-effective. Provided that the optimal binding of gangliosides on membranes can be achieved, dot or line immunoassays may be a good alternative

for the multiparametric determination of antiganglioside autoantibodies. Here we describe the first evaluation of a novel line immunoassay by comparing the results from patients with immune-mediated PNP and those from control groups.

PATIENTS AND METHODS

Determination of Antiganglioside Autoantibodies

A novel line immunoassay using 11 different gangliosides (GM1, GM2, GM3, GM4, GD1a, GD1b, GD2, GD3, GT1a, GT1b, and GQ1b) was developed in cooperation between Attomol GmbH (Lipten, Germany), GA Generic Assays GmbH (Dahlewitz, Germany), and Rene-Louis Humbel (LLIP Laboratoire Luxembourgeois d'Immuno-Pathologie, Esch-sur-Alzette, Luxembourg). This commercially available "antiganglioside dot" assay was applied to analyze the antiganglioside autoantibody profile in sera of 77 patients with clinically defined or suspected immune-mediated PNP, 30 patients with SLE, and 60 healthy blood donors according to the instructions of the manufacturer. Briefly, patients and control sera, stored at -30°C , were agitated gently after thawing in order to ensure homogeneity. Sera were diluted at 1:100 and incubated 2 h at 4°C to allow sufficient binding of autoantibodies to the gangliosides immobilized on the solid phase (membrane). Unbound sample components were removed by the following wash step. The bound autoantibodies reacted specifically with anti-IgG or anti-IgM conjugated to horseradish peroxidase (POD) during a second incubation step of 60 min at 4°C . Excessive conjugate was separated from the solid-phase immune complexes by an additional wash step. After substrate addition for staining of the immune reaction the stripes were read off after drying for at least 30 min. A sample was considered to be positive in respect to one of the gangliosides if the coloration of the test line was discriminable from the background (FIG. 1).

Patient Selection

Sera tested by routine examinations for antiganglioside antibodies with enzyme immunoassays were collected and stored at -30°C from 1997 until 2005. Sera of this collection were recruited for this study from patients suffering from acute or chronic immune-mediated or probable immune-mediated neuropathies by the following criteria: (1) clinically defined syndrome comprising hyporeflexia, reduced vibration senses and neurographic signs of motor and/or sensory PNP and (2) no alternative or contributing causes of neuropathy (e.g., diabetes mellitus, vitamin deficiency, vasculitis, exposure to neurotoxins). In 18 cases a definite diagnosis of GBS ($n = 7$), MFS ($n = 7$), GBS–MFS overlap

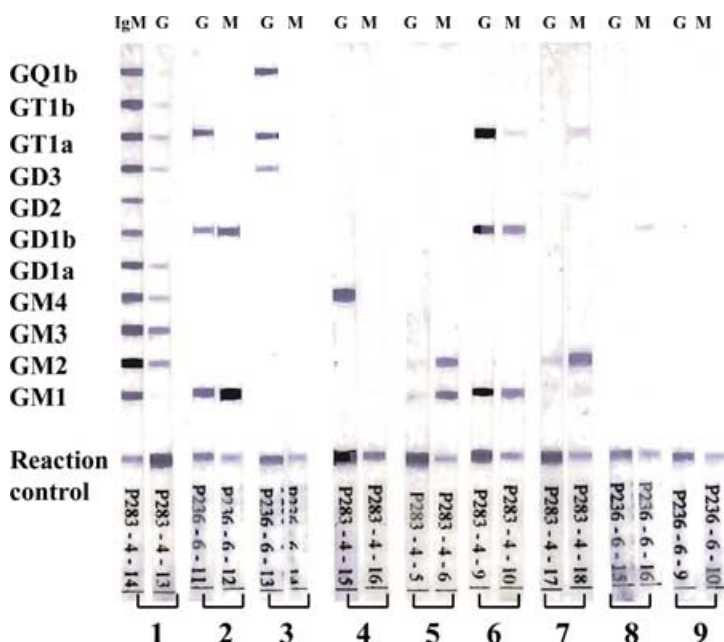


FIGURE 1. Examples of antiganglioside autoantibody reactivities in sera of patients with neuropathies and systemic lupus erythematoses (SLE): (1) serum from a patient with multifocal motor neuropathy served as positive control because of IgM reactivity (first lane) with all gangliosides; (2) Guillain-Barré syndrome (GBS) patient showing anti-GM1, anti-GD1b (IgG and IgM), and anti-GT1a (IgG only); (3) Miller-Fisher syndrome (MFS) patient with anti-GQ1b, anti-GT1a, and anti-GD3 IgG only; (4–6) patients with unclassified peripheral neuropathies (PNP) showing different autoantibody reactivities: one patient with anti-GM4 (IgG only), one patient with anti-GM1 and anti-GM2 (strong IgM and weak IgG reactivity), and one patient with anti-GM1, anti-GD1b, and anti-GT1a (strong IgG and weaker IgM reactivity); (7,8) two SLE patients: with the exception of the anti-GM2 IgM, staining of the other antiganglioside reactivities is weaker than those found in patients with neuropathies; (9) negative control (serum from healthy blood donor): this picture was seen with the majority of control sera.

syndrome ($n = 1$), MMN ($n = 3$), and CIDP ($n = 1$) could be made according to established criteria.^{3,4,8,11,12} In the remaining cases ($n = 59$) the retrospective analyses of the clinical, electrophysiological, and histological data were not sufficient to include patients definitely in one of the subgroups of immune-mediated neuropathies.

GBS

Based on the potentially life-threatening character and the need of an immediate beginning of therapy, the diagnosis of GBS was primarily made by

clinical and cerebrospinal fluid (CSF) evaluation. The clinical syndrome suggestive of GBS consists of ascending flaccid paresis, hypo- or areflexia, limb paresthesia, and in part of involvement of lower cranial nerves. CSF protein levels were elevated in most patients and cell counts were within normal levels. After exclusion of an IgA deficiency, intravenous immunoglobulin (IVIG) therapy was started (0.4 g per kg/day for 5 consecutive days), usually when the patient was not able to walk less than 5 m. Alternatively, in some patients plasma exchange therapy was initiated (five times, usually every other day). Electrophysiological testing was done in all patients in the following days and not later than 3 days after admission. Motor and sensory nerve conduction studies including F wave latency measurements were performed in at least two nerves of an upper limb and two nerves of a lower limb. Sera of patients were collected during first 2 weeks after symptom onset.

MFS and GBS–MFS Overlap Syndromes

Patients with a clinical syndrome suggestive of MFS were included in this subgroup (ophthalmoplegia, hypo- or areflexia, ataxia). Electrophysiological tests were performed as described for GBS to evaluate patients for MFS and GBS–MFS overlap syndromes. All patients had a CSF evaluation and a brain MRI to exclude other pathologies.

CIDP

Patients with a clinical neuropathy syndrome lasting more than 8 weeks were suggestive of a chronic autoimmune-mediated neuropathy. If electrophysiological tests showed a mainly demyelinating polyneuropathy and a clinical response to treatment with IVIG or plasmapheresis was observed, patients were considered to be included in this subgroup. CSF of candidate patients was tested to exclude other pathologies. In some patients a suralis nerve biopsy was performed and only those patients with a typical histopathological pattern were included. Only one of the patients of this study fulfilled all criteria of CIDP.

MMN

Patients were considered for this subgroup if they had a clinical syndrome with an involvement of more than one motor nerve and without or with very mild sensory deficits. Electrophysiological testing showing at least one conduction block was required for inclusion. Another prerequisite was responsiveness to IVIG or plasmapheresis. If performed, a suralis biopsy showing pathological affection of the nerve was a reason for exclusion.

Control Groups

Sera from 60 blood donors of ages between 20 and 30 years and sera of 30 patients with systemic lupus erythematoses (SLE) served as controls. SLE was classified according to the ACR criteria.¹³

RESULTS

Frequencies of Detectable Antibodies

In 75 of the 77 analyzed patients with PNP (97.4%) at least one antiganglioside autoantibody was detectable compared to 12.2% of the controls. The most frequent antibodies in the studied group of PNP were anti-GM1 and anti-GT1a followed by anti-GQ1b, anti-GM2 (IgM only), anti-GM3, anti-GM4, anti-GD1b, and anti-GD3 (TABLE 1). Anti-GM2 (IgG only), anti-GD2, and anti-GT1b were not found in neuropathies or were detectable only at frequencies below 2%. The number of detectable antibody specificities is associated with PNP. Only one antiganglioside antibody was found in 39.0% of PNP patients versus 6.7% in control sera, two antibodies were found in 28.6 versus 3.3%, and more than two antibodies were found in 29.9 versus 2.2%, respectively. In the control group more than two antibodies were detected only in two SLE patients but not in sera of blood donors. The reactivities were weak or very weak compared to those in sera with neuropathies (FIG. 1).

Diagnostic Specificity

In the sera of blood donors only IgG against GM3 (1.7%) and GM4 (3.3%) and IgM against GM1 (8.3%) and GD1b (1.7%) were found. Sera of SLE

TABLE 1. Frequency of antiganglioside antibodies in immune-mediated peripheral neuropathies (PNP) and controls (CON=blood donors plus SLE patients)

Isotype Ganglioside specificity	IgG PNP // CON	IgM PNP // CON	IgG + IgM PNP // CON
GM1	22.1 // 0	37.7 // 6.7	19.5 // 0
GM2	1.3 // 0	18.2 // 2.2	0 // 0
GM3	13.0 // 1.1	6.5 // 0	0 // 0
GM4	13.0 // 2.2	10.4 // 0	2.6 // 0
GD1a	6.5 // 1.1	2.6 // 0	0 // 0
GD1b	10.4 // 0	15.6 // 2.2	7.8 // 0
GD2	0 // 0	1.3 // 1.1	0 // 0
GD3	7.8 // 1.1	7.8 // 0	2.6 // 0
GT1a	32.5 // 1.1	23.4 // 0	13.0 // 0
GT1b	1.3 // 1.1	0 // 0	0 // 0
GQ1b	19.5 // 0	3.9 // 0	2.6 // 0

patients showed weak IgG reactivities against GD1a, GD3, and GT1a in one patient and IgM reactivities against GM1, GM2, GD1b, GD2, or GD3 in three other patients. No control sera demonstrated both IgG and IgM to the same ganglioside compared to 48.1% of sera from patients with PNP. Therefore, the diagnostic specificity to each antiganglioside antibody for immune-mediated neuropathies is 100% if the ganglioside is detected by IgG and IgM as well. If only IgG or IgM is reactive to gangliosides, the specificities range from 93.3% to 100%. With the exception of anti-GM1 IgM, the diagnostic specificities for the other antiganglioside antibodies in this study are higher than 97% versus the combined control group of blood donors plus SLE patients.

Antiganglioside Antibody Profiles in GBS Patients

In all patients anti-GM1 was positive (one IgG only, four IgM only, and two IgG plus IgM). In two cases anti-GM1 IgM was the only detectable antibody. The remaining patients expressed from two to six different antiganglioside antibodies: anti-GM1 in combination with anti-GM2, anti-GM3, anti-GM4, and anti-GD1b autoantibodies. In one case anti-GM1 IgM was found together with anti-GM4, anti-GT1a and anti-GQ1b IgG.

Antiganglioside Antibody Profiles in MFS Patients

Interestingly, only antiganglioside antibodies of the IgG isotype could be found in our group of 7 MFS patients. In all patients anti-GQ1b in combination with anti-GT1a were positive. In addition, anti-GD3 antibodies were detectable in three cases (FIG. 1). One of these patients also expressed anti-GM3 and anti-GM4.

DISCUSSION

Autoimmune neuropathies are a heterogeneous group of acute and chronic disorders of the peripheral nervous system. In most patients of these acquired neuropathies autoantibodies against a set of different gangliosides are detectable in the sera.^{2,5,14,15} Attempts have been made to associate antiganglioside antibodies with specific clinical and pathological features, such as lo-coregional predilection, the kind of fiber involvement (motor versus sensory), and pathology (axonal versus demyelinating). A multiparametric approach allowing the determination of a broad range of IgG and IgM antiganglioside antibodies is required to improve the basis for serological diagnostics of autoimmune neuropathies. Enzyme immunoassays with highly purified gangliosides have been introduced in the routine diagnostics for some of the most diagnostic relevant antibodies (e.g., anti-GM1, anti-GQ1b). However, for antibody profiling one has to test each antibody with a separate assay (if available)

or one can use combiassays with different gangliosides immobilized in separate wells of microtiter plates. With dot or line immunoassays 10 or more autoantibodies can be analyzed simultaneously on one strip. In the novel line immunoassay described here 11 different gangliosides (GM1, GM2, GM3, GM4, GD1a, GD1b, GD2, GD3, GT1a, GT1b, and GQ1b) were efficiently bound to a membrane allowing the simultaneous detection of corresponding antiganglioside autoantibodies. The test is easy to perform and easy to interpret because of a good ratio of background to positive staining. In almost all tested sera (with the exception of one SLE patient) no background membrane staining was visible and a positive staining of the ganglioside lines can be definitely interpreted. Although many neuropathy cases of this study could not be differentiated into known disease entities because of limitations of the retrospective collection of clinical, electrophysiological, and histological data, the results obtained with the described line assay can be interpreted as follows: (a) With the exception of anti-GM1 IgM the antiganglioside antibodies detectable with this assay are highly specific for inflammatory neuropathies. (b) The diagnostic specificity for neuropathies is even higher if both IgG and IgM are reactive against a defined ganglioside or if more than one antiganglioside antibody is detectable. (c) A broad spectrum of reactivities with more than two antiganglioside autoantibodies including all tested gangliosides except GD2 and GT1b was found in nearly one-third of the patients with neuropathies. (d) For the first time, anti-GM4 antibodies were detected either as the only antibody or in combination with other reactivities. Taken together, the described assay allows an easy to perform as well as cost-effective analysis of antiganglioside autoantibody profiles. The data support previous reports suggesting antiganglioside autoantibodies to be pathognomonic for autoimmune PNP.²

Given the heterogeneous clinical presentation and course, diagnosis and treatment of autoimmune neuropathies remain still a clinical challenge.¹⁶ Based on the probable underestimation of autoimmune neuropathies and the available therapeutic options like IVIG or plasmapheresis, it is necessary to identify affected patients to prevent disease progression by adequate therapy. Especially in chronic autoimmune neuropathies, well-defined serological markers like antiganglioside antibody profiles may facilitate the identification of those patients and provide the basis for a specific therapy. In clinical routine no definite cause can be detected for a proportion of polyneuropathies. Using a multiparametric assay as a screening method, new entities or subtypes of autoimmune neuropathies may be identified in combination with clinical and electrophysiological data.

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