

Anti-sulphatide antibodies in peripheral neuropathy

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Abstract

A study was carried out on 135 patients with chronic idiopathic neuropathy (63), neuropathy associated with monoclonal gammopathy (51, including eight with anti-MAG antibody activity) and the Guillain-Barré syndrome (GBS) (21). Serum IgM, IgG and IgA anti-sulphatide antibody titres were compared with titres in 304 patients with other neurological or immunological diseases and in 50 normal subjects. Titres were presented a) as the highest serum dilution at which reactivity could be detected, and b) in the linear region of the optical density curve. A substantial number of patients with neurological or immunological diseases had higher titres than normal subjects. Compared with normal and disease controls, five patients with neuropathy associated with IgMk monoclonal gammopathy had raised titres of IgM anti-sulphatide antibodies and one patient with GBS had raised IgM, IgG and IgA anti-sulphatide antibodies in the acute phase of the disease. Two patients had a predominantly axonal sensory neuropathy with presenting symptoms of painful paresthesiae and minimal neurological deficit. Three patients had a predominantly demyelinating sensorimotor neuropathy associated with anti-MAG antibody activity. The patient with GBS had extensive sensory loss and antibody titres returned to normal within three weeks. Raised titres of anti-sulphatide antibodies occurred in several types of neuropathy, but all had some degree of sensory impairment and associated immunological abnormality.

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Raised titres of serum anti-sulphatide antibodies were initially reported in patients with multiple sclerosis, idiopathic thrombocytopenic purpura and chronic active hepatitis.¹⁻³ Recently, these antibodies have been found in predominantly sensory axonal neuropathy, demyelinating neuropathy associated with anti-MAG antibodies and also in the Guillain-Barré syndrome (GBS).⁴⁻⁷

To define which subtypes of peripheral neuropathy are associated with raised anti-sulphatide antibodies, we studied patients with neuropathy and other neurological and immunological diseases, and normal subjects.

MATERIALS AND METHODS

Patients

Included in the study were 135 patients with chronic idiopathic axonal polyneuropathy—CIAP [sensory (26), sensorimotor (35), motor (2)], neuropathy associated with monoclonal gammopathy [IgM (19, including eight with anti-MAG antibody activity), IgG (28), IgA (4)] and GBS (21).⁸ All patients were diagnosed by clinical, electrophysiological and laboratory examination at the Departments of Neurology of the University Hospitals of Utrecht and Groningen.⁸⁻¹⁰ Controls included patients with various other neuropathies (vitamin B deficiency (4), diabetes mellitus (6), Sjögren's syndrome (1), and paraneoplastic (3) and hereditary (6) neuropathy), motor neuron disease (36; including 21 with classical amyotrophic lateral sclerosis and 15 with lower motor neuron disease), multiple sclerosis (20), myasthenia gravis (20), as well as other neurological diseases (OND (100); including Alzheimer's disease (20), Parkinson's disease (20), stroke (20), epilepsy (20), head trauma (20)), other immunological diseases (OID (48); including rheumatoid arthritis (18), systemic lupus erythematosus without neurological symptoms (20), chronic active hepatitis (7), Sjögren's syndrome (3)), monoclonal gammopathy without neurological disease (MGWND; IgM (20), IgG (20), IgA (20)) and normal subjects (50).

Serum was obtained from all patient and control groups and stored at -70°C until use.

ANTI-SULPHATIDE ANTIBODY ASSAY

Anti-sulphatide antibodies were measured by enzyme-linked immunosorbent assay (ELISA), as previously described.¹⁰ Briefly, microwells were coated with $50\ \mu\text{l}$ of methanol containing $5\ \mu\text{g/ml}$ of sulphatide (Sigma) and evaporated overnight. Uncoated microwells were used as controls. Wells were saturated with $100\ \mu\text{l}$ of PBS buffer ($0.15\ \text{M NaCl}$, $0.01\ \text{M NaH}_2\text{PO}_4$, pH 7.4) containing 1% BSA (ELISA solution) for four hours. Serum serially diluted in $50\ \mu\text{l}$ of ELISA solution beginning with a dilution of 1:200 was added in triplicate to sulphatide-coated wells and uncoated control wells, and incubated overnight at 4°C . After washing, peroxidase-conjugated rabbit antibodies to human IgM, IgG, IgA, kappa or lambda light chains (Sigma), diluted 1:1000 in ELISA solution were added for two hours. Reaction products were visualised with O-phenylene-

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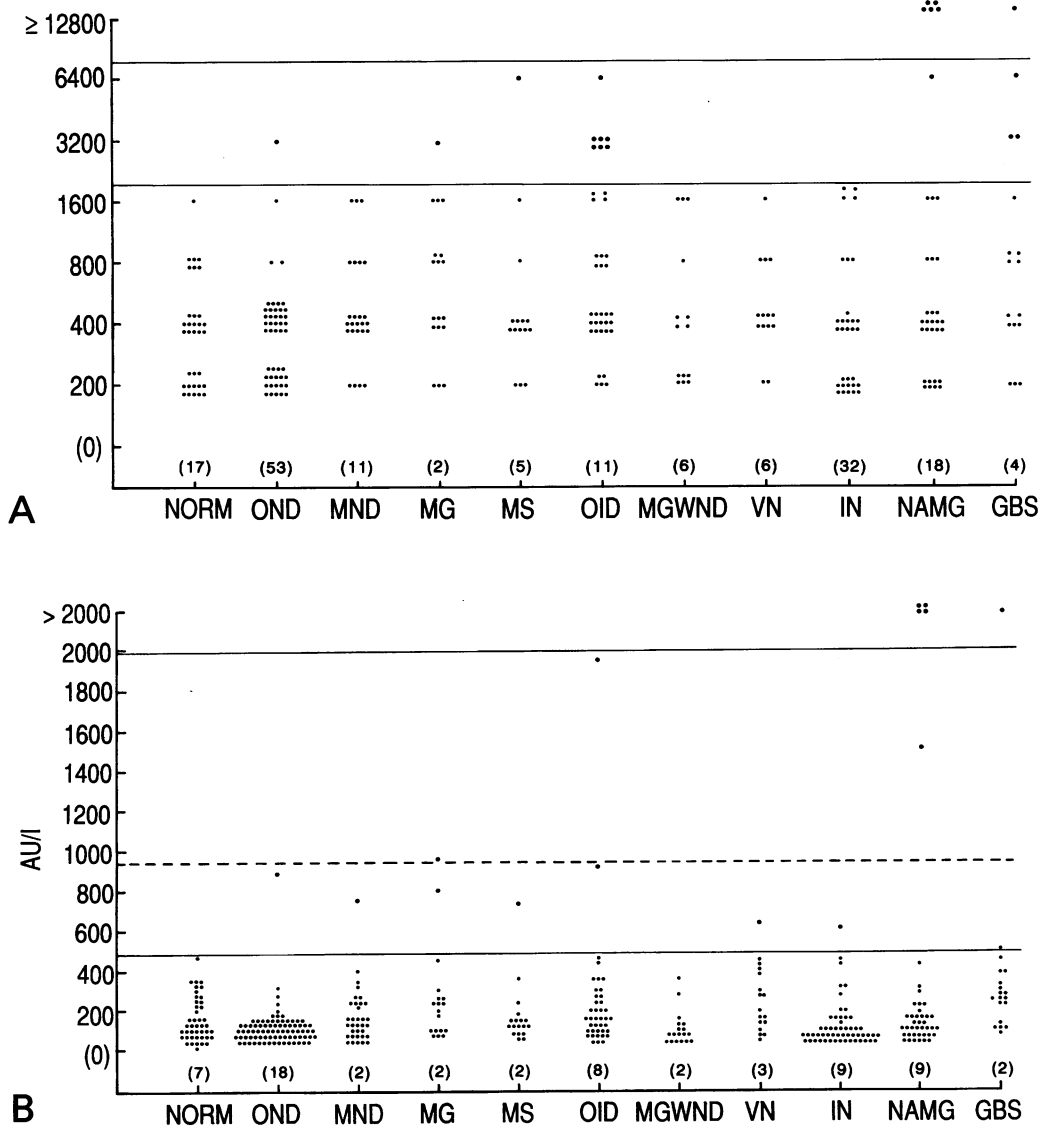
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Figure A (top): IgM anti-sulphatide antibody titres determined by method A. Lower horizontal line represents the highest titre of normal controls. Upper horizontal line represents the highest titre of disease controls; B (bottom): IgM anti-sulphatide antibody activity in Arbitrary Units per litre (AU/l) determined by method B. Lower horizontal line represents the highest antibody titre (490) as well as the mean value plus 3 SD of normal controls (493). Dotted line represents the mean value plus 3 SD of normal and disease controls (952). Upper horizontal line represents the highest antibody titre of disease controls.

NORM = normal subjects; OND = other neurological diseases; MND = motor neuron disease; MG = myasthenia gravis; MS = multiple sclerosis; OID = other immunological disease; MGWND = monoclonal gammopathy without neurological disease; VN = various neuropathies; IN = idiopathic neuropathies; NAMG = neuropathy associated with monoclonal gammopathy; GBS = Guillain Barré Syndrome.

Numbers given along the X-axis represent numbers of patients with values of 0.



diamine as substrate and read spectrophotometrically at 492 nm in a multiscan reader (Bio-Rad).

DETERMINATION OF ANTIBODY TITRES

Anti-sulphatide antibody titres were presented in two ways to increase the validity of abnormal results and for better comparison with previously reported anti-sulphatide antibody titres⁴:

A) Readings were taken from the last part of the optical density (OD) curve. For each patient the titre was taken as the highest serum dilution at which spectrophotometric optical density readings for sulphatide-coated wells were 0.05 units greater than in corresponding uncoated control wells.

B) Readings were also taken from the linear region of the OD curve as previously described.¹⁰ Serum of a patient with high titre of anti-sulphatide antibodies was used as an internal control sample in each experiment and all test sera were normalised against it. The binding of this serum was given the value of 10.000 Arbitrary Units per litre (AU/l). Antibody titres were determined as the relative level of immunoglobulin binding compared to this positive control. Values in

uncoated control wells were subtracted from values in sulphatide-coated wells.

DETECTION OF ANTI-MAG ANTIBODIES

Anti-MAG antibodies were measured in all patients with peripheral neuropathy by ELISA using sulphated glucuronic acid paragloboside (SGPG) as antigen (kindly provided by Dr N Latov, Columbia University, New York), as previously described.¹¹ Anti-MAG antibody activity was confirmed in patients with high titres by Western Blot after separation of myelin proteins by SDS-PAGE as described previously.¹²

ABSORPTION OF PATIENT SERA

Serum from patients who had raised anti-MAG and anti-sulphatide antibodies was absorbed with sulphatide by the method of Hirabayashi *et al.*¹³ Briefly, sulphatide was conjugated with Octyl-Sepharose 4B beads, suspended in patient serum at various dilutions and left overnight at 4°C.

The mixture was centrifuged for 5 minutes at 1000 rpm to remove the Sepharose beads and the remaining antibody activity of the supernatant was measured by ELISA and

Table Clinical features of patients with raised anti-sulphatide antibody titres.

Patient number	Age/Sex	Diagnosis		Disease Duration	Clinical features	Electro-physiol	Anti-sulphatide		
		Neurologic	Immunologic				IgM	IgG	IgA
A1	61/M	Sensory neuropathy	WM: IgMk	8 year	Numbness, nocturnal paresthesia in LEs. Impairment of all sensory modalities distally in LEs. Normal strength. Reflexes intact.	A	51200/ 4900	—	—
A2	68/M	Predominantly sensory neuropathy	NMG: IgMk	23 year	Numbness, painful nocturnal paresthesia in LEs. Impairment of all sensory modalities distally in LEs, impaired vibratory sensation in hands. Weakness distally in LEs and UEs (MRC 5-). Absent DTRs in LEs.	A	102400/ 10000	—	—
B1	65/M	Sensorimotor neuropathy	NMG: IgMk anti-MAG	19 year	Numbness, painful nocturnal cramps and paresthesias in LEs and UEs. Impairment of all sensory modalities distally in LEs and UEs. Weakness distally in LEs (MRC 3). Absent DTRs in LEs and UEs.	D + A	12800/ 1450	—	—
B2	83/M	Sensorimotor neuropathy	NMG: IgMk anti-MAG	29 year	Numbness, painful paresthesia in LEs and UEs. Impairment of all sensory modalities distally in LEs and UEs. Weakness in LEs (MRC 4). Ataxia in LEs and UEs. Tremor in UEs. Absent DTRs in LEs and UEs.	D	51200/ 3500	—	—
B3	69/M	Sensorimotor neuropathy	NMG: IgMk anti-MAG	11 year	Numbness, paresthesia in LEs. Impairment of all sensory modalities distally in LEs. Weakness in LEs (MRC 3). Absent DTRs in LEs.	D	25600/ 2945	—	NR
C1	36/M	GBS	IgM ↑ IgG ↑ IgA ↑	6 months	Numbness in LEs and UEs. Impairment of all sensory modalities in LEs and UEs. Weakness in LEs (MRC 2) and LEs (MRC 4). Absent DTRs in LEs and UEs.	D	12800/ 3122	6400/ 640	3200/ 984

M = male; GBS = Guillain Barré Syndrome; NMG = non-malignant monoclonal gammopathy; WM = Waldenstrom Macroglobulinaemia; LEs = lower extremities; UEs = upper extremities; MRC = Medical Research Council; DTRs = deep tendon reflexes; D = demyelination; A = axonal; anti-sulphatide = anti-sulphatide antibody titre: method A/method B; NR = not raised.

compared with serum absorbed with unconjugated Octyl-Sepharose beads. Patient serum with raised anti-MAG antibodies without anti-sulphatide antibody activity served as a control.

Results

ANTIBODY ELISA ASSAY

Serum anti-sulphatide antibodies were measured by ELISA and antibody titres were presented in two ways, as shown in fig A (method A) and B (method B) for IgM anti-sulphatide antibodies.

Method A

IgM anti-sulphatide antibody titres in normal individuals ranged from 0 to 1600. Ten disease controls had IgM anti-sulphatide antibody titres in the range of 1600 to 6400. This range was considered borderline, not specific for one disease or syndrome. IgM anti-sulphatide antibody titres of greater than 6400 were thus considered raised, and were only seen in five patients with neuropathy associated with monoclonal gammopathy and in one patient with GBS. IgG and IgA anti-sulphatide antibody titres (not shown) were generally lower than IgM and ranged from 0 to 800 for IgG and from 0 to 400 for IgA in normal controls, and from 0 to 1600 for IgG and from 0 to 800 for IgA in disease controls. Anti-sulphatide antibody titres greater than 1600 for IgG and greater than 800 for IgA were considered raised and were found only in the GBS patient who also had raised IgM titres.

Method B

Normal controls had IgM anti-sulphatide

antibody activity from 0 to 490 and disease controls from 0 to 1950 AU/l (fig B). Antibody activity of greater than 1950 AU/l was found in four patients with neuropathy associated with monoclonal gammopathy and one patient with GBS, all of whom had raised anti-sulphatide antibodies by method A above.

IgG and IgA antibody activity (not shown) ranged from 0 to 292 for IgG and 0 to 83 AU/l for IgA and disease controls from 0 to 1518 for IgG and 0 to 750 AU/l for IgA. All patients with neuropathy had IgG antibody activity in the range of disease controls and only the patient with GBS (previously identified by method A) had a greater antibody activity for IgA.

Mean value plus 3 SD of controls as normal range

To compare the results to previously published work⁴ we determined the mean value plus 3 SD for normal controls by method B, which was 493 AU/l for IgM anti-sulphatide antibodies. When this value was taken as the upper limit of normal, 11 patients with values between 493 and 1950 AU/l had raised antibody activity, of which eight were disease controls. The GBS patient had higher antibody activity for both IgG and IgA, but also seven disease controls for IgG and 24 disease controls for IgA.

We also determined the mean value plus 3 SD of normal and disease controls, which was 952 AU/l for IgM antibodies. One patient with neuropathy associated with monoclonal gammopathy (also positive by method A) and two disease controls had antibody activity between 953 and 1950 AU/l.

The GBS patient had raised antibody activity for IgG and IgA.

PATIENTS WITH RAISED ANTI-SULPHATIDE ANTIBODIES

A summary of the clinical and laboratory data of patients with raised anti-sulphatide antibodies is presented in the table. Six patients had raised anti-sulphatide antibodies compared with normal and disease controls by method A. Five of them also had raised anti-sulphatide antibodies compared with disease controls by method B, and one of them (patient B1, table) had raised antibodies compared with the mean value plus 3 SD of normal and disease controls. Three clinical presentations were associated with raised anti-sulphatide antibodies:

A) Painful sensory neuropathy

Patients A1 and A2 had identical presenting complaints of painful nocturnal paresthesiae. Otherwise neurological deficit was minimal and the clinical course was very slowly progressive.

B) Sensorimotor neuropathy associated with anti-MAG antibody activity

Three out of eight patients with neuropathy associated with anti-MAG antibody activity also had raised titres of anti-sulphatide antibodies. The anti-MAG and anti-sulphatide antibodies were of the IgMk isotype and absorption of patient serum with sulphatide bound to Octyl-Sepharose resulted in approximately 80% reduction of antibody binding to sulphatide as well as to MAG, suggesting that the IgMk monoclonal antibody reacted with both antigens. As control, absorption of sera with anti-MAG antibodies without anti-sulphatide antibodies did not reduce antibody binding to MAG.

C) GBS

One of 21 patients with GBS had raised anti-sulphatide antibody titres. This patient had raised IgM, IgG and IgA anti-sulphatide antibodies and had the most profound sensory loss among the 21 patients. Detailed clinical data of the 21 GBS patients were previously published.¹⁰ Titres were highest in the acute phase of the disease and returned to the normal range within three weeks.

Discussion

A substantial number of patients with neurological or immunological diseases had higher anti-sulphatide antibody titres compared with normal subjects. Six patients with peripheral neuropathy had raised anti-sulphatide antibodies compared with normal and disease controls. However, neuropathy associated with raised anti-sulphatide antibodies does not appear to constitute one clinical syndrome. Two patients had a predominantly axonal sensory neuropathy with presenting symptoms of painful paresthesiae and minimal neurological deficit. Three patients had a severe sensorimotor neuropathy with

demyelination and raised anti-MAG antibodies and one patient had acute GBS with extensive sensory loss. The chronic neuropathies were all associated with IgM monoclonal gammopathy.

Absorption studies in the three patients with raised titres of anti-MAG and anti-sulphatide antibodies suggest that the IgMk monoclonal gammopathy has antibody activity to both antigens. Ilyas *et al* reported similar results in two of ten patients with anti-MAG antibodies.⁵

By using thin-layer chromatography (TLC) anti-sulphatide antibodies were previously found in 65% of patients with GBS, however, disease controls were not studied and 15% of healthy controls also had anti-sulphatide antibodies.⁶ In contrast, Ilyas *et al* did not find raised anti-sulphatide antibodies in GBS by TLC and ELISA, when titres were compared with normal and disease controls.⁷ In our study only one of 21 GBS patients had raised anti-sulphatide antibody titres. Titres were highest in the acute phase of the disease and returned to the normal range within three weeks suggesting that the antibodies were related to the disease. Raised titres of IgM, IgG or IgA anti-sulphatide antibodies may be an infrequent finding in GBS patients and may be peculiar to patients with extensive sensory loss. However, a larger number of GBS patients will have to be tested to confirm these observations.

From the present study it may be concluded that raised anti-sulphatide antibody titres are not as common as previously suggested by Pestronk *et al*, who found raised titres in 18 of 64 (28%) patients with sensory ± motor neuropathy.⁴ The discrepancy may result from the following differences in our assay procedures: a) Pestronk *et al* defined high titres as those more than 3 SD above the mean value of normal controls. We found that using this method a substantial number of patients with other diseases would have raised titres. We considered antibody titres to be raised when compared with normal and disease controls. b) A significant number of patient sera have relatively high binding to uncoated control wells. In our study values in uncoated control wells were subtracted from values in sulphatide-coated wells for each patient. Using this method only antibody binding to sulphatide was measured. In our study two methods of calculating antibody titres were compared. In method A readings were taken from the last part and in method B from the linear part of the OD curve. The OD curve of each patient has a different shape, which explains intra-individual variations of results from method A or B. Although both methods detect highly elevated antibody titres, we prefer method A for routine use, as values at higher serum dilutions suggest higher affinity antibody binding and may be more accurate. In addition values from method B are more difficult to compare with other laboratories, as not every laboratory may use the same positive control.

The role of anti-sulphatide antibodies in

the pathogenesis of neuropathy is still unknown. Sulphatide is a common glycolipid found in spinal cord and peripheral nerve tissue, and is highly enriched in myelin.^{14 15} It is unlikely that the antibodies arise as a result of tissue breakdown, as raised anti-sulphatide antibodies were not found in the disease controls, who also had nerve damage. In addition, in the patient with GBS, the antibodies were present in high titres early in the acute phase of the disease before significant tissue breakdown. Several sulphated molecules are present in peripheral nerve tissue and cross-reactivity with antibodies binding to sulphatide may occur. The binding site and the fine specificity of anti-sulphatide antibodies may determine the particular clinical syndrome. Thus in patients who have predominantly axonal sensory neuropathy, binding may occur to sensory axons whereas in patients with demyelinating neuropathy (anti-MAG associated or GBS) antibodies may be directed against the sulphatide in myelin. However, sensory loss as the common feature of all six patients with raised anti-sulphatide antibodies may be explained by antibody binding to dorsal root ganglia.¹⁶ Additional motor deficit or demyelination may result from binding of these antibodies to cross-reactive sulphated epitopes, the presence of associated anti-MAG activity or to other underlying immune abnormalities that may occur in GBS. In five of the six patients the anti-sulphatide antibodies occurred as monoclonal gammopathy suggesting a primary B-cell response. In GBS the presence of IgG and IgA antibodies suggests T-cell involvement. Testing for anti-sulphatide antibodies in patients with peripheral neuropathy may be useful to study possible pathogenic mechanisms in a subgroup of patients with sensory neuropathy. Treatment strategies in these patients could be evaluated by measuring antibody titres. Additional testing for anti-MAG antibodies is necessary, since cross-reactive antibodies occur and initial symptoms of neuropathy associated with anti-MAG antibodies may be similar.¹⁷

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