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THE PATHOPHYSIOLOGICAL ROLE OF ANTI-GANGLIOSIDE ANTIBODIES IN PERIPHERAL NERVOUS SYSTEM DISORDERS

A thesis submitted to the University of Glasgow for the Ph.D. degree

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CONTENTS

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I believe that to start and finish a Ph.D. is more than simply carrying out a piece of research. It is not just to write a successful Ph.D. thesis. It is the whole process of learning and adventure. Throughout this journey I have had the assistance of distinguished people whose help and encouragement were decisive for me. I am exceedingly indebted to my supervisor Dr Hugh J. Willison for his support during all these years of study and research; without his encouragement I would never finish my Ph.D. studies. I am deeply grateful to Dr Margaret Gladden who introduced me into research work and helped me with her suggestions and to Dr Edward Rowan for his unlimited help and advice during the more than two years experimental work at Strathclyde University. Also, I would like to express my special thanks to Mrs Jean Veitch for performing ELISAs for anti-ganglioside antibodies and estimating complement activity and to Dr Graham O' Hanlon for his help on the immunohistochemistry chapter of my thesis. Finally, I will never forget the smile of the city of Glasgow.

ABSTRACT

In this study we investigated the neurophysiological effects, as well as the binding properties and complement activating ability of neuropathy-associated human anti-ganglioside antisera and monoclonal antibodies on isolated nerves and nerve-muscle preparations. Two categories of antibodies were studied: anti-GM1 antisera and monoclonal antibodies from patients with chronic motor neuropathies and Guillain-Barré syndrome, and anti-GQ1b and related anti-disialosyl antisera and monoclonal antibodies from patients with chronic ataxic neuropathies and Miller Fisher syndrome. *In vitro* recordings for up to 4 hours of compound nerve action potentials, latencies, rise times and stimulus thresholds from isolated ensheathed and desheathed sciatic nerve and rabbit desheathed sural nerve as well as twitch tension from mouse phrenic nerve-hemidiaphragm preparation were performed, in the presence of anti-ganglioside antibodies and fresh human serum as an additional source of complement. No changes were observed over this time course, with all electrophysiological parameters being within 15% of the starting values for both normal and antibody containing sera. Immunohistological evaluation of desheathed nerves exposed to anti-ganglioside antibodies demonstrated antibody deposition with complement activation at up to 90% of nodes of Ranvier in some preparations. These data indicate that anti-ganglioside antibodies can diffuse into nerve, bind to nodes of Ranvier and fix complement *in vitro* without resulting in any acute physiological deterioration, suggesting that the node of Ranvier is relatively resistant to acute anti-ganglioside antibody mediated injury. The data also suggest that anti-ganglioside antibodies are unlikely to exert major direct pharmacological effects on nodal function and neuromuscular transmission. The models used in this study failed to give an *in vitro* approval of the suggested pathophysiological effects of anti-ganglioside antibodies on nerve and nerve-muscle function, possibly

because their limited viability does not allow time for the evolution of a full inflammatory lesion, and *in vivo* models may be more suitable for identifying the effects of these antibodies on nerve conduction.

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABBREVIATIONS

AIDP=Acute inflammatory demyelinating Guillain-Barré syndrome

ALS=Amyotrophic lateral sclerosis

AMAN=Acute pure motor axonal Guillain-Barré syndrome

AMSAN=Acute motor-sensory Guillain-Barré syndrome

CANOMAD=Chronic ataxic neuropathy, ophthalmoplegia, monoclonal IgM protein, cold agglutinins and disialosyl antibodies

CIDP=Chronic inflammatory demyelinating polyradiculopathy

CNS=Central nervous system

CSF=Cerebrospinal fluid

CT=Cholera toxin

EAE=Experimental allergic encephalomyelitis

EAN=Experimental allergic neuritis

ELISA=Enzyme-linked immunoabsorbent assay

GBS=Guillain-Barré syndrome

MADSAM=Multifocal acquired demyelinating sensory and motor neuropathy (Lewis-Sumner syndrome)

MAG=Myelin associated glycoprotein

MBP=Myelin basic protein

MFS=Miller Fisher syndrome

MMN=Multifocal motor neuropathy

MGUS=Monoclonal gammopathy of undetermined significance

PNS=Peripheral nervous system

PPN=Paraproteinemic neuropathy

RT=Room temperature

SGLPG=Sulfoglucuronyl lactosaminyl paragloboside

SGPG=Sulfoglucuronyl paragloboside

STX=Saxitoxin

TLC=Thin layer chromatography

TTX=Tetrodotoxin

PART ONE – GENERAL INTRODUCTION

Preface

At the brink of the new millennium neuroscience has become one of the great frontiers of science and the progress that has been made in the last decades in understanding the biology of the neurological disorders is extraordinary. Indeed, the significant advances in the fields of basic neuroscience give new approaches to the basic pathogenic mechanisms of nervous system disorders and several of these approaches have changed our understanding of their pathophysiology and hence their treatment.

Neurological diseases affect the qualities that make the lives of human beings so special and distinct. Among these diseases, the autoimmune peripheral neuropathies have attracted the interest and enthusiasm of researchers from different scientific disciplines. Immune responses have been postulated to be involved in the pathogenesis of autoimmune peripheral neuropathies and glycolipids have been shown to play a central role in autoimmune processes affecting the nervous system. The human nervous system not only expresses large quantities of glycolipids but also contains glycolipid structures that are absent or less frequent in other organs. These molecules are thus potential antigenic targets for circulating autoantibodies in autoimmune nervous system disorders. Autoimmune peripheral neuropathies cover a set of clinical syndromes in which idiopathic neuropathy causes acute, subacute or chronic weakness affecting peripheral nerves. Our understanding of the pathogenic mechanisms leading to these clinical syndromes is growing rapidly through advances in epidemiology, microbiology, and immunology. A list of some neuropathies that have been reported to be associated with antiglycolipid antibodies is presented in the following Table 1:

Table 1 *Antiglycolipid autoantibodies in neuropathies*

Clinical syndrome	Glycolipid antibody reactivity	References
Acute demyelinating Guillain-Barré syndrome (AIDP) Acute pure motor axonal Guillain-Barré syndrome (AMAN) Acute motor-sensory axonal Guillain-Barré syndrome (AMSAN)	IgG, IgM and IgA antibodies to GM1, GD1a, LM1, GT1a, GM1b, GM2, sulfatide, and other glycolipids	(Ilyas et al. 1988; McKhann et al. 1993; Yuki et al. 1993d; Visser et al. 1995; Rees, Gregson & Hughes 1995a; Rees et al. 1995b; Hafer-Macko et al. 1996; Hahn 1998; Ho et al. 1999; Hughes et al. 1999; Kaida et al. 2000)
Miller Fisher syndrome (MFS), acute oropharyngeal palsy, and Bickestaff's brainstem encephalitis	IgG antibodies to GQ1b and GT1a	(Chiba et al. 1992; Chiba et al. 1993; Yuki et al. 1993a; Yuki et al. 1993b; Willison et al. 1993c; O'Leary et al. 1996; Willison et al. 1997)
Multifocal motor neuropathy (MMN)	IgM antibodies to GM1 and related Gal(β1-3)GalNAc bearing glycolipids	(Freddo et al. 1986; Pestronk et al. 1988a; Pestronk et al. 1988b; Pestronk et al. 1990; Santoro et al. 1990; Krarup et al. 1990; Pestronk 1991; Lange et al. 1992; Griffin 1994; Kinsella et al. 1994; Wolfe et al. 1997)
Chronic axonal and demyelinating predominantly sensory neuropathy	IgM antibodies to sulfatide	(Ilyas et al. 1992a; van den Berg et al. 1993; Lopate et al. 1997a)
Chronic large fibre predominantly sensory neuropathy with ataxia	Monoclonal IgM antibodies to gangliosides containing NcuNAc(α2-8)NeuNAc epitopes	(Ilyas et al. 1985; Freddo et al. 1986; Younes-Chennoufi et al. 1992; Daune et al. 1992; Yuki et al. 1992a; Willison et al. 1994; Latov 1994; Dalakas & Quarles 1996; Willison et al. 1996; Kusunoki et al. 1996b; O'Leary & Willison 1997)
Neuropathy associated with IgM gammopathy	Monoclonal IgM antibodies to SGPG, SGLPG, MAG, P_0, sulfatide, and gangliosides	(Latov et al. 1980; Braun, Frail & Latov 1982; Ilyas et al. 1984; Ilyas et al. 1985; Vital et al. 1989; Kyle & Dyck 1993; Nobile-Orazio et al. 1994; Pestronk et al. 1994a; Latov 1995; van den Berg et al. 1996; Ropper & Gorson 1998)
Insulin-dependent and non-insulin-dependent diabetes mellitus	Gangliosides, sulfatide	(Buschard et al. 1993; Shigeta et al. 1997)

Antiglycolipid antibodies have been extensively studied in patients with neuropathy associated with IgM paraproteinemia. It appears clear from clinical, electrophysiological

and experimental data, that the target glycolipid or glycolipid epitope for the IgM is related to the type of neuropathy, purely sensory or predominantly sensory or uniquely motor. About half of the patients with peripheral neuropathy and IgM monoclonal gammopathy have serum antibodies reacting with the myelin-associated glycoprotein (MAG), which shares an oligosaccharide epitope (HNK-1) with the peripheral nerve protein P_0, a sulfated glucuronyl paragloboside (SGPG), and its related sulfated glucuronyl lactosaminyl paragloboside (SGLPG). This epitope is also present in human natural killer cells. It seems that there is a close correlation between the sensory demyelinating neuropathies and the presence of anti-MAG and anti-SGPG antibodies. Also, anti-GD1b IgM monoclonal antibodies have been found in sensory ganglionopathies or chronic large fibre sensory neuropathies with ataxia, and strong evidence supports the pathogenic role of the anti-GD1b ganglioside antibody in this neuropathy.

Guillain-Barré syndrome (GBS) is another autoimmune neuropathy that has been studied by several research groups. The term GBS defines a recognisable clinical entity that is characterised by rapidly evolving symmetrical limb weakness, loss of tendon reflexes, sensory signs and variable cranial nerve involvement and autonomic dysfunction. GBS is the most common cause of acute neuromuscular paralysis in developed countries. GBS has undergone considerable revision in recent years with the addition of axonal GBS (AMAN-acute motor axonal neuropathy, and AMSAN-acute motor and sensory axonal neuropathy) to the traditional view of acute inflammatory demyelinating polyradiculoneuropathy. Several glycolipid antigens, including GM1, LM1, GM1b, GM2, GT1a, GD1a, and sulfatide have been found to be immune targets in GBS and its subgroups. Since *Campylobacter jejuni* infection often precedes the

disease, it has been proposed that this infection may be involved in the initiation of the antiglycolipid response.

Moreover, it seems clear that the IgG anti-GQ1b antibody is associated with the syndrome of acute ophthalmoplegia, areflexia and ataxia defined as Miller Fisher syndrome (MFS). Anti-GQ1b antibody has also been demonstrated in serum from GBS patients with ophthalmoplegia and from patients with acute paralysis of extraocular muscles, suggesting that the anti-GQ1b antibody might be of pathogenic importance in an immune-mediated extraocular muscle paresis. Also, the antiglycolipid reactivity in sera from patients with chronic inflammatory demyelinating polyradiculopathy (CIDP), a chronic form of GBS is another issue of investigation.

The role of antiglycolipid antibodies in multifocal motor neuropathy (MMN), a newly recognised chronic neuropathy that has clinical features in common with both CIDP and lower motor neuron disease, has been extensively investigated. The diagnostic hallmark of MMN is the electrophysiological demonstration of persistent localised motor conduction block. Although the pathogenesis of MMN has not been elucidated, there is increasing evidence for an autoimmune mechanism. Anti-GM1 antiganglioside antibody has been the most extensively studied antigen and its clinical significance and its potential pathogenic role are controversial. Another common antigen found in several neurological disorders is sulfatide, sulfated galactosylceramide. Sulfatide is also a potential antigen in insulin and non-insulin-dependent diabetes mellitus, a disease where late complications are neuropathy and retinopathy.

CHAPTER ONE: GANGLIOSIDES AND PERIPHERAL NERVOUS SYSTEM

1. Distribution and biochemistry of gangliosides

Knowledge of basic biochemistry of gangliosides is important in understanding their physiological significance and their implication in many current areas of interest in autoimmune peripheral neuropathies. Glycosphingolipids are amphipathic components of plasma membranes of all vertebrate cells and they also occur in intracellular membranes of the secretory and endocytotic pathways, e.g., in the Golgi, endosomal and lysosomal membranes (Marks, Marks & Smith 1996; Voet & Voet 1995). They are widely distributed in nervous tissue, particularly in the outer leaflet of the plasma membrane, where they contribute to cell surface carbohydrates.

Gangliosides are a family of sialic acid containing glycosphingolipids (Svennerholm 1963; Svennerholm 1994; Marks et al. 1996; Voet & Voet 1995). Gangliosides show great molecular diversity with numerous novel minor components and are composed of lipid and carbohydrate moiety (Svennerholm 1956; Svennerholm & Raal 1961; Ledeen 1985). The lipids are a group of biologically important compounds related more by their physical than by their chemical properties. In the body, lipids serve as an efficient source of energy, and, also, as a thermal insulator in the subcutaneous tissues and around certain organs, including the nervous system. They have the common property of being relatively insoluble in water and nonpolar lipids act as electrical insulators allowing rapid propagation of depolarisation waves along myelinated fibres. The fat content of nerve tissue is particularly high. Carbohydrates are widely distributed in animals having both structural and important metabolic roles. The sugar glucose is the most significant carbohydrate. The simplest type of sugar is a monosaccharide - a single sugar unit. Monosaccharides may be joined together to form disaccharides, trisaccharides, tetrasaccharides, and polymers or polysaccharides. Glycosphingolipids contain a fatty

acid, the alcohol sphingosine, and carbohydrate. The major glycolipids found in animal tissue are glycosphingolipids. They contain ceramide and one or more sugars. The two simplest are galactosylceramide and glycosylceramide. Galactosylceramide is a major glycosphingolipid of nervous system, but it is found in relatively low amounts elsewhere. Galactosylceramide can be converted to sulfogalactosylceramide (classic sulfatide), which is a major component of peripheral nerve myelin and an autoantigen in predominantly sensory neuropathies (Willison 1994).

Ganglioside comprise at least one sialic acid residue. Sialic acid is a generic term for N-acetylneuraminic acid, usually abbreviated to NeuNAc or NANA. The sialic acid(s) are linked to the internal or terminal galactose of an oligosaccharide core composed of up to four sugars with the following sequence: ceramide-glucose-galactose-N-acetylgalactosamine-galactose (Ledeen 1985; Willison 1994). Ceramide is the lipid moiety in ganglioside molecules, a fatty acid linked to a long base chain, sphingosine. Ceramide is synthesised in the endoplasmic reticulum. First, the aminoacid serine, following activation by a combination with pyridoxal phosphate, combines with palmitoyl-CoA to form 3-ketosphinganine. This is converted to dihydrosphingosine in a reductive step utilising NADPH. Dihydroceramide is formed by a combination with acyl-CoA followed by desaturation to form ceramide (Voet & Voet 1995). There is evidence that ceramide may act as a lipid mediator (second messenger), activating a protein kinase and opposing some of the actions of diacylglycerol. The acyl group is represented frequently by long-chain saturated or monoenoic acids (Voet & Voet 1995). Gangliosides are synthesised from ceramide by a stepwise addition of activated sugars and a sialic acid, usually N-acetylneuraminic acid. Gangliosides have an amphipathic structure because they contain polar groups. The lipid moiety represents the hydrophobic or water-insoluble part of the molecule that is embedded into the

membrane. This anchors the hydrophilic or water-soluble carbohydrate moiety to the outer surface of the membrane. The carbohydrate portion of gangliosides is a series of two or more sugars with at least one sialic acid. The sialic acid(s) are attached to the internal or the terminal galactose of the oligosaccharide chain (Svennerholm 1956; Svennerholm & Raal 1961; Svennerholm 1994).

Glycosphingolipids are inserted into the membrane lipid bilayer as is illustrated in the following Figure 1, which also indicates the structure of several carbohydrate chains in the commonly occurring glycosphingolipids. The ceramide group is contained within the lipid bilayer, with the sphingosine and fatty acid hydrocarbon chain parallel to and interacting with the fatty acyl chains of the sphingolipids. The carbohydrate group projects out from the surface of the bilayer, interacting with the surrounding water (Montgomery et al. 1996).

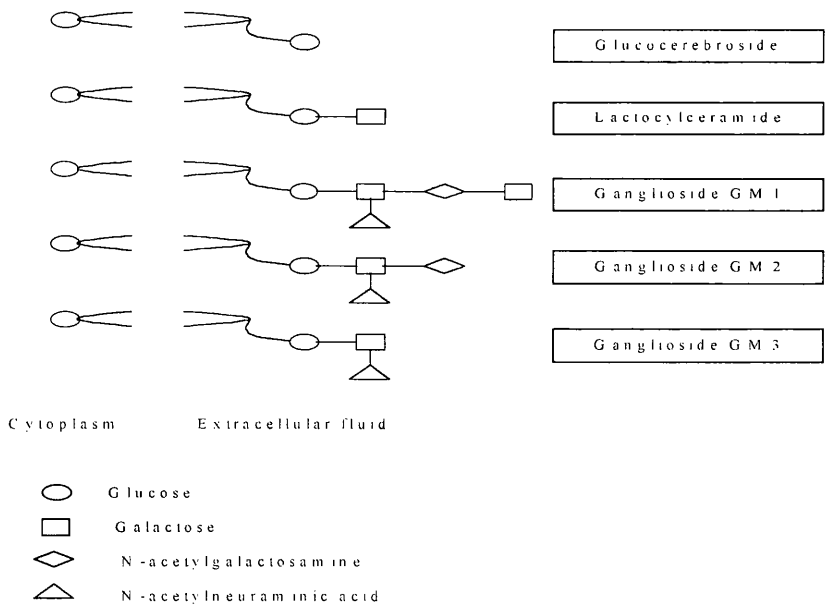

Fig. 1 Insertion and orientation of glycosphingolipids in the membrane lipid bilayer. The ceramide group is inserted between phospholipids, and the carbohydrate projects into the extracellular fluid. The carbohydrate chains of different kinds of glycosphingolipids vary in number and type of monosaccharide residues.

2. Terminology of gangliosides

The problems in the nomenclature of gangliosides arise from the complexity of the carbohydrate moiety of these compounds. The systematic names of the oligosaccharides linked to the hydrophobic portion, namely ceramide, are very cumbersome and they cannot be used for oral communication. Today, the code system, suggested by Svennerholm, is generally accepted (Svennerholm 1963; Svennerholm 1994). According to his suggestion gangliosides are named with the following method: G stands for ganglio; M, D, T, and Q refers to the number of sialic acids residues (mono-, di-, tri-, and quad-, respectively); arabic numerals and lower case letters refers to the sequence of migration in thin layer chromatography (TLC).

The older methods of separation and identification of lipids, based on classic chemical procedures of crystallisation, distillation, and solvent extraction, have now been largely supplanted by chromatographic procedures. TLC is particularly useful for the separation of the lipid moiety of gangliosides (Svennerholm 1963). The migration of gangliosides in TLC depends upon their oligosaccharide core and the number of attached sialic acids. Small gangliosides migrate more slowly than gangliosides with a longer oligosaccharide core and more sialic acids. For example, GM1 ganglioside (that has an oligosaccharide core consisted of four sugars, Cer-Glc-Gal-GalNAc-Gal) migrates more slowly than the three sugar oligosaccharide GM2 ganglioside (Cer-Glc-Gal-GalNAc). On the other hand GD1b runs ahead of GT1b, which runs ahead of GQ1b, as they contain two, three, and four sialic acids respectively (Willison 1994).

There are four major gangliosides of the ganglio- series in the brain tissue (GM1, GD1a, GD1b, GT1b) (Svennerholm 1956; Svennerholm & Raal 1961; Pestronk 1991; Svennerholm 1994; Willison 1994). They each contain the same four sugar chain, but vary in the number of sialic acids; GM1 with one, GD1a with two, GD1b with two, and

GT1b with three. One ganglioside not found in peripheral neurons is GM4, sialosylgalactosylceramide, which appears to be a marker for myelin and oligodendrocytes of the CNS (Ledeen 1985). Numerous minor gangliosides in brain, nerve, and myelin have been described.

All antiglycolipid antibodies associated with neuropathy react with epitopes located on the carbohydrate region of glycolipid molecules. Because the carbohydrate structures are frequently present on several different glycolipids, glycoproteins and other carbohydrate composed molecules, such as bacterial lipopolysaccharides, there is considerable possibility for shared reactivity (Willison 1994). As an example, the Gal(β1-3)GalNAc epitope found on GM1 ganglioside is also present on asialo-GM1 and GD1b, and the complete GM1 structure is present on *Campylobacter jejuni* lipopolysaccharide (Yuki et al. 1993c; Aspinall et al. 1994; Moran 1997; Hahn 1998; Sheikh et al. 1998b). Also, autoantibodies reacting with the sulfated glucuronic acid epitope on SGPG reacts with similar epitopes on many other glycoproteins of nerve tissue including the myelin associated glycoprotein (MAG) and the peripheral nerve compact myelin protein P_0 (Braun et al. 1982; Burger et al. 1990; Pestronk et al. 1994a; van den Berg et al. 1996).

3. Ganglioside storage disease in Clinical Neurology

Many neurological disorders have been associated with gangliosides. A block at the various intermediate stages of ganglioside metabolism causes the accumulation of the specific gangliosides within the nervous system and other tissue (Sandhoff & Conzelmann 1984; Marks et al. 1996; Voet & Voet 1995). Their excessive storage is responsible for the pathophysiology and clinical features of these diseases, and its identification on biopsy specimens is a useful screening procedure.

Tay-Sachs disease, or infantile GM2 gangliosidosis, is the prototype for the ganglioside storage diseases; it is caused by a severe deficiency of hexosaminidase A (Sandhoff & Conzelmann 1984; Marks et al. 1996; Voet & Voet 1995) The patient presents between the third and sixth month of life with marked irritability and hyperacusis; by the age of six months weakness and hypotonia become evident, followed by progressive blindness, deafness, seizures, and spasticity; death occurs before the age of three years in these children (Evans et al. 1991). Also, juvenile GM2 gangliosidosis, adult-onset GM2 gangliosidosis, and Sandhoff's disease, which is phenotypically similar to Tay-Sachs disease, are all GM2 gangliosidoses (Sandhoff & Conzelmann 1984). GM1 gangliosidosis (Kohlschutter 1984) is an autosomal recessive disorder caused by a deficiency of lysosomal acid beta galactosidase. The severity of this disease correlates with the degree of enzyme deficiency. In addition to the above the distribution of gangliosides is affected in Niemann-Pick disease, a group of disorders caused by sphingomyelinase deficiency (Sandhoff & Conzelmann 1984; Evans et al. 1991; Marks et al. 1996).

Gangliosides are also involved in other disorders. For instance, an experimental autoimmune multiple sclerosis-like disease was induced in rabbits after immunisation with gangliosides (Konat et al. 1982). Moreover, exogenous ganglioside injections can be immunogenic, triggering an IgG anti-GM1 anti-ganglioside antibody response, resulting in acute axonal Guillain-Barré syndrome (Illa et al. 1995). On the other hand, a number of studies have found increased titres of anti-ganglioside antibodies in the sera and cerebrospinal fluid of patients with multiple sclerosis, even though the significance of these findings is unclear (Sadatipour, Greer & Pender 1998). Also, experimental allergic peripheral neuritis was observed in the peripheral nerves of animals by immunising them with gangliosides (Nagai et al. 1976).

4. Functions and therapeutic effects of gangliosides

The functional roles of gangliosides are still poorly understood. They have been implicated in a variety of molecular mechanisms of important biological phenomena of the nervous system (Nagai 1995). Gangliosides are the most specific lipids of neuronal membranes (Svennerholm 1994; Ledeen 1985). Gangliosides of the central nervous system undergo characteristic changes during the stages of brain development, short-term stimulation and long-term thermal adaptation. At the very early stages of brain development the less polar mono- and disialo- gangliosides are synthesised followed later by biosynthesis of the more polar ones. Short-term light, electrical stimulation and extreme temperature conditions affect the metabolism of gangliosides (Pankkezhum & Brewer 1990). An abundance of literature suggests that exogenous administration of gangliosides could enhance neurite outgrowth and recovery from injury (Karpiak 1984; Toffano et al. 1984; Roisen et al. 1984; Tsuji et al. 1988; Riggot & Matthew 1997) .

Many authors suggest the possible use of gangliosides as a therapeutic agent for the treatment of many neurological problems (Pankkezhum & Brewer 1990). Initially, ganglioside mixtures were not considered immunogenic when administered parenterally (Asbury 1994), but subsequent reports suggested that exogenous ganglioside injections can elicit immune mediated side effects triggering a strong IgG GM1 antibody response (Illa 1995). The therapeutic effects of gangliosides have been well documented by several investigators in *in vitro* studies. In experimental animals, gangliosides have shown a considerable capacity to repair and regenerate damaged peripheral or central nervous tissue (Ceccarelli, Aporti & Finesso 1976). Several studies have extended the therapeutic role of gangliosides to diseases such as diabetic neuropathy (Horowitz 1984; Hallett et al. 1984; Naarden et al. 1984). Gangliosides were administered in several clinical trials in the treatment of acute stroke and amyotrophic lateral sclerosis, although

without therapeutic effect (Hallett et al. 1984; Hoffbrand et al. 1988). The effect of GM1 ganglioside on the amplitude of the electroencephalogram, neurologic function and histology has been studied in chronic middle cerebral artery occlusion in cats (Komatsumoto et al. 1988) and also, GM1 ganglioside has been tested in patients with Parkinson's disease (Schneider et al. 1995). Some of these data give promising results with clear benefits for some patients, although the therapeutic potential of gangliosides needs further extensive investigation (Abraham, Abraham & Wynn 1984; Fedele, Crepaldi & Battistin 1984; Schneider et al. 1995).

5. Involvement of gangliosides in biological interactions

Gangliosides have been associated with Ca++ interactions (Milani et al. 1992). Ca++ ions have been implicated in the initiation and propagation of neuronal activity and they play a central role in the release of neurotransmitter from nerve terminals. Also Ca++ ions regulate many other stages of neuronal activity, such as interaction of the neurotransmitter with the postsynaptic membrane, axoplasmic flow, and axonal growth (Kandel 1991). Ganglioside micelles have been reported to bind Ca++ with a high affinity and this may have physiological significance in synaptic transmission. For example, blocking ganglioside activity by antibody, toxin or competition may block synaptic transmission (Pankkezhum & Brewer 1990). So far there is not sufficient evidence to establish the involvement of gangliosides in neural transmission and extensive studies in this direction are necessary.

Another important role of gangliosides is their interaction with neuroactive substances and enzymes. It has been suggested that gangliosides act as receptors for serotonin (Woolley & Gommi 1965), and they also can bind LSD, tryptamine, ergometrine, morphine, naloxone, chloropromazine, tubocurarine, colchicine, tetanus toxin,

botulinium toxin, and bilirubin (Pankkezhum & Brewer 1990). The inhibitory effect of gangliosides on Na+ /K+ ATPase of guinea pig brain microsomal fraction (Pankkezhum & Brewer 1990) was reported long ago. Gangliosides have been shown to bind specifically to calmodulin (Higashi, Omori & Yamagata 1992), and influence the activity of various types of protein kinases (Ca++ -calmodulin-dependent, Ca++ -phospholipid-dependent, cAMP-dependent and Ca++ -independent forms) and, also, they can interact with Mg++ - and Ca++ - ATPases (Fukunaga, Miyamoto & Soderling 1990; Guérold et al. 1992; Higashi & Yamagata 1992). So, gangliosides may contribute to neuronal activity through their effects on ion channels and ion pumps (LoPachin et al. 1993).

Wieraszko and Seifert suggested the involvement of gangliosides in cholinergic transmission in the striatum; rat brain striatal slices, treated with neuroaminidase from *Vibrio cholerae* abolished synaptic transmission within 90 min (Wieraszko & Seifert 1984). The injection of exogenous neuraminidase into frog spinal cord segments caused a significant increase of motorneuron activity in the corresponding spinal nerve, and moreover neuroaminidase treatment increases the amplitude of postsynaptic potentials in fish optic tectum (Romer & Rahmann 1979). In rat hippocampal slices, GM1 seems to be implicated in synaptic transmission as it can be reduced by GM1 specific probes such as GM1 antiserum, cholera toxin, and neuroaminidase from *Arthrobacter ureafaciens* (Wieraszko & Seifert 1986). Exogenously added gangliosides delay the action of ruthenium red which blocks synaptic transmission in rat hippocampal slices (Wieraszko 1986). In addition, gangliosides have been shown to influence the effect of anaesthetics on membranes (Harris et al. 1984; Harris & Groh 1985). Gangliosides are likely to be involved in the uptake of choline, and ganglioside-protein complexes may serve as choline binding sites and carriers (Massarelli et al. 1982; Ferretti & Borroni 1984). The

presence of gangliosides in both presynaptic and postsynaptic membranes could explain their possible functional involvement in synaptic transmission (Pankkezhum & Brewer 1990).

6. Gangliosides and bacterial toxins

Because of their structural diversity, gangliosides have been implicated in various cell recognition and signalling phenomena (Pankkezhum & Brewer 1990; Willison 1994; Nagai 1995). Many bacteria adhere to and colonise their hosts through production of toxins, which are often proteins that can bind to receptors on cell membranes (Willison & Kennedy 1993; Fishman, Pacuszka & Orlandi 1993). Some of these toxins frequently use gangliosides or cross-reactive glycoproteins as their cell surface receptors (Fishman et al. 1993; Holmgren 1994). When injected into the nervous system, cholera toxin produced a similar pattern of neuropathological lesions to those induced by anti-GM1 antibodies (Schwerer et al. 1986).

6a. GM1 ganglioside, the membrane receptor for cholera toxin

It is now known that the GM1 ganglioside is the specific and only natural biological receptor for cholera toxin; this was first suggested by van Heyningen et al (van Heyningen et al. 1971), who observed that crude preparations of gangliosides inactivated choleragen. Since then several groups showed that ganglioside GM1 was the most potent inhibitor of toxin binding and action (Cuatrecasas 1973a; Cuatrecasas 1973b; Holmgren 1981; Fishman 1982). Cholera toxin is a protein with two types of subunits: a single "heavy" subunit of molecular weight (MW) 28,000 non covalently attached to a 58,000-MW aggregate of "light" subunits. The "light" subunits are responsible for cell binding (B subunits) and the "heavy" subunits for the direct toxic activity (A subunit) of

cholera toxin (Lönnroth & Holmgren 1973; van Heyningen 1974; Holmgren 1981; Holmgren 1994). The binding receptor within the GM1 ganglioside (Figure 2) is the sequence Galβ1-3GalNAcβ1-4(NeuAcα2-3)Gal (Holmgren, Lönnroth & Svennerholm 1973; Holmgren 1994).

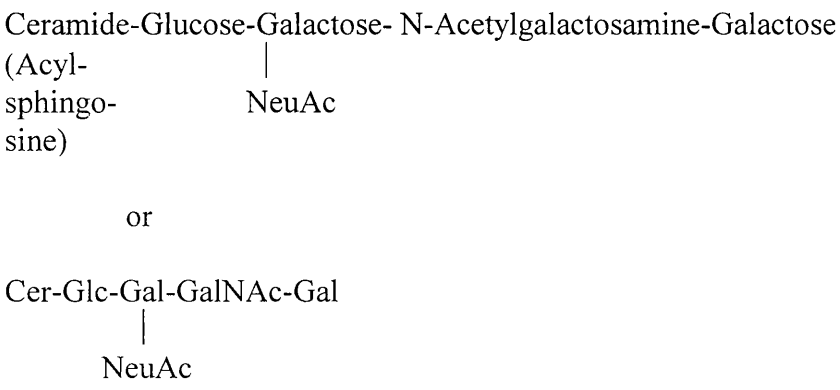

Fig. 2 GM1 ganglioside, a monosialoganglioside, the receptor in human intestine for cholera toxin.

6b. Gangliosides and other than CT bacterial toxins

Toxins produced by strains of *Campylobacter jejuni* and *Escherichia coli* can bind to GM1 ganglioside and variably to other gangliosides and glycoproteins (Fishman et al. 1993). Certain strains of *E. coli* produce a heat-labile enterotoxin (LT) which is similar in size to choleragen and is composed of A and B subunits which appear to be structurally and antigenically similar to the subunits of choleragen (Holmgren 1994). LT can be inactivated by GM1 ganglioside, although it is unclear if GM1 ganglioside is the native or only receptor for LT (Fishman et al. 1993). It is established that *Campylobacter jejuni* is a frequent preceding infection in GBS associated with anti-GM1 IgG Ab. *Campylobacter jejuni* strains contain cell wall lipopolysaccharides cross-reactive with GM1 ganglioside (Yuki et al. 1993c; Aspinall et al. 1994; Willison 1994).

Tetanus toxin is the neurotoxin produced by *Clostiridium tetani* (van Heyningen 1968). Tetanus toxin seems to mediate its neurotoxic effect on the peripheral and central nervous system by blocking the release of neurotransmitter from inhibitory presynaptic membranes. Some studies demonstrated the interaction between tetanus toxin and gangliosides (van Heyningen 1968; Fishman 1982). Tetanus toxin binds with high affinity to GQ1b and GT1b gangliosides, less well to GD1b, and with low affinity to GD1a and monosialo-gangliosides (Fishman 1982).

Also, *Clostiridium botulinium* toxin causes a disease similar to Miller Fisher syndrome but affecting all neuromuscular junctions (Willison & Kennedy 1993). Since the anti-GQ1b antibody is strongly linked to MFS (Chiba et al. 1992; Chiba et al. 1993; Yuki et al. 1993b; Willison et al. 1993c), it is likely that the toxin and the anti-GQ1b Ab bind to the same GQ1b (or GQ1b cross-reactive) receptor which is particularly highly concentrated in ocular nerves (Chiba et al. 1997). The similarities between botulism and MFS substantiate the role of anti-GQ1b antibodies in the syndrome of ophthalmoplegia, ataxia and areflexia (Willison & Kennedy 1993).

7. Gangliosides in central and peripheral nervous system

Some studies have demonstrated that peripheral nervous system myelin contains a significantly high concentration of glycosphingolipid antigens, that may be targets for immunoglobulins detected in patients with several peripheral neuropathies (Svennerholm et al. 1994). The immunoglobulins from these patients have been found to react with specific sites in glycolipid molecules, and antigens of the patients' autoantibodies can be separated into several groups correlated with their clinical conditions (Table 1). Therefore, there is considerable interest in elucidating the characteristic and specific composition of glycolipids in the nervous tissue.

Schwann cells in the peripheral nervous system (PNS) develop plasma membrane processes that spirally ensheath neuraxons in segments between nodes of Ranvier (internodes). In the central nervous system (CNS) oligodendrocytes play a similar role. Compared to unmyelinated axons, the fatty insulated sheath surrounding the axons of myelinated fibres is essential for the high speed conduction of action potentials. Cholesterol, complex phospholipids and glycophospholipids form the lipid bilayer, comprising about 70% of the dry weight of both CNS and PNS myelin (Schwartz 1991). The glycolipids galactocerebroside (GalC) and galactosulfatide (sGalC) are typical major myelin classes, together with galactoglyceride (GalDG) and gangliosides. These components are assembled with myelin-specific membrane proteins, the most abundant of which are the integral membrane proteins proteolipid protein (PLP) in the CNS and P_0 in the PNS and the myelin basic protein (MBP), with myelin-associated glycoprotein (MAG) as a minor component (Schwartz 1991). One ganglioside not found in peripheral nerves is GM4, sialosylgalactosylceramide, which appears to be a marker for myelin and oligodendrocytes of the CNS of mammals and other vertebrates (Ledeen 1985; Chiba et al. 1997). Also, the glycolipid antigens of peripheral nerve differ from those of CNS; large proportions of lactotetraose and lactohexaose gangliosides and sulfoglucuronyl glycosphingolipids are found in PNS myelin, which occur only in negligible amounts in CNS myelin (Svennerholm et al. 1994).

Peripheral nerves usually consist of both motor and sensory nerve fibres. The ganglioside composition of the peripheral nervous system might be of great importance in understanding the autoimmune mechanisms in peripheral neuropathies. Ganglioside analysis of human motor and sensory nerves revealed that ceramide composition of sensory nerve GD1a, GD1b, and GM1 differed apparently from those in the motor nerve; sensory nerve gangliosides contained a larger amount of long-chain fatty acids in

the gangliotetraose gangliosides than motor nerve (Ogawa-Goto et al. 1990; Svennerholm et al. 1994). Furthermore, the predominant ganglioside in the human peripheral nervous system myelin, both for motor and sensory nerves is LM1 (sialosylneolactotetraosyl ceramide) (Ogawa-Goto et al. 1992; Chiba et al. 1997). Sialosyl-nLc6Cer and disialosyl-nLc4Cer, GD3, GM3, and GD1b are detected as common components of the two nerve myelins (Ogawa-Goto et al. 1992). The same study showed that the content of GM1 ganglioside in the motor nerve myelin was higher than that in the sensory nerve myelin (Ogawa-Goto et al. 1992), in contrast with the results reported by Svennerholm et al. (Svennerholm et al. 1994).

Moreover, the frequent association of anti-GQ1b antibodies with diseases characterised by ophthalmoparesis (Chiba et al. 1992; Chiba et al. 1993; Willison et al. 1993c; Yuki et al. 1993a; Yuki 1993b; Yuki 1996a) correlates with the abundant expression of GQ1b ganglioside in the paranodal regions of the extramedullary portions of all three cranial nerves involved in oculomotion (Chiba et al. 1997). Ganglioside composition of the human cranial nerves showed higher proportion of GQ1b ganglioside in all three nerves (IIIrd, IVth, and VIth) that control the ocular muscles involved in the ocular movement as compared with the other cranial nerves (Chiba et al. 1997). This finding may support the role of serum anti-GQ1b antibody in the pathogenic mechanisms of ophthalmoplegia in MFS and GBS.

Table 2 *Chemical structure of glycolipids of biological interest* (Svennerholm 1994)

Glycolipid nomenclature	Carbohydrate sequence
GM4	NeuAcα2→3Galβ1→Ceramide
GM3	NeuAcα2→3Galβ1→4Glcβ1→Ceramide
GM2	GalNAcβ1→4(NeuAcα2→3) Galβ1→Ceramide
GM1	Galβ1→ 3GalNAcβ1→4(NeuAcα2→3) Galβ1→4Glcβ1→Ceramide
GM1b	NeuAcα2→3Galβ1→ 3GalNAcβ1→4 Galβ1→4Glcβ1→Ceramide
GD3	NeuAcα2→8 NeuAcα2→3Galβ1→4Glcβ1→Ceramide
GD2	GalNAcβ1→4(NeuAcα2→8NeuAcα2→3) Galβ1→4Glcβ1→Ceramide
GD1a	NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→3) Galβ1→4Glcβ1→Ceramide
GD1b	Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→3) Galβ1→4Glcβ1→Ceramide
GT3	NeuAcα2→ 8NeuAcα2→ 8NeuAcα2→3Galβ1→4Glcβ1→Ceramide
GT1a	NeuAcα2→8NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→3) Galβ1→4Glcβ1→Ceramide
GT1b	NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→3) Galβ1→4Glcβ1→Ceramide
GT1c	Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→8NeuAcα2→3) Galβ1→4Glcβ1→Ceramide
GQ1b	NeuAcα2→8NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→3) Galβ1→4Glcβ1→Ceramide
GQ1c	NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→8NeuAcα2→3) Galβ1→4Glcβ1→Ceramide
Glycosyl-ceramide	Glcβ1→Ceramide
Galactosyl-ceramide	Galβ1→Ceramide
Globoside	GalNAcβ1→3Galα1→4 Galβ1→4Glcβ1→Ceramide
Paragloboside	Galβ1→4GalNAcβ1→3Galβ1→4Glcβ1→Ceramide
LK1 Sulfoglucuronyl neolactotetrao sylceramide (SGPG)	3-sulfateGlcUAβ1→3Galβ1→4GalNAcβ1→3Galβ1→4Glcβ1→Ceramide

Abbreviations: Gal=galactose, Glc=glucose, GalNAc=*N*-acetylglucosamine, GlcNAc=*N*-acetylglucosamine, NeuAc=*N*-acetylneuraminic acid. Terms within brackets () represent a branching point in the molecule.

CHAPTER TWO: PERIPHERAL NEUROPATHIES - AN OVERVIEW

1. A brief anatomic view of the peripheral nervous system

The nervous system is divided into central nervous system (CNS) and peripheral nervous system (PNS). The CNS includes the brain and spinal cord and the PNS consists of the cranial and spinal nerves and their associated ganglia. There are 12 pairs of cranial nerves, which leave the brain and pass through foramina in the skull, and 31 pairs of spinal nerves, which leave the spinal cord and pass through intervertebral foramina in the vertebral column. Each spinal nerve is connected to the spinal cord by the anterior and posterior roots, which are bundles of nerve fibres carrying nerve impulses away from and to the CNS, respectively. The anterior root contains efferent nerve fibres that go to the skeletal muscle (motor fibres) and their cells lie in the gray matter of the anterior horn of the spinal cord. The posterior root consists of afferent fibres that carry information about sensations of touch, pain, temperature, and vibration to the central nervous system (sensory fibres) and their cell bodies are situated in a spindle-shaped enlargement of the posterior root called the spinal ganglion or dorsal root ganglion (DRG). The spinal nerves contain a mixture of motor and sensory fibres. Similarly, some of the cranial nerves are composed of afferent nerve fibres (olfactory, optic, and vestibulo-cochlear), others are composed of efferent fibres (oculomotor, trochlear, abducent, accessory, and hypoglossal), and the remainder contains both afferent and efferent fibres (trigeminal, facial, glossopharyngeal, and vagus).

The peripheral nervous system is generally considered to begin at the nerve root or exit zones of the spinal cord (and at the Obesteiner-Redlich junctions for the cranial nerves) and to extent distally to the peripheral motor or sensory endings. Structures also included in the peripheral nervous system are portions of the postganglionic autonomic fibres that lie outside of the spinal cord and brainstem, the autonomic ganglia and the

postganglionic fibres. Another way of describing the peripheral nervous system is that which is synonymous with the distribution of the Schwann cells. Nevertheless, the intraspinal portion of the central processes of the primary sensory neurons, the cell bodies of the motor neurons and the proximal portion of their axons and the preganglionic autonomic neurons lie within the central nervous system and therefore, are protected by the blood brain barrier. The dorsal root ganglia are barrier free structures. These anatomic facts have major implications for the distribution of disease and selective vulnerabilities of neural elements that make up the peripheral nervous system (Kahle 1986; Gardner & Bunge 1993).

2. Basic microscopic anatomic considerations of nerve fibres

Nerve fibres are surrounded by connective tissue, which is subdivided into endoneurium, perineurium, and epineurium. The endoneurium forms the supporting structure found around individual axons within each fascicle. The perineurium consists of collagenous tissue, which binds each fascicle with elastic fibres and mesothelial cells. This layer rather provides a diffusion barrier to regulate intrafascicular fluid. The epineurium, composed of collagen tissue, elastic fibres, and fatty tissue, tightly binds individual fascicles together.

The nerve trunks contain myelinated and unmyelinated fibres. All axons in the peripheral nervous system (PNS) and central nervous system (CNS), both myelinated and nonmyelinated, are surrounded by, or ensheathed in, accessory or satellite cells. In the PNS, these are the Schwann cells and in the CNS, the oligodendrocytes. Although important anatomical, biochemical, and morphological differences occur in the nerve fibres of the PNS and CNS, the fundamental structure of central and peripheral myelinated fibres, and their electrophysiological characteristics, are essentially similar.

In the myelinated fibres of the PNS, the Schwann cell is spirally wrapped around the axon forming a multilayered membrane specialisation, the myelin sheath. The nodes of Ranvier are located at junctions between adjacent Schwann cells and represent myelin-free fibre segments. The internodes are the long fibre segments corresponding to the extension of one Schwann cell. But whereas in the PNS a single satellite cell ensheathes each myelinated axon between one node and the next, in peripheral non-myelinated nerves, many axons are ensheathed by a single Schwann cell. In myelinated fibres of the CNS, a single oligodentrocyte may ensheath 30-50 internodes. One consequence of this arrangement is that in some pathological conditions, the death of a single myelin-forming cell in the periphery affects only one internode, whereas in the CNS many internodes are destroyed (Thomas, Berthold & Ochoa 1993).

3. Composition and molecular organisation of the myelin membrane

A detailed analysis of the biochemical properties and organisation of myelin components is required in order to understand the immunopathogenic aspects of peripheral neuropathies. The biochemical composition of myelin is simple, having a high proportion of lipids and relatively few individual protein components.

The major protein in mature peripheral protein is the P_0 glycoprotein, which represents more than 50% of the total membrane protein. CNS lacks P_0 protein, which is truly specific for peripheral nerve myelin. Analysis of myelin membranes indicates the presence of two other basic proteins designated P_1 and P_2 which account for a further 15-20% of the membrane protein. However, these proteins are not specific for peripheral nerve because they are also expressed in the CNS. Most of the other quantitatively minor peripheral nerve myelin proteins, such as myelin-associated glycoprotein (MAG), are probably also common to both peripheral and central myelin. P_0 and MAG are

members of the immunoglobulin superfamily. In comparison to P_0 protein, less is known about the functions of P_1 and P_2 proteins in peripheral nerve myelin. The P_1 basic protein is identical to the CNS myelin basic protein (MBP), which is the major CNS autoantigen responsible for inducing experimental allergic encephalomyelitis (EAE). EAE has been extensively investigated because of its similarities to multiple sclerosis. The P_2 basic protein is unrelated to MBP and its function within the PNS myelin remains unclear. Immunocytochemical analyses have shown that P_2 protein is concentrated within the cytoplasmic domains of the PNS myelin sheath rather than in compact internodal myelin and P_2 basic protein is believed to play a metabolic rather than structural role in peripheral nerve myelin. Of the remaining proteins MAG, present in both PNS and CNS myelin, is a highly glycosylated transmembrane glycoprotein concentrated in the periaxonal and outer regions of the peripheral nerve myelin sheath.

In addition to the proteins discussed so far, PNS myelin contains a number of other minor proteins, which may be highly specific for the PNS and may be considered as potential antigen targets in autoimmune peripheral neuropathies. On the other hand, the importance of PNS myelin lipids and in particular glycolipids as autoantigenic targets in human autoimmune neuropathies has been extensively recognised. The major myelin lipids are cholesterol, ethanolamine glycerophosphatide, sphingomyelin, and galactocelebroside. Ethanolamine plasmalogens, serine choline glycerophosphatides, and sulfatide are also present in smaller amounts. More importantly PNS myelin contains several different gangliosides that have been implicated in the pathogenesis of GBS and antibody-mediated polyneuropathy. The extracellular display of the sialyated oligosaccharide core of gangliosides makes them potential target antigens for pathogenic autoantibodies in neurological diseases (Brostoff et al. 1972; Trapp, McIntyre & Quarles 1979; Norton & Cammer 1984; Schwartz 1991; Thomas et al. 1993).

4. Physiology of nerve conduction

Ion channels are an important class of membrane glycoproteins that govern the flow of ions across the membranes (Siegelbaum & Koester 2000). In nerve fibres they control the rapid changes that occur in the generation of membrane action potentials. Channels can be distinguished from each other on the basis of their ion selectivity and the factors that determine their opening and closing, or gating. Various ion blockers, toxins and drugs can influence the activity of ion channels. Also, ion channels are important targets in various diseases. Myasthenia gravis, Lambert-Eaton syndrome and acquired neuromyotonia are thought to be autoimmune neurological disorders resulting from the actions of specific antibodies against ion channels (Vincent, Lily & Palace 1999). With the increasing understanding and detailed knowledge of ion channel function it seems likely that other diseases due to channel dysfunction will be identified and new pharmacological therapies will be developed.

Nerve cells are capable of self-generation of electrochemical impulses at their membranes and they can transmit signals along the membranes, so they are "excitable" cells. The membrane potential of large nerve fibres when they are not transmitting nerve signals is about –90 millivolts (mV). That means, the potential inside the fibre is 90 mV more negative that the potential in the interstitial fluid on the outside of the fibre. This potential is due to the sodium-potassium pump which actively transports sodium to the outside of the fibre and potassium to the inside (three Na+ ions to the outside for each two K+ ions to the inside causing the negative charge inside the cell membrane). The sodium-potassium pump also causes the different concentration gradients for sodium and potassium across the resting nerve membrane. The contribution of the potassium and sodium diffusion through the nerve membrane and the continuous pumping by the

electrogenic Na+ - K+ pump are the most important factors that give the normal resting membrane potential of –90 mV.

Nerve signals are transmitted by rapid changes in the membrane potential, which are called action potentials. At the time of depolarization stage, the opening of sodium channels makes membrane very permeable to sodium ions, allowing tremendous numbers of sodium ions to flow to the interior of the axon causing the potential to rise rapidly from –90 mV in the positive direction. The depolarization process travels along the entire extent of the nerve fibre and the transmission of depolarization gives rise to a nerve impulse. The increase Na+ influx into the cell produces the rising phase of the action potential. The falling phase of the action potential is caused by the subsequent closing of the Na+ channels, which reduces the Na+ influx into the cell, and by the opening of voltage gated K+ channels, which allows increased K+ efflux from the cell. This rapid diffusion of potassium ions to the exterior re-establishes the normal negative resting potential and the membrane is repolarized. Voltage-gated channels are opened when the membrane potential is near the action potential threshold. They open in an all-or-none fashion, and when open, they permit a pulse of current to flow with a variable duration but constant amplitude. At the juncture between each two successive Schwann cells along the axon, i.e. the area of the node of Ranvier, ions can still flow easily between the extracellular fluid and the nerve membrane, while along the myelin sheath the ions flow is considerably decreased. Thus, action potentials can occur only at the nodes of Ranvier.

In myelinated axons the action potential is conducted from node to node (saltatory conduction) instead of continuous propagation that occurs in ummyelinated fibres. In other words, electrical current flows through the surrounding extracellular fluid and through the axoplasm from node to node, exciting successive nodes one after another

and the nerve impulse jumps down the fibre. Saltatory conduction is of important value for myelinated nerve fibres, because this mechanism increases the conduction velocity of nerve impulse transmission and conserves energy for the axon (Guyton 1991; Ganong 1993).

Internodal capacitance and conductance decrease with increasing myelin thickness and myelinated fibres fulfil all the conditions required for maximal conduction velocity. The demyelinated or remyelinated fibres have an increased capacitance and conductance due to thinner myelin sheaths; so, more local current is lost to charge the capacitors and by leakage through the internodal membrane, and the demyelinated axons characteristically have conduction failure, decrease velocity, and temporal dispersion. The impairment of nerve conduction by demyelination is considerable. In humans, myelinated sensory fibres conduct up to a maximum of 87m/sec, and motor axons conduct between 5 and 10% slower. In contrast, in mammalian unmyelinated axons, the conduction velocity of the nerve impulses is of the order of 1m/sec; it varies in proportion to the square root of the fibre diameter (Koester 1991; Thomas et al. 1993).

5. Properties of multifibre nerve trunks - The compound nerve action potential

Because of the practical difficulty of isolating single mammalian fibres, most of the electrical data on such fibres come from studies of the extracellularly recorded compound nerve action potential, resulting from simultaneous activation of all or some of the population of fibres comprising the nerve tract. The compound nerve action potential is merely the algebraic sum of individual fibres action potentials. With subthreshold stimuli, none of the nerve fibres are stimulated and no response occurs. When the stimuli are of threshold intensity, axons with low thresholds depolarise. As the intensity of the stimuli is increased, the axons with higher threshold fire and the

electrical response increases proportionately until the stimulus is strong enough to excite all the fibres of the nerve. The stimulus that results in excitation of all axons is the maximal stimulus, and further application of greater supramaximal stimuli produces no further increase in the size of the obtained action potential.

Mammalian nerve fibres are divided into three groups: A, B, and C; the A group is further subdivided into α, β, γ, and δ fibres. The A and B fibres are all myelinated, whereas the C fibres are unmyelinated. Because of the relations between conduction velocity, diameter, and threshold the A fibres are the largest and also have the lowest threshold. The B fibres cannot be distinguished from small A fibres either histologically or in terms of conduction velocity because both groups have similar diameters. C fibres can be easily distinguished from either A and B fibres because they are all unmyelinated and of small diameter and hence of very slow conduction velocity. In the following Table 3, the various fibre types are listed with their functions, diameters and conduction velocities (Ganong 1993).

Table 3 *Nerve fibre types in mammalian nerve*

Fibre type		Function	Fibre Diameter (μm)	Conduction Velocity (m/s)
A	α	Proprioception; somatic motor	12-20	70-120
	β	Touch, pressure	5-12	30-70
	γ	Motor to muscle spindles	3-6	15-30
	δ	Pain, cold, touch	2-5	12-30
B		Preganglionic autonomic	<3	3-15
C	Dorsal root	Pain, temperature, some mechanoreceptors, reflex responses	0.4-1.2	0.5-2
	Sympathetic	Postganglionic sympathetics	0.3-1.3	0.7-2.3

6. Neuropathological aspects

It is important in the study of a peripheral neuropathy to recognise the underlying pathological nature of the condition because it influences subsequent management. In general, there are three basic pathological processes by which peripheral nerve fibres are affected:

-Wallerian degeneration

-segmental demyelination, and

-axonal degeneration

6a. Wallerian degeneration

This is the response when nerve axons are interrupted. Motor and sensory loss in the distribution of the transected fibres occurs immediately, the axon and the myelin disintegrate distally to the site of transection, conduction fails, and recovery is slow and incomplete. Denervation atrophy occurs in muscles to which the motor nerve fibres have been interrupted. Although Wallerian degeneration is usually caused by physical or mechanical injury of nerve fibres, nerve trunk ischaemia can produce focal axonal damage and distal Wallerian degeneration stems from widespread pathological processes affecting small vessels and capillaries of the size of vasa nervorum. Multifocal nerve trunk ischaemia in systemic vasculitis, is a frequent basis for multiple mononeuropathy (mononeuropathy multiplex) encountered clinically (Bosch & Mitsumoto 1991; Asbury 1994).

6b. Segmental demyelination

This pathological process implies breakdown of myelin sheath or the myelin-producing Schwann cells with preservation of axonal continuity. Conduction block is the hallmark

of segmental demyelination. In practice this occurs most commonly in immune mediated inflammatory demyelinating neuropathies and rarely in hereditary disorders of Schwann cell myelin metabolism. Myelin damage can be produced experimentally by myelinotoxic agents such as diphtheria toxin and chronic lead intoxication. The physiological expression of segmental demyelination is conduction block, resulting in the clinical deficits of motor weakness and sensory loss. Block can occur with a lesion restricted to a single node of Ranvier. This is because sodium channels are highly concentrated at the axon membrane at the nodes of Ranvier, whereas the membrane of the internodal regions contains potassium channels that stabilise the membrane potential, thus reducing excitability. Nerve conduction studies can provide confirmation of demyelination. As a rule, demyelination is likely when conduction velocities of motor and sensory nerves are reduced to less than 70% of normal. Other electrodiagnostic features of demyelinating polyneuropathies are temporal dispersion and desynchronization of compound evoked potentials and prolonged distal latencies and late responses.

In current clinical practice, conduction block is usually accepted when the amplitude or the area of an evoked action potential, recorded through surface electrodes, is reduced with proximal stimulation, compared with the response to distal stimulation and in the absence of temporal dispersion. The extent of the reduction has been put at 20% to 60% in published papers. Several articles have emphasised the problems caused by temporal dispersion, which not only can lead to prolongation of the evoked action potential, but can also cause cancellation of the negative phase of one motor unit action potential by the positive phase of another, as a result of desynchronization. This can reduce the amplitude or the area of the compound muscle action potential, which can be misinterpreted as evidence of conduction block. Temporal dispersion and phase

cancellation must be excluded as a cause of reduction. Conduction block is not found in axonal disorders or primary lesions of nerve cell bodies. Recovery from segmental demyelination can be rapid and dramatic, because all that is required is remyelination of those sites that are demyelinated and blocked (Lafontaine et al. 1982; Cornblath et al. 1991; Bosch & Mitsumoto 1991; Parry & Sumner 1992; Oh, Kim & Kuruoglu 1994).

6c. Axonal degeneration

This pathological process involves distal axonal breakdown, resembling Wallerian degeneration, presumably due to metabolic derangement within the neurons. The myelin sheath breaks down concomitantly with the axon in a pattern that starts at the most distal part of the nerve and progresses towards to the cell body, hence the term dying-back neuropathy. The distinction between Wallerian degeneration and axonal degeneration may not always be evident, even histologically, but the processes are quite different. Wallerian degeneration implies transection of otherwise healthy nerve fibres, but axonal degeneration results from systemic metabolic disorders and toxins. Electrodiagnostic features of axonal degeneration are striking decreases in the amplitudes of nerve and muscle compound action potentials with minor degrees of slowing of conduction velocity. When explored by electromyography, the distal muscles show acute or chronic changes of denervation. Recovery from axonal degeneration may be delayed and often incomplete (Bosch & Mitsumoto 1991; Asbury 1994).

7. Clinical symptoms and signs in peripheral neuropathies

Peripheral neuropathy is defined as the condition in which there is deranged function and structure of peripheral motor, sensory, and autonomic neurons. The numerous and varied neuropathic symptoms and signs are usually a mixture of sensory, motor and

autonomic disturbances. Damage to motor nerve fibres either by demyelination or axonal degeneration causes weakness of the affected muscles. Muscle cramps, fasciculations and muscle atrophy are all manifestations of motor neuron dysfunction. Tendon reflexes are reduced due to the muscle weakness, and may disappear completely if there is co-existing damage to the large sensory group Ia axons from muscle spindles. Sensory disturbances can be categorised as those resulting from the involvement of small or large sensory fibres. They frequently co-exist. Among the many varied positive sensory symptoms affecting the small sensory fibres are those of burning dysesthesias and paresthesias, usually painful. Hypoesthesia to pin prick and thermal stimuli is another frequent finding on sensory examination. The prominent sign of large fibre neuropathy is sensory ataxia. On examination, reduced vibration and position sense are present, together with areflexia and disturbances in balance particularly in the dark or with eyes closed (positive Romberg's test). Autonomic dysfunction may present with a variety of symptoms and signs (hypotension, anhidrosis or hyperhidrosis, hypothermia, cardiac dysrythmias etc.) and is characteristically associated with Guillain-Barré syndrome, amyloidosis, diabetes, porphyria, and certain familiar sensory and autonomic neuropathies (Bosch & Mitsumoto 1991; Thomas & Ochoa 1993).

CHAPTER THREE: NEUROPATHIES ASSOCIATED WITH ANTI-GLYCOCONJUGATE ANTIBODIES

Introduction

It is well established that the majority of peripheral nerve myelin proteins are immunogenic, and antibodies specific for MAG, MBP, P_0, and P_2 have been extensively studied. Demyelinating activity has been observed after animal immunisation with peripheral nerve tissue or myelin and this activity implies either immunoglobulin or cell mediated immune attack following recognition of antigenic determinants exposed on the outer surface of the myelin sheath (Saida et al. 1978; Hahn, Gilbert & Feasby 1980). In the rabbit the major target antigen for the demyelinating antibody response is the major myelin glycolipid galactocylceramide (GC). Sera from patients with GBS also contain reactive autoantibodies that recognise peripheral nerve glycolipids (Ilyas et al. 1988). These antibodies can initiate demyelination, either *in vivo* after intraneural injection (Harrison et al. 1984), or *in vitro* in myelinating cultures of peripheral nerve (Koski 1990). These antibodies recognise a number of different carbohydrate epitopes on myelin glycolipids (Quarles, Ilyas & Willison 1984).

Gangliosides are important carbohydrate determinants for autoimmune activity and many forms of peripheral neuropathy with a suspected autoimmune basis are associated with serum antibodies against gangliosides. Multifocal motor neuropathy (MMN) and Guillain-Barré syndrome including its variant Miller Fisher syndrome (MFS) are two characteristic examples of antiganglioside antibodies associated syndromes. MMN and motor forms of Guillain-Barré syndrome are associated with anti-GM1 IgM and IgG antibodies respectively; ataxic neuropathies (including the CANOMAD phenotype - chronic ataxic neuropathy, ophthalmoplegia, monoclonal IgM protein, cold agglutinins

and disialosyl antibodies (Willison et al. 1996)), and Miller Fisher syndrome are associated with anti-GQ1b IgM and IgG antibodies respectively.

1. Neuropathies associated with paraproteinemia

The association between monoclonal gammopathy and peripheral neuropathy is now well recognised (Kelly 1985; Kyle & Dyck 1993; Nobile-Orazio et al. 1994; Latov 1995; van den Berg et al. 1996; Ropper & Gorson 1998). Some common disorders of the peripheral nervous system are closely connected with the presence of excessive amounts of an abnormal immunoglobulin in the blood. These peripheral neuropathies are termed paraproteinemic neuropathies and electrophoresis or the more sensitive immunofixation test can detect the abnormal paraprotein (Kyle 1992; Kyle & Dyck 1993).

Approximately 10% of patients with idiopathic peripheral polyneuropathy are of this type and the anomalous blood immunoglobulins are usually monoclonal (termed M protein or M spike) as they are the product of a single clone of plasma cells (Latov 1995). If such a paraprotein is identified in the serum or the urine of a patient with neuropathy, haematological studies, including bone marrow aspiration, skeletal survey and bone scan, and appropriate tissue biopsies may be done to determine whether the monoclonal gammopathy is associated with an underlying lymphoproliferative disorder. The main diseases that may be considered are primary amyloidosis, multiple myeloma, osteosclerotic myeloma, Waldeström's macroglobulinemia, cryoglobulinemia, non-Hodgkin's lymphoma, Castleman's disease and related lymphatic diseases, and chronic leukaemias (Kyle & Dyck 1993; Latov 1995; Ropper & Gorson 1998). Frequently, none of these are found and the patient is considered as having a monoclonal gammopathy of undetermined significance (MGUS), and the associated neuropathy is labelled MGUS neuropathy (Kyle & Dyck 1993; Latov 1995; Chalk 1997).

Neuropathies associated with IgM M-proteins are distinguished from those with IgG or IgA M-proteins because of evidence for the pathogenicity of IgM M-proteins. IgG or IgA monoclonal gammopathies are associated with neuropathy in patients with osteosclerotic myeloma or the POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, and skin changes). The neuropathy in Waldeström's macroglobulinemia is similar to MGUS IgM neuropathy. Amyloid or cryoglobulinemia neuropathies can occur with IgM, IgG or IgA M-protein. In some patients with non-malignant IgG or IgA monoclonal gammopathy malignant disease may develop, mostly myeloma, after some years. In the majority of patients the IgM M-protein shows autoantibody activity that reacts with oligosaccharide determinants of glycolipids and glycoproteins in peripheral nerve (Kyle & Dyck 1993). The incidence of this type of neuropathy is estimated to be between 1 to 5 per 10000 adult population. Patients typically present with a slowly progressive distal and symmetrical sensory or sensorimotor neuropathy that usually affects the arms and then the legs. Cranial nerves and autonomic function are normal. Many patients have a predominantly sensory neuropathy. A few patients may present with a mainly motor neuropathy characterised by rapid progression with profound weakness. The examination of cerebrospinal fluid (CSF) usually shows increased protein concentration (Kyle & Dyck 1993).

Myelin-associated glycoprotein (MAG) is a minor protein (glycoconjugate) component of nerve and is central to understanding the paraproteinemic neuropathies. In 1980, Latov et al. reported a patient with severe demyelinating neuropathy and high levels of monoclonal IgM kappa immunoglobulin that bound to peripheral nerve myelin. Immunosuppressive therapy resulted in clinical improvement and significant reduction of circulating monoclonal antibodies (Latov et al. 1980). The constituent to which the IgM immunoglobulin bound was later shown to be MAG, a specific glycoprotein

component of myelin (Braun et al. 1982). Soon after, in patients with paraproteinemia and peripheral neuropathy it was observed that the IgM that bound to MAG might also bind to gangliosides (Ilyas et al. 1984) and that monoclonal IgM kappa from a patient with a long-standing progressive sensory polyneuropathy bound to polysialosyl gangliosides but did not react to MAG (Ilyas et al. 1985).

One year later, Freddo et al. reported a patient with an IgM lambda dysproteinemia and a progressive weakness of all four extremities, normal sensation and absent reflexes diagnosed as lower motor neuron disease; the monoclonal immunoglobulin from this patient bound avidly to GM1 and GD1b and asialo-GM1 gangliosides (Freddo et al. 1986). Since then, many more patients have been identified (Willison et al. 1993b; Brindel et al. 1994; Herron et al. 1994; Oka et al. 1996). Thus the question was raised that gangliosides might be potential autoantigens or could act as tissue specific binding sites for monoclonal proteins.

MAG is concentrated in periaxonal Schwann-cell membranes and paranodal loops of myelin and acts as an adhesion molecule for interactions between the Schwann cells and the axons. Its unique structure consists of five immunoglobulin-like domains and a carbohydrate epitope. In approximately one-half of patients with IgM M-protein and neuropathy, the M-protein binds to a carbohydrate determinant that is shared by the proteins myelin-associated-glycoprotein (MAG), the main P_0 glycoprotein of myelin and peripheral myelin protein-22 (PMP-22), and several complex glycosphingolipids (Braun et al. 1982; Burger et al. 1990; Pestronk et al. 1994a; van den Berg et al. 1996). Of these complex glycosphingolipids the best characterised are sulfoglucuronylparagloboside (SGPG) and sulfoglucuronyl lactosaminyl paragloboside (SGLPG), which are present in both myelin and axon in peripheral nerve (Yu et al. 1990). The same determinant is also

recognised by the mouse monoclonal antibody HNK-1, which shows a higher affinity compared with most of the human antibodies (Burger et al. 1990).

The majority of patients with anti-MAG IgM activity have a slowly progressive, predominantly sensory, demyelinating neuropathy, whereas patients with unknown IgM reactivity do not have an homogeneous pattern of neuropathy, suggesting that variable pathogenic mechanisms may implicated in the later group (Nobile-Orazio et al. 1994). Recent studies suggest that the neuropathy associated with anti-MAG and anti-SGPG antibodies are more heterogeneous than was previously thought, and that although most of these antibodies react with both MAG and SGPG, some may react with MAG or SGPG alone (van den Berg et al. 1996).

The relationship between anti-MAG autoantibodies and neuropathy is generally accepted (Griffin 1994; Latov 1994; van den Berg et al. 1996), but the precise role of the monoclonal protein in the immunopathogenesis of the neuropathy remains to be elucidated (Wolfe et al. 1997). Electrophysiological studies show demyelinating features with slowed nerve conduction velocities and prolonged distal latencies (Kelly 1990). Pathological studies of biopsed nerves in patients with high titres of anti-MAG antibodies display pronounced demyelination, loss of myelinated fibres, widening of the myelin lamellae, and deposits of IgM and complement on the myelin sheaths (Trojaborg et al. 1995). Abnormal widening of myelin lamellae is almost pathognomonic for patients with anti-MAG antibodies (Vital et al. 1989; Steck & Kappos 1994). Passive transfer of patients' serum intraneurally into cats' median and ulnar nerves (Willison et al. 1988) and sciatic nerve (Hays et al. 1987; Trojaborg et al. 1989) induces demyelination. Immunofluorescence studies demonstrated deposition of the injected M-protein on the surface of the myelin sheath, implying that the M-protein showed reactivity with epitopes of myelin (Hays et al. 1987).

The experimental study by Tatum showed the capacity of IgM anti-MAG antibodies to produce demyelination and the characteristic wide-spacing of myelin in chickens and proved that this type of neuropathy is an antibody-mediated disease (Tatum 1993). In all these instances the case for pathogenic activity of IgM antibodies directed against MAG and other glycosphingolipids including gangliosides is better recognised than for other antigens and for IgG and IgA antibodies (Ropper & Gorson 1998). Despite the debate about the precise pathogenic role of the paraprotein, therapeutic reduction of antibody titres is associated with clinical improvement (Latov 1995; Notermans 1996; van den Berg et al. 1996; Chalk 1997).

2. Monoclonal gammopathy of undetermined significance

Two thirds of patients with paraproteinemic neuropathy have what has been termed "monoclonal gammopathy of undetermined significance". MGUS was once considered benign, but it is now known that a number of patients (approximately 20%) will develop in time a malignant plasma-cell disorder, usually multiple myeloma (Kyle 1992; Kyle & Dyck 1993; Ropper & Gorson 1998). Epidemiological evidence suggest that the prevalence of MGUS is higher in patients with idiopathic neuropathy than in those with an identifiable cause of the neuropathy and the incidence of neuropathy is higher in patients with monoclonal gammopathy than in the general population (Notermans 1996).

These polyneuropathies affect usually but not exclusively, men over 50 years of age. The symptomatology in patients includes foot numbness, paresthesias, imbalance, and gait ataxia that progress over a period of months. Large diameter sensory fibres are most affected (joint position, vibration, and touch). Weakness of the distal muscles of the legs with atrophy occurs as the disease advances. Only a small minority of the patients has a pure motor disorder resembling motor neuron disease. Neuropathy associated with

MGUS is typically a predominantly demyelinating disorder or mixed with findings of demyelination and axonal loss, but a pure axonal type of neuropathy with distinct clinical and electrophysiological features has been described (Gorson & Ropper 1997). Although IgG is the most common class of paraprotein in patients with MGUS, in those with neuropathy the most frequent type of immunoglobulin is IgM (60 percent), followed by IgG (30 percent) and IgA (10 percent) (Ropper & Gorson 1998). Finally, anti-GM1 antibody levels are rarely raised in MGUS and probably play a minor or no role in the pathogenesis of these conditions (Finsterer et al. 1996).

3. Guillain-Barré syndrome and autoimmunity

The importance of antiglycolipid antibodies in the pathogenesis of GBS and other diseases of the peripheral nervous system with suspected autoimmune basis is indicated by the fact that they have been a field of intensive study over the past years (Ilyas et al. 1988; McFarlin 1990; Yu et al. 1990; Pestronk 1991; Ropper 1992; Griffin 1994; Latov 1994; Kornberg & Pestronk 1995; Rees et al. 1995b; Baumann et al. 1998; Fredman 1998; Hahn 1998). In particular, many forms of peripheral neuropathy are associated with increased levels of activated T cells in the peripheral blood, have circulating antimyelin autoantibodies, and respond well, in some cases, to plasma exchange or intravenous immunoglobulin infusions (Dalakas 1995). However, the aetiology of these diseases remains obscure, and although prodromal infections have been reported to play an important role, other factors such as vaccination or surgery are also reported to trigger some of these diseases (Arnason & Soliven 1993). To date, there is increasing evidence that antimyelin autoantibody activity may be involved in their pathogenesis. The presence of demyelinating autoantibodies that recognise peripheral nerve myelin glycolipids has been demonstrated in sera of patients with GBS. In some cases a

decrease in autoantibody titre correlates with the patient's clinical improvement, suggesting that the antibody is possibly involved in the pathogenesis of the disease (Ilyas et al. 1988).

So far, experimental and clinical studies of the inflammatory demyelinating neuropathies demonstrate that very different pathogenic mechanisms may be responsible for the initiation of the autoimmune attack to the nerve. Experimental animal models emphasise the role of the T cell response, whereas clinical studies also indicate the involvement of antimyelin autoantibodies. For antibody-mediated demyelination, the target epitopes of the myelin membrane must be accessible to the circulating antibodies and other substances and the blood nerve barrier must be disrupted. Once these minimal requirements are met, the demyelination will be mediated by antibody-induced activation of complement or by an antibody-dependent cell-mediated cytotoxic response. In contrast, in the case of T cell-mediated disease the endoneurial presence of antigen-presenting molecules is required. Since endoneurial inflammation is the hallmark of AIDP, demyelination might be the consequence of the inflammatory mediators secreted by activated macrophages or of direct cytotoxicity by T cells (Arnason & Soliven 1993; Hughes et al. 1999). The association of GBS with prodromal infections may suggest that a pathogen may be involved in the initiation of an autoimmune response, but further detailed studies of the immune response in human peripheral neuropathies are necessary to clarify the understanding in these diseases.

An autoimmune pathogenesis for GBS was first suggested after the demonstration that rabbits immunised with peripheral nerve tissue develop an inflammatory demyelinating disease of the peripheral nervous system, experimental allergic neuritis (EAN) (Waksman & Adams 1955). This experimental disease (EAN) is considered the *in vivo* model of GBS, with similar clinical features and inflammatory lesions. EAN appears to

be predominantly cell-mediated and can be induced by lymphocytes from sensitised animals but not by serum; autoreactive T cells that recognise specific peripheral nerve myelin proteins are responsible for the inflammatory demyelinating pathology in EAN (Waksman & Adams 1955). However, whether such an autoreactive T cell plays an active role in the pathogenesis of GBS is still unclear.

Indeed, there is increasing evidence implicating antimyelin autoantibody activity in the pathogenesis of GBS. GBS is considered an acute immune-mediated peripheral neuropathy in which an immune response following an infection is directed against components of peripheral nerve. The presence of demyelinating autoantibodies has been demonstrated in the sera of patients with GBS. Autoantibody responses against peripheral nerve myelin glycolipids are also involved in the pathogenesis of certain neuropathies associated with IgM gammopathies. An antibody mediated animal model of EAN can also be induced in rabbits by immunisation with galactocerebroside, the major myelin glycolipid (Saida, Saida & Dorfman 1979). EAN can be induced in the Lewis rat by immunisation with peripheral nerve homogenate, bovine peripheral nerve myelin, myelin basic proteins P_0 and P_2, and neutrogenic peptides (synthetic peptides derived from P_2) (Hartung, Stoll & Toyka 1993).

Campylobacter jejuni infection has been recognised as a frequent prodromal event in GBS and is associated with the generation of serum antibodies to gangliosides that can bind to specific gangliosides epitopes in peripheral nerves and cross-react with carbohydrate antigens in bacterial polysaccharides (Latov 1994; Oomes et al. 1995; van der Meché & van Doorn 1995; Yuki 1997; Sheikh et al. 1998a; Sheikh et al. 1998b). Both the demyelinating form and the recently identified axonal GBS can follow *Campylobacter jejuni* infection frequently associated with demonstrable anti-ganglioside antibodies (Hahn 1998). However, the in vivo model of GBS (EAN) cannot be induced

in the Lewis rats by *Campylobacter jejuni*; also, immunisation with gangliosides does not produce EAN in the Lewis rats, and the addition of gangliosides to the immunogen does not alter this experimental disease (Ponzin et al. 1991).

In other species, immunisation with gangliosides has been more successful. Rabbits immunised with GM1 developed electrophysiological conduction block and Gal(b1-3)GalNAc deposition at the nodes of Ranvier in the sciatic nerve but not clinical symptoms (Thomas et al. 1991), while rabbits immunised repeatedly with GD1b ganglioside developed a sensory ataxia with loss of dorsal root ganglion cells (Kusunoki et al. 1996b). One of 8 rabbits immunised with *Campylobacter jejuni* isolated from a Chinese patient with the acute motor axonal neuropathy (AMAN) form of GBS developed axonal neuropathy. Feeding the same *Campylobacter jejuni* isolate to chickens led to paralysis in 33 of 100 chickens. In the same study sciatic nerve histology showed Wallerian degeneration and only occasional paranodal demyelination. Also, several fibres had intra-axonal macrophages (Li et al. 1996). The pathologic changes were similar to AMAN in humans and this study represents the most direct animal model of GBS following *Campylobacter jejuni* infection, although it needs to be investigated more extensively.

4. The anti-ganglioside antibody in lower motor neuron syndromes

4a. History and clinical observations

Increased titres of antiganglioside antibodies reacting with GM1, GD1b, and asialo-GM1 ganglioside were first reported in a patient with a lower motor neuron syndrome without conduction block, but with a serum IgM monoclonal gammopathy (Freddo et al. 1986). In this report the association of the motor neuropathy with an autoantibody directed

against a neural antigen suggested that the disease may be immune mediated and that patients with IgM M protein and motor syndrome may form a distinct clinical syndrome.

Two years later Pestronk et al. described two patients with multifocal conduction block in motor but not in sensory fibres and high titres of anti-GM1 anti-ganglioside antibodies (Pestronk et al. 1988b). The initial diagnosis was a lower motor neuron form of amyotrophic lateral sclerosis. Both patients had progressive asymmetrical weakness in the hands with fasciculations. Upper motor neuron or bulbar signs were absent. Sensory examination was normal. The patients did not show improvement with corticosteroid treatment and the titres of the antibodies remained high during this treatment. However they showed significant improvement and the antibody titre was reduced after treatment with cyclophosphamide. This report underscored the fact that some patients diagnosed as having lower motor neuron forms of amyotrophic lateral sclerosis may have had a motor neuropathy with serum antibodies to GM1 ganglioside, which is immune mediated and treatable (Pestronk et al. 1988b).

In fact, in the past, numerous cases of pure motor syndromes without signs of upper motor neuron involvement or types of focal weakness and atrophy such as monomelic amyotrophy that occasionally persisted for decades, were usually considered variants of amyotrophic lateral sclerosis (Parry & Clarke 1988; Pestronk et al. 1988b; Kaji et al. 1992; Kornberg & Pestronk 1995; Parry 1996). These studies described the cardinal features of this condition: insidious onset, male preponderance, asymmetrical wasting and weakness usually confined to the upper limbs, slow progression of the disease and lack of involvement of the cranial nerves, cerebellum, brainstem, or sensory system suggesting that the focus of the disease was on cell bodies in the ventral horn (Riggs, Schochet & Gutmann 1984; Gourie-Devi, Suresh & Shankar 1984).

Since the first full description of this disease subsequent studies appeared, reporting patients with increased titres of monoclonal or polyclonal IgM anti-GM1 antibodies and motor neuropathy or motor neuron disease syndromes (Pestronk et al. 1988a; Pestronk et al. 1990; Santoro et al. 1990; Sadiq et al. 1990; Krarup et al. 1990; Lange et al. 1992; Kaji et al. 1992; Adams et al. 1993; Kinsella et al. 1994; Chaudhry et al. 1994; Pestronk et al. 1994b). Nerve conduction studies in these patients showed blockade of nerve impulses along the course of motor fibres suggested that the motor axon in peripheral nerve, rather than the cell body in spinal cord is the primary focus of the disease. These reports suggest that either conduction block or serum antibodies to GM1 ganglioside could be useful markers in the diagnosis and management of lower motor neuron syndromes (Pestronk et al. 1988a; Pestronk et al. 1990; Sadiq et al. 1990; Kinsella et al. 1994; Kornberg & Pestronk 1994).

Patients with immune-mediated motor neuropathies typically have slow progressive weakness with muscular atrophy. The weakness is frequently asymmetric and involves the arms more than the legs; more often it begins distally and its distribution is usually multifocal, localised within the territory of the individual peripheral nerves. With long-existing disease, muscle atrophy becomes more generalised and symmetrical with axonal loss. Deep tendon reflexes are often preserved in the affected areas in the early phase but they reduce with the course of the disease. Fasciculations and cramps may be present in the disease, and also bulbar signs may occasionally be present. The average age of onset is in the fifth decade with a range of 20 to 70 years. The symptoms of the patients may progress slowly for periods of up to 20 years. Conduction studies frequently show one or more areas with conduction block in motor nerves, although sometimes motor conduction could be normal. Sensory signs or abnormal sensory conduction are typically absent and cerebrospinal fluid protein is usually normal. At

present the suggested diagnostic criteria for this newly recognised clinical entity (multifocal motor neuropathy) include progressive lower motor neuron weakness, multifocal motor demyelination with motor conduction block and normal sensory responses on electrodiagnostic testing. The asymmetry of the weakness and the loss of tendon reflexes with relatively strong muscles are two useful clinical features. The presence of elevated antiglycolipid antibodies is not an essential criterion for the diagnosis of the disease, although they are present in approximately 50 - 70% of patients with the clinical and electrophysiological picture of MMN (Pestronk et al. 1990; Kinsella et al. 1994; Chaudhry et al. 1994; Parry 1996; Griffin et al. 1996a; Pestronk et al. 1997; Wolfe et al. 1997). In patients where the electrodiagnosis and clinical evaluation are inconclusive, a high anti-GM1 antibody titre may be useful in supporting the diagnosis of MMN. Security of diagnosis is an important issue because patients with MMN can improve remarkably to intravenous immunoglobulin administration as a first line of treatment, or alternatively to cyclophoshamide, whereas MMN does not respond to plasmapheresis or corticosteroids (Pestronk et al. 1989; Nobile-Orazio et al. 1993; Azulay et al. 1994; van der Meché & van Doorn 1997; Dalakas 1999).

MMN was previously not distinguished from chronic inflammatory demyelinating polyradiculopathy (CIDP) (Lewis et al. 1982; Parry & Clarke 1988; Kaji et al. 1992). There is still some controversy as to whether MMN and CIDP represent distinct or overlapping disorders (Lewis et al. 1982). However clinical, electrophysiological, and immunological data suggest that the differences between these two diseases are more prominent than the similarities. In CIDP proximal symmetrical weakness, the remitting-relapsing course of the disease, sensory symptoms, and increased protein concentration in the majority of patients distinguish this clinical entity from MMN (Pestronk 1991; Lange et al. 1992; Griffin 1994; Kinsella et al. 1994; Kaji et al. 1994; Chaudhry et al.

1994; Taylor et al. 1996; Parry 1996; Ho et al. 1999). Also, anti-GM1 antibody is not often found in CIDP (Kornberg & Pestronk 1995; Melendez-Vasquez et al. 1997; Case Records of the Massachusetts General Hospital (Case 13-1998) 1998) and patients with MMN show an extremely poor response to corticosteroids (with less than 3% of reported patients responding) (Pestronk et al. 1990; Chaudhry et al. 1994; Saperstein et al. 1999). Sensory abnormalities in MMN are unusual (Krarup et al. 1990; Adams et al. 1993; Parry 1996). The clinical, histopathological, and electrodiagnostic hallmarks suggest selective involvement of motor fibres (Kaji et al. 1994). However, some studies have demonstrated that MMN is not an exclusively motor abnormality, although it seems to be so with relation to clinical and electrophysiological criteria. The pathological process in MMN appears also to affect sensory fibres frequently, albeit to a lesser degree than motor fibres (Ho et al. 1999).

The first report of a multifocal demyelinating neuropathy with persisting conduction block was published by Lewis et al. in 1982. Their patients showed motor and sensory symptoms and they have been considered to represent the first described cases of MMN, although the authors regarded this type of neuropathy as a CIDP variant (Lewis et al. 1982). Recently, a new type of multifocal motor neuropathy with involvement of sensory nerves has become known as the Lewis-Sumner syndrome or multifocal acquired demyelinating sensory and motor neuropathy (described by the acronym MADSAM (Saperstein et al. 1999)). Patients with this disorder appear to have important differences from those with MMN: shorter duration of symptoms, abnormal nerve conduction studies showing multifocal motor and sensory conduction block, definite sensory abnormalities including pathological features of prominent sensory nerve demyelination, high incidence of elevated CSF protein, no elevated titres of anti-GM1 antibodies, and good response to corticosteroids (Saperstein et al. 1999). As to

whether this clinical disorder represents a distinct nosologic entity is a matter of controversy. Multifocal acquired demyelinating sensory and motor neuropathy is thought to be distinct from MMN syndrome, and may represents a variant of CIDP (Saperstein et al. 1999) but some authors believe that it is premature to draw such definite conclusions (Parry 1999).

4b. The clinical significance of anti-GM1 antibodies

The clinical significance of anti-GM1 antibodies is still debated (Sadiq et al. 1990; Lange et al. 1992; Adams et al. 1993; Parry 1994; Kinsella et al. 1994; Lange & Trojaborg 1994; Taylor et al. 1996; Parry 1996). Antibodies to GM1 and other gangliosides have been described in a wide variety of neurologic and non-neurologic disorders. Antibodies to GM1 and asialo-GM1 have been found in the sera of patients with multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and even in patients with cranial trauma and cerebrovascular accidents or normal controls (Endo et al. 1984; Willison et al. 1993a; Lange & Trojaborg 1994; Taylor et al. 1996; Salih et al. 1996; Matsuki et al. 1999; Hashimoto 1999). Some reports describe the occurrence of anti-GM1 antibodies in patients with ALS, motor neuron syndromes with gammopathy or thyroid abnormalities, cervical spondylosis simulating ALS, other motor neuropathies and CIDP (Pestronk et al. 1988a; Lamb & Patten 1991; Willison et al. 1993a; Taylor et al. 1996). The possibility that anti-GM1 antibodies might be useful in the diagnosis of ALS was considered initially, but at the present time they do not play a role in either diagnosis or treatment decisions in ALS (Griffin et al. 1996a). Although anti-GM1 antibodies are not necessary for the diagnosis of MMN, it is important to realise, however, that testing for anti-GM1 antibodies is useful when a treatable motor neuropathy is suspected but is difficult to distinguish from ALS (Wolfe et al. 1997).

Whether anti-GM1 antibodies are primarily or secondarily associated with neural tissue damage or whether they represent significant indicators of autoimmune reactivity is not yet clearly known.

4c. Antecedent infections, neurological disease and antibodies to gangliosides

The relationship between preceding infections and antibodies against GM1 ganglioside has been extensively investigated. Guillain-Barré syndrome is an acute demyelinating disease of the peripheral nervous system with accompanying rise in CSF albumin while the cell count remains normal (Honavar et al. 1991; Ropper 1992; Arnason & Soliven 1993; van der Meché & van Doorn 1995). The classical syndrome is usually an acute monophasic neuropathy that usually affects the motor, sensory and autonomic nerves (van der Meché & van Doorn 1995; Hughes & Rees 1997). The symptoms are usually symmetrical and more marked in the lower limbs and the paralysis appears to ascend. The advancing disease may involve the cranial nerves, especially the facial nerves, and in almost 25% of patients the disease affects the respiratory muscles, so that ventilatory support is necessary (Hughes & Rees 1997; Hahn 1998). Typically the progressive phase lasts up to three weeks and by definition must last no longer than 4 weeks. Although its cause and pathogenesis remain unknown, the neurological symptoms are preceded by clinical signs and symptoms of infection 1-3 weeks before the neuropathy (Ropper 1992; Arnason & Soliven 1993; van der Meché & van Doorn 1995).

The clinical picture of GBS is usually produced by an acute inflammatory demyelinating polyradiculoneuropathy, but recently a specific subgroup, separable from poliomyelitis and demyelinating GBS, with distinctive clinical, electrophysiological and laboratory findings has been recognised that is due to acute axonal neuropathy (McKhann et al. 1993; Yuki 1994; Feasby 1994; Visser et al. 1995; Hafer-Macko et al.

1996; Powel & Myers 1996; Hahn 1997; Hughes & Rees 1997; Feasby & Hughes 1998; Hahn 1998; Ho & Griffin 1999). The distinctive clinical pattern of acute motor axonal Guillain-Barré syndrome (AMAN) is characterised by a more rapid onset of paralysis, an earlier nadir, an initially predominant distal weakness, no sensory involvement, and a sparing of the cranial nerves. The affected patients have significantly high titres of anti-GM1 antibodies with no electromyographic evidence for demyelination, and the disease often follows a gastro-intestinal illness usually caused by prodromal *Campylobacter jejuni* infection (Visser et al. 1995).

GBS with severe axonal degeneration involving motor nerves has also been reported in association with anti-GD1a antiganglioside antibody, which may play a significant role in the pathogenesis of AMAN (Yuki et al. 1992b; Yuki et al. 1993d; Carpo et al. 1996; Lugaresi et al. 1997; Ho et al. 1999). Moreover, recently IgG anti-GalNAc-GD1a antibody has been reported to be a possible marker of this pure motor axonal variant of GBS and must be tested together with anti-GM1 in order to diagnose AMAN (Ang et al. 1999; Kaida et al. 2000). Hyperreflexia has been described in some patients with AMAN and anti-GM1 antibodies (Kuwabara et al. 1999). Autopsy studies have shown Wallerian-like degeneration of motor fibres (McKhann et al. 1993). Patients with AMAN seem to respond better to intravenous immunoglobulin than to plasma exchange (Visser et al. 1995).

At present the Guillain-Barré syndrome should be considered to be pathologically heterogeneous, with a primary demyelinating variant, which is most common in North America and Europe, a primary axonal motor variant, described in northern China, and a rare primary axonal sensory and motor variant (Case Records of the Massachusetts General Hospital (Case 39-1999)1999). The variability in the clinical spectrum of GBS may be caused by different pathogenic mechanisms (Kuwabara et al. 1998b; Sheikh et

al. 1998b), although at the present time this seems to have no significant importance in determining the therapeutic approaches for the management of GBS (Hadden et al. 1998). The high effectiveness of intravenous immunoglobulin has been proven in clinical trials and its efficacy appears to be equal to or, in some aspects, superior to plasmapheresis (van der Meché, Schmitz & the Dutch Guillain-Barré study group 1992; Plasma exchange/Sandoglobulin Guillain-Barré syndrome study group 1997; Hadden et al. 1998; Dalakas 1999).

Campylobacter jejuni has been identified as an important cause of prodromal infection in GBS (Yuki et al. 1990; Gregson et al. 1993; Mishu et al. 1993; Oomes et al. 1995; van der Meché & van Doorn 1995; Rees et al. 1995b; Jacobs et al. 1996; Hughes & Rees1997; Nishimura et al. 1997; Feasby & Hughes1998; Hahn 1998; Jacobs et al. 1998; Sheikh et al. 1998b; Case Records of the Massachusetts General Hospital (Case 39-1999)1999), and patients with recent *Campylobacter jejuni* infection had antibody to GM1 ganglioside (Gregson et al. 1993; Oomes et al. 1995; Rees et al. 1995a; Jacobs et al. 1996; Yuki et al. 1997; Hahn 1998; Hao et al. 1998; Jacobs et al. 1998). The frequency of anti-GM1 antibody associated GBS in different series varies from 2% to 30% (Ilyas et al. 1988; Yuki et al. 1990; Nobile-Orazio et al. 1992; van den Berg et al. 1992; Gregson et al. 1993; Rees et al. 1995a; Jacobs et al. 1996; Hughes & Rees 1997; Nevo & Pestronk 1997; Feasby & Hughes 1998; Hao et al. 1998; Kuwabara et al. 1998b). Some studies have confirmed the association between *Campylobacter jejuni* infection, IgG antibodies to GM1 ganglioside and severe axonal degeneration with poor prognosis in patients with the AMAN form of Guillain-Barré syndrome (Gregson et al. 1993; Yuki 1994; Oomes et al. 1995; van der Meché & van Doorn 1995; Rees et al. 1995b; Jacobs et al. 1996; Hahn 1997; Hughes & Rees 1997; Nevo & Pestronk 1997; Jacobs et al. 1998), although other studies suggest that the presence of anti-GM1

antibody is not necessarily a marker of poor prognosis (Rees et al. 1995a; Kuwabara et al. 1998a; Dalakas 1999; Case Records of the Massachusetts General Hospital (Case 39-1999)1999).

The molecular mimicry between GM1 ganglioside and *Campylobacter jejuni* lipopolysaccharides isolated from patients with GBS may play a key pathogenic role in the production of antiganglioside antibodies and the development of the GBS (Latov 1994; Oomes et al. 1995; van der Meché & van Doorn 1995; Yuki 1997; Sheikh et al. 1998a; Sheikh et al. 1998b). It has been shown that GM1-oligosaccharide structure is present in the lipopolysaccharide of *Campylobacter jejuni* (Yuki et al. 1993c; Aspinall et al. 1994; Moran 1997; Hahn 1998; Sheikh et al. 1998b). Although the mechanism of molecular mimicry remains an issue of controversy (Jacobs et al. 1997a), the association of specific gangliosides with distinct forms of GBS lends support to this notion.

The IgG anti-GT1a antibody may be associated with the pharyngeal-cervical-brachial variant or oropharyngeal palsy in GBS and can further contribute in diagnosis of this variant of GBS (Koga et al. 1998; Koga et al. 1999). Also LM1 (Ilyas et al. 1992b) and GM1b (Kusunoki et al. 1996a) gangliosides are immune antigenic targets in GBS, but their possible pathogenic effect remains unknown.

Certain micro-organisms apart from *Campylobacter jejuni* have been associated with particular autoantibodies: *Cytomegalovirus* is associated with GM2 ganglioside (Irie et al. 1996; Jacobs et al. 1998), although the role of anti-GM2 antibody in the development of *Cytomegalovirus*-related GBS is unknown (Hahn 1998; Yuki & Tagawa 1998; Khalili-Shirazi et al. 1999). More recently Mori et al. reported a patient with an axonal GBS associated with anti-GM1 antibodies after *Haemophilus influenzae* infection. Using ELISA inhibition studies and cytochemical staining of the bacteria strain they postulated that *H. Influenzae* has a Gal(β1-3)GalNAc epitope which is shared by GM1

or GD1b gangliosides. They suggest that the syndrome could occur because of a crossed immune reaction between a component of the bacterium and the axolemma of the motor nerves, as has been speculated in *Campylobacter jejuni*-related GBS (Mori et al. 1999b).

4d. *In vitro* and *in vivo* studies on anti-GM1 antibodies

The pathogenic relationship between autoimmune peripheral neuropathies and antiganglioside antibodies is still a matter of great controversy (Latov 1994; Hahn 1998; Kaji & Kimura 1999). Nevertheless, abundant clinical and epidemiological observations support the notion of a pathogenic role for these antibodies (Table 1). However, as described above, antibodies to GM1 and other gangliosides have been found in a variety of neurologic and non-neurologic autoimmune conditions. In addition, a wide variety of electrophysiological and morphological studies on anti-GM1 antibodies have shown divergent findings.

Experimental conduction block was described in demyelination induced by topical application of anti-galactocerebroside serum (Lafontaine et al. 1982), and after intraneural injection of anti-galactocerebroside (Saida et al. 1979; Sumner et al. 1982) or anti-MAG antibodies (Hays et al. 1987; Willison et al. 1988; Trojaborg et al. 1989). Intraneural injection of anti-GM1 serum from a patient who had motor neuron disease and multifocal motor conduction block into rat sciatic nerve produced acute conduction block with significant fall in the amplitude ratio and deposits of immunoglobulin were observed at the nodes of Ranvier (Santoro et al. 1992). Similar studies using intraneural injection into rat tibial nerve showed that anti-GM1 sera obtained from patients with MMN produce conduction block and demyelination, whereas, in the same study, injection of sera with high titres of anti-GM1 obtained from patients with spinal

muscular atrophy did not produced block and pathological analysis did not showed demyelination (Uncini et al. 1993).

In contrast, Harvey et al. (Harvey et al. 1995) failed to induce conduction block after intraneural injection into rat tibial nerves of immunoglobulin fractions with high titres of anti-GM1 IgG or IgM antibodies from patients with Guillain-Barré syndrome or MMN. In the same study, histological examination of the injected nerves did not show demyelination despite the binding of the anti-GM1 antibody to the nodes of Ranvier. Similarly, antisera against GM1 have previously failed to induce demyelination after injection into rat sciatic nerves, in contrast to the marked demyelination produced by anti-galactocerebroside and anti-P_0 sera (Hughes et al. 1985).

In similar studies, immunisation of rabbits with GM1 ganglioside has been reported to induce electrophysiological abnormalities, mild axonal degeneration and immunoglobulin deposits at the nodes of Ranvier (Thomas et al. 1991). Axonal degeneration and conduction block was observed after intraneural injection of the B subunit of cholera toxin (CT-B) into sciatic nerves of rats in which anti-CT-B antibodies were previously generated by immunisation with the B subunit of CT, which avidly binds to GM1 ganglioside (Wirguin et al. 1995). The study implies that CT binds to GM1 both at the nodal gap and paranodal myelin (Corbo et al. 1993) and complement activation by immune complexes may damage the myelin sheath and axons at the nodes of Ranvier, producing both conduction block and demyelination. In another study using mouse phrenic nerve-hemidiaphragm preparation multifocal motor neuropathy sera containing anti-GM1 antibodies blocked nerve conduction at distal motor nerves, but also, multifocal motor neuropathy anti-GM1 negative sera produced similar results (Roberts et al. 1995), suggesting that anti-GM1 antibodies may are not exclusively the cause of conduction block.

In numerous immunohistological studies using anti-GM1 ganglioside sera (Freddo et al. 1986; Gregson et al. 1991; Harvey et al. 1995; Kusunoki et al. 1997) and monoclonal antibodies (O' Hanlon et al. 1996; Molander et al. 1997; O' Hanlon et al. 1998), binding to peripheral nerve structures, including nodes of Ranvier has been convincingly demonstrated (Santoro et al. 1990; Santoro et al. 1992; Adams et al. 1993; Nardeli et al. 1994; Hafer-Macko et al. 1996; Paparounas et al. 1999).

Also, incubation of an isolated nerve preparation *in vitro* with anti-GM1 antibodies produced acute conduction block of myelinated nerve fibres in excised and desheathed rat sciatic nerves (Arasaki et al. 1993). However, in a more recent study the same authors observed conduction block with IgM antibodies whereas IgG antibody failed to block myelinated nerve conduction (Arasaki et al. 1998), suggesting a difference in action between IgG and IgM antibodies. Takigawa et al. (Takigawa et al. 1995) found that anti-GM1 antibody increased the potassium current elicited by step depolarisation, and in the presence of active complement blocked sodium channels irreversibly, suggesting that anti-GM1 antibodies block sodium channels, causing conduction block (Waxman 1995). However these data have recently been challenged in a study in which the application of the same sera with high titres of anti-GM1 antibody did not cause conduction block or block sodium channels (Hirota et al. 1997). It was therefore concluded that the physiological action of the antibody alone is insufficient to explain conduction block in human disorders. More recently, as discussed later in this thesis, Paparounas et al. showed that anti-GM1 antibodies can bind to the nodes of Ranvier, and activate the complement cascade without causing acute conduction block *in vitro* (Paparounas et al. 1999). This also suggests that antiganglioside antibodies do not act as a primary blocker of sodium channels.

5. The role of anti-GQ1b antibody in autoimmune neuropathies

5a. Anti-GQ1b antibody: an immunological marker for Miller Fisher syndrome

Over the last years there has been an extensive discussion related to the role of anti-GQ1b antibodies in the pathogenesis of Miller Fisher syndrome. The syndrome of total external ophthalmoplegia, severe ataxia and loss of tendon reflexes was first reported in three patients by M. Fisher; he noticed that the cause of this syndrome was obscure but the rise in the protein of the cerebrospinal fluid in the late stages of the disease indicated a close relation to the Guillain-Barré syndrome, and since then the MFS has been described as an unusual variant of Guillain-Barré syndrome with benign prognosis (Fisher 1956).

The first report involving the antiganglioside antibodies in the pathogenesis of MFS was published in 1992. Chiba et al. found increased serum anti-GQ1b IgG activity in all six patients with MFS in the early phase of the illness. The titre of the antibody reduced with time. They suggested an association between anti-GQ1b antibodies and MFS, emphasising that this antibody could be a possible immunological marker of great clinical significance in this syndrome (Chiba et al. 1992). Since then several studies have found increased anti-GQ1b antibodies titres in Miller Fisher syndrome with the immunoglobulin being of IgG class rather than IgM; the antibody titre decreased with the clinical course of the illness (Chiba et al. 1993; Yuki et al. 1993b; Willison et al. 1993c; Jacobs et al. 1995; Yuki 1996a; Carpo et al. 1998; Suzuki et al. 1998; Schwerer et al. 1999).

5b. The link of anti-GQ1b antibody with acute ophthalmoplegia

Some studies have shown that anti-GQ1b IgG antibody is related to the severity of the cerebellar-like ataxia of the syndrome (Yuki et al. 1993b). Selective staining of the

human cerebellar molecular layer by serum IgG anti-GQ1b antibodies from patients with Miller Fisher syndrome or Guillain-Barré syndrome with ophthalmoplegia and ataxia give further evidence that anti-GQ1b antibody is related with ophthalmoplegia and ataxia in MFS and similar syndromes, and supports the hypothesis for a central origin of the ataxia in MFS (Kornberg et al. 1996). In contrast, a number of studies suggest the close correlation between anti-GQ1b IgG antibody and acute ophthalmoplegia in typical Miller Fisher syndrome, in atypical (without ataxia) Miller Fisher syndrome with ophthalmoplegia, or Bickerstaff's brainstem encephalitis, and in Guillain-Barré syndrome with ophthalmoplegia (Chiba et al. 1993; Yuki 1996a; Goffette et al. 1998; Suzuki et al. 1998), or isolated ophthalmoplegia (Yuki, Koga & Hirata 1998; Radziwill et al. 1998). Some authors recommend that diseases which show external ophthalmoplegia, should be labelled as "IgG anti-GQ1b antibody syndrome" (Yuki 1996a), and this proposal is supported by the fact that the anti-GQ1b antibody activity reflects the severity of the patients' symptoms, especially ophthalmoplegia (Mizoguchi 1998). Extensive investigations including large numbers of patients have confirmed the close association of IgG anti-GQ1b antibody with both ataxia and ophthalmoplegia (Kusunoki, Chiba & Kanazawa 1999). In contrast, other reports suggest that anti-GQ1b IgG antibody is not necessarily associated with ophthalmoplegia. They presented patients with acute oropharyngeal palsy (O'Leary et al. 1996), acute demyelinating polyradiculoneuropathy (Mizoguchi et al. 1997) or Miller Fisher-like syndrome (Mori et al. 1999a) with high titres of anti-GQ1b IgG antibody activity without ophthalmoplegia.

Immunohistochemical studies have shown staining in the paranodal regions of the extramedullary portions of the human oculomotor, trochlear and abducens nerves; such an accumulation of GQ1b epitope was not present in the other cranial nerves or in the peripheral and central nerve tissues examined (Chiba et al. 1993). The same authors

more recently (Chiba et al. 1997) investigated the ganglioside composition of all the human cranial nerves. They found a significantly higher percentage of GQ1b ganglioside in the optic, and all three ocular motor nerves than in all the other cranial nerves.

5c. Anti-GQ1b antibody and neuromuscular transmission

Recent electrophysiological evidence has revealed that sera from patients with Miller Fisher syndrome who were anti-GQ1b positive cause a transient rise in miniature end plate potential frequency, followed by transmission block, in the mouse phrenic nerve-hemidiaphragm preparation; this suggests that the GQ1b antibody is likely to cause a disturbance of acetylcholine release from motor nerve terminals (Roberts et al. 1994). Clinical electrophysiological recordings in anti-GQ1b positive patients with tetraparesis suggest that motor nerve terminal block was the cause of weakness and thus the anti-GQ1b antibody may be the blocking factor (Uncini & Lugaresi 1999). Plomp et al. have recently published the effects of anti-GQ1b antibody on mouse neuromuscular junctions *in vitro*; they found that the antibody binds at neuromuscular junctions, inducing a massive quantal release of acetylcholine from nerve terminals, and blocks neuromuscular transmission, an effect that closely resembles the action of the paralytic neurotoxin α-latrotoxin at the mouse neuromuscular junctions. The report strongly suggests that anti-GQ1b antibodies, in conjunction with activated complement components, are the principal pathophysiological factors of motor symptoms in MFS (Plomp et al. 1999).

5d. Molecular mimicry and anti-GQ1b antibody

In some anti-GQ1b IgG antibody positive sera, additional anti-GT1a IgG antibody activity has been detected (Chiba et al. 1993); this indicates that anti-GQ1b and anti-GT1a antibodies cross-reacted with each other's antigen, suggesting that the same IgG antibody bound to both antigens, which are structurally similar (Chiba et al. 1993; Willison et al. 1997). Cross-reactivity of anti-GQ1b IgG antibody with surface epitope on *Campylobacter jejuni* strains from patients with Miller Fisher syndrome supports the hypothesis of molecular mimicry between bacteria and neural tissue (Jacobs et al. 1995; Yuki 1997; Neisser et al. 1997; Jacobs et al. 1997a; Hahn 1998; Yuki & Miyatake 1998; Ohtsuka et al. 1998a).

5e. Benign brainstem encephalitis and anti-GQ1b antibody

The nosological condition with which Miller Fisher syndrome has clinical similarities is Bickerstaff's brainstem encephalitis. Bickerstaff described 8 patients who had a clinical syndrome of gradual onset with almost total paralysis of all motor function originating in the brainstem, but without cardiac or respiratory abnormality; the syndrome is accompanied by mild pyramidal and long-tract sensory disturbances with severe drowsiness. All these patients had a prodromal infection. Seven patients had a dramatic total recovery without neurological sequelae. He characterised it as a grave syndrome but with benign prognosis (Bickerstaff & Cloake 1951; Bickerstaff 1957). Whether Miller Fisher syndrome and Bickerstaff's encephalitis are distinct diseases is a matter of controversy. Whether the lesions responsible for the clinical findings of these diseases are in the peripheral or central nervous system is a matter of disagreement as well (Phillips, Stewart & Anderson 1984; Al-Din 1987; Taphoorn et al. 1989; Yaqub et al.

1990; Petty et al. 1993; Al-Din et al. 1994; Urushitani, Udaka & Kameyama 1995; Miwa et al. 1995).

Interestingly, sera taken from patients with Bickerstaff's brainstem encephalitis with ophthalmoparesis have increased titres of anti-GQ1b IgG antibody during the acute phase of the syndrome (Yuki et al. 1993a; Yuki 1995; Yuki 1996a; Shimokawa et al. 1998). The antibody titres decrease during the clinical course of the disease. These findings suggest that a common autoimmune mechanism is likely in Miller Fisher syndrome and Bickerstaff's encephalitis and that both illnesses possibly represent a distinct disease with a wide spectrum of symptoms, which include ophthalmoplegia and ataxia. Plasmapheresis has been suggested as a successful treatment in Miller Fisher syndrome and in Bickerstaff's brainstem encephalitis with anti-GQ1b IgG antibody (Yuki 1995; Yuki 1996a; Shimokawa et al. 1998). Immunoadsorption using the tryptophan-immobilized polyvinyl alcohol gel column is a new alternative type of plasmapheresis in these illnesses (Yuki 1996b; Tagawa, Yuki & Hirata 1998; Ohtsuka et al. 1998b).

6. Sensory neuropathy with antibodies to GD1b and disialosyl gangliosides

6a. Clinical implications

Monoclonal IgM antibodies that reacted with GD1b and disialosyl gangliosides were first reported in a patient with a predominantly sensory ataxic demyelinating neuropathy associated with paraproteinemia. The monoclonal IgM reacted strongly with GD1b and other gangliosides containing disialosyl groups, such as GT1b, GD3, and GD2, but not with GM1, GM3, GD1a, and MAG (Ilyas et al. 1985). Since then several patients have been described with predominantly sensory neuropathy and elevated titres of IgM or IgG antibodies with activity against disialosyl residue (Younes-Chennoufi et al. 1992; Daune

et al. 1992; Yuki et al. 1992a; Willison et al. 1993b; Willison et al. 1994; Brindel et al. 1994; Herron et al. 1994; Wicklein et al. 1997).

All patients had a large-fibre neuropathy with areflexia and most had gait ataxia and elevated CSF protein levels. In most cases the neuropathy was demyelinating, but also mixed, axonal and demyelinating, has been described in some patients (Latov 1995). Also, antibodies against GD1b ganglioside have been found in many patients with motor neuron syndromes and mixed sensorimotor neuropathies where the IgM antibody also recognises GM1 or other gangliosides without disialosyl groups (Latov 1994).

6b. Experimental studies on anti-GD1b antibodies - Immunoreactivity against dorsal root ganglion neurons and sensory neuropathy

Anti-GM1/GD1b IgM M-immunoglobulins from patients with motor neuron syndromes can bind presynaptically to neuromuscular junctions in guinea pig gastrocnemius muscle (Schluep & Steck 1988), and moreover can destroy human spinal cord neurons co-cultured with muscle (Heiman-Patterson et al. 1993). Sera from patients with a clinically pure sensory ataxic neuropathy containing high titres of IgM anti-disialosyl antiganglioside antibodies (reacting against GD1b, GD3, GT1b, GQ1b, GM3, and GD1a but not against GM1) showed cytoplasmic staining in about 80% of human dorsal root ganglion cells. This study suggests that the immunostaining of dorsal root ganglion neurons is likely to be due to the reactivity with GD1b ganglioside (Oka et al. 1996).

Also, other important studies have revealed cytotoxic effects of GD1b antibody that appear to be destructive to ganglionic neurons *in vitro*; sera from patients with acute relapsing sensory neuropathy and high titres of monoclonal IgM antibody recognising disialosyl groups of gangliosides produced cell death of rat dorsal root ganglion neurons

in cultures (Ohsawa et al. 1993). Interestingly, the cell damage occurred without the addition of complement (Ohsawa et al. 1993).

Willison et al., using a monoclonal antibody cloned from a patient with an IgM paraproteinemic neuropathy which was reactive with GD1b, GT1b, GQ1b and GT3 gangliosides, demonstrated immunolocalization to the dorsal root ganglia, and to motor nerve terminals, muscle spindles, myelinated axons and nodes of Ranvier in rodent tissue (Willison et al. 1996). Moreover they showed that this antibody can cause neurophysiological effects by impairing nerve excitability and reducing neurotransmitter release in the phrenic nerve-hemidiaphragm preparation (Willison et al. 1996). Kusunoki et al., using two mouse monoclonal antibodies each monospecific to GM1 and GD1b gangliosides, found that the GD1b bound to neurons in dorsal root ganglia and sympathetic ganglia, and some parts of the peripheral myelin, mainly to paranodal areas, whereas no significant immunoreactivity was observed with GM1 antibody (Kusunoki et al. 1993). Furthermore, GD1b is localized to dorsal root ganglia neurons and to paranodal myelin of human peripheral nerve, and these places could be the binding sites for anti-GD1b antibodies (Kusunoki et al. 1997).

GD1b ganglioside shares with GM1 the Gal (β1-3) GalNAc epitope, whereas the NeuNAc (α2-8) NeuNAc moiety is present only in the GD1b structure (Dalakas & Quarles 1996). It is likely that when IgM immunoglobulin recognises the Gal (β1-3) GalNAc epitope of GM1 and GD1b gangliosides, patients present with a motor neuropathy (Freddo et al. 1986; Corbo et al. 1993). In contrast, if IgM reacts with epitopes containing disialosyl groups found only in the GD1b structure there is clinically a sensory neuropathy.

The report by Kusunoki et al. that immunisation of rabbits with GD1b ganglioside can cause sensory ataxic neuropathy resembling the human disease was the first study in

which an animal model for autoimmune neuropathy was induced by sensitisation with ganglioside. High anti-GD1b antibody titre and the absence of inflammatory lymphocytic infiltration suggested an antibody-mediated pathogenic process (Kusunoki et al. 1996b). In the same study pathological analysis showed axonal degeneration in the dorsal column of the spinal cord, in the dorsal roots, and sciatic nerve; some of the nerve cell bodies in the dorsal root ganglia had degenerated and disappeared (Kusunoki et al. 1996b). In a more recent study the same investigators immunised increased numbers of rabbits with GD1b and confirmed the induction of sensory ataxic neuropathy in more than one-half of them. Moreover, they found that IgG antibody monospecific to GD1b may be crucial to induce this experimental neuropathy, whereas antibodies that react with both GD1b and GM1 by binding to the terminal Gal-GalNAc residue may be of no importance in pathogenesis (Kusunoki et al. 1999). The primary sensory neurons that mediate proprioceptive sensation depend mainly on neurotrophin-3, which is particularly essential for the survival and maintenance of large sensory neurons in the dorsal root ganglion (Chen et al. 1999). Each neurotrophin has its receptor and trkC is the preferred receptor for neurotrophin-3 (Chen et al. 1999). Hitoshi et al. studied the expression of trkC in dorsal root ganglia of experimentally diseased rabbits after immunisation with GD1b ganglioside and found markedly reduced expression of trkC in dorsal root ganglia of affected rabbits in the acute phase. This suggested that the GD1b antibody-mediated down-regulation of trkC expression could be important in the pathogenesis of this experimentally induced sensory ataxic neuropathy (Hitoshi et al. 1999).

Despite the strong evidence supporting the pathogenic role of anti-GD1b antibody in inducing sensory ataxic neuropathy further studies are needed to elucidate thoroughly the pathophysiological mechanisms and the immunopathological aspects of this antibody-mediated autoimmune neuropathy. Finally, the convincing evidence that

GD1b antibody is causally related to the development of sensory ataxic neuropathy is very encouraging, and improves the prospects that future therapies will successfully inactivate or remove the antibody (Dalakas & Quarles 1996; Quarles & Dalakas 1996; O'Leary & Willison 1997).

7. Predominantly sensory neuropathy and anti-sulfatide antibodies

Increased titres of circulating monoclonal or polyclonal IgM antibodies to sulfatide have been reported in a variety of chronic axonal and demyelinating polyneuropathy syndromes (Ilyas et al. 1992a; Lopate et al. 1997a; Ferrari et al. 1998), associated with a predominantly sensory neuropathy (van den Berg et al. 1993; Latov 1995; Lopate et al. 1997a). Immunocytochemical studies have shown that the antibodies bound to the surface of rat dorsal root ganglia neurons (Quattrini et al. 1992; Nardeli et al. 1995); other studies demonstrated the presence of IgM antibody and complement product C3d bound to myelin sheath, suggesting that pathological changes in some types of demyelinating neuropathy associated with antisulfatide antibodies are complement mediated (Ferrari et al. 1998). In several other studies the antisulfatide antibody cross reacted with MAG (Ilyas et al. 1992a; van den Berg et al. 1993), and patients had a sensorimotor neuropathy with demyelination, widened myelin lamellae, and deposits of IgM on myelin sheaths (Latov 1995). Passive transfer in new-born rabbits of anti-sulfatide antibodies from a patient has produced demyelinating nerve lesions resembling the patient's polyneuropathy (Nardeli et al. 1995). Also, sera from patients with insulin-dependent diabetes mellitus (IDDM) were anti-sulfatide positive and sulfatide as an antigen may be involved in the pathology of IDDM, being an immunological marker of this disorder (Buschard et al. 1993). In addition, serum autoantibodies against sulfatide were detected in non-insulin-dependent diabetes mellitus (NIDDM) patients with

diabetic neuropathy, so that autoimmune mechanisms may play a role in this type of neuropathy (Shigeta et al. 1997).

The role of anti-sulfatide antibodies in the pathogenesis of neuropathy is still unknown (van den Berg et al. 1993). Sulfatide is a common glycolipid of the spinal cord and peripheral nerve tissue, and is highly enriched in myelin (van den Berg et al. 1993). Recent studies suggest that anti-sulfatide antibody related polyneuropathy syndromes do not appear to constitute a distinct syndrome with specific clinical, electrophysiological, histopathological, and antibody binding findings (van den Berg et al. 1993). Some patients seem to have a predominantly axonal sensory neuropathy, while others present with severe demyelinating sensorimotor neuropathy (van den Berg et al. 1993). It is likely that the antisulfatide antibodies differ in their fine specificity, binding sites and cross-reactivity. Maybe these parameters could determine the particular clinical syndromes and, also, explain the heterogeneity of anti-sulfatide antibody associated neuropathy. Thus in predominantly axonal sensory neuropathy, binding may occur to sensory axons whereas in patients with demyelinating neuropathy, antibodies may be directed against the sulfatide in myelin (Lopate et al. 1997a; Lopate et al. 1997b). Moreover the predominant sensory disturbances in these patients may be explained by antibody binding to dorsal root ganglia (Quattrini et al. 1992), although recent studies have found that IgM anti-sulfatide antibodies show several different tissue binding patterns in the peripheral and central nervous systems and no strong binding to structures in dorsal root ganglia (Lopate et al. 1997b).

PART TWO – THE STUDY

Introduction

In the first two chapters of this study we attempted to clarify the electrophysiological effect of anti-ganglioside antibodies on nerve conduction. For this purpose we used two different isolated nerve preparations: a) the mouse sciatic nerve preparation, where the greater part of this work was conducted and b) the rabbit sural nerve prerараtion, a pure sensory nerve from another animal species. Subsequently, in the immunohistochemistry chapter we investigated the immunoglobulin and complement deposition at the nodes of Ranvier following *in vitro* incubation of mouse sciatic nerve teased fibres with anti-ganglioside IgM antibodies. Finally, we completed this study by examining the physiological effect of antibody on neuromuscular transmission with the aid of mouse phrenic nerve-hemidiaphragm preparation.

CHAPTER ONE: ELECTROPHYSIOLOGICAL EFFECT OF ANTIBODIES AGAINST GANGLIOSIDES ON MOUSE SCIATIC NERVE PREPARATION

A. Method and materials

1. Anti-ganglioside antisera and monoclonal antibodies

Sera were collected from patients with autoimmune neuropathies and normal controls and stored at -70°C until use (as approved by the Southern general Hospital NHS Trust Ethics Committee). Screening for anti-ganglioside antibodies using a panel of gangliosides comprising GM1, GM2, GM3, GD1a, GalNAc-GD1a, GD1b, GD3, GT1a, GT1b and GQ1b, was performed by ELISA (enzyme-linked immunoabsorbent assay) and thin layer chromatography overlay (Willison et al. 1996). We studied nine samples of human sera or plasma (see Table 4) positive for anti-ganglioside antibodies taken from the following groups of patients: a) three patients with multifocal motor neuropathy that showed anti-GM1 IgM antibody reactivity; b) three patients with paraproteinemic and anti-disialosyl IgM antibodies; c) three patients with Guillain-Barré syndrome or Miller Fisher syndrome and anti-GM1 or GQ1b IgG antibodies. We also examined three monoclonal IgM anti-GM1 antibodies cloned from peripheral blood lymphocytes of MMN patients with elevated titres of anti-GM1 antibody (Paterson et al. 1995). In order to maintain experimental consistency throughout these studies, a single human normal control serum, which did not contain anti-ganglioside antibodies, was used as a source of complement and confirmed as having classical pathway (CH_{50} =120 units/ml) and alternate pathway (CH_{50} =71 units/ml) complement levels within the normal ranges. Immunoassay studies of these sera were performed by Mrs J Veitch in the Neuroimmunology Laboratory, Southern General Hospital.

Table 4 *Binding properties and origin of anti-ganglioside antibodies*

Code	Diagnosis	Ab class	Ganglioside binding profile (titre)			
			GM1	GM2	GD1b	GQ1b
GA	MMN	IgM	1/17000	1/35000	neg	neg
JW	MMN	IgM	1/12800	neg	1/7400	neg
PS	MMN	IgM	1/1000	neg	1/800	neg
CH	PPN	IgM	Neg	neg	1/100,000	1/450,000
VP	PPN	IgM	Neg	neg	1/120,000	1/170,000
BK	PPN	IgM	neg	neg	1/1300	>1/5000
MI	GBS	IgG	>1/5000	neg	>1/5000	neg
JY	GBS	IgG	1/1600	1/1400	1/600	neg
AS	MFS	IgG	Neg	neg	neg	1/3300
Monoclonal antibodies*						
Wo1	MMN	IgM	9100	neg	1700	neg
Sm1	MMN	IgM	6500	neg	neg	neg
Bo1	MMN	IgM	1200	neg	250	neg

neg signifies reactivity undetectable; MMN = multifocal motor neuropathy; PPN = paraproteinemic neuropathy; GBS = Guillain-Barré syndrome; MFS = Miller Fisher syndrome; Ab = antibody. *Titre is expressed as the reciprocal of the antibody concentration that gave half maximal binding.

2. Electrophysiological recordings from mouse sciatic nerve

Male BALB/c mice (weighing 25-30g) were killed by inhalation of carbon dioxide, according to United Kingdom Home Office guidelines. A 5-7 cm length of sciatic

nerve, comprising proximal and distal regions was dissected out and desheathed under a dissecting microscope (x50). The preparation was then mounted into a recording chamber consisting of three cylindrical compartments of 1ml capacity each (Figure 3).

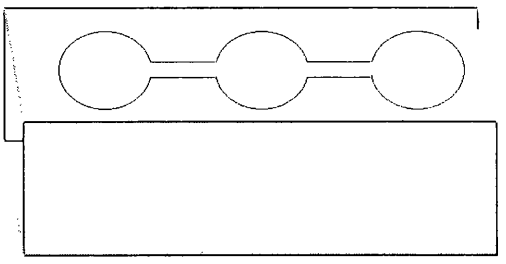

Fig. 3 Diagram of the recording chamber.

Electrical isolation between the compartments was achieved using vaseline. The two external compartments were filled with physiological buffer 154mM NaCl, 5mM KCl, 1.25mM $MgCl_2$, 11mM glucose, 5.46mM HEPES (*N*-[2-hydroxyethyl]piperazine-N^1-[2-ethanesulfonic acid]), 1.8mM $CaCl_2$, pH 7.4; the central compartment (volume=1mL) contained the test reagents. Before each experiment the solution was saturated with 100% O_2 for 30mins. Standard extracellular recording techniques were employed to record compound nerve action potential events. Pellet type silver electrodes were dipped into each of the compartments of the recording chamber with stimulation taking place between the central and one of the external compartments. Recordings were obtained from the central pool. The ground electrode was dipped into the central pool. A Grass S48 stimulator was used to supply electrical impulses (0.4Hz, 0.04msec duration) supramaximally via a stimulus isolation unit, model SIU 5A (Grass Instrument Co., Quincy, Mass., USA). Signals were amplified with a CED 1902 transducer, digitised by an analogue-digital converter CED 1401 and analysed with a microcomputer program (Dempster 1988).

In each experiment the amplitude, latency, rise time, and threshold of the obtained action potentials were measured. Before the addition of test compounds, the sciatic nerve preparation was incubated in physiological buffer for 45 min under constant supramaximal stimulation to demonstrate viability in the preparation and consistency in the recordings. All test solutions were dialysed against physiological buffer at pH 7.4. Osmotic pressure was maintained at 320±10 mOsm/kg. The pH and osmotic pressure were re-checked at the beginning and end of all recordings since in preliminary experiments we noted markedly deleterious effects of high and low pH and osmolarity on the physiological stability of the preparation. All test sera and plasma, including the normal serum used as a source of supplementary complement, were diluted 1:10 in physiological buffer. Monoclonal anti-GM1 antibodies and normal human IgM were diluted in physiological buffer and used at a concentration of 20μg/ml. Cholera toxin B subunit (Sigma C9903) a high affinity ligand for GM1 (Holmgren 1981) was used at 20μg/ml, and the sodium channel blockers saxitoxin (STX) and tetrodotoxin (TTX) at 0.5nM and 1μg/ml concentration, respectively.

All the experiments on ensheathed nerve and the initial experiments on desheathed nerve were carried out at a room temperature (RT) of 20±2°C. In later experiments, one anti-GQ1b IgM (CH) and one anti-GM1 IgG (MI) sera were tested after changing temperature conditions, and also two anti-GM1 sera (GA and JW) were studied at 37°C, maintained using a circulating constant temperature bath. Nerves were incubated with test sera for 180-240mins, with supplementary normal serum as a fresh source of active complement being applied for a further 90-120mins. In the later experiments performed at 37°C, fresh serum was applied together with the two anti-GM1 sera from the start of the experimental recordings. Electrophysiological studies were performed with the operator blinded to the nature of the test compound.

The data are presented as mean ± standard error mean. All measurements were normalized according to the control values at time 0 min, without the presence of anti-ganglioside antibody. Statistical significance was tested with non-parametric statistics (Mann – Whitney's test) and X^2 test as appropriate. All analyses were performed using Minitab 10.5 for Windows statistical package.

B. Results

1. Pitfalls that can influence the electrophysiological recordings

In early electrophysiological recordings on ensheathed and desheathed mouse sciatic nerve preparations the reproducibility of some experiments was doubtful; we found acute conduction block after the application of patients' sera or plasmas and we hypothesized that it was due to a direct effect of anti-ganglioside antibody activity. However, we observed similar changes in nerve action potentials with the preparation incubated only with physiological salt solution, and also we recorded acute conduction block after the application of other normal control compounds.

Initially, we were unable to give a satisfactory explanation of these contradictory results and we tried to identify and elucidate those factors and parameters that could influence the stability and consistency of the electrophysiological recordings. We performed a significant number of experiments with control and anti-ganglioside compounds and we found that several factors can affect and alter the experimental process and give incorrect results. Only after having identified these factors, were we able to achieve good reproducibility of the experiments and trustworthiness of the recordings. In the next pages, we briefly discuss these observations.

1a. High osmolarity and changes in bath pH can induce negative effects on compound nerve action potential *in vitro*

In preliminary experiments we observed that some time after the antibody application, a progressive decrease in the amplitude of the action potential occurred. This was more apparent with some samples, in particular with some batches of monoclonal antibodies (Figure 4A shows such an extreme example). When we checked the osmotic strength of the bathing solution before and after the antibody application, we found that it was considerably higher compared to normal osmolarity of the physiological salt solution used (Normal osmolarity: 320±10 mOsm/kg; Table 5). We also obtained similar results with some control compounds (Figure 4B). In order to look at the effects of high osmolarity reagents on nerve conduction, we removed a part of the volume (100μL) of a hypertonic solution and substituted it with an equal amount of normal osmolarity control compound; then, an increase in nerve action potential was observed (Figure 5A, and Table 5). As expected, the application of normal isotonic compounds did not influence the real physiological effect of STX solution which was of normal osmolarity (Figure 5B). We assumed that the high osmotic pressure of test compounds and solutions can produce acute conduction block and, in subsequent experiments, the osmolarity of bathing solutions was maintained at 320±10 mOsm/kg.

We also observed that significant changes in pH of bathing solutions and compounds (Table 5) can alter the electrophysiological recordings (Figures 6A, 6B, 7, and 8), and in all later experiments the pH was at 7.4 level, checked at the beginning and end of recordings.

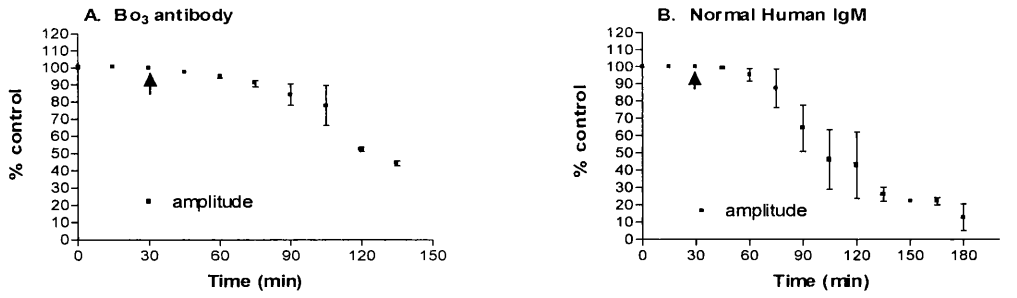

Fig. 4 Effect of high osmotic strength compounds on the amplitude of action potentials of desheathed mouse sciatic nerve preparation. Arrows signify the time of application of Bo3 anti-GM1 antibody (Fig. 4A, n=2), and normal human IgM (Fig.4B, n=2). The final osmolarities were 776mOsm/Kg and 885 mOsm/Kg respectively. Points are mean values and SEM are indicated by error bars unless they were smaller than symbols.

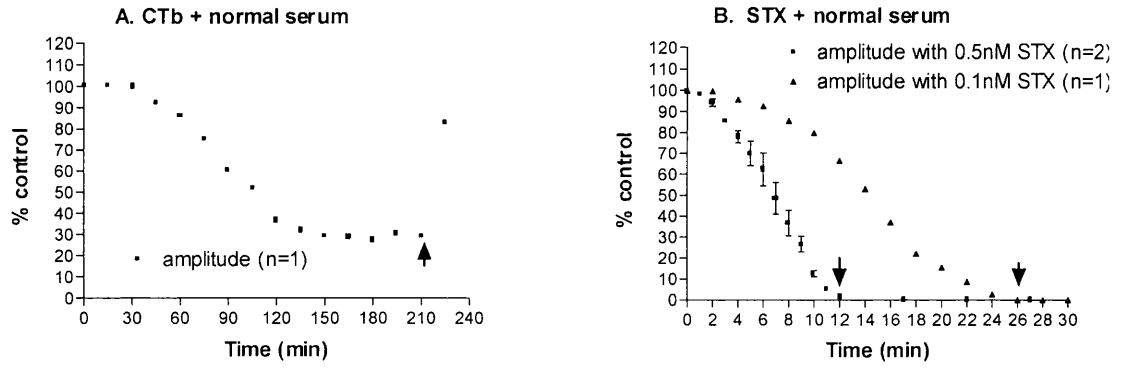

Fig. 5 Removal (arrows) of 100 μL of the bathing solution (1/10 of the total bath volume) and application (arrows) of 100 μL normal human serum of normal osmolarity can reverse the conduction block induced by high osmolarity Cholera toxin B subunit (Fig. 5A, and Table 5), but does not influence the physiological effect of normal osmolarity saxitoxin (STX) solution (Fig. 5B). Total bath volume=1mL.

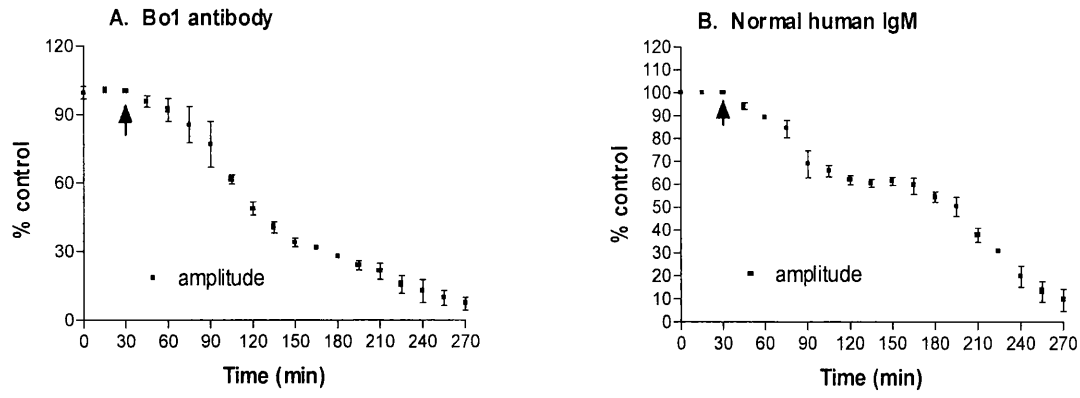

Fig. 6 Effect of pH=9.4 on the amplitude of desheathed mouse sciatic nerve action potential. The pH of physiological salt solution before application of test compounds was 7.4. Arrows signify the time of application of Bo1 anti-GM1 IgM antibody (Fig. 6A, n=2), and normal human IgM (Fig. 6B, n=2). SEM are indicated by error bars unless they were smaller than symbols.

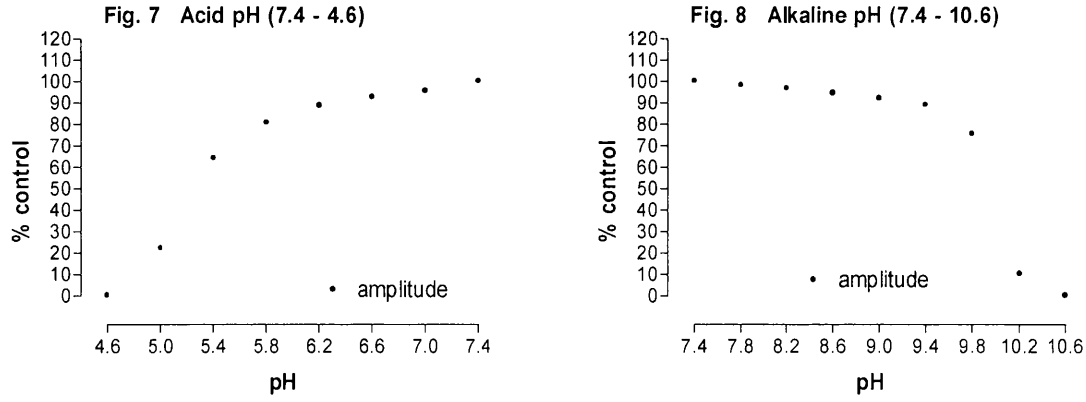

Fig. 7, 8 Incubation of desheathed mouse sciatic nerve in different acid and alkaline physiological buffers. For each pH value a different preparation was used, and each preparation was exposed to a different pH for 15 min.

Table 5 *Osmolarities and pH changes of bathing solutions before and after application of some monoclonal anti-ganglioside antibodies (Bo1, Bo3, Sm1, Wo1) and control compounds*

Sample	**Values before application of test reagents (physiological buffer)**		**Values at end of experiments (after application of test reagents)**	
	Osmolarity	**pH**	**Osmolarity**	**pH**
Bo1	318 mOsm/kg	7.4	322 mOsm/kg	9.4
Bo3	324 mOsm/kg	7.4	776 mOsm/kg	7.3
Sm1	325 mOsm/kg	7.4	538 mOsm/kg	7.2
Wo1	310 mOsm/kg	7.4	472 mOsm/kg	7.6
Human IgM (1^{st} sample)	328 mOsm/kg	7.4	885 mOsm/kg	7.7
CTb subunit	312 mOsm/kg	7.4	515 mOsm/kg	7.4
Human IgM (2^{nd} sample)	320 mOsm/kg	7.4	344 mOsm/kg	9.4

1b. Additional experimental factors that can affect nerve fibres physiology *in vitro*

In some experiments, even though we have controlled the osmotic pressure and the pH of used solutions, we observed progressive reduction in action potentials amplitude leading to acute conduction block and we regarded that as a true effect of anti-ganglioside antibody containing sera. The following Figure 9 shows such an example.

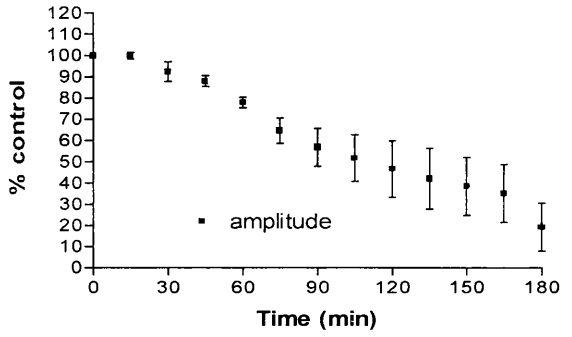

Fig. 9 Progressive reduction of nerve action potential 15 min after the nerve set up and antibody application, although osmolarity and pH were at normal levels (representative experiments n=3. Osmolarity was 320±10 mOsm/Kg and pH=7.4 and they were checked at the beginning and at the end of the experiments).

Because of poor reproducibility in our findings, we suspected that other factors, apart from osmotic strength and pH, could affect the stability of the preparation and so the reliability of our results.

i) Poor electrical isolation can cause conduction block

As has been described in method, the preparation was mounted into a recording chamber consisting of three cylindrical compartments (Figure 3); the central compartment communicated with each of the extreme ones by means of a small groove and electrical isolation between the compartments was achieved using vaseline on these grooves.

Impaired electrical isolation among the experimental bath compartments can influence the experiment and give conduction block as Figure 10 shows. Therefore, care was taken to achieve hermetically sealed bath compartments.

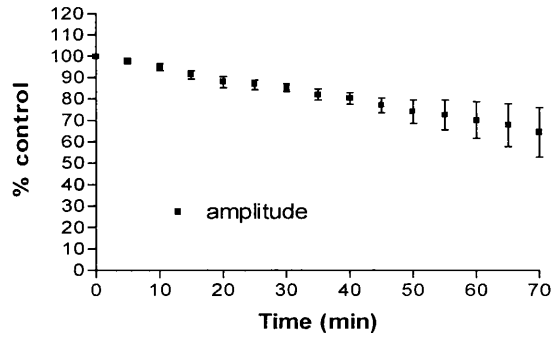

Fig. 10 Impaired electrical isolation (creating small pores among the recording chamber compartments) can lead to acute conduction block (n=2).

ii) Stretch of the nerve and conduction block

Figure 11 shows the result of nerve stretching in the amplitude of the obtained action potentials in ensheathed and desheathed preparations. Stretch of the nerve during the dissection can damage or traumatize nerve fibres and, especially, clumsy desheathment of the nerve can eliminate the viability of the preparation.

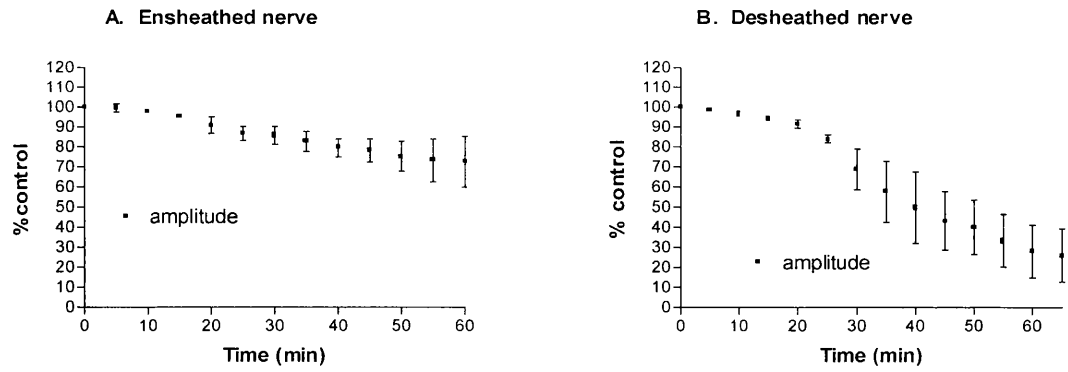

Fig. 11 Effect of stretching on ensheathed (n=2) and desheathed (n=2) nerves (slight pulling of nerve fibres during the dissection).

iii) Dry nerve and conduction block

It was important to keep all the length of the nerve in moist environment throughout the experiment. In preliminary experiments with anti-ganglioside antibody or control solutions, we observed that it was not sufficient to cover with vaseline only the two narrow grooves of the experimental chamber but also the very small portions of the nerve at the edges of each groove. Figure 12 depicts this important detail, which, if overlooked, could lead to a dry nerve and progressive conduction block.

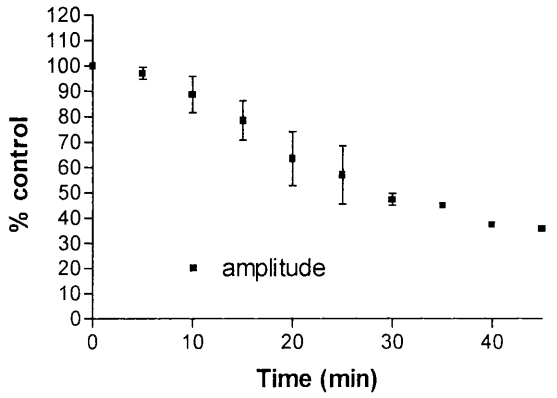

Fig. 12 The dissected nerve has to be kept in moist environment and even a short period of exposure into dry conditions can change the elctrophysiological recordings (n=2).

iv) Pressure on chamber grooves and conduction block

Moreover, we observed that during the nerve placement in to experimental bath it was important to press gently the two square glass coverslips, which were placed at the top of each vaseline and nerve-containing groove. Even minor pressure can traumatize the nerve fibres into the narrow grooves of the chamber and produce progressive decrease in the amplitude of the obtained action potentials (Figure 13).

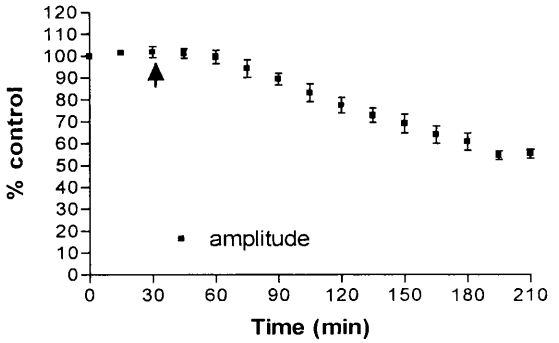

Fig. 13 Pressure on glass coverslip in experimental chamber can damage the nerve fibres into small bath grooves and lead to conduction block. Arrow signifies the time of application of excessive pressure (n=3).

v) Experimental instability the first 30-45 min

Finally, in preliminary experiments, immediately after the nerve set up and during the first 30 to 45 min of recordings we often observed a gradual increase in the amplitude of the electrophysiological recordings (Figure 14). Thus, before the addition of test compounds, the preparation was incubated for up to 45 min under constant supramaximal stimulation in order to demonstrate consistency in the recordings.

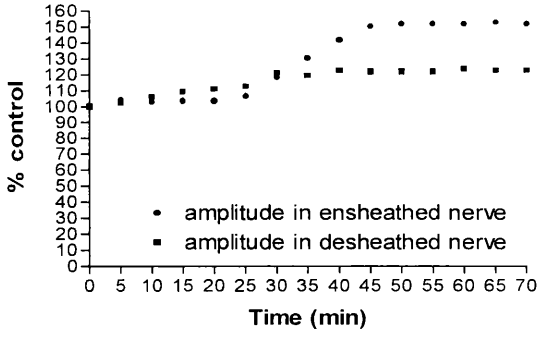

Fig. 14 Before the addition of test compounds, during the first 30-45 min of the experiment an increase in nerve action potential was observed. Consistency in recordings could be achieved after this "stabilization" period.

2. Ensheathed nerve

2a. Control experiments

In electrophysiological recordings from mouse ensheathed sciatic nerve, we first performed a significant number of control experiments:

- with physiological salt solution (Figure 15),
- with normal human serum, which did not contain anti-ganglioside antibodies (Figure 16),
- with normal guinea pig serum as a source of complement (Figure 17), and
- with pentoglobin (commercially prepared normal human IgM) (Figure 18).

The data are summarized in Table 6.

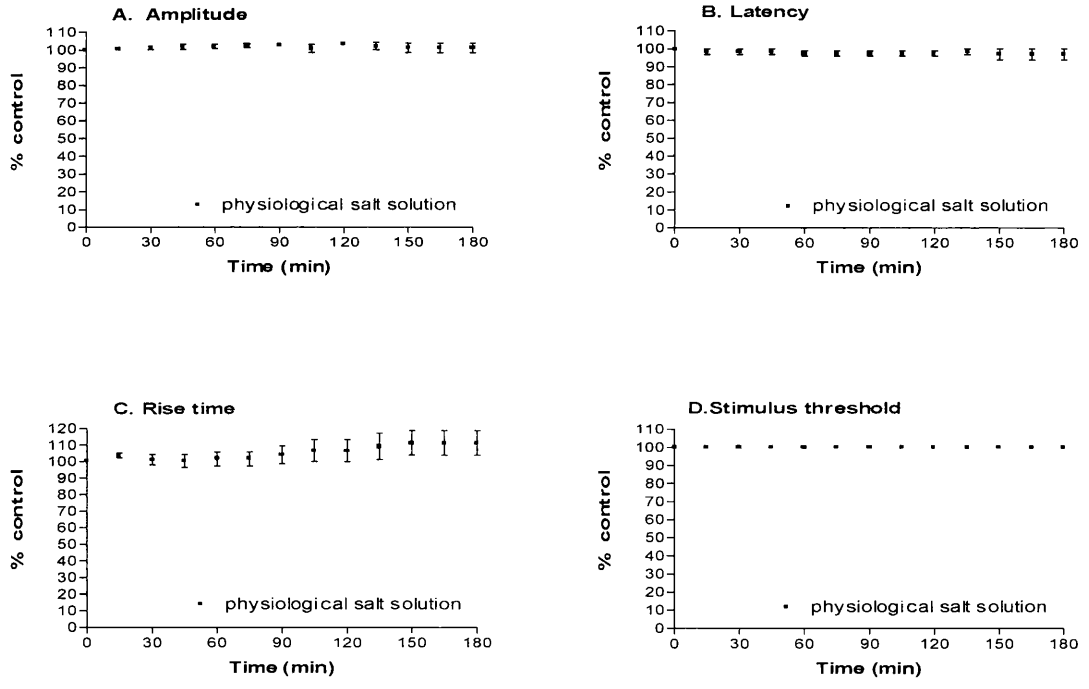

Fig. 15 Electrophysiological recordings performed at RT on ensheathed mouse sciatic nerve exposed to physiological salt solution (n = 4). SEM are indicated by error bars unless they were smaller than symbols.

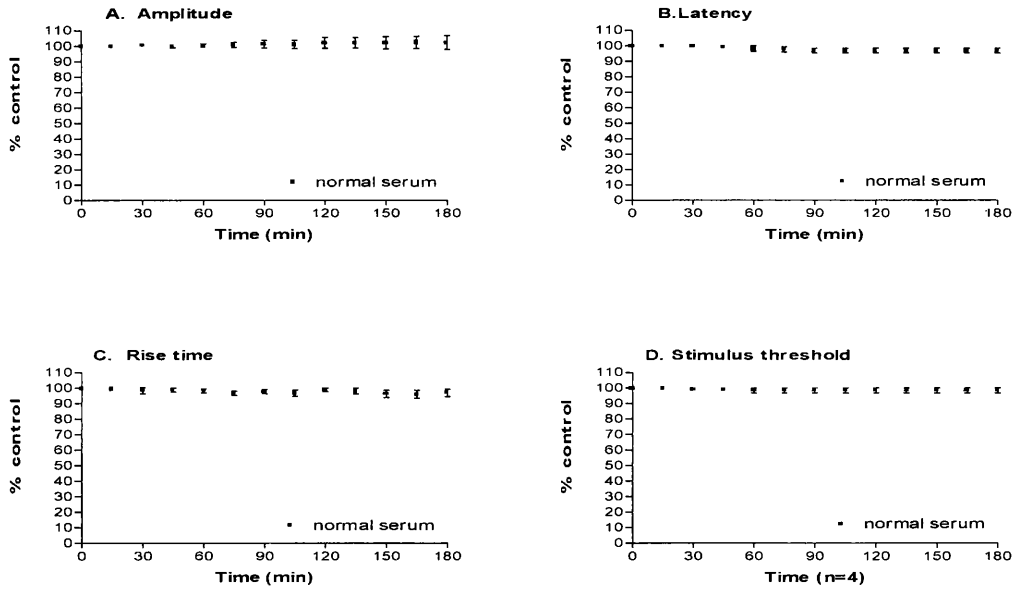

Fig. 16 Electrophysiological recordings performed at RT on ensheathed mouse sciatic nerve exposed to normal human serum (n = 4). SEM are indicated by error bars unless they were smaller than symbols.

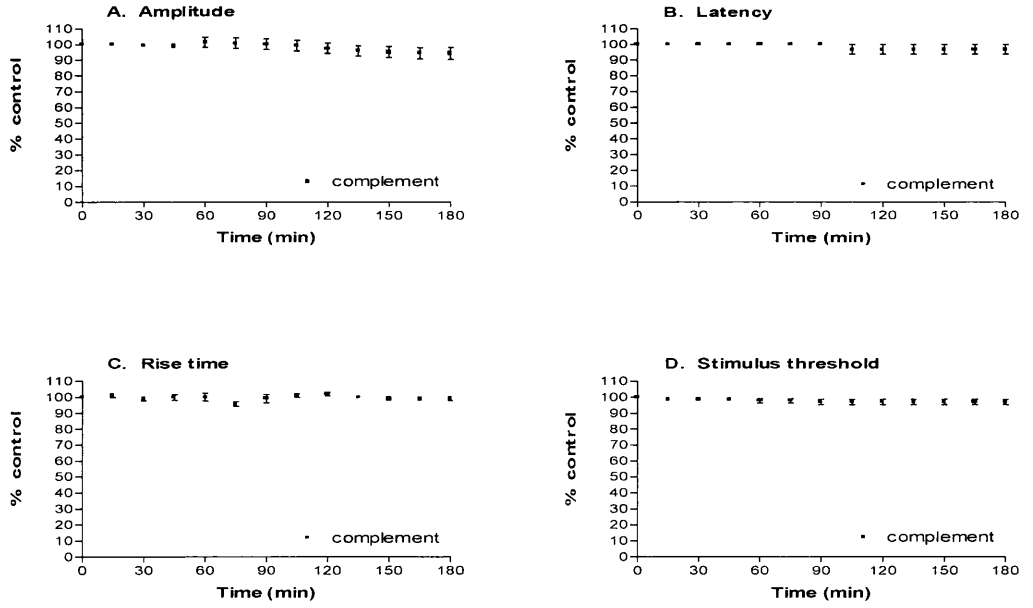

Fig. 17 Electrophysiological recordings performed at RT on ensheathed mouse sciatic nerve exposed to normal guinea pig serum as a source of complement (n = 4). SEM are indicated by error bars unless they were smaller than symbols.

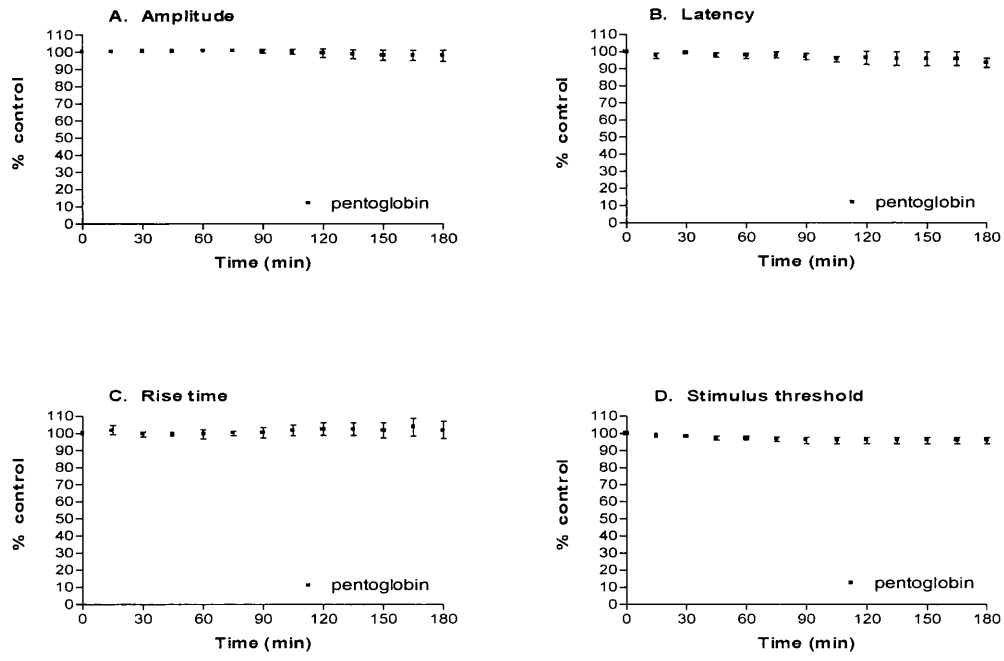

Fig. 18 Electrophysiological recordings performed at RT on ensheathed mouse sciatic nerve exposed to normal human IgM (n = 4). SEM are indicated by error bars unless they were smaller than symbols.

Finally, we tested the effects of tetrodotoxin (TTX), a sodium channel blocker and the anti-GQ1b IgM serum that obtained from a patient with PPN (CH).

2b. Conduction block induced by TTX

Nerves treated with TTX showed acute conduction block of the compound nerve action potentials. In 45 min the amplitude of the compound nerve action potentials was decreased in about 90%; also, a significant prolongation of the latency was observed; the rise time was equally reduced and an increase to the stimulus threshold was noticed (Figure 19 and Table 6).

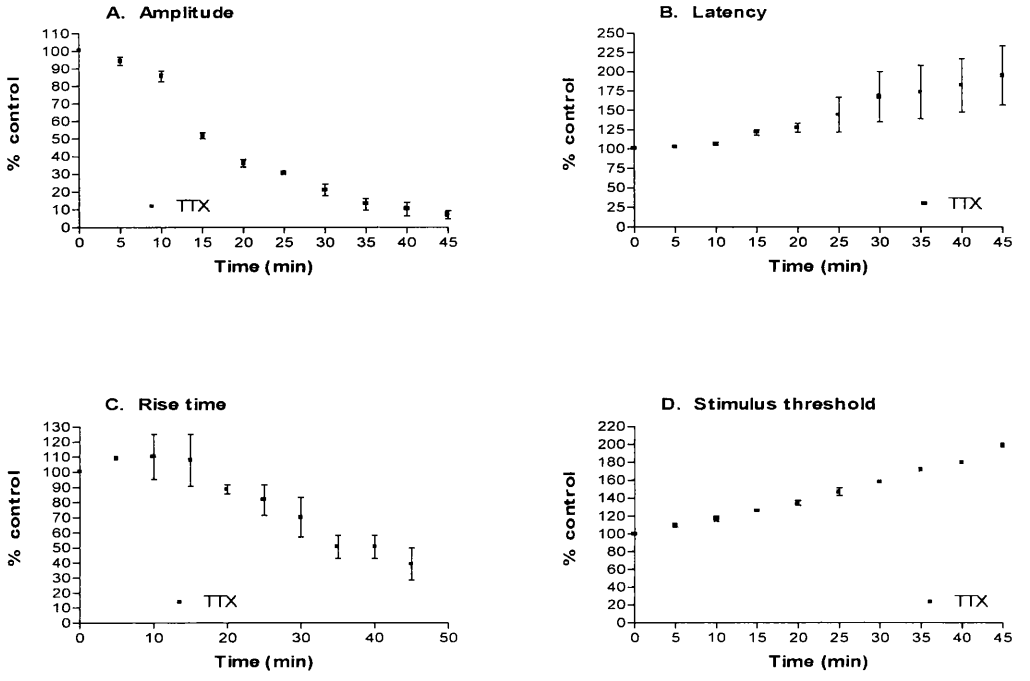

Fig. 19 Electrophysiological recordings performed at RT on ensheathed mouse sciatic nerve exposed to sodium channel blocker tetrodotoxin (TTX: n = 2). SEM are indicated by error bars unless they were smaller than symbols.

2c. The effect of anti-GQ1b IgM antibody

The application of anti-GQ1b anti-ganglioside serum did not produce conduction block in three hours of recording at room temperature (Figure 20 and Table 6). No significant changes in amplitude, latency, rise time, and stimulus threshold of the recorded nerve action potentials were documented.

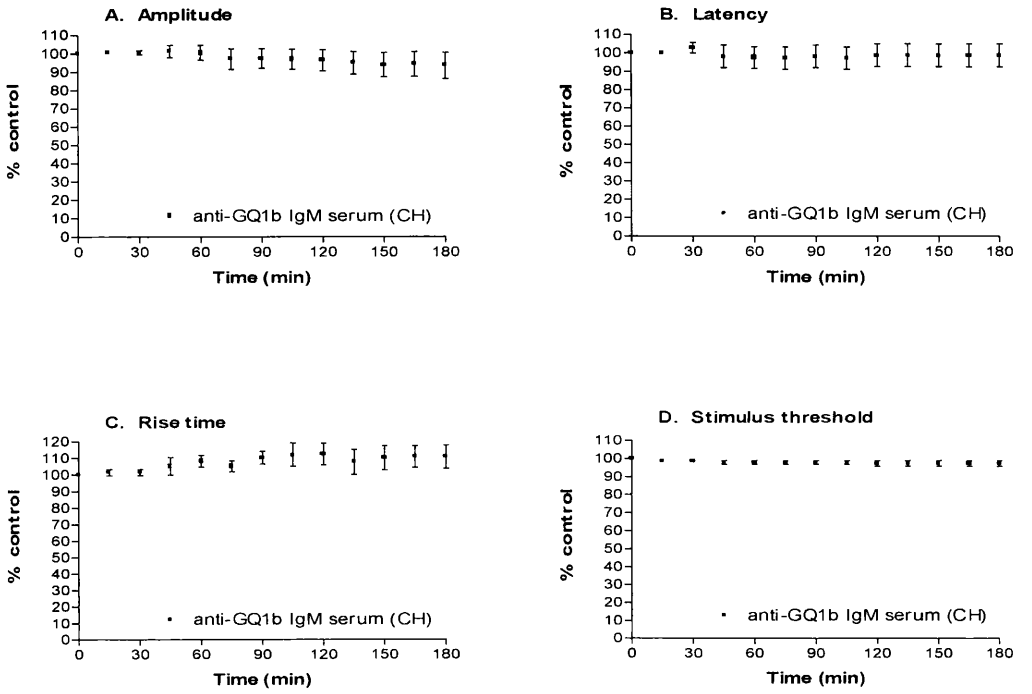

Fig. 20 Electrophysiological recordings performed at RT on ensheathed mouse sciatic nerve exposed to anti-GQ1b IgM serum from a patient with PPN (CH: n = 5). SEM are indicated by error bars unless they were smaller than symbols.

Table 6 *Electrophysiological parameters recorded from the ensheathed mouse sciatic nerve at room temperature*

SAMPLE	AMPLITUDE (mV)		LATENCY (ms)		RISE TIME (ms)		THRESHOLD (V)	
	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)
CONTROL MATERIALS								
PSS (n=4)	4.4±1.9	101.3±2.7	0.9±0.1	96.9±3.1	0.9±0.1	111.4±7.5	3.3±0.2	100.0±0.0
NS (n=4)	0.9±0.2	102.2±4.4	1.1±0.3	96.7±1.6	1.1±0.2	96.7±2.5	3.2±0.2	98.3±1.7
NGPS (n=4)	1.1±0.1	94.1±3.9	1.2±0.2	96.9±3.1	0.9±0.0	98.9±1.1	3.4±0.1	97.1±1.7
IgM (n=4)	1.2±0.1	97.9±3.3	1.1±0.3	93.3±2.7	1.2±0.2	102.0±5.0	3.8±0.2	95.9±1.9
TTX (n=2)	0.7±0.1	7.1±2.2	0.7±0.2	194.9±38.4	0.7±0.2	39.3±10.7	3.2±0.3	198.6±1.4
ANTI-GANGLIOSIDE SERUM								
CH (n=5)	1.9±1.2	93.4±7.1	0.8±0.1	98.8±6.2	0.8±0.0	110.8±7.0	3.6±0.4	97.3±1.6

PSS = physiological salt solution; NS = normal serum; NGPS = normal guinea pig serum; IgM = normal human IgM; TTX = tetrodotoxin.

3. Desheathed nerve

3a. The effect of nerve desheathment on saxitoxin penetration

In early electrophysiological recordings from isolated ensheathed nerves during which no effects of anti-ganglioside antibody were observed we were concerned that antibody may be poorly penetrating. We compared the rate of onset of saxitoxin action (STX, a sodium channel blocking agent) in sheathed and desheathed nerve (Figure 21) and noted clear differences between the two preparations, indicating the nerve sheath acts as a significant mechanical diffusion barrier to small molecules delivered to the extraneural environment. Therefore, all subsequent incubations were carried out on desheathed nerve preparations.

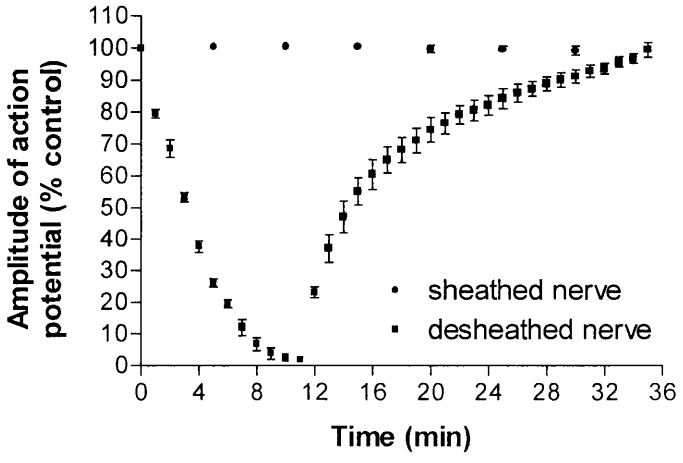

Fig. 21 Electrophysiological effect of saxitoxin (STX), a sodium channel blocker on sheathed (n=3) and desheathed (n=3) mouse sciatic nerves with the same concentration of STX (0.5 nM) of STX. Desheathed preparations were washed out after 12 min. SEM are indicated by error bars unless they were smaller than symbols.

3b. Electrophysiological recordings at room temperature

1. Control experiments

We first applied fresh normal human serum (as a control for the test sera) to the preparation for periods up to 4hrs and observed no significant changes in any of our recorded parameters (Figure 22 and Table 7). Then we obtained similar results with the nerve exposed to normal human IgM (Figure 23 and Table 7). We also observed no effect with cholera B subunit, a high affinity multimeric ligand for GM1 ganglioside, applied under similar conditions (Figure 24 and Table 7).

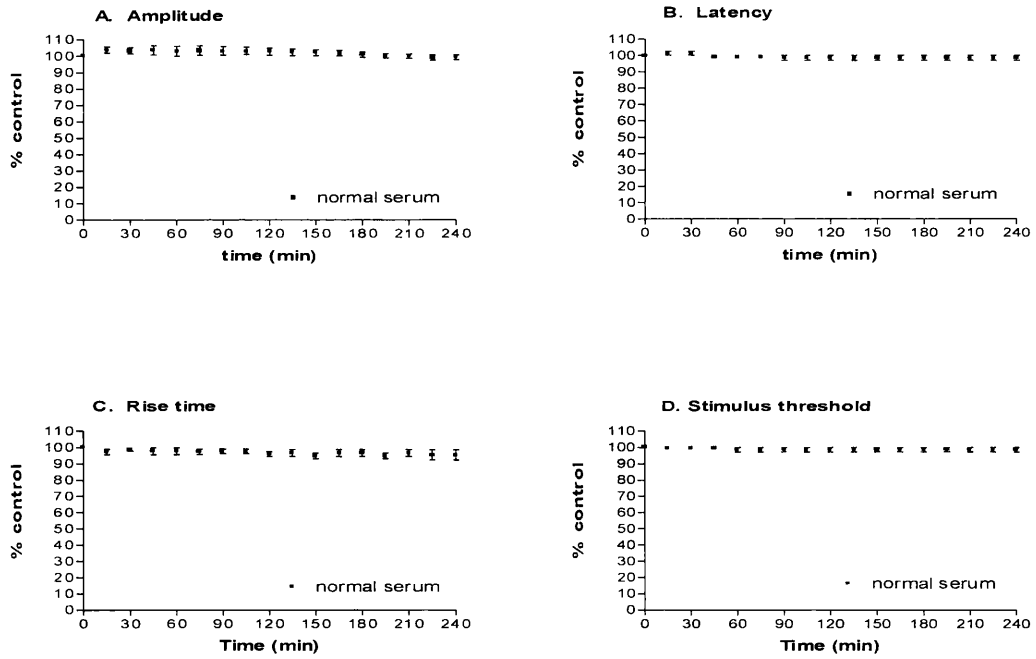

Fig. 22 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to normal human serum (n = 4). SEM are indicated by error bars unless they were smaller than symbols.

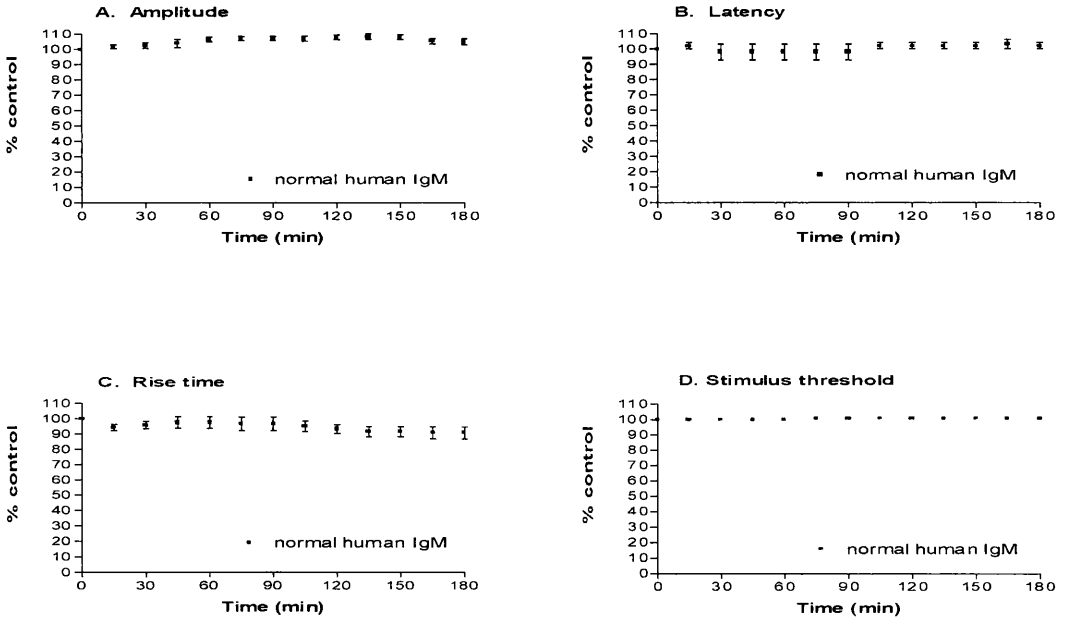

Fig. 23 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to normal human IgM (n = 4). SEM are indicated by error bars unless they were smaller than symbols.

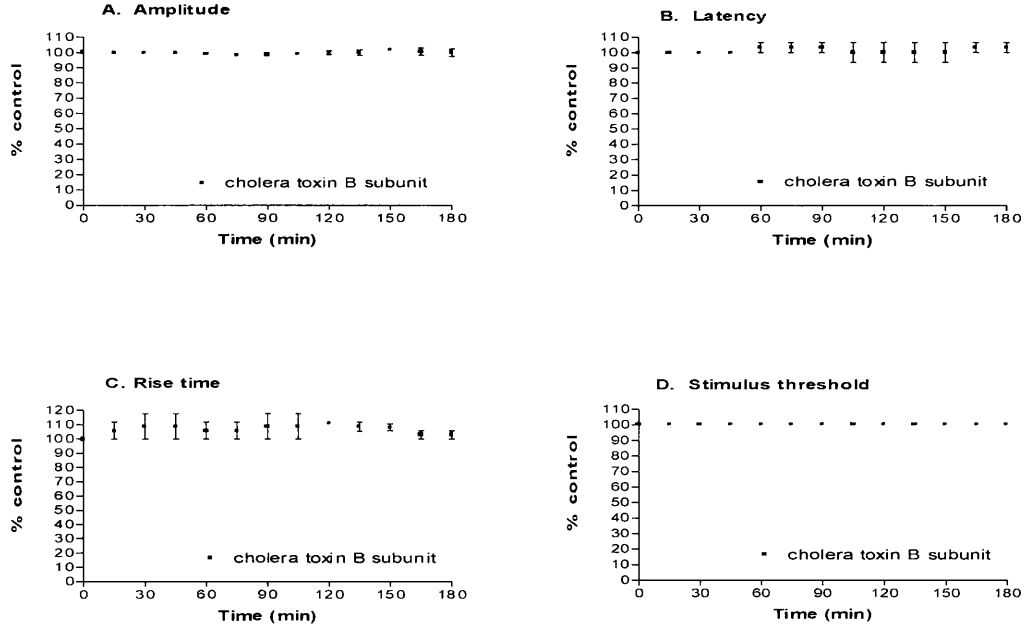

Fig. 24 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to cholera toxin B subunit (n = 2). SEM are indicated by error bars unless they were smaller than symbols.

Table 7 *Electrophysiological parameters recorded from the desheathed mouse sciatic nerve in control experiments*

SAMPLE	AMPLITUDE (mV)		LATENCY (ms)		RISE TIME (ms)		THRESHOLD (V)	
	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)
NS (n=4)	2.0±0.1	99.4±1.4	0.9±0.2	98.5±1.5	0.8±0.1	95.4±3.1	3.5±0.1	98.6±1.4
IgM (n=4)	3.8±1.0	104.9±2.0	0.9±0.1	102.1±2.1	1.1±0.2	90.6±3.9	3.6±0.1	100.6±0.6
CT (n=2)	3.0±0.3	99.9±2.5	0.8±0.0	103.3±3.3	0.9±0.0	102.9±2.9	3.5±0.1	100.0±0.0

NS = normal serum; IgM = normal human IgM; CT = cholera toxin B subunit.

2. Physiological effect of anti-GM1 IgM sera

When we applied anti-GM1 IgM containing sera obtained from three different MMN patients (GA, JW, PS), we again observed no significant changes from baseline levels of the amplitude of the compound nerve action potential, latency, rise time or stimulus threshold (Figures 25, 26, 27 and Table 8). In order to ensure sufficient complement was available (if required to induce an effect) we added a fresh source at 3 or 4 hrs for a further 90-120mins and again did not observed any changes in the recorded parameters.

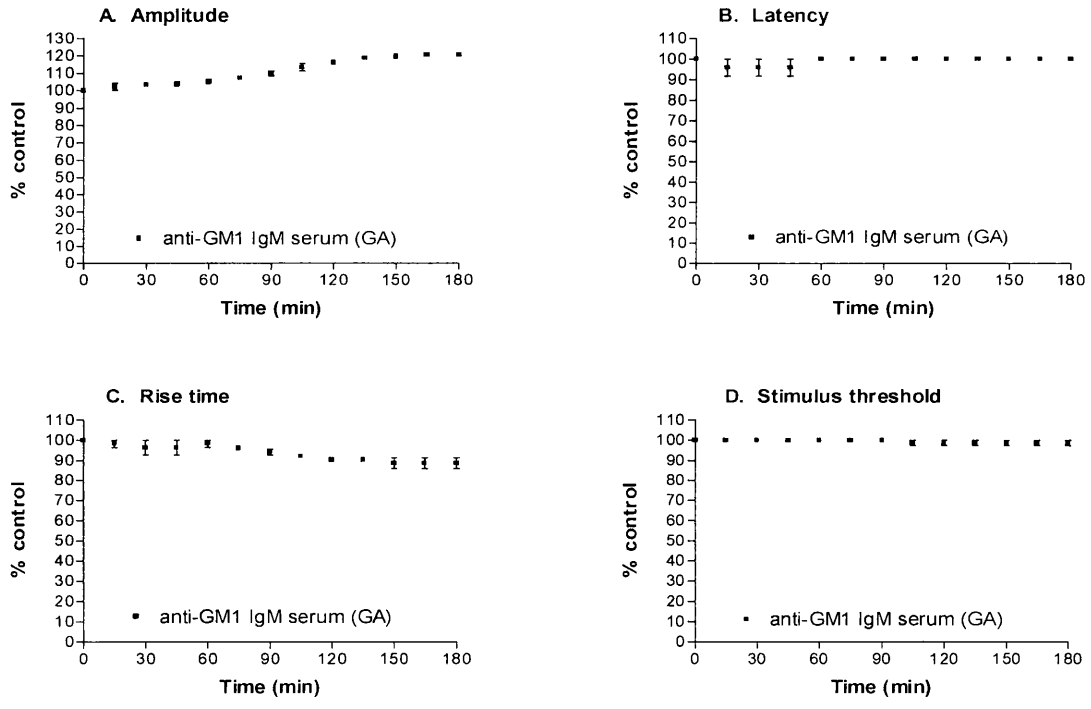

Fig. 25 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to anti-GM1 IgM serum from a patient with MMN (GA: n = 2). SEM are indicated by error bars unless they were smaller than symbols.

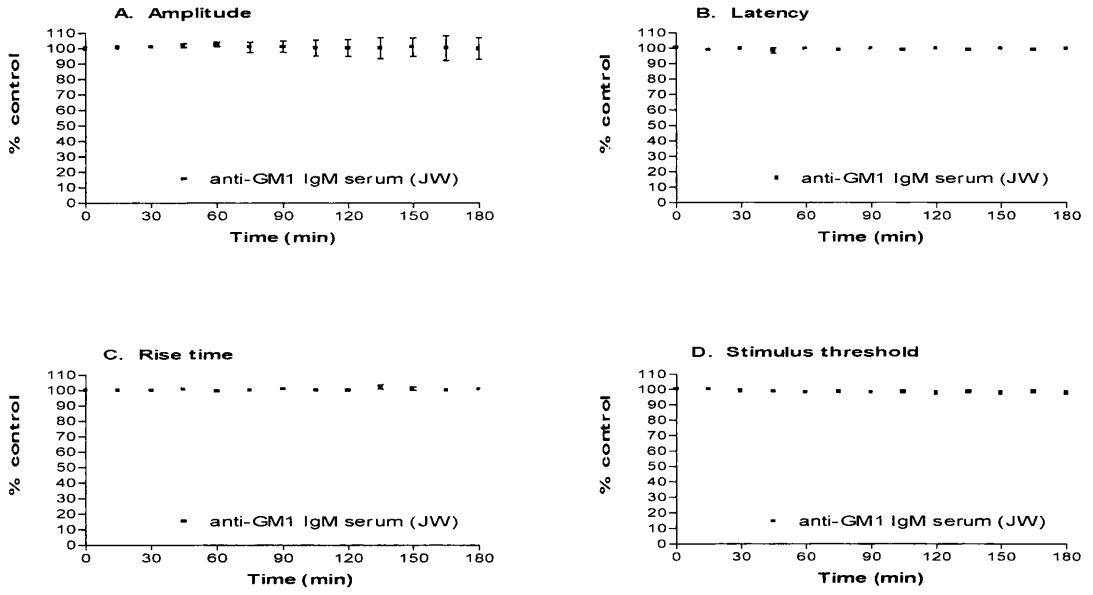

Fig. 26 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to anti-GM1 IgM serum from a patient with MMN (JW: n = 6). SEM are indicated by error bars unless they were smaller than symbols.

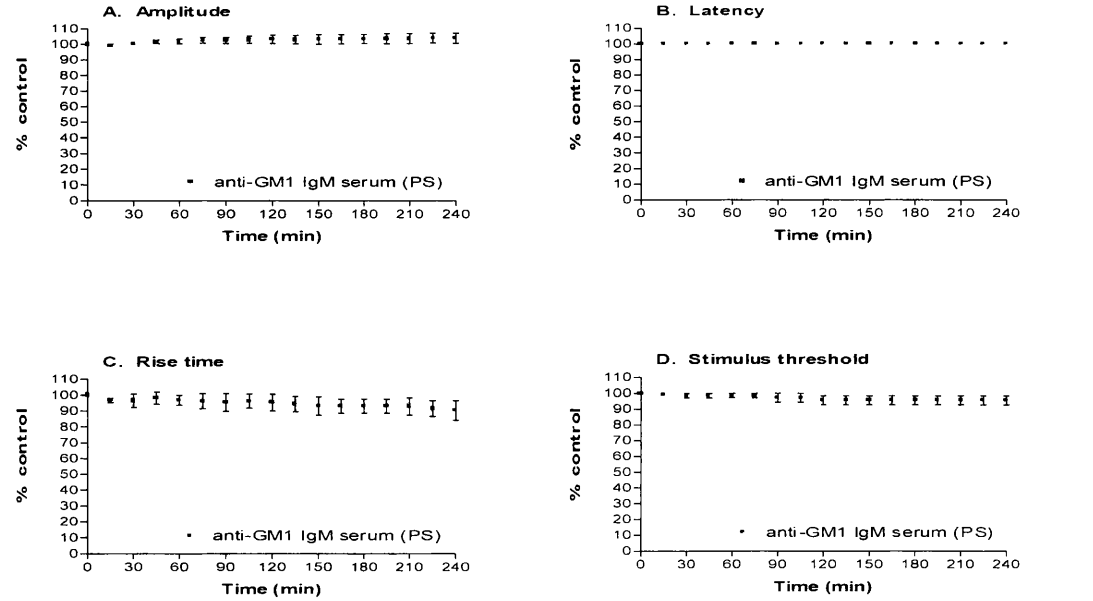

Fig. 27 Electrophysiological recordings performed at RT on desheathed mouse sciatic nerve exposed to anti-GM1 IgM serum from a patient with MMN (PS: n = 4). SEM are indicated by error bars unless they were smaller than symbols.

Table 8 *Electrophysiological parameters recorded from the desheathed mouse sciatic nerve incubated with anti-GM1 IgM sera*

SAMPLE	AMPLITUDE (mV)		LATENCY (ms)		RISE TIME (ms)		THRESHOLD (V)	
	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)
GA (n=2)	3.3±0.9	120.6±0.7	0.8±0.2	100.0±0.0	1.3±0.1	88.5±2.8	3.7±0.0	98.6±1.3
JW (n=6)	5.7±2.1	99.9±7.0	1.1±0.1	99.2±0.8	0.9±0.1	100.8±0.8	3.6±0.1	97.6±0.9
PS (n=4)	2.7±0.8	103.8± 3.1	0.6±0.0	100.0±0.0	0.7±0.0	90.2±6.2	3.0±0.1	96.1±2.3

3. Physiological effect of anti-GQ1b IgM sera

We studied the acute physiological effects of three anti-GQ1b IgM containing sera obtained from three different patients with PPN (CH, VP, and BK). We used again normal human serum as a source of complement that applied for a further 90-120 mins. During the 4h of nerve incubation none of the measured parameters has again significantly changed (Figures 28, 29, 30 and Table 9).

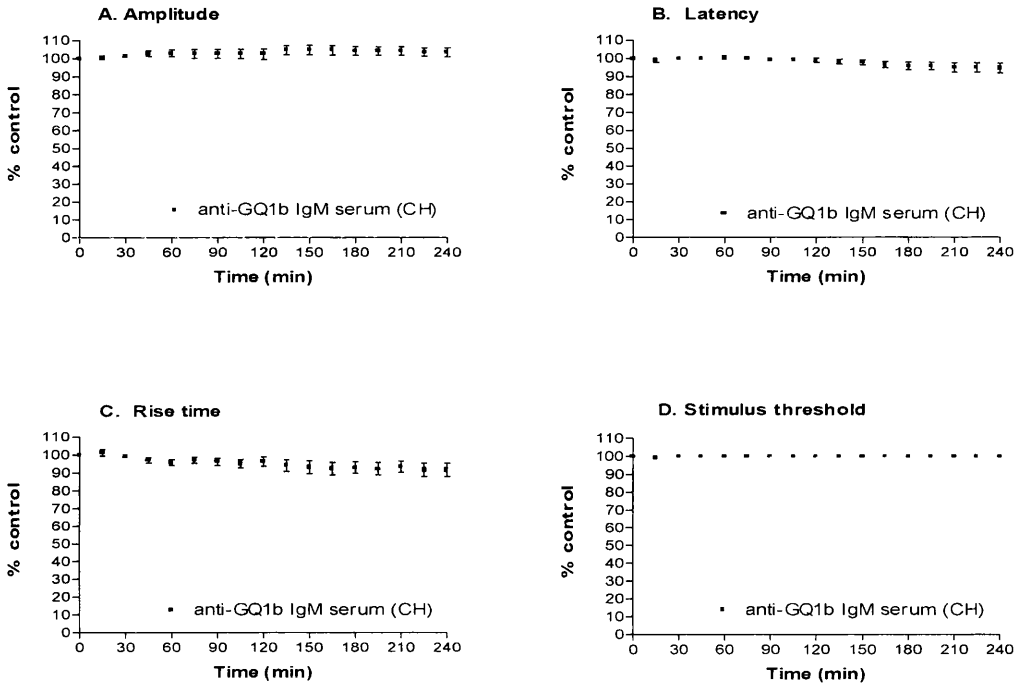

Fig. 28 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to anti-GQ1b IgM serum from a patient with PPN (CH: n = 10). SEM are indicated by error bars unless they were smaller than symbols.

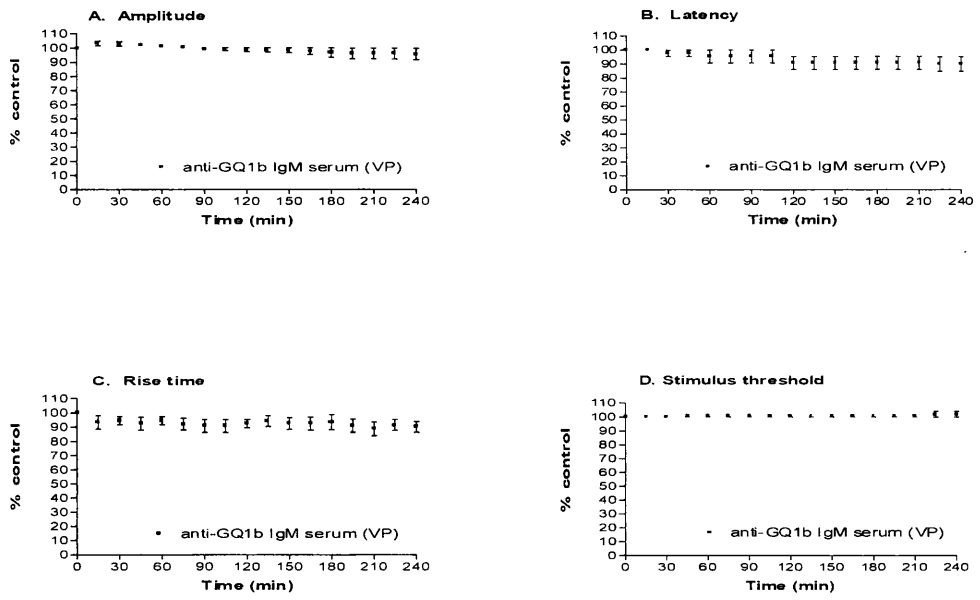

Fig. 29 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to anti-GQ1b IgM serum from a patient with PPN (VP: n = 3). SEM are indicated by error bars unless smaller than symbols.

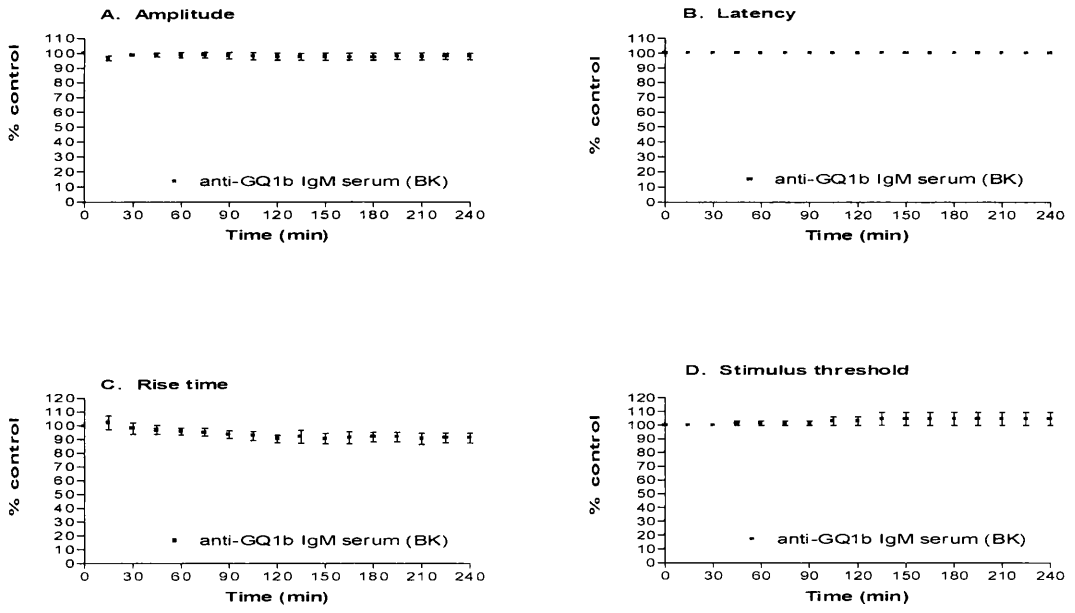

Fig. 30 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to anti-GQ1b IgM serum from a patient with PPN (BK: n = 4). SEM are indicated by error bars unless they were smaller than symbols.

Table 9 *Electrophysiological parameters recorded from the desheathed mouse sciatic nerve incubated with anti-GQ1b IgM sera*

SAMPLE	AMPLITUDE (mV)		LATENCY (ms)		RISE TIME (ms)		THRESHOLD (V)	
	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)
CH (n=10)	2.9±0.3	103.3±2.5	0.9±0.1	94.5±2.8	0.9±0.0	92.0±3.7	3.7±0.3	99.9±0.7
VP (n=3)	2.5±0.3	95.5±3.9	0.7±0.1	89.8±5.1	0.7±0.0	90.1±3.8	3.2±0.1	102.1±2.1
BK (n=4)	3.4±1.4	97.9±2.1	0.7±0.1	100.0±0.0	0.7±0.1	91.1±3.6	3.0±0.1	104.7±4.7

4. Physiological effect of IgG anti-GM1 and anti-GQ1b sera

Having not observed any acute physiological effect with IgM anti-ganglioside sera, we investigated the role of IgG anti-ganglioside sera under the same experimental conditions. We studied the acute effects of two different anti-GM1 IgG antibodies obtained from two patients with GBS (MI, and JY) and a anti-GQ1b IgG antibody from a third patient with MFS (AS). Again human serum was used as an additional source of complement and the nerve was incubated for 4 hrs. The IgG antibody did not induce any significant changes in the recording parameters (Figures 31, 32, 33 and Table 10).

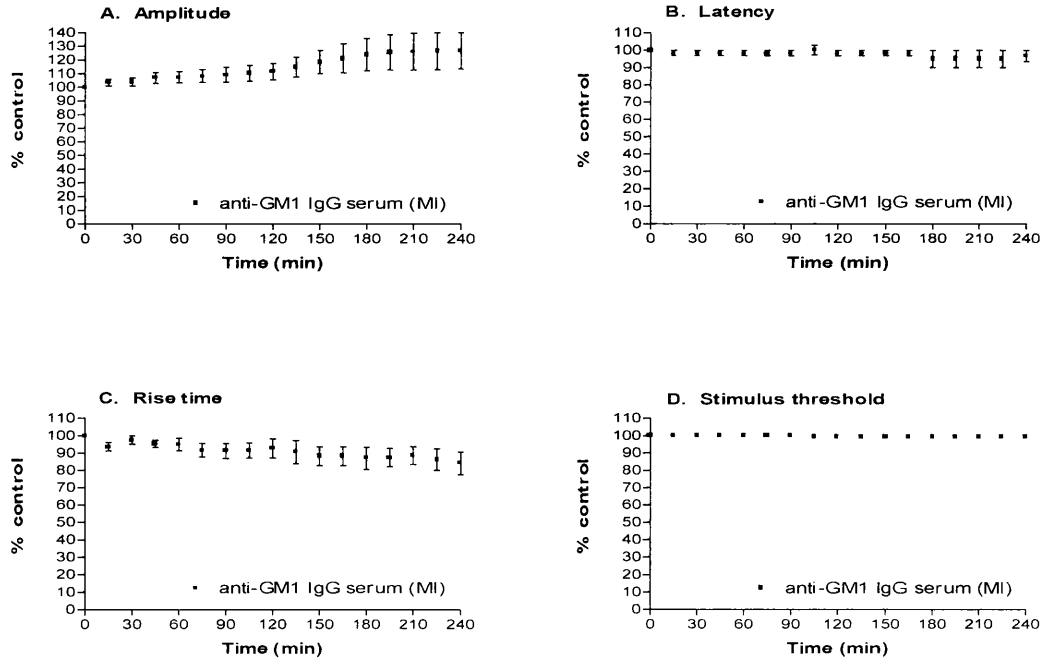

Fig. 31 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to anti-GM1 IgG antibody from a patient with GBS (MI: n = 4). SEM are indicated by error bars unless they were smaller than symbols.

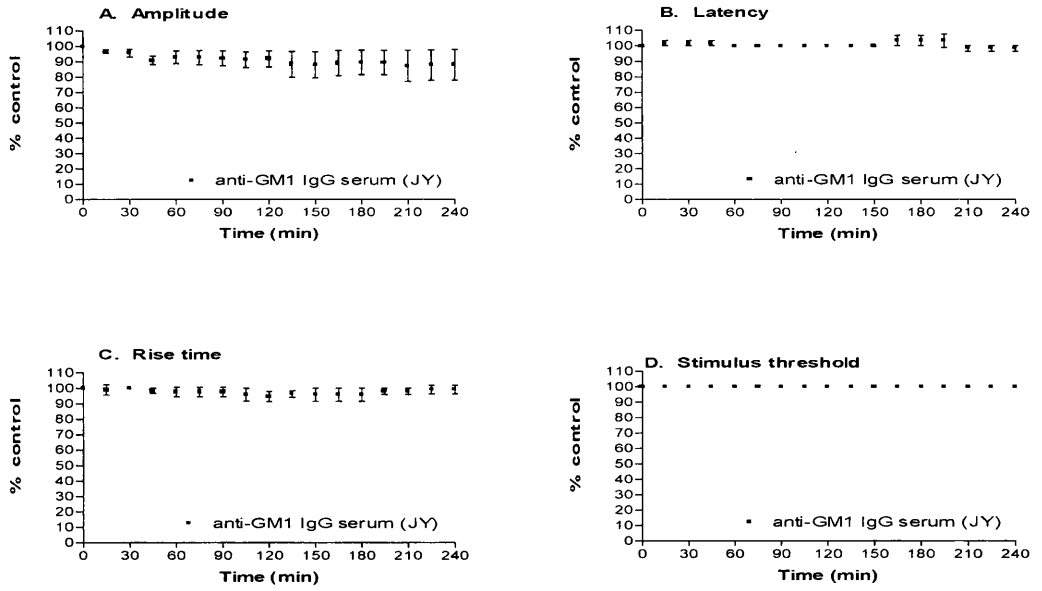

Fig. 32 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to anti-GM1 IgG antibody from a patient with GBS (JY: n = 4). SEM are indicated by error bars unless they were smaller than symbols.

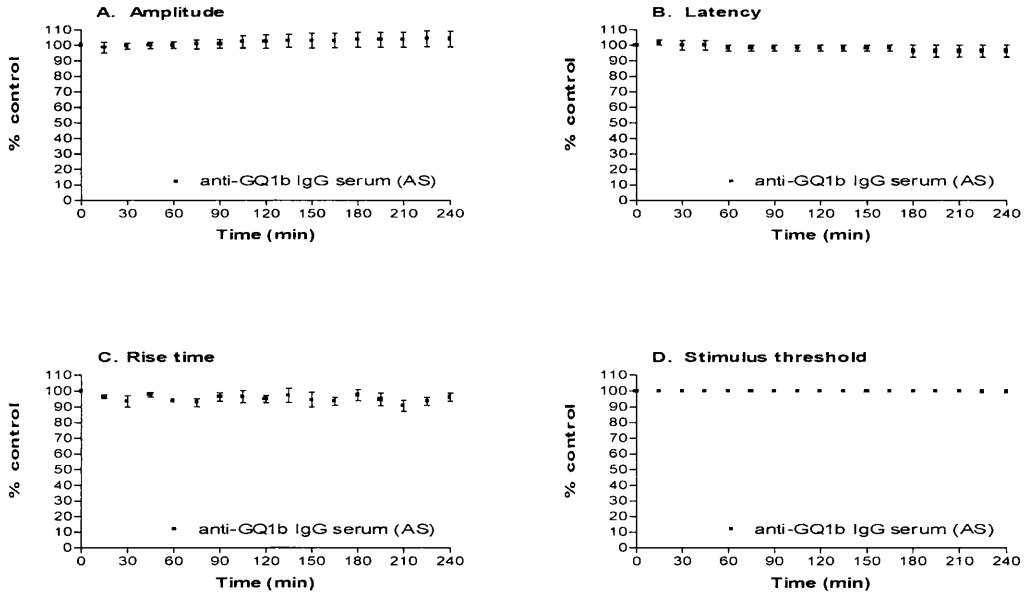

Fig. 33 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to anti-GQ1b IgG antibody from a patient with MFS (AS: n = 4). SEM are indicated by error bars unless they were smaller than symbols.

Table 10 *Electrophysiological parameters recorded from the desheathed mouse sciatic nerve incubated with anti-GM1 and anti-GQ1b IgG sera*

SAMPLE	AMPLITUDE (mV)		LATENCY (ms)		RISE TIME (ms)		THRESHOLD (V)	
	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)
MI (n=4)	2.2±0.5	126.7±13.4	0.7±0.1	96.7±3.3	1.1±0.1	84.1±6.6	3.6±0.1	99.3±0.7
JY (n=4)	3.4±0.7	87.7±9.9	0.7±0.0	98.2±1.8	0.7±0.1	99.2±2.8	3.3±0.2	100.0±0.0
AS (n=4)	2.4±0.3	103.9±5.1	0.6±0.0	96.1±3.8	1.0±0.1	96.0±2.6	4.5±0.3	99.5±0.5

5. Physiological effect of anti-GM1 monoclonal antibodies

In order to determine the acute electrophysiological effects of anti-ganglioside monoclonal antibodies we tested three different anti-GM1 IgM monoclonal antibodies that came from three different patients with MMN (Wo1, Sm1, and Bo1). The monoclonal antibodies did not induce any acute significant effect on desheathed mouse sciatic nerve. Again all measured parameters (amplitude, latency, rise time, and stimulus threshold of the action potentials) remained without considerable alterations (Figures 34, 35, 36 and Table 11).

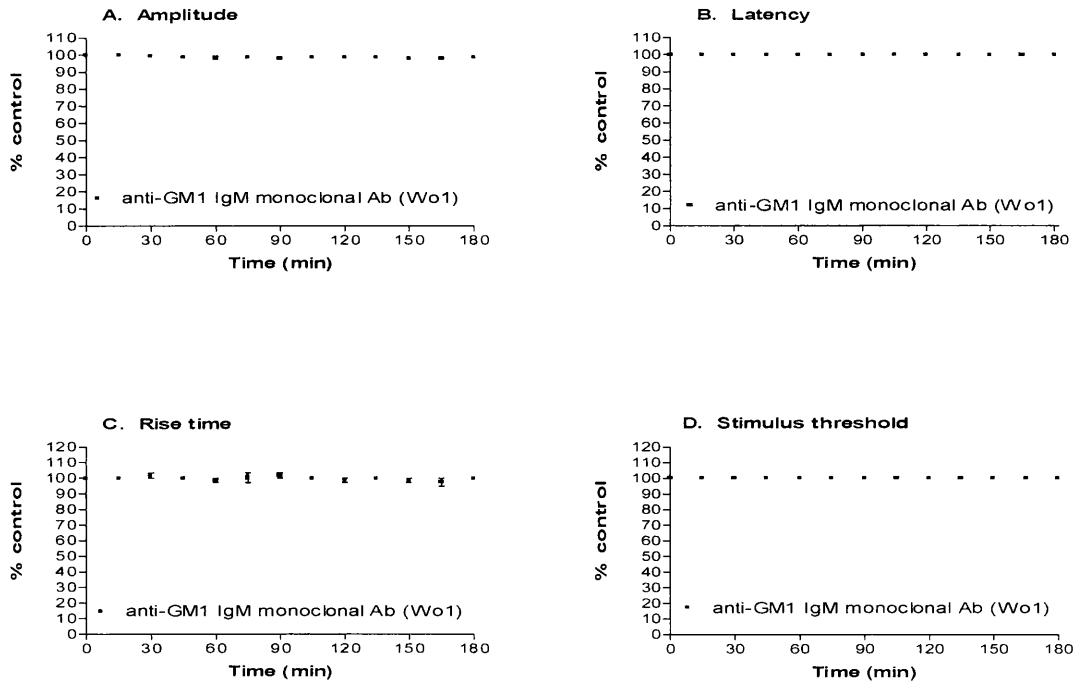

Fig. 34 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to anti-GM1 IgM monoclonal anti-ganglioside antibody from a patient with MMN (Wo1: n = 2). SEM are indicated by error bars unless they were smaller than symbols.

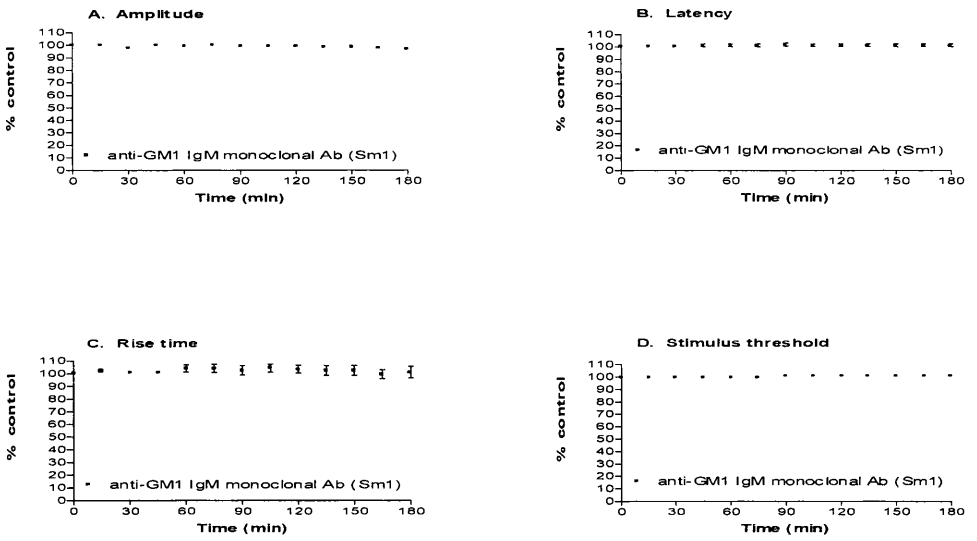

Fig. 35 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to anti-GM1 IgM monoclonal anti-ganglioside antibody from a patient with MMN (Sm1: n = 6). SEM are indicated by error bars unless they were smaller than symbols.

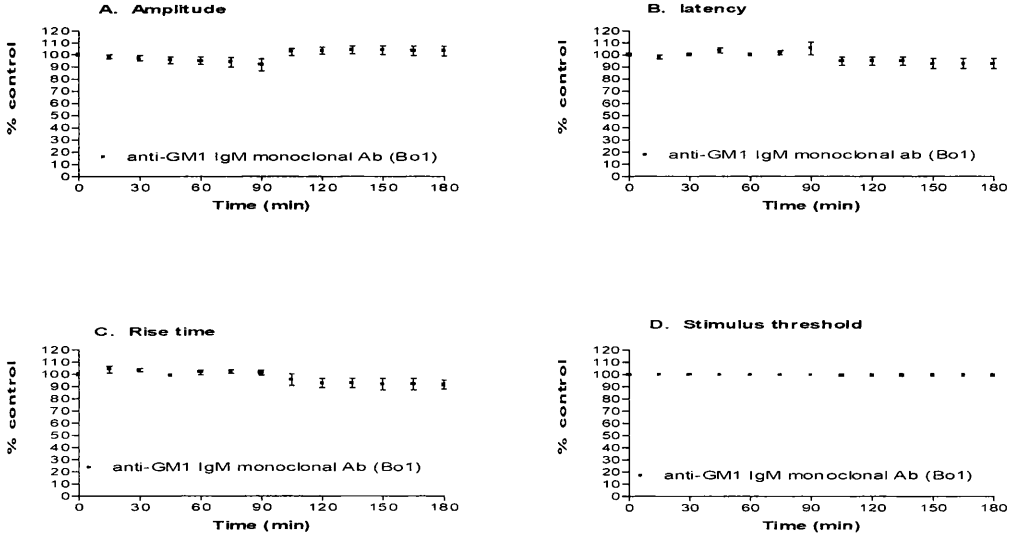

Fig. 36 Electrophysiological recordings at RT on desheathed mouse sciatic nerve exposed to anti-GM1 IgM monoclonal anti-ganglioside antibody from a patient with MMN (Bo1: n = 4). SEM are indicated by error bars unless they were smaller than symbols.

Table 11 *Electrophysiological parameters recorded from the desheathed mouse sciatic nerve exposed to anti-GM1 IgM monoclonal antibodies*

SAMPLE	AMPLITUDE (mV)		LATENCY (ms)		RISE TIME (ms)		THRESHOLD (V)	
	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)
Wo1 (n=2)	3.1±0.2	98.3±0.7	0.7±0.0	100.0±0.0	0.6±0.0	100.0±0.0	2.7±0.2	100.0±0.0
Sm1 (n=6)	3.7±0.8	97.2±5.9	0.7±0.1	101.2±1.2	0.7±0.0	101.1±4.5	3.6±0.4	100.8±0.6
Bo1 (n=4)	2.5±0.6	103.2±4.0	0.6±0.1	92.8±4.1	0.9±0.2	91.6±3.7	3.3±0.2	99.3±0.7

3c. Effect of temperature on anti-ganglioside antibody activity

1. Electophysiological recordings under different temperature conditions

So far, our results have not confirmed the hypothesis that anti-ganglioside antibodies could induce acute conduction block *in vitro*. We suspected that temperature may play an important role in antibody binding and affinity and decided to record at different temperature conditions. We tested two different anti-ganglioside sera: one anti-GQ1b IgM from a patient with PPN (CH), and one anti-GM1 IgG from a second patient with GBS (MI). In the first case we applied the antibody at RT for 1 hour, then we warmed up the preparation and we recorded at 37°C in the presence of complement (Figure 37 and Table 12); in the second case the nerve was incubated for 1 h at 37°C with the IgG antibody, then the temperature was decreased and we recorded at RT after complement application (Figure 38 and Table 12). Despite that, none of these two antibodies produced conduction block.

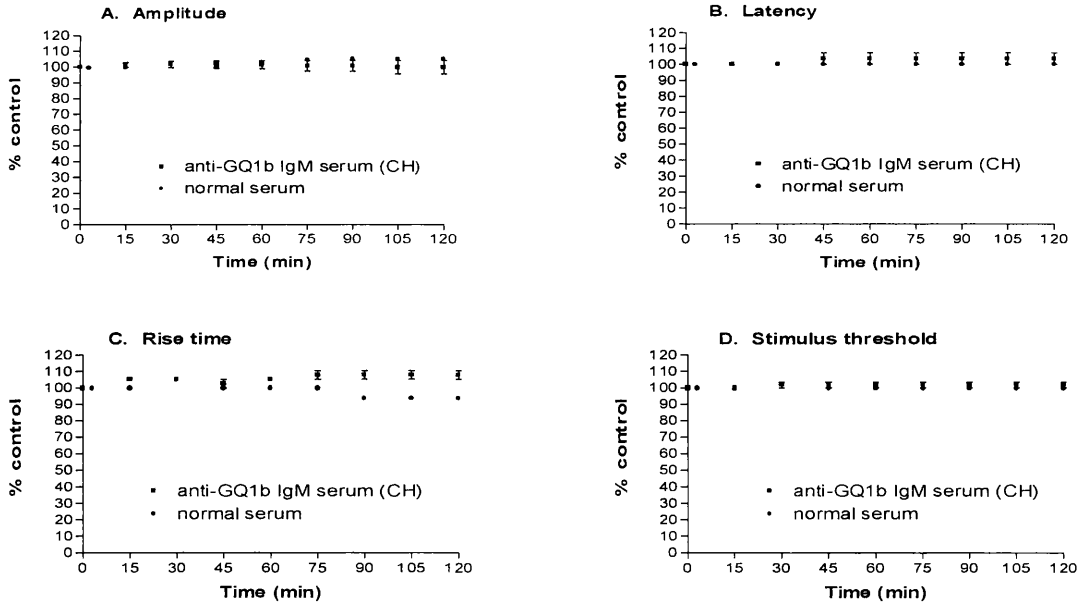

Fig. 37 Electrophysiological recordings at 37°C after incubation of desheathed mouse sciatic nerve at RT for 1h with complement. We tested anti-GQ1b IgM serum (n=2, CH).

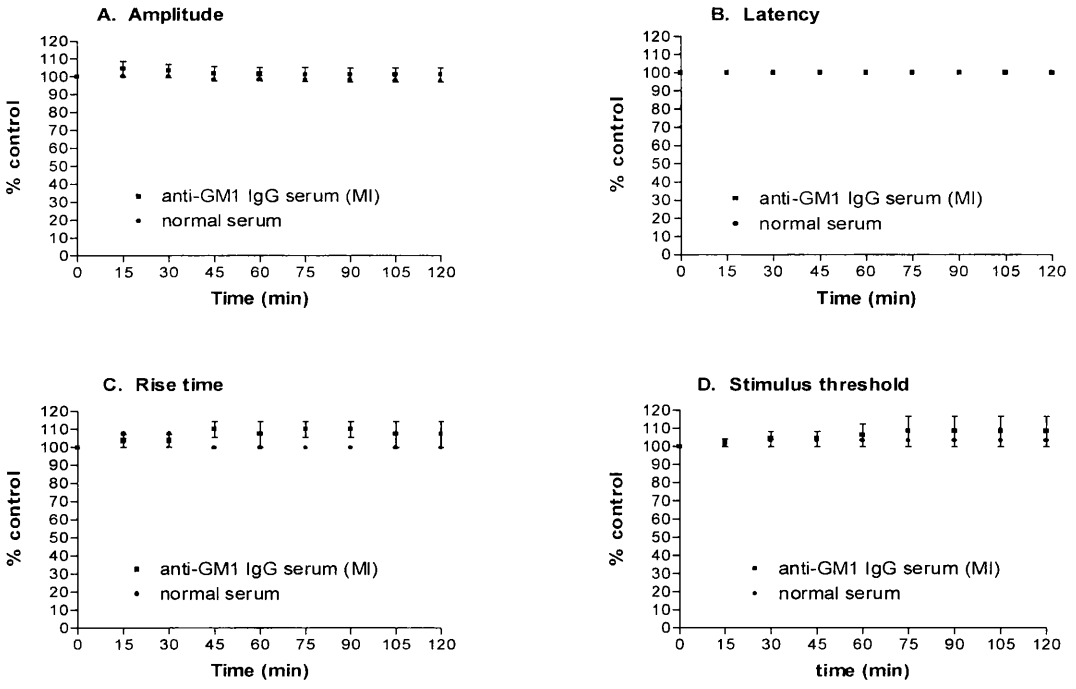

Fig. 38 Electrophysiological recordings at RT after incubation of desheathed mouse sciatic nerve at 37°C for 1 h with IgG anti-GM1 serum (MI) and complement (n=2).

Table 12 *Electrophysiological parameters recorded from the desheathed mouse sciatic nerve under different temperature conditions*

SAMPLE	AMPLITUDE (mV)		LATENCY (ms)		RISE TIME (ms)		THRESHOLD (V)	
	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)
CH (n=2)	5.3±0.4	99.8±4.3	0.65±0.0	103.6±3.6	1.0±0.0	107.9±2.7	3.1±0.3	101.8±1.8
NS (n=1)	8.8	105.2	0.7	100.0	0.8	93.8	2.9	100.0
MI (n=2)	6.8±0.4	100.8±4	0.6±0.0	100±0.0	0.8±0.1	107.1±7.1	2.7±0.3	108.3±8.3
NS (n=1)	4.0	97.6	0.5	100.0	0.7	100.0	2.9	103.5

NS = Normal serum.

2. Electrophysiological recordings at 37°C

Having determined from immunofluorescence studies (see pages 133-145) that some anti-GM1 antibodies possessed extensive nodal immunoreactivity with complement activation at 37°C, we obtained further electrophysiological recordings under these incubation conditions. We observed in preliminary experiments that nerve viability (as defined by electrophysiological stability of our recorded parameters) began to deteriorate after 4hrs at 37°C and we thus restricted our recording period to 3hrs, plus 45 mins stabilization period prior to antibody and complement application. We examined the acute effects of two anti-GM1 IgM containing sera (GA, and JW) that showed the highest immunoreactivity in teased fibres. Under these conditions, considered optimal in terms of antibody binding for observing any potentially deleterious effects on nerve conduction in this experimental paradigm, we again observed no effects of anti-GM1 antibody containing sera. During the time course described the amplitude, latency, rise time, and stimulus threshold of the recorded nerve action potentials showed no significant alteration (Figures 39, 40 and Table 13).

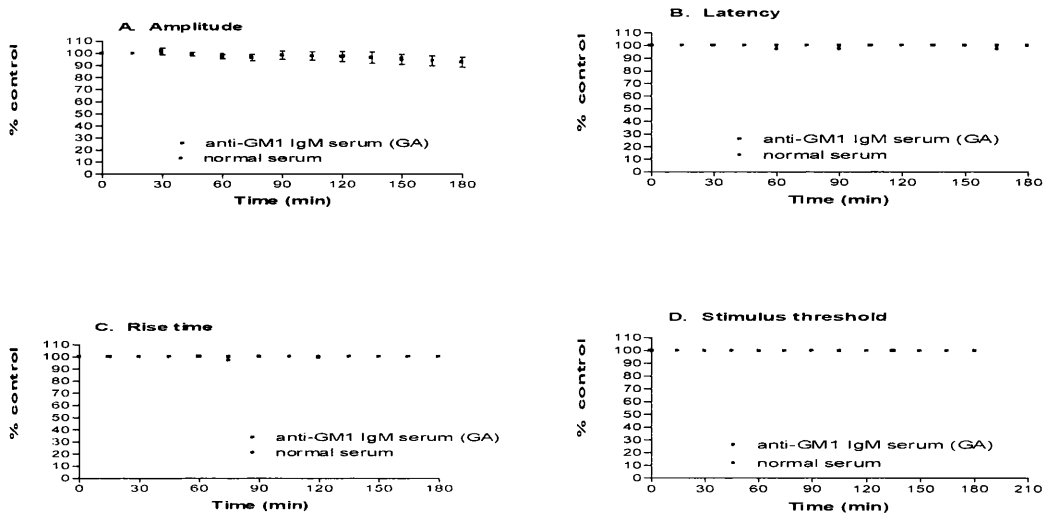

Fig. 39 Electrophysiological recordings at 37°C on desheathed mouse sciatic nerve exposed to anti-GM1 IgM serum from a patient with MMN (GA). Points are means of 4 experiments. SEM are indicated by error bars unless they were smaller than symbols.

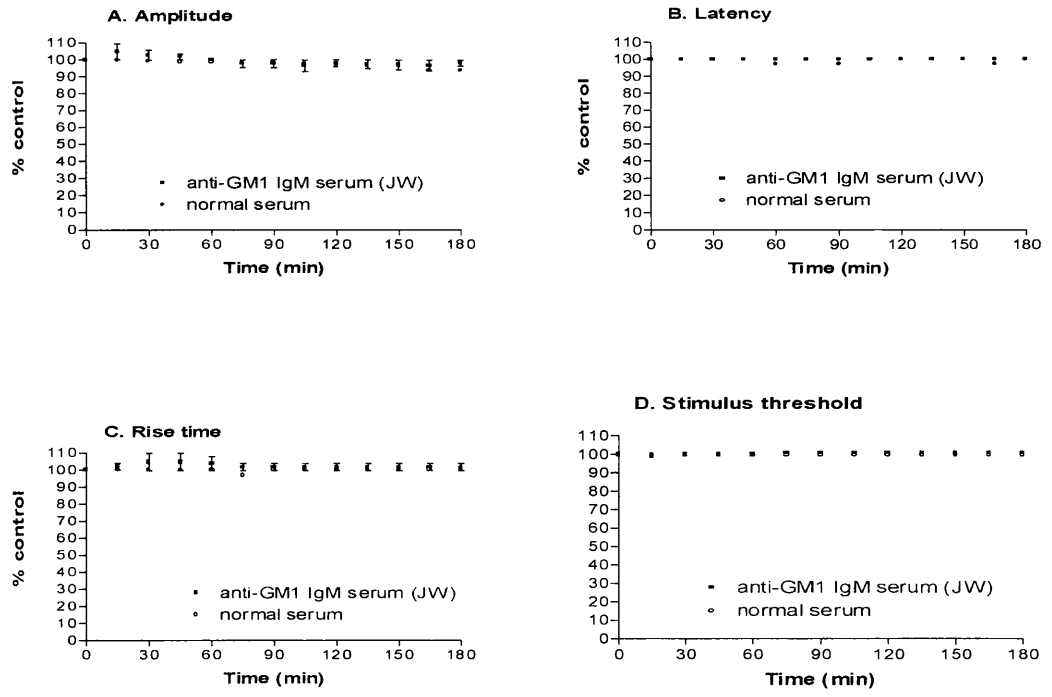

Fig. 40 Electrophysiological recordings at 37°C on desheathed mouse sciatic nerve exposed to anti-GM1 IgM serum from a patient with MMN (JW). Points are means of 4 experiments. SEM are indicated by error bars unless they were smaller than symbols.

Table 13 *Electrophysiological parameters recorded at 37°C from the desheathed mouse sciatic nerve exposed to anti-GM1 IgM sera from two patients*

SAMPLE	AMPLITUDE (mV)		LATENCY (ms)		RISE TIME (ms)		THRESHOLD (V)	
	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)
GA (n=4)	6.1±0.6	92.7±4.8	0.3±0.2	100.0±0.0	1.1±0.2	100.0±0.0	3.4±0.1	100.0±0.0
JW (n=4)	4.0±1.2	97.7±1.7	0.1±0.0	100.0±0.0	1.4±0.3	101.7±2.1	3.5±0.2	100.8±0.8
NS (n=1)	6.4	93.3	0.7	100.0	0.7	100.0	4.5	100.0

NS = normal serum.

C. Discussion

The physiological role of anti-ganglioside antibodies in the pathogenesis of the peripheral neuropathies with which they are associated remains uncertain. It has yet to be firmly established whether their existence is related with the basic pathogenic mechanism or they are simply an associated epiphenomenon of the human autoimmune response. Although several reports have suggested that some peripheral neuropathy syndromes are immune mediated and anti-ganglioside antibodies are the responsible cause for these diseases other data have not confirmed that theory.

One arm of this study was to examine the acute physiological effects of anti-GQ1b and structurally related disialosyl gangliosides on nerve conduction *in vitro*. It has been previously shown in electrophysiological studies using the mouse phrenic nerve-hemidiaphragm preparation *in vitro* that the motor nerve terminals appear to be important site for their action in producing muscle weakness at room temperature (Roberts et al. 1994; Willison et al. 1996; Plomp et al. 1999). Although an important site, the motor nerve terminal is unlikely to be the exclusive site of action for several reasons. Firstly, clinical electrophysiological data from affected patients with both the acute Miller Fisher syndrome and chronic sensory ataxic neuropathy associated with anti-disialosyl antibodies indicates that peripheral nerve demyelination can occur (Willison et al. 1993b; Herron et al. 1994; Jacobs et al. 1997b). Secondly, pathological studies on biopsy and post-mortem material have confirmed the peripheral nerve demyelination (Phillips et al. 1984). Thirdly, GQ1b immunoreactive glycoconjugates have been shown in immunohistological studies with GQ1b and anti-disialosyl antibodies to be localized to peripheral nerve nodes of Ranvier, both in the human extraocular nerves (Chiba et al. 1993) and the mouse sciatic nerve (Willison et al. 1996).

There are thus strong grounds for predicting that these antibodies might have pathophysiological effects in the *in vitro* mouse sciatic nerve model system.

The second arm of the study concerned the role of anti-GM1 antibodies on nerve conduction. Clinical studies strongly support the view that there is an association between anti-GM1 antibodies and motor nerve failure. In addition, immunohistological studies have demonstrated that GM1 is localized to peripheral nerve nodal regions (Ganser, Kirschner & Willinger 1983; Santoro et al. 1990; Gregson et al. 1991; Thomas et al. 1991; Corbo et al. 1993). However the relationship of the anti-GM1 antibody to the pathogenesis of acute and chronic autoimmune peripheral neuropathies remains a matter of controversy and a wide variety of electrophysiological and morphological studies on anti-GM1 antibodies have shown contradictory findings (Hughes et al. 1985; Thomas et al. 1991; Santoro et al. 1992; Arasaki et al. 1993; Uncini et al. 1993; Harvey et al. 1995; Takigawa et al. 1995; Roberts et al. 1995; Hirota et al. 1997).

Under the experimental conditions described here, we did not observed any acutely deleterious effect of anti-ganglioside antibodies on mouse ensheathed and desheathed sciatic nerve conduction. The experiments reported here were conducted after extensive preliminary studies aimed at creating a highly stable nerve preparation within the duration of the electrophysiological recordings. We described the negative effect that can exert changes of bath osmotic pressure and pH. Moreover, we found that great care had to be taken with the tissue desheathment and placement of the desheathed nerve in the recording bath in order to ensure a stable recording preparation; for this reason the stability of the preparation was always checked and monitored for 45 min prior to application of test compounds. We notice that a large number of experiments were rejected because they were not in keeping with those preconditions. We also repeated

all experiments on mouse desheathed sciatic nerve preparation at least three occasions to accommodate any experimental variability.

In our study we used IgM and IgG anti-ganglioside antibodies and both failed to induce acute conduction block. Recently, Arasaki et al. (Arasaki et al. 1998) applied sera from 14 patients with AIDP, MMN, and CIDP to isolated lengths of rat sciatic nerve and found that 4 produced conduction block in some axons. The effect was complement dependent and authors suggest that it was associated with the presence of IgM antibodies to ganglioside GM1, although in the same study sera from other patients with IgM anti-ganglioside antibody activity did not produce conduction block and in an older report the same authors found acute conduction block with rabbit IgG anti-ganglioside sera (Arasaki et al. 1993). Our study indicates that it is unlikely that the type of the antibody (IgM or IgG) plays a crucial role in the production of conduction block or, in an other respect, we cannot support the concept that anti-ganglioside antibodies of only IgM or only IgG subclass are involved in the pathogenesis of autoimmune peripheral neuropathies.

Moreover, in order to ensure that we had not overlooked any electrophysiological abnormalities we re-investigated the 2 anti-GM1 antibodies that showed the highest degree of binding to the majority of nodes (see pages 133-145). In these later experiments, incubations and recordings were conducted at physiological temperatures (37^{o}C) under which antibody binding had been identified as maximal and complement activation would also be maximal. Even under these conditions, no effects were confirmed up to 3 hours incubation, after which time recordings were terminated because of the potential for non-specific deterioration in the viability of the preparation.

Ideally, prolonged electrophysiological recordings would have been conducted, particularly since our immunodeposition studies suggested that to observe full antibody

and complement fixation required overnight incubation, compared with the poorer and less consistent immunodeposition signals obtained after 3 hours incubation. However this was clearly not possible and a compromise position was thus reached. The study described here cannot provide evidence that anti-ganglioside antibodies play a critical role in the pathogenesis of autoimmune peripheral neuropathies. Moreover, our data are not in favour of the suggestion that anti-ganglioside antibodies may interfere or directly block sodium channels. Anti-ganglioside antibodies are not necessarily the cause in autoimmune peripheral neuropathies and maybe the presence of other unidentified factors plays a primary role in these disorders.

Our conclusion is that anti-ganglioside sera do not produce conduction block under carefully control conditions when applied locally to short nerve segments *in vitro* for small periods. Further studies are necessary to demonstrate whether anti-ganglioside antibodies have a key pathogenic role, on their own or in association with other undetermined factors in causing human autoimmune peripheral neuropathy syndromes. The significance of antibodies to gangliosides remains a challenging subject for further investigation.

CHAPTER TWO: ELECTROPHYSIOLOGICAL EFFECT OF ANTI-GANGLIOSIDE ANTIBODY ON RABBIT SURAL NERVE

Introduction

In the experiments described so far, we attempted to clarify the pathophysiological role of anti-ganglioside antibodies from many different aspects. The electrophysiological recordings from mouse sciatic nerve preparation clearly showed that these antibodies were without effect in this *in vitro* model. We decided to use rabbit sural nerve, a pure sensory nerve, in order to test the hypothesis that CANOMAD anti-ganglioside sera (Willison et al. 1996) might have some deleterious effects on sensory neurons. Rabbit may have different ganglioside composition at the nodes of Ranvier and more vulnerability to human complement than mouse. Also, several studies support the role of serum anti-ganglioside antibodies against GD1b and related gangliosides containing disialosyl moieties in the pathogenesis of autoimmune ataxic neuropathies (Ilyas et al. 1985; Daune et al. 1992; Dalakas & Quarles 1996; O'Leary & Willison 1997). Moreover, rabbit has been proven vulnerable to anti-GD1b antibodies in sensory ataxic neuropathy model (Kusunoki et al. 1996b).

A. Method and materials

1. Anti-ganglioside serum

We examined the acute physiological effect of anti-GQ1b and anti-GD1b IgM anti-ganglioside antibodies-containing serum from a patient who suffered from a chronic, large fiber sensory PPN (CH: see Table 4). This serum showed no detectable GM1 or/and GM2 activity. As a source of complement and as a control compound we used the same human normal serum described in mouse sciatic nerve preparation, which did not contain anti-ganglioside antibodies.

2. Electrophysiological recordings from desheathed rabbit sural nerve

Rabbits were killed according to United Kingdom Home Office guidelines. A 5-6 cm length of sural nerve (which originates from the union of the medial sural cutaneous branch of the tibial nerve and the sural communicating branch of the common peroneal nerve below the popliteal space) was dissected out and desheathed under a dissecting microscope (x50). The preparation was then mounted into the same recording chamber used in mouse sciatic nerve preparation (Figure 3), and the physiological buffer solution that we used was of the same composition as previously described (see pages 84-87). We also followed exactly the same method of extracellular recording as in mouse sciatic nerve preparation and we controlled all conditions that could influence the reliability of our results, as it has already been described in the previous chapter. All the experiments were carried out at room temperature (RT) of 20±2°C. The preparation was incubated for 180 mins in the test serum and normal human control serum was applied the last 90 mins of each experiment as a supplementary source of complement. Control serum, complement and patient's serum were diluted 1:10 in physiological buffer. The sodium channel blocker, tetrodotoxin (TTX) was used at a concentration of 1mg/ml. Finally, we performed the same analysis of the data as has been described in the previous chapter.

B. Results

1. Acute conduction block induced by TTX

We first applied tetrodotoxin (TTX), which selectively blocks sodium channels, and we observed acute reversible conduction block in about 3 min (Figure 41).

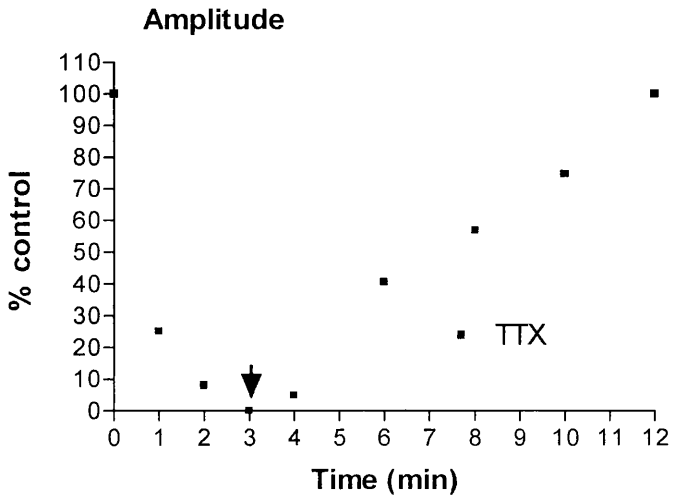

Fig. 41 Desheathed rabbit sural nerve treated with 1mg/ml tetrodotoxin (TTX), a reversible sodium channel blocker (n=1). Arrow shows the start of the washout phase.

2. Physiological effect of IgM anti-GQ1b and anti-GD1b serum

When we applied the patient's serum containing GQ1b and GD1b anti-ganglioside antibodies we noticed no significant changes in the amplitude, latency, rise time or stimulus threshold of the action potentials. When we added fresh normal human serum in order to ensure that sufficient complement was available, again we did not observed any changes in the recorded parameters (Figure 42 and Table 14).

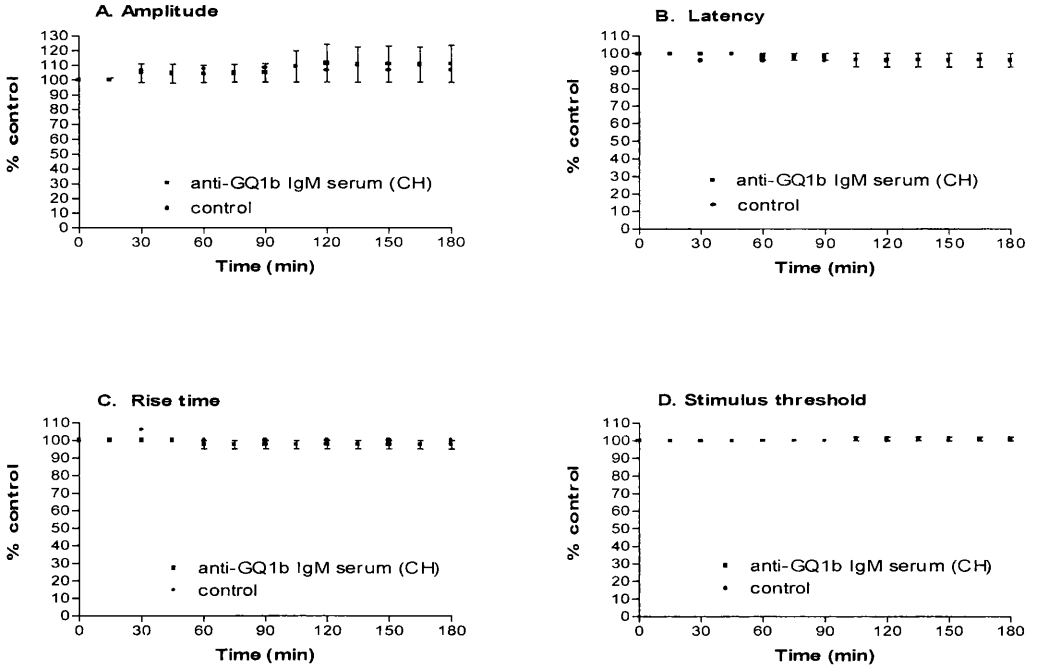

Fig. 42 Desheathed rabbit sural nerve recordings at RT. Exposure of the nerve to anti-GQ1b and anti-GD1b IgM serum from a patient with PPN (CH). Points are means of 3 experiments. SEM are indicated by error bars unless they were smaller than symbols.

Table 14 *Electrophysiological parameters recorded at RT from the desheathed rabbit sural nerve exposed to anti-GQ1b and anti-GD1b IgM serum*

SAMPLE	AMPLITUDE (mV)		LATENCY (ms)		RISE TIME (ms)		THRESHOLD (V)	
	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)	Time zero (absolute values)	End of incubation (% time zero)
CH (n=3)	3.0±1.2	110.9±12.6	0.5±0.2	96.1±3.9	0.7±0.0	97.6±2.4	4.0±0.8	101.1±1.1
NS (n=1)	2.1	106.3	0.9	95.8	0.6	100.0	3.9	100.0

NS = normal serum.

C. Discussion

The recent literature about autoimmune peripheral neuropathies has been dominated by the discovery of antibodies to a wide variety of gangliosides. Clinical and electrophysiological data from affected patients have shown that serum antibodies against gangliosides may have a significant contribution in the pathogenic mechanism of some peripheral neuropathy syndromes. In particular, several reports have suggested an association between sensory neuropathy syndromes and antibodies which immunoreact with disialosyl gangliosides (Ilyas et al. 1985; Daune et al. 1992; Yuki et al. 1992a; Brindel et al. 1994; Dalakas & Quarles 1996). Also, it has been reported that IgM antibody recognizing B-series gangliosides including GQ1b and GD1b causes cell death of rat dorsal root ganglion neurons and it has been proposed the anti-B-series ganglioside-directed antibody is the causal agent for the human sensory polyneuropathy (Ohsawa et al. 1993).

In addition, it is well established that the anti-GQ1b antibody is the best example of a close association between antibodies to gangliosides and neuropathy and it is detected in the majority of patients with Miller Fisher syndrome (MFS) (Chiba et al. 1992; Chiba et al. 1993; Yuki et al. 1993b; Willison et al. 1993c; Jacobs et al. 1995; Yuki 1996a; Carpo et al. 1998; Suzuki et al. 1998; Schwerer et al. 1999). The anti-GQ1b ganglioside antibody is highly specific for this syndrome of acute sensory ataxic neuropathy with ophthalmoplegia (MFS), and it is not found in normal sera. The only other cases in which it is found are patients with related conditions, which might share the same pathogenic mechanism, as the acute oropharyngeal palsy variant of GBS (O' Leary et al. 1996) and the benign brain stem encephalitis (Yuki et al. 1993a). Strong evidence supporting a pathogenic role for anti-GQ1b antibody comes from the demonstration that ganglioside GQ1b is particularly abundant in the ocular motor nerves (Chiba et al. 1993;

Chiba et al. 1997). However, the clinical and neurophysiological findings in Fisher syndrome do not match entirely with the distribution of GQ1b ganglioside (which is not present in high quantities in the lower cranial nerves that may be affected in atypical MFS whereas its concentration is high in optic nerves that are not affected (Chiba et al. 1997)). In some studies sera positive for anti-GQ1b antibody have been found to impair neuromuscular transmission in the mouse phrenic nerve-hemidiaphragm model at room temperature (Roberts et al. 1994; Plomp et al. 1999), although MFS sera without any detectable anti-GQ1b activity have also been shown to block evoked quantal release at mouse neuromuscular junction (Buchwald et al. 1998b). On the other hand the anti-GD1b ganglioside antibody has been proven to cause sensory ataxic neuropathy in rabbits due to antibody-mediated damage to the sensory neurons in the first reported animal model for autoimmune neuropathy mediated by anti-ganglioside antibody (Kusunoki et al. 1996b).

In this study we examined the acute physiological effect of the anti-GQ1b and the structurally related anti-GD1b ganglioside antibody on a pure sensory nerve preparation for several reasons. Firstly, it has been reported that the anti-GQ1b antibodies are associated with selective and more severe damage of sensory nerves (Jacobs et al. 1997b), and the GD1b epitope is present on dorsal root ganglion neurons and paranodal myelin (Kusunoki et al. 1993; Oka et al. 1996; Willison et al. 1996; Kusunoki et al. 1997). Secondly, studies on human peripheral nerve tissues have demonstrated the differences in biochemical composition, content and concentration of gangliosides between motor and sensory nerves which may explain the variations in peripheral nerve disorders (Ogawa-Goto et al. 1990; Ogawa-Goto et al. 1992; Svennerholm et al. 1994). Thirdly, electrodiagnostic and pathological studies from affected patients with sensory neuropathy syndromes and anti-disialosyl antibodies have suggested the selective

vulnerability of sensory nerve fibers (Dalakas 1986; Willison et al. 1993b). Fourthly, it is well recognized that sensory and motor axons exhibits ion channels with different properties and this diversity may explain the regional and selective immune attack in different types of peripheral polyneuropathies (Waxman 1995). Moreover, we selected rabbit sural nerve because rabbit nodes of Ranvier contain a set of ionic channels that are very similar to those in human nodes as it has proven with voltage-clamp methods (Schwarz, Reid & Bostock 1995). The tested antibody came from a patient who suffered from chronic paraproteinemic neuropathy with predominantly sensory rather than motor symptoms.

Our study described here did not reveal any changes on recorded electrophysiological parameters within the 3 hours time frame. The IgM anti-GQ1b and anti-GD1b antibodies failed to exert any acute dysfunction on sensory neurons conduction and these results are in keeping with the previous study on mouse sciatic nerve preparation. Also, these negative results failed to give an *in vitro* support of the suggested pathogenic importance of disialosyl antibodies in autoimmune sensory polyneuropathies. Moreover, we failed to confirm that these antibodies in the presence of complement could block sodium channels, as has been postulated (Waxman 1995), compared with the acute block induced by the tetrodotoxin (TTX). Despite our negative results, we remain in favour of the view based on collective data from clinical studies that these antibodies are likely to be pathogenic *in vivo*. Moreover, more extensive studies than the *in vitro* electrophysiological model described here will be necessary to convincingly elucidate the role of these antibodies in causing conduction block.

CHAPTER THREE: IMMUNOHISTOCHEMICAL STUDY OF ANTI-GANGLIOSIDE ANTIBODIES

A. Method and materials

1. Immunofluorescence studies on mouse sciatic nerve preparations

Male BALB/c mice (15-20 g) were sacrificed by exposure to carbon dioxide and the sciatic nerves were dissected and desheathed as for the electophysiology preparation described above. The nerves were incubated overnight at RT or at 37°C in 0.1 ml of pre-oxygenated physiological buffer containing the experimental sera or monoclonal antibodies. In preliminary studies we found that incubation for up to 4 hours was insufficient to achieve equilibration in antibody binding and produced antibody binding and complement fixation with high inter-experimental variability; we therefore performed overnight incubations to compensate for this effect. We tested three different anti-GM1 monoclonal antibodies (Wo1, Bo1, Sm1; 20 μg/ml), two different red cell eluate preparations (RCE, prepared on red cell affinity columns as described in Willison et al., (Willison et al. 1996)) with anti-GQ1b IgM activity (CH, VP; 20 μg/ml), and three different plasmas with activity against GQ1b (BK; diluted 1:10), GM1 (PS; diluted 1:10) or GM1/GM2 (GA; diluted 1:50). The active antibodies in each preparation were IgM (see Table 4). We used normal human serum as both a serum control (at the appropriate dilution) and a source of complement (diluted 1:10). Samples were coded and studied blind. After the incubation period, the nerves were subjected to three 15 min washes with clean buffer, and then teased out into bundles containing as few fibres as possible, using fine syringe needles. The separated fibres were allowed to air dry onto 3-aminopropyltriethoxysilane coated slides before use or storage at –20°C. To identify immunoglobulin and complement deposits on the fibres, the slides were incubated for 1 hour at 4°C in PBS containing 10% lamb serum, 0.3% rhodamine-

labelled anti-human IgM antibody (Southern Biotechnology Associates; diluted 1:300), and either a fluorescein-labelled antibody to the C3c complement activation product (C3c; Dako) or 0.1% fluorescein-labelled cholera toxin B subunit (CT; Sigma). The CT was used to help identify Schwann cell architecture, especially the paranodal regions that stain strongly. The slides were again rinsed in cold PBS before being mounted in Citifluor antifade (Citifluor Products, Canterbury, UK), ringed with nail-varnish to minimise drying and stored at 4°C in the dark prior to viewing. Controls consisting of second antibody alone were run in parallel with every staining batch.

2. Imaging

Images were obtained by means of a Sony colour 3CCD camera mounted onto a Zeiss Axioplan fluorescent microscope, linked to a PC driven image archiving system (Sirrius VI; Optivision). The threshold of the image acquisition equipment was set up such that the control level of staining obtained with secondary antibodies alone was zero. The model staining counts and micrographs shown in this study are based on the signal detected above the threshold level. The time involved in making individual teased fibre preparations for use for this study meant that the screening of large numbers of control samples was impractical. It was decided to control this study with a single, well characterised normal serum used extensively in other studies within the group (Plomp et al. 1999). In preliminary studies conducted by Dr. Graham O'Hanlon to assess the range of background signals produced by "normal" serum on neuronal membranes, a panel of 11 serum samples (including the control for this study), each lacking anti-ganglioside or known anti-neural activity, were screened in a quantifiable assay. The control serum was not significantly different from the pooled results from the other 10 samples (2-tailed Mann Whitney U test; $p = 0.42$), and as such taken to be a reasonable

representative of the population. For the fluorescein (C3c staining) and rhodamine (IgM) channels, images were integrated to obtain unambiguous signal intensities for counting nodes. On each slide, 25 consecutive nodes were identified at random under phase microscopy and scored either positive or negative for IgM and C3c. Bitmap processing and annotation were conducted on PhotoMagic and Windows Draw (both by Micrographx Inc.). Images were printed directly using a photographic-quality colour printer (Kodak ColourEase).

Data were analysed using Chi-square or Fisher exact test as appropriate. All analyses were performed using the Minitab 10.5 for windows statistical package.

B. Results

1. Preliminary immunohistochemical observations from the nerves used in electrophysiological recordings

We performed the electrophysiological experiments on mouse desheathed sciatic nerve preparation at the Department of Physiology and Pharmacology of Strathclyde University, and the nerves were incubated in ganglioside antibody antisera for periods up to 4 hrs. In order to examine immunoglobulin and complement deposition at the nodes of Ranvier, we kept some of these preparations at 4°C in paraformaldeyde. From these we prepared teased fibres, and we performed our first immunofluorescence study using nerves that were incubated with IgM anti-ganglioside sera, because of the very high background signals observed with immunostaining for IgG deposits. We examined four different IgM anti-ganglioside sera (GA, JW, BK, and PS) and two different monoclonal anti-ganglioside antibodies (Bo1/Bo3, and Sm1). As control we used nerves incubated with normal human IgM. In this study we did not perform a quantitative analysis for immunopositive and immunonegative nodes. However, GA,

and JW sera show some staining at a proportion of nodes of Ranvier in many experiments. Also, BK, and PS sera gave rise to some immunodeposits at the nodes compared with normal human IgM, whereas Bo1/Bo3 and Sm1 monoclonal antibodies showed very weak or no immunoreactivity.

2. Immunoglobulin and complement deposition following *in vitro* incubation with IgM anti-ganglioside antibody

In order to determine whether nerves incubated with sera contained immunoglobulin and complement deposits at nodes of Ranvier we investigated separately the effect of each Ab used in the electrophysiology section, following the same incubation period and concentration as described in that section. We examined the effects at two different temperatures, RT and 37°C. The results were quite ambiguous; monoclonal antibodies and anti-ganglioside sera showed characteristic diversity, although some were again proved highly immunoreactive (GA, and JW).

We wanted to have a clear view of the immunodeposition of the anti-ganglioside antibody. The incubation period was increased from 4 hrs to overnight and for each serum or/and monoclonal Ab examined, an observer blinded quantitative immunoanalysis was performed. Studies were again restricted to sera containing IgM anti-ganglioside activity because of the very high background signals observed when immunostaining for IgG deposits, with either normal of disease-associated sera at these low dilutions (1/10). The data are demonstrated in Figures 43, 44 and Table 15. The immunodeposits were more prominent at 37°C compared to RT with a statistically significant difference ($p=0.007$). Also, under the imaging conditions described above, 25 of nodes of Ranvier (15%) stained positive for human IgM following incubation at RT with normal human serum, whereas this dropped to 16 positive nodes (8%) at 37°C

(p=0.048). One of the monoclonal antibodies (Wo1) and one of the anti-GM1 antibody containing sera studied (GA) gave rise to abundant immunodeposits at 60-90% nodes of Ranvier at both RT and 37°C (Table 15), although statistically significant difference between 37°C and RT was achieved only for one of these two samples (Wo1, p=0.001). Five (GA, PS, BK, CH, Wo1) out of eight anti-ganglioside samples showed statistically significant difference versus control serum at 37°C, whereas only two of them (GA, and Wo1) had a similar effect at RT. Three tested samples (VP, Bo1, and Sm1) failed to achieve significant differences in the frequency of nodal staining at either temperature. The vast majority of IgM positive nodes were also positive for the complement activation product C3c. Examples of these immunostaining patterns are shown in Figure 45.

Table 15 *Immunofluorescence studies on teased fibres prepared from desheathed mouse sciatic nerve pre-incubated with anti-ganglioside antibodies*

Antibody code	Immunopositive nodes of Ranvier										
	37°C					RT					
	Pos	Neg	tot	%	Chi$^{2\#}$ (+/-vs NS)	Pos	Neg	tot	%	Chi$^{2\#}$ (+/-vs NS)	Chi$^{2\#}$ (+/-vs 37°C)
NS	16	173	189	8	nsd	25	139	164	15	nsd	*
Total (GA-Sm1)	114	97	211	54	***	85	123	208	41	***	**
GA	60	15	75	80	***	40	20	60	67	***	nsd
PS	5	10	15	33	*	4	11	15	27	nsd	nsd
BK	5	10	15	33	*	4	11	15	27	nsd	nsd
CH	4	11	15	27	*	8	20	28	29	nsd	nsd
VP	7	23	30	23	nsd	9	21	30	30	nsd	nsd
Wo1	29	1	30	97	***	18	12	30	60	***	**
Bo1	3	13	16	19	nsd	2	13	15	13	nsd	nsd
Sm1	1	14	15	7	nsd	0	15	15	0	nsd	nsd

$^{\#}$ or Fisher exact test where required. Nsd; no significant difference.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$

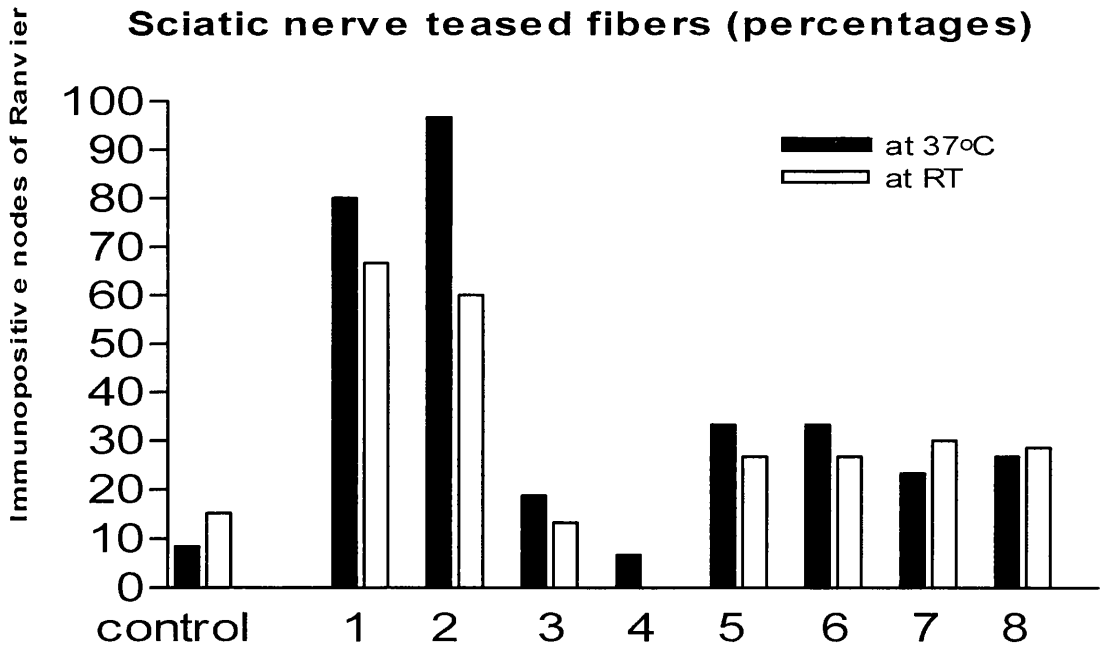

Fig. 43 Immunofluorescense studies on mouse sciatic nerve teased fibres (Data from Table 15). Three different anti-GM1 monoclonal antibodies (2, 3, 4) and five different sera, of which two anti-GM1 (1,5) and three anti-GQ1b (6, 7, 8) positive, were tested. All the tested antibodies were of IgM type. In the control experiments we used a single normal human serum (see page 134).

1=GA, **2**=Wo1, **3**=Bo1, **4**=Sm1, **5**=PS, **6**=BK, **7**=VP, and **8**=CH.

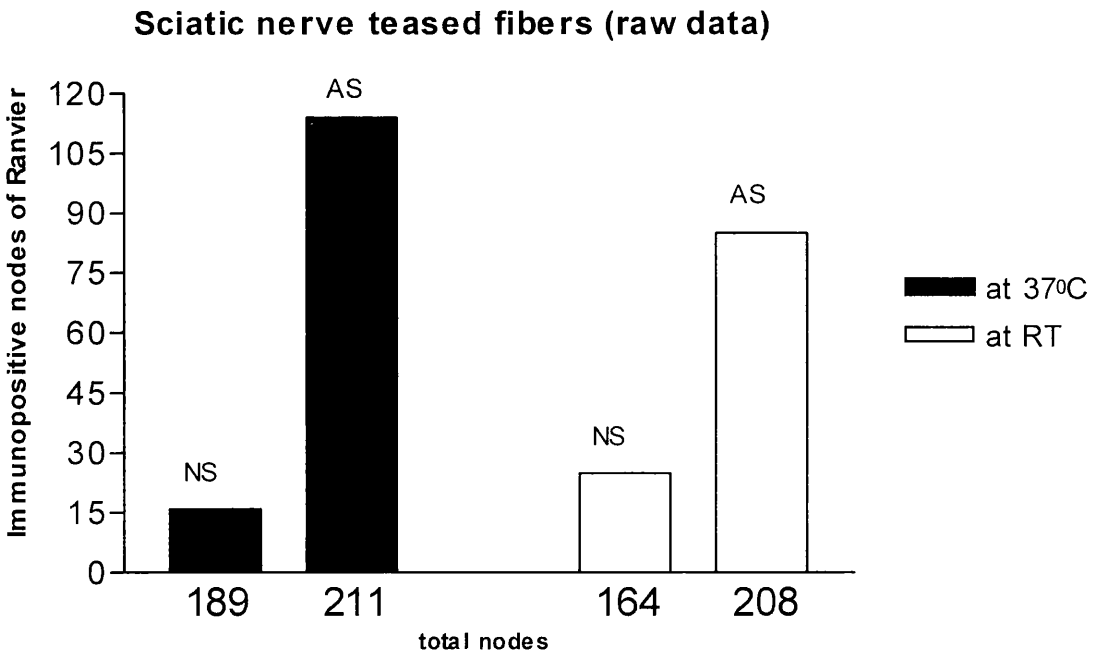

Fig. 44 A significantly increased deposition of IgM at nodes of Ranvier was observed in nerves exposed to anti-ganglioside antibodies ($p<0.001$). For anti-ganglioside samples (AS) the immunodeposits were more prominent at 37°C compared to RT ($p<0.01$), whereas a significant increase in staining at RT versus 37°C for control serum (NS) was also observed ($p<0.05$) (data from Table 15).

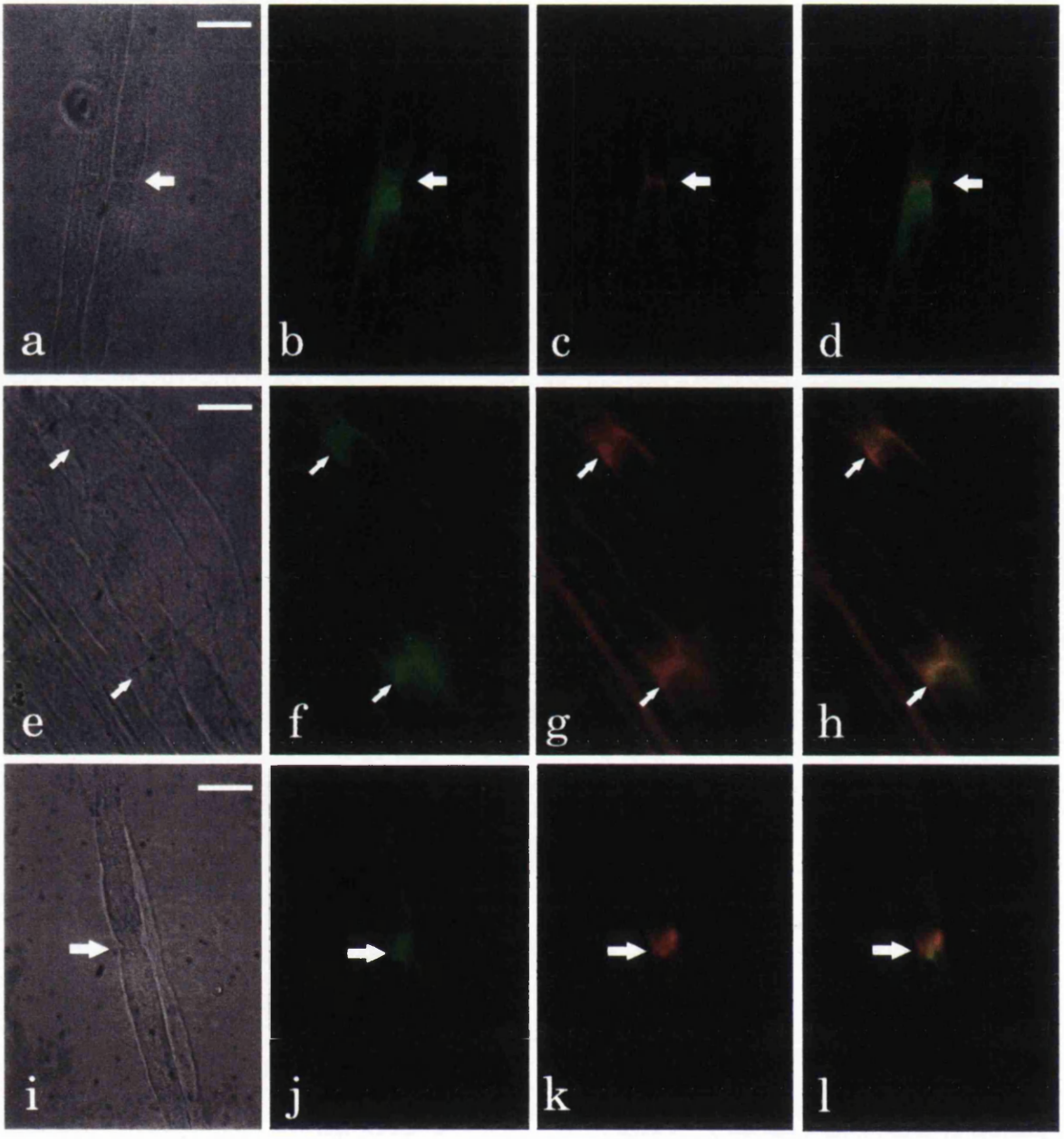

Fig. 45 Deposition of antibody and complement in mouse sciatic nerve teased-fibre preparations. Phase-contrast images (panels **a, e,** and **i**) of positively stained nodes of Ranvier (arrows). GA anti-GM1 IgM serum treated nerve (**a-d**) was stained with fluorescein-labelled cholera toxin B subunit (**b**) which binds the paranodal myelin and for human IgM deposits (**c**) which are deposited at the nodal gap. Wo1 anti-GM1 monoclonal antibody treated nerve (**e-h**) was stained with fluorescein-labelled cholera

toxin B subunit (**f**) and for human IgM deposits (**g**) which occupy a nodal and paranodal distribution. Wo1-treated nerve (**i-l**) stained for complement C3c (**j**) and human IgM (**k**) which again occupy a nodal and paranodal distribution. Scale bar = 20 μm; x100, under oil).

C. Discussion

Over the last decade immunohistochemistry has been an intense field in investigating the role of anti-glycoconjugate antibodies in peripheral neuropathies and numerous immunohistological studies have suggested that some of these disorders are anti-glycolipid antibody-mediated syndromes. Circulating monoclonal IgM anti-MAG antibodies have been proved to be associated with a specific type of human peripheral nerve demyelination. Positive serum immunofluorescence studies and the presence of widening of myelin lamellae have demonstrated that human anti-MAG neuropathy is likely to be antibody mediated (Hays et al. 1987; Willison et al. 1988; Trojaborg et al. 1989; Vital et al. 1989; Tatum 1993; Vallat et al. 1996).

On the other hand the anti-ganglioside antibodies have been postulated to be implicated in the pathogenesis of some autoimmune peripheral neuropathies. It remains unclear whether these antibodies are primarily responsible for causing damage to the nerves and conflicting results have been obtained from electrophysiological and pathological studies (Thomas et al. 1991; Santoro et al. 1992; Arasaki et al. 1993; Uncini et al. 1993; Harvey et al. 1995; Takigawa et al. 1995; Hirota et al. 1997). However, several immunohistochemical studies provide strong evidence that supports their implication in the pathogenesis of human neuropathies and anti-ganglioside antibody deposits have been detected in peripheral nerve structures, including nodes of Ranvier (Santoro et al. 1990; Gregson et al. 1991; Thomas et al. 1991; Santoro et al.

1992; Adams et al. 1993; Kusunoki et al. 1993; Nardeli et al. 1994; Harvey et al. 1995; Hafer-Macko et al. 1996; Oka et al. 1996; Willison et al. 1996; Kusunoki et al. 1996b; Kusunoki et al. 1997; Paparounas et al. 1999). The purpose of the present immunofluorescence study was to investigate the anti-ganglioside antibody and complement deposition at nodes of Ranvier. The node of Ranvier is a narrow gap of axon along a myelinated fibre that generates currents for nerve impulses. This nodal region is a mere 0.5-1.0 μm wide and contains the highest density of ion channels of all known excitable membranes (Waxman & Ritchie 1985).

The data presented in this study suggest that antibody and complement products can be deposited at a proportion of nodes of Ranvier and this finding may explain the possible pathophysiological effect of anti-ganglioside antibody. It is possible that these antibodies block sodium channels at the node of Ranvier which is highly differentiated in that region, exhibiting unique morphological, electrophysiological and cytochemical properties compared to other sites along the fibre (Waxman & Ritchie 1985). GM1 ganglioside has been localized at the node of Ranvier (Ganser et al. 1983; Corbo et al. 1993) where sodium channels are clustered (Waxman 1995) and IgM deposits have been observed at nodes of Ranvier in our study. We do not know the mechanism that may elicit the production of antibodies that may block sodium channels. Also, the initial events that cause exposure of the GM1 epitopes to the antibodies are similarly unknown. Previously, binding of anti-GM1 antibodies to the nodal region has been suggested to block ion channels and disrupt the membrane at the node of Ranvier, causing conduction block (Takigawa et al. 1995), although this hypothesis has been recently disputed (Hirota et al. 1997).

The fine specificities and the differences in the sources of the antibodies may also play an important role in the nature and extent of nodal binding. It is known from

immunohistological studies that different anti-GM1 antibodies are highly diverse in their peripheral nerve staining patterns, including the presence or absence of binding to nodes of Ranvier (O' Hanlon et al. 1998). Similarly there are marked species differences in the distribution and immunoreactivity of gangliosides, including differences in peripheral nerve between rat and mouse (O' Hanlon et al. 1996). For these reasons we checked for the binding of antibody and complement deposition in the same preparations from which electrophysiological recordings have been made and observed high degrees of nodal binding with some anti-ganglioside antibodies. Also, the period of time during which the nerve fibre is exposed to antibody may be another important factor, as we observed substantial differences in antibody binding between overnight and four hours incubation periods. This could offer a possible explanation for our negative electrophysiological results, where the exposure of the nerve to antibody was no longer than four hours.

Our findings suggest that the antibodies might exert their effect by binding to the nodal regions of peripheral nerve. This might set into motion a cascade of sequential reactions through complement activation. The activation of terminal complement components (membrane attack complex) might lead to the insertion of pore forming molecules into the nodal membrane. Thus, ions can move through these pores and the antibodies may disturb conduction of action potentials through direct or indirect interference with sodium channels or other structures, which are located at the nodes of Ranvier in peripheral nerve. The reason for the increased binding at 37°C is unknown, but the antigen may be more accessible at 37°C than at RT. Moreover, we do not know if there was any preferential involvement of motor or sensory fibres since the nerve we used was a mixed peripheral nerve. This study revealed that one of the tested anti-GM1 sera and one of the monoclonal antibodies gave rise to antibody and complement deposits at over 50% of nodes of Ranvier in some preparations. In contrast, some other

antibodies derived from different patients showed a lower affinity for their target epitope and in some other cases the immunostaining at the nodes of Ranvier was absent. The effect of antibodies might also depend on their size or local factors, which allow them to penetrate most cryptic and unavailable epitopes or not. Also, the ability of the antibody to access an antigenic site may be determined by the epitope density and interactions with neighbouring molecules. In addition, the nature and severity of the disease and antibody titre may play another significant role.

Thus, although the hypothesis that these antibodies may play a pathogenic role in autoimmune peripheral nerve disorders is still disputable, this study clearly shows that anti-ganglioside antibody could cause conduction abnormalities through binding at the nodes of Ranvier. However the data are insufficient to conclude that the anti-ganglioside antibodies are responsible for the nerve damage and new efforts are needed to further investigate their pathogenic effects against peripheral nervous system targets.

CHAPTER FOUR: TWITCH TENSION IN MOUSE PHRENIC NERVE-HEMIDIAPHRAGM PREPARATION AFTER ANTI-GANGLIOSIDE ANTIBODY APPLICATION

Introduction

Our immunochemistry studies clearly showed that two anti-GM1 positive sera (GA, and JW) were at high percentages immunoreactive at body temperature, and also the anti-GQ1b containing serum (CH) had a significant immunolocalization at nodes of Ranvier at the same temperature (see Table 15). However, these sera did not have any deleterious effect on isolated mouse sciatic nerve physiology at 37°C (see pages 120-121). We wished to detect any acute effect of these antibodies on neuromuscular transmission at physiological temperature. We used mouse phrenic nerve-hemidiaphragm preparation, which is a useful model in investigating the antibody-mediated disorders of neuromuscular junction.

A. Method and materials

Phrenic nerve-hemidiaphragm preparations were dissected from male Balb-C mice, 20-25g that were killed by exposure to carbon dioxide. The preparation was mounted in a 10 ml tissue bath containing a physiological salt solution of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $KHPO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 11.1. The solution was maintained at 37°C and was aerated throughout the experiment with gas containing 95% oxygen and 5% CO_2. The experiments were carried out at pH=7.4 and osmotic pressure 320±10 mOsm/kg. The pH of the solution was checked after the initial 100% saturation and also at the end of each experiment. Phrenic nerve was stimulated with pulses of 0.01 msec duration and 10 V stimulus strength, once every 10 sec via a platinum ring electrode. Twitches were recorded on

Grass 79B and Washington ink writing polygraphs using Grass FT03 or SRI transducers. We employed low voltage and short duration pulses in order to ensure that the evoked twitch was due to indirect (via the nerve) stimulation; if with such stimulation the elicited tension of the hemidiaphragm was of very small amplitude or unrecorded the preparation was discarded. Before the optimization of the muscle length the resting tension of 1 g was used. Preparations were allowed to stabilize for about 20 min before the addition of the compounds.

Two anti-GM1 IgM anti-ganglioside antibodies from two different patients with MMN (GA, and JW) were highly immunoreactive in mouse sciatic nerve teased fibres at 37°C, as previously described. We examined these two anti-GM1 IgM highly positive sera and one anti-GQ1b and anti-GD1b IgM from a patient with PPN (CH) (see Table 4). The source of complement was normal human serum, which was also used as control compound. Patients' sera and normal serum were diluted 1:40 and complement 1:50. Both were applied together from the start of each experiment.

The data are presented as mean±SEM. All measurements were normalized according to the control values at time zero, without the presence of anti-ganglioside antibody. Statistical significance was tested with non-parametric statistics (Mann-Whitney's test) and Chi-square test as appropriate. All analyses were performed using the Minitab 10.5 for Windows statistical package.

B. Results

1. Neuromuscular blockade induced by curare

In preliminary control experiments we studied the acute physiological effects of tubocurarine on neuromuscular transmission. Tubocurarine combines with acetylcholine receptors postjuctionally producing neuromuscular block (competitive blockade). Figure 46 is a characteristic example of the effect of tubocurarine on mouse phrenic nerve-hemidiaphragm twitch tension. Also this Figure clearly depicts the completely reversible effect of the drug after the washout of the preparation.

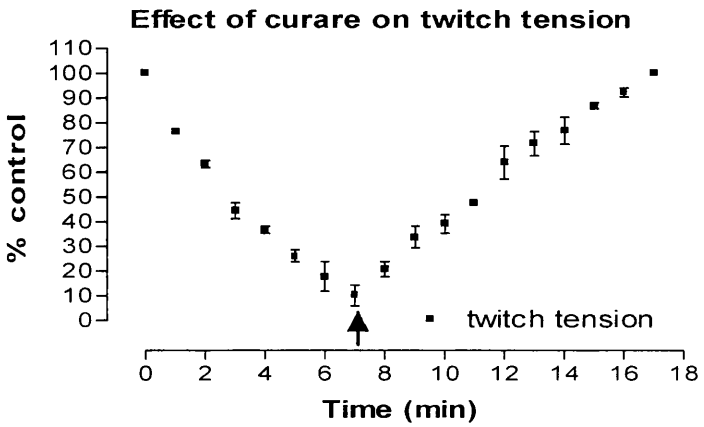

Fig. 46 Acute neuromuscular block in mouse phrenic nerve-hemidiaphragm preparation produced by tubocurarine (10^{-8} M) at 37°C. Preparations were washed out after 7min (arrow). Points are means of two experiments and SEM are indicated by error bars unless they were smaller than symbols.

2. Pitfalls in mouse phrenic nerve-hemidiaphragm recordings

In early twitch tension recordings, in order to reveal any subtle change in neuromuscular transmission due to anti-ganglioside antibody activity, we decided to induce a partial block by curare in order to increase the sensitivity of observing an effect. Thus, a 70% reduction in the twitch height was induced by tubocurarine (10^{-8} M). The neuromuscular block may be antagonized by any compound that increases the release of

acetylcholine by nerve impulses. With the safety factor reduced, any alteration in the release of acetylcholine would be more apparent. With this method, in about 15 mins after the application of anti-ganglioside antibody, a gradual increase in twitch tension was observed despite the presence of tubocurarine, and it was thought that this effect was due to anti-ganglioside antibody activity (Figure 47A). The same phenomenon was observed in the corresponding control experiments after the application of normal human (Figure 47B). Therefore, the method of investigating the acute physiological effects of anti-ganglioside antibodies in partially curarized neuromuscular junction was abandonded. We postulate that the recovery observed after the application of normal or anti-ganglioside sera was most likely a dilution phenomenon.

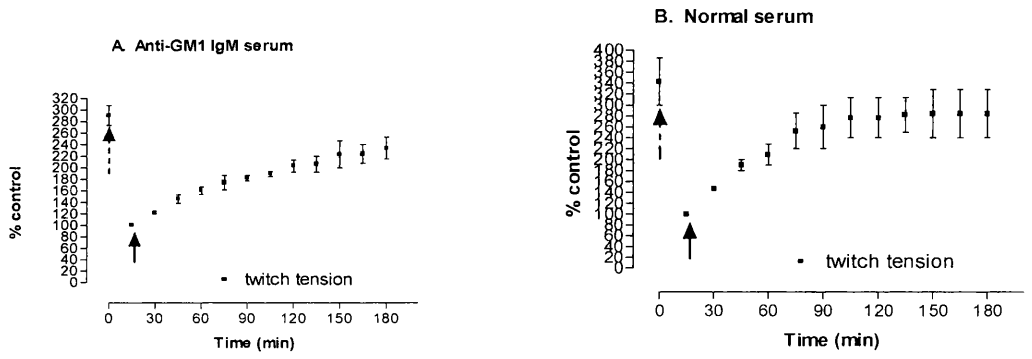

Fig. 47A Misleading effect of anti-ganglioside Ab on mouse phrenic nerve-hemidiaphragm twitch tension experiments at 37°C with the safety factor reduced. Dotted and solid arrows show the application of tubocurarine (10^{-8} M) and anti-GM1 IgM serum (diluted 1/40), respectively. SEM are indicated by error bars unless they were smaller than symbols.

Fig. 47B When we applied normal human serum (diluted again 1/40) we again observed the same phenomenon as with anti-ganglioside sera.

3. Physiological effect of anti-ganglioside antibody on muscle twitch tension

We first applied fresh normal human serum to the preparation for periods up to 3 hrs and we observed no significant changes in the recording twitch tension (Tables 16, 17 and Figure 48). When we applied the patients' IgM anti-ganglioside containing sera, we again observed no significant changes in phrenic nerve twitch tension in up to 3 hrs of recording (Figure 48 and Tables 16, 17). Table 16 shows the absolute values of all performed experiments, and Table 17 the percentage changes after application of test compounds for up to 3 hrs.

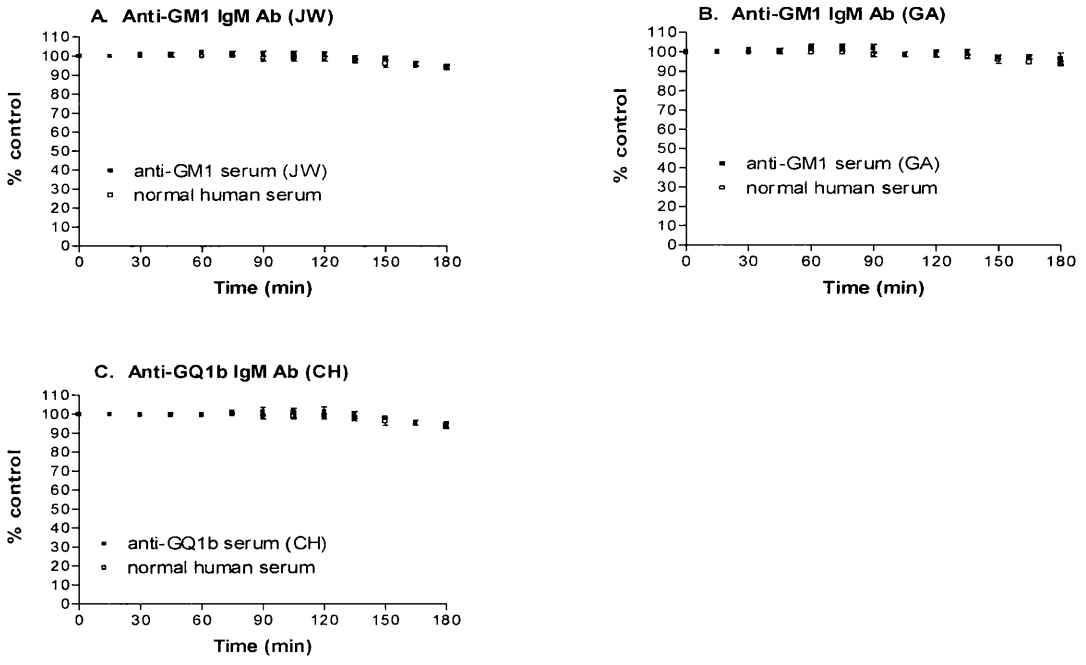

Fig. 48 Results from mouse phrenic nerve-hemidiaphragm twitch tension experiments at 37°C. We tested the following anti-ganglioside sera: **A.** Anti-GM1 IgM from a patient with MMN (JW, n=5) **B.** Anti-GM1 IgM from another patient with MMN (GA, n=5) and **C.** Anti-GQ1b IgM from a third patient with PPN (CH, n=5). Normal human serum was used as control serum (n=3) and as a source of active complement. Complement was added with patient serum at the beginning of incubation period. SEM are indicated by error bars unless they were smaller than symbols.

Table 16 *Twitch tension in absolute values (g) recorded from mouse phrenic nerve-hemidiaphragm preparation at 37°C after the application of anti-ganglioside sera for up to 3 hrs*

TIME (min)	SAMPLES			
	NS (n=3)	**JW** (n=5)	**GA** (n=5)	**CH** (n=5)
0.0	1.6±0.2	1.8±0.2	1.3±0.2	1.7±0.1
15.0	1.6±0.2	1.8±0.2	1.3±0.2	1.7±0.1
30.0	1.6±0.2	1.8±0.2	1.3±0.2	1.7±0.1
45.0	1.6±0.2	1.8±0.1	1.3±0.2	1.7±0.1
60.0	1.6±0.2	1.8±0.1	1.4±0.2	1.7±0.1
75.0	1.6±0.2	1.8±0.2	1.4±0.2	1.7±0.1
90.0	1.6±0.2	1.8±0.2	1.3±0.2	1.7±0.1
105.0	1.6±0.2	1.8±0.1	1.3±0.2	1.7±0.1
120.0	1.6±0.2	1.8±0.2	1.3±0.1	1.7±0.1
135.0	1.6±0.2	1.7±0.2	1.3±0.2	1.7±0.1
150.0	1.6±0.2	1.7±0.2	1.3±0.2	1.6±0.1
165.0	1.6±0.2	1.7±0.2	1.3±0.2	1.6±0.1
180.0	1.5±0.2	1.7±0.1	1.3±0.2	1.6±0.1

NS = normal serum; JW = serum with anti-GM1 IgM antibody; GA = serum with anti-GM1 IgM antibody; CH = serum with anti-GQ1b IgM antibody.

TABLE 17 *Mouse phrenic nerve-hemidiaphragm preparation. Twitch tension experiments at 37°C. The table shows the percentage changes after application of test compounds for up to 3 hrs*

TESTED SERA	TIME ZERO	END OF INCUBATION (% TIME ZERO)
Anti-GM1 Ab (**JW**, n=5)	100.0% (1.8g±0.2)	94.7±1.2
Anti-GM1 Ab (**GA**, n=5)	100.0% (1.3g±0.2)	96.6±2.9
Anti-GQ1b Ab (**CH**, n=5)	100.0% (1.7g±0.1)	94.1%±1.8
NS (n=3)	100.0% (1.6g±0.2)	94.0±1.1

Values are means ±SEM. The absolute values at time zero are indicated in parentheses. NS = normal serum.

C. Discussion

Two anti-ganglioside antibodies, anti-GM1 and anti-GQ1b, have been described to be associated with specific neurological syndromes and much of the recent literature supports their pathogenic role in human autoimmune peripheral neuropathies. The clinical presentation of neuropathy may depend on whether the nerve dysfunction occurs in myelin, the nodes of Ranvier, or neuromuscular junction. In this study we examined the acute neurophysiological effects of anti-GM1 and anti-GQ1b positive sera using a motor nerve-muscle preparation.

Electrophysiological studies have previously implied the possible presynaptic effect of anti-GQ1b antibody on acetylcholine release from motor nerve terminals in mouse phrenic nerve-hemidiaphragm preparation at room temperature (Roberts et al. 1994), and mouse neuromuscular junctions have been found important sites of antibody action in conjunction with activated complement (Plomp et al. 1999). However, the aetiopathogenic role of anti-GQ1b antibody in causing dysimmune clinical syndromes cannot be established with certainty since immunoglobulin G as well as its Fab

fragments from anti-GQ1b negative MFS patients has been shown to depress evoked quanta end plate currents from mice hemidiaphragms (Buchwald et al. 1998b). Similarly, the neuromuscular blockade induced by IgG antibodies from patients with GBS was independent of the presence or absence of detectable anti-GM1 or anti-GQ1b antibodies (Buchwald et al. 1998a). Moreover, sera and plasma from patients with MMN can interfere with distal motor nerve function and block nerve conduction at distal motor nerves, although the anti-GM1 antibody may not be the cause of such dysfunction since anti-GM1 negative sera from patients with MMN can have the same activity in motor nerve-muscle preparation (Roberts et al. 1995). Also, Miller Fisher sera/plasmas showed no effect on transmitter release from PC12 cells (Benatar, Willison & Vincent 1997).

This study failed to demonstrate the neuromuscular blockade induced by these antibodies. Our results imply that the anti-GQ1b or anti-GM1 antibodies are not sufficient by their own in inducing muscle weakness in MFS and other related neuropathies. Maybe there are other or additional serum factors or antibodies against undetermined antigens which interfere with presynaptic calcium inflow or prevent the activation of post synaptic channels and so, play a primary but still unknown role in these neurological disorders. The causal role of an antibody (not necessarily the GQ1b or anti-GM1) responsible for muscle weakness observed in some of these syndromes seems more likely since muscle strength improves rapidly after therapeutic plasmapheresis or immunoadsorption (Yuki 1996a; Yuki 1996b; Ohtsuka et al. 1998b). It must be emphasised that we took all necessary precautions to minimise artificial effects since it is well known that neurotransmitter release from motor nerve terminals can be influence by many factors (Hubbard, Jones & Landau 1968). Also, the experiments described here were performed at body temperature and we investigated the

effects of IgM antibodies; maybe we would obtain different results at room temperature or with antibodies of IgG type. These may more easily reach nerve terminals and the nodes of Ranvier would be easier accessible antigenic targets. Perhaps only the high affinity anti-ganglioside antibodies can bind to the presynaptic membranes and nodes of Ranvier and, therefore, exert their neurophysiological effects. By contrast, the presence of anti-ganglioside antibodies might be a secondary phenomenon to the destruction of neurons and following exposure of neuronal antigens to the immune system. May be humoral immunity to other antigens, or cell-mediated immunity and immune complexes are of pathogenic significance in autoimmune polyneuropathies.

In conclusion, although our observations cannot support the hypothesis that anti-GQ1b and/or anti-GM1 anti-ganglioside antibodies have a primary effect on neuromuscular junction and subsequently play a key pathogenic role in some neuromuscular disorders, additional studies may provide clues to the events that stimulate these antibodies and may further clarify their pathophysiological relevance and their potential role in neuromuscular transmission.

PART THREE - GENERAL DISCUSSION

Summary

This study has examined the binding, complement activating potential and acute physiological effects of neuropathy-associated human anti-ganglioside antibodies. We tested *in vitro* a range of human anti-ganglioside antisera and monoclonal antibodies with high titers of IgM or IgG antibodies to GM1 and GQ1b (and structurally related) gangliosides. Sera were obtained from nine different patients with multifocal motor neuropathy, Guillain-Barré syndrome or Miller Fisher syndrome. Monoclonal antibodies were cloned from peripheral nerve lymphocytes of three different patients with anti-GM1 associated neuropathies. No abnormal physiological effects were observed on nerve conduction in isolated mouse sciatic or rabbit sural nerve organ bath preparations. Neither the anti-ganglioside sera nor the monoclonal antibodies produced acute conduction block; even with the presence of activated complement products nerve conduction remained unchanged. Moreover, anti-ganglioside antibodies were without effect on muscle twitch tension recorded from mouse phrenic nerve-hemidiaphragm preparation. However, quantitative immunohistochemical analysis of mouse sciatic nerve teased fibres exposed to anti-ganglioside antibodies, revealed a significant increase in IgM (immunoglobulin M) and complement deposition at nodes of Ranvier. These data indicate that anti-ganglioside antibodies cannot induce any overt physiological injury on nerve conduction *in vitro*, despite that antibody and complement products can deposite at a significant proportion of nodes of Ranvier.

1. Autoimmunity in peripheral nervous system disorders: a brief introduction

The pathogenic role of autoantibodies in causing peripheral neuropathies and neuromuscular diseases is well established in some disorders (Dalakas 1995; Vincent et al. 1999). The classical antibody-mediated neurological disease is myasthenia gravis. In this disease, circulating autoantibodies to acetylcholine receptors are found in more than 80% of patients and their pathogenicity has been demonstrated by "passive transfer" of the disease into experimental animals, resulting in impairment of neuromuscular transmission postsynaptically with weakness and loss of muscle acetylcholine receptors (Toyka et al. 1977). Lambert-Eaton myasthenic syndrome is another disorder associated with antibodies to voltage-gated calcium channels (Vincent, Lang & Newsom-Davis 1989) and, also, antibodies to voltage-gated potassium channels have been demonstrated to be of pathogenic importance in acquired neuromyotonia (Isaacs' syndrome) (Newsom-Davis & Mills 1993; Vincent 2000).

Much of the research efforts over the past ten years have focused on lipid and glycoconjugate antigens and substantial bibliographic evidence implicates them as major targets for antibody responses in some peripheral neuropathy syndromes with suspected autoimmune basis. The autoimmune pathogenesis seems to be well characterised in the IgM-associated polyneuropathy with anti-MAG antibodies where the pathological process (Vital et al. 1989; Steck & Kappos 1994; Trojaborg et al. 1995) can be transferred to experimental animals causing demyelination and conduction block (Hays et al. 1987; Willison et al. 1988; Trojaborg et al. 1989; Tatum 1993). On the other hand, although strong circumstantial evidence support the link between antibodies to gangliosides and autoimmune responses in some peripheral nerve disorders, the precise role which these antibodies play in the disease process is not understood. It remains to be elucidated to what extent these antibodies are responsible for autoimmune peripheral

neuropathies, including Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS). Moreover, important aspects of polyneuropathies associated with immunoglobulins that bind to gangliosides remain unclear. Can they initiate the disease process and are they indicators of primary immune attack, or are the antibody responses merely secondary to neural tissue damage by other mechanism(s)?

2. Autoimmune responses in peripheral nerve gangliosides

It has been known for more than two decades that peripheral nerve contains gangliosides (Svennerholm 1963; Svennerholm 1994; Svennerholm et al. 1994). Gangliosides are complex glycosphingolipids which contain at least one sialic acid residue (N-acetylneuraminic acid) attached to the internal and/or the terminal galactose of an oligosaccharide core composed for up to four sugars. Gangliosides reside in the outer layer of plasma membrane and the hydrophilic sugars are located on the outer surface of the membrane. They are linked to the cell by the hydrophobic lipid moiety ceramide, which is immersed into the membrane. There is an abundance of literature suggesting that gangliosides may play a role in membrane and cell functions. The binding of cholera toxin to GM1 ganglioside is well documented (van Heyningen et al. 1971; Cuatrecasas 1973a; Holmgren 1981) and gangliosides on nerve terminals may also act as receptors for tetanus and botulinium toxin (Fishman 1982; Willison & Kennedy 1993).

The abundance of gangliosides in the nervous system and the extracellular display of their sugars make them attractive potential antigenic targets in autoimmune neurological disorders. Many attempts have been made to link anti-ganglioside antibody responses with the pathogenesis of these disorders and to explain specific features of these diseases: whether they are entirely motor or sensory in presentation, demyelinating or axonal on electrophysiological study or pathological examination or associated with a

particular regional distribution of deficits. The subject of polyneuropathies associated with immunoglobulins that bind to gangliosides has produced considerable controversy and despite efforts, the presence of measurable antibodies to gangliosides in some peripheral neuropathy syndromes has not yet provided proof that they are pathogenic.

3. Anti-ganglioside antibodies in the present study

In this study we attempted to clarify the role of anti-ganglioside antibodies in mediating acute experimental conduction block *in vitro*; we investigated their binding and complement fixing capacity to nodes of Ranvier, in conjuction with physiological recordings from isolated mouse sciatic nerve, rabbit sural nerve and mouse phrenic nerve-hemidiaphragm preparations under a range of experimental conditions.

We first investigated the very acute physiological effects of the anti-GQ1b and structurally related disialosyl antibodies. The anti-GQ1b antibody may be of pathogenic importance in MFS. Since the first report involving the anti-GQ1b antibody in the pathogenesis of MFS (Chiba et al. 1992), several studies have found increased anti-GQ1b antibody titres in MFS with the immunoglobulin being of IgG class rather than IgM. The antibody titre decreases with the clinical course of the illness (Chiba et al. 1993; Yuki et al. 1993b; Willison et al. 1993c; Jacobs et al. 1995; Yuki 1996a; Carpo et al. 1998; Suzuki et al. 1998; Schwerer et al. 1999). Electrophysiological evidence has revealed that anti-GQ1b positive sera from patients with Miller-Fisher syndrome induce severe defects in acetylcholine release from motor nerve terminals and lead to block neuromuscular transmission on mouse phrenic nerve-hemidiaphragm preparation. This suggests that the GQ1b antibody, in the presence of activated complement, plays the principal role in MFS motor symptomatology (Roberts et al. 1994; Plomp et al. 1999). However, contrary to the generally accepted view that the GQ1b antibody plays a crucial

role in MFS, another recent study demonstrated that serum without any detectable ganglioside antibodies was as effective as serum IgG containing GQ1b antibody in blocking evoked quantal release of acetylcholine in mouse phrenic nerve-hemidiaphragm preparation (Buchwald et al. 1998b).

Initially, we examined the electrophysiological effects of this antibody on ensheathed mouse sciatic nerve preparation at room temperature and we did not note any abnormality. Having observed overt differences of saxitoxin action in ensheathed and desheathed nerves, we assumed that the sheath of the nerve acted as a significant diffusion barrier for antibody penetration and all subsequent electrophysiological recordings were performed on desheathed nerve preparations. We tested both IgM and IgG antibody from four different patients and we did not observe any defect on nerve conduction at room temperature. Also, the same antibody in the presence of active complement did not have any abnormal effect on other two different preparations: on rabbit desheathed sciatic nerve at room temperature and on twitch tension in mouse phrenic nerve-hemidiaphragm preparation at physiological temperature. However, the anti-GQ1b IgM antibody does bind to a significant proportion peripheral nerve nodes of Ranvier; two out of three anti-GQ1b antibodies from different patients demonstrated significant ability of binding to the nodes of Ranvier in sciatic nerve teased fibres preparations after overnight incubation at 37^{o}C.

In this study, the anti-GM1 antibody was also included. The role of this antibody in autoimmune peripheral neuropathies is controversial. Previous electrophysiological and immunohistological studies have shown divergent findings. Intaneural injection of antibody into rat sciatic nerve produced acute conduction block with significant fall in the amplitude ratio (Santoro et al. 1992) and rabbits immunised with GM1 ganglioside revealed electrophysiological abnormalities, mild axonal degeneration and

immunoglobulin deposits at the nodes of Ranvier (Thomas et al. 1991). Uncini et al. showed that sera from patients with MMN containing anti-GM1 antibodies produced conduction block and demyelination when injected into rat tibial nerve. However, in the same study, injection of sera with high titres of anti-GM1 antibodies obtained from patients with spinal muscular atrophy did not produce block and pathological analysis did not indicate demyelination (Uncini et al. 1993). On the contrary, other electrophysiological and morphological studies have revealed different findings. Harvey et al. (Harvey et al. 1995) injected intraneurally into rat tibial nerve immunoglobulin fractions with high titres of anti-GM1 IgG or IgM anti-ganglioside antibodies from patients with GBS or MMN; the antibody did not induce acute conduction block, and histological examination of the injected nerves did not show demyelination despite the binding of the anti-GM1 antibody to the nodes of Ranvier. Similarly, previous studies using antisera against GM1 ganglioside failed to induce demyelination after injection into rat sciatic nerves, in contrast to the marked demyelination produced by anti-galactocerebroside and anti-P_0 antisera (Hughes et al. 1985).

In vitro studies on the effects of anti-GM1 antisera have also been conflicting. Arasaki et al. reported a minor reduction in the amplitude of compound nerve action potential in desheathed rat sciatic nerve (Arasaki et al. 1993) and more recently they found conduction block with 4 out 14 examined anti-ganglioside sera (Arasaki et al. 1998). Takigawa et al. (Takigawa et al. 1995) found that anti-GM1 antibody increased potassium current elicited by step depolarisation, and in the presence of active complement blocked sodium channels irreversibly. This finding supported the hypothesis that Na^+ channels may be targets of immune attack in some neurological diseases (Waxman 1995). However, this hypothesis has recently been disputed; the application of the same sera with high titres of anti-GM1 antibody did not cause

conduction block or block sodium channels, suggesting that it is insufficient to conclude that anti-GM1 antibody alone play a pathogenic role in some neuropathies (Hirota et al. 1997). Also, another *in vitro* study applying MMN sera to the mouse phrenic nerve-hemidiaphragm preparation found dysfunction at motor nerve terminals with anti-GM1 positive and anti-GM1 negative sera (Roberts et al. 1995).

We investigated the acute physiological effects of IgM and IgG anti-GM1 sera and IgM monoclonal antibodies in two different preparations. Also, we examined the nodal binding ability of anti-GM1 IgM sera and monoclonal antibodies using immunofluorescence techniques. Exposure for up to four hours of the desheathed sciatic nerve to either IgM monoclonal anti-ganglioside antibody or sera of IgM and IgG classes did not induce any significant alteration in the amplitude, latency, rise time, and threshold of the obtained compound nerve action potentials. The presence of complement did not change the nerve conduction. In contrast, the immunofluorescence studies demonstrated significant reactivity of the antibody with nodes of Ranvier in mouse sciatic nerve teased fibres preparations. The results from two anti-GM1 antibodies were impressive; they showed intense staining at the nodes of Ranvier in high percentages and characteristic ability to fix complement, especially at 37°C. But, surprisingly, these two antibodies did not have any positive physiological effect on nerve conduction when we test them again in sciatic nerve preparation at 37°C. Moreover, neither of these two antibodies, in the presence of complement, demonstrated any acute harmful effect in muscle twitch tension through indirect stimulation in mouse phrenic nerve-hemidiaphragm preparation.

4. Anti-ganglioside antibodies in peripheral neurological disorders: a mystery?

Although our electrophysiological findings do not support the pathogenic role of anti-ganglioside antibodies, the immunofluorescence studies have clearly shown that the node of Ranvier is capable of binding antibodies and fixing complement. Our findings are entirely consistent with those reported by Harvey et al. (Harvey et al. 1995) and Hirota et al. (Hirota et al. 1997) and not with those reported by others (Arasaki et al. 1993; Takigawa et al. 1995). But the controversy still exists. Do the anti-ganglioside antibodies produce conduction block *in vitro* or not? And why the experimental studies have shown so great diversity? And more importantly, are the anti-ganglioside antibodies of primary pathogenic importance in autoimmune peripheral neuropathies or does their existence simply signify a nerve tissue breakdown epiphenomenon? Do they cause the human disease or are other as yet unidentified factors responsible for the disease process?

4a. Variability in experimental studies: some considerations

The divergent findings from the *in vitro* and *in vivo* experimental studies may have many explanations. Technical factors were of great importance in our studies. In particular, it is well established the effect of osmotic pressure changes upon transmitter release from mammalian motor nerve terminals. Hyperosmotic solutions initially increase miniature end-plate potentials transmitter release from motor nerve terminals and then block neuromuscular transmission (Hubbard et al. 1968). Also, pH and osmotic changes can significantly influence nerve impulse propagation in isolated nerve *in vitro* (Orchardson 1978; Minwegen & Friede 1984). We noticed profoundly negative effects on electrophysiological parameters following alterations in bath pH, osmolarity, and ionic strength of incubation solutions and took great care to control these variables. Early in

the course of this work, a significant amount of preliminary experimental data were collected in order to control these technical factors. It is possible that technical and operator dependent factors may be contributing to discrepancies between some of the previously published data.

The route of access by which antibody penetrates nerve is another important factor in these types of experiments. Organ bath preparations may have advantages over *in vivo* models in this respect since direct application of a fixed antibody concentration to intact or desheathed preparations can be studied. Another advantage of organ bath physiology is that ionic and buffering conditions, electrode placement and stimulation/recording conditions can be carefully controlled under direct visualisation. *In vivo* studies of a disease induced in an experimental animal could provide evidence that a disorder is mediated by circulating factors.

Intraneural injection of patients' sera or various solutions has been widely used in research studies in order to examine various neuropathologic and demyelinating effects. Intraneural injection has been used to examine the pathogenic role of sera from patients with anti-MAG (Hays et al. 1987; Willison et al. 1988; Trojaborg et al. 1989) or anti-sulfatide antibodies (Quattrini et al. 1992), and also the *in vivo* demyelination and nerve conduction block induced by anti-galactocerebroside antibodies (Saida et al. 1979; Sumner et al. 1982). Intraneural injection studies were employed to study the effects of anti-ganglioside antibodies and the results were contradictory, as it has already been described (Santoro et al. 1992; Uncini et al. 1993; Harvey et al. 1995; Wirguin et al. 1995). However, passive transfer by intraneural injection of antibodies is a highly artificial and poorly controllable experimental model (Low et al. 1982; Oomes et al. 1991). Multiple technical factors associated with the intraneural injection technique itself have to be taken into account. Increasing needle size, rapid injection, and large

volumes of testing compound may contribute to or even cause pathological changes of the injected nerve. Also, extraneous movement of the needle or the animal may increase the rate of axonal degeneration and segmental demyelination, as interesting studies have shown (Dyck et al. 1982). On the other hand systemic injection of antibody may require large amounts of antibodies to bypass the blood nerve barrier which is relatively impermeable to some macromolecules such as IgM antibody.

However, the experimental models are different and certainly not identical to human disease. The experiments performed in this study have examined only the very acute effects of exposure to anti-ganglioside antibody. The duration of antibody exposure to the target sites does not correspond to the human disease and binding to the target appeared to be more efficient after overnight incubation which may have limited the potential of the *in vitro* approach. Moreover, the pathophysiological environment of the human disease is very different. In normal nerve, the blood-nerve barrier prevents the free passage of immunoglobulins from the blood into the endoneurial space. The blood-nerve barrier formed by the endoneural capillaries with their tight junctions and the perineurium protects the nerve by its relative impermeability. In experimental models the blood-nerve barrier either does not exist, as in peripheral nerve cultures *in vitro*, or is bypassed, as in intraneural injection. Maybe, some degree of dysfunction in blood-nerve barrier *in vivo* seems to be a prerequisite for anti-myelin antibody to penetrate into peripheral nerve environment, although some gangliosides, in particular GM1, are enriched in the membrane of the motor nerve terminals which lack the blood-nerve barrier and so anti-neural antibodies can easily gain access. How this may be triggered in these disorders is unknown, and whether the anti-ganglioside antibody responses play a predominant or secondary role in the pathogenesis of demyelination remains uncertain. In human disease an impaired blood-nerve barrier would permit the chronic exposure of

nerve fibres to serum constituents, while in the experimental model nerve fibres are exposed to antibody activity for a brief period. Patients with some chronic autoimmune neuropathies suffer for years, or even decades, from the disease. Thus, to induce conduction block or other electrophysiological abnormalities acutely in up to four hours is a different model.

Also, the affinity of the antibody and their ability to fix complement is likely to be different in experimental models in comparison to human disease. Our immunohistochemistry studies of desheathed sciatic nerves exposed to anti-ganglioside antibodies demonstrated antibody deposition and complement activation in the majority of nodes of Ranvier in some but not all preparations. The apparent discrepancies may be explained by the fact that different or the same clinical syndromes affected different patients because of differences in antibody affinity, and/or ability to fix complement. Additionally, circulating factors other than, or in addition to, anti-ganglioside antibodies may be present and play an important role in patients with autoimmune peripheral neuropathies. Maybe these still unidentified factors contribute to the disease process or play an important pathogenic role and then the detected anti-ganglioside antibody is simply a consequence of a still unknown mechanism.

Perhaps we would detect electrophysiological failure of nodal and neuromuscular conduction if we were able to conduct longer-term recordings under experimental circumstances in which nodes and neuromuscular junctions were exposed to prolonged pro-inflammatory insults. Unfortunately, it was not feasible because our experience has shown that a good and well-controlled preparation can be alive and reliable for not more than 5-6 hours.

4b. Molecular mimicry in antibody-mediated peripheral neuropathies

Recent neurophysiological and pathological studies have led to a reclassification of GBS into acute inflammatory demyelinating poluneuropathy (AIDP), acute motor axonal neuropathy (AMAN), and acute motor sensory axonal neuropathy (AMSAN) (McKhann et al. 1993; Powel & Myers 1996; Hahn 1998). Significant antecedent infections include *Campylobacter jejuni* (4-66%), *Cytomegalovirus (CMV)* (5-15%), *Epstein-Barr virus* (2-10%), and *Mycoplasma pneumoniae* (1-5%). These infections are not obligatorily associated with any clinical subtype, although severe axonal degeneration follows *Campylobacter jejuni* infection and severe sensory deficits is more common following *CMV*.

Strong evidence supports the important role of antibodies to gangliosides in GBS pathogenesis. Ganglioside-like epitopes exist in the lipopolysaccharide bacterial wall of *Campylobacter jejuni*. In particular, the *Campylobacter jejuni* lipopolysaccharide fraction contains side chains with the same structure as gangliosides GM1, GQ1b, GD1a, GD3 and GT1a, and these findings support the hypothesis of molecular mimicry between gangliosides and surface epitopes on *Campylobacter jejuni* strains from patients with GBS or MFS (Yuki et al. 1993c; Aspinall et al. 1994; Jacobs et al. 1995; Oomes et al. 1995; Yuki 1997; Jacobs et al. 1997a; Hao et al. 1998). Also, antibodies to GM1 ganglioside are frequent in cases with severe axonal degeneration following *Campylobacter jejuni* infection, although not all *Campylobacter jejuni* associated GBS patients have anti-GM1 antibodies (Yuki et al. 1990; Rees et al. 1995a; Jacobs et al. 1996; Feasby & Hughes 1998). Additionally, only half of patients with GBS and anti-GM1 antibodies have had preceding enteritis suggesting a recent *Campylobacter jejuni* infection. Recent studies have found that sera from GBS patients without evidence of *Campylobacter jejuni* infection also frequently reacted with GM1 ganglioside (Hao et al.

1998). Studies for the presence of cross-reactive antigens in other than *Campylobacter jejuni* infectious agents would be an interesting subject. A very recent report suggests the presence of a GM1-like structure on the surface of a particular strain of *Haemophilus influenzae* isolated from a patient with axonal GBS associated with anti-GM1 antibody after *Haemophilus influenzae* infection (Mori et al. 1999b). Attempts to match the subtypes of GBS to the fine specificity of anti-ganglioside antibodies continue but, so far have not fully explained the pathogenesis. Some studies underscore the great diversity of lipopolysaccharide structures in *Campylobacter jejuni* (Penner & Aspinall 1997), and the substantial heterogeneity among *Campylobacter jejuni* strains in their expression of GM1-like epitopes and among the fine specificities of different neuropathy-associated anti-GM1 antibodies (Prendergast, Willison & Moran 1999).

Although *Campylobacter jejuni* strains from GBS patients can induce antibodies that cross-react with gangliosides and *Campylobacter jejuni* lipopolysaccharides, the question whether these antibodies are pathogenic remains unanswered. Even though these antibodies can bind to peripheral nerve structures, as others and we have shown, again this crucial question remains unanswered. In a recent study, all rabbits immunised with *Campylobacter jejuni* lipopolysaccharides from GBS-associated strains that contain a GM1-like epitope produced high cross-reactive antibodies, but none of the animals developed any neurological sign of the disease (Ang et al. 2000). Thus, the hypothesis of molecular mimicry of *Campylobacter jejuni* lipopolysaccharides with gangliosides has been confirmed from many results, but whether the anti-ganglioside antibodies are of pathogenic value in the development of neurological disorders remains to be determined.

4c. Immune responses directed against sodium channels?

The interesting hypothesis that anti-ganglioside antibodies may induce immune-mediated changes and may block ion channels is another matter of controversy and needs further investigation. Maybe the diversity of Na^+ channels provide a basis for selective immune attack in different types of axons: different types of sodium channels in different types of sensory and motor axons may explain the selective involvement for large versus small sensory fibres or pure motor or myelin involvement. The striking selection of motor over sensory axons in AMAN (McKhann et al. 1993) implies that motor axons may be antigenically distinct targets (Hafer-Macko et al. 1996). Motor axons are known to have distinctive electrophysiological properties, derived from a distinctive assembly of ion channels. Since antibodies to ganglioside GM1 are more common in patients with AMAN, this ganglioside may be considered to be the most likely target for the autoantibodies. Although high IgM anti-GD1a antibody titres may be found in several motor syndromes including pure motor demyelinating neuropathy associated with IgM MGUS (Carpo et al. 1996), most recently an association between IgG antibodies to GD1a ganglioside and predominantly motor axonal GBS was suggested (Yuki et al. 1992b; Lugaresi et al. 1997; Ang et al. 1999; Ho et al. 1999; Kaida et al. 2000). The hypothesis that the anti-ganglioside antibodies may block ion channels cannot be supported from our electrophysiological data. Moreover this hypothesis cannot apply to the anti-GQ1b antibody-mediated attack in MFS, since important studies have emphasised that sodium channels are practically absent from the terminal part of the presynaptic endings in mouse motor nerve terminals (Brigant & Mallart 1982).

4d. T cell responses in autoimmune peripheral neuropathies

Cellular phenomena may involve in the pathogenesis of autoimmune human neuropathies. Migration of activated T cells across the blood-nerve barrier, recruitment of macrophages into the endoneurium, and macrophage-mediated demyelination may are important factors in the pathophysiology of these diseases. On the other hand, humoral factors including antibodies and complement may enhance the peripheral nerve damage induced by T cells. The autoimmune hypothesis in demyelinating GBS is based largely on the close clinical and histopathologic similarities between GBS and experimental allergic neuritis (EAN) (Vriesendrop 1997) and the continuing failure of epidemiological studies to associate a single infectious agent with the disease. Furthermore, extensive studies over many years have failed to associate autoimmune responses with a defined peripheral myelin autoantigen in this disease. It implies that not one but a number of peripheral nerve myelin autoantigens may be implicated in the pathogenesis of GBS, if indeed this disease does involve an autoimmune basis. T cells responses to any of myelin proteins, P_2, P_0 and PMP_{22} may be responsible for some or perhaps all forms of GBS, but identification of the specificity of these T cells is still in a preliminary stage (Hughes et al. 1999). T cell-mediated cytotoxic attack against ganglion neurons in patients with acute sensory neuronopathy has been proposed (Minwegen & Friede 1984).

The variation in disease severity of both primary demyelinating GBS and primary axonal GBS is likely to be dependent on the intensity of the immune response provoked by the preceding event (Griffin et al. 1996b). Transfer of antigen-specific T cells from EAN rats to naive rats also induces similar disease, and the clinical and histological severity is dependent on the antigen dose in EAN induced by immunisation (Hahn et al. 1988). Transfer a low number of cells causes mild disease that is predominantly

characterised by demyelination, whereas transfer of a high number of cells causes severe paralysis and widespread axonal degeneration (Hahn et al. 1988). Although good recovery is described in Chinese children with axonal GBS (AMAN), several clinical studies from other geographically different areas emphasised the correlation of antecedent *Campylobacter jejuni* infection with axonal GBS and poor recovery (Vriesendrop et al. 1993; Hartung et al. 1995; Visser et al. 1995; Rees et al. 1995b). This may reflect axon-specific anti-ganglioside antibody-mediated primary axonal injury in some patients.

5. Future research studies and conclusions

The anti-ganglioside antibodies are likely to be pathogenic. However, we failed to prove that in our electrophysiological study, despite the immunohistological results of antibody binding and complement activation at the nodes of Ranvier in up to 90% of nodes in some preparations. Our data indicate that the node of Ranvier is relatively resistant to acute anti-ganglioside antibody-mediated damage. These data also suggest that anti-ganglioside antibodies are unlikely to have major pharmacological effects on nodal function.

So, the relationship between autoimmune peripheral neuropathy syndromes and anti-ganglioside antibodies remains controversial. In particular the role of anti-GM1 antibodies in the pathogenesis of multifocal motor neuropathy (MMN) is still debated. These antibodies are not specific for MMN or even for lower motor neuron syndromes. High titres of antibodies to GM1 ganglioside are present in only about 50% of patients with MMN and the absence of anti-GM1 antibodies does not rule out the diagnosis of MMN if the clinical and electrophysiological findings are characteristic. Also, anti-ganglioside antibodies are found in normal population even at low titres, in neurological

disorders other than lower motor neuron syndromes and also in a variety of non-neurological diseases in which there is evidence for an autoimmune pathogenic mechanism. Thus anti-ganglioside antibodies have been reported in patients with SLE, rheumatoid arthritis, myasthenia gravis, polymyositis, and cervical spondylosis (Sadiq et al. 1990; Lamb & Patten 1991; Willison et al. 1993a; Taylor et al. 1996).

The presence of anti-ganglioside antibodies might be a secondary phenomenon to destruction of neurons and following exposure of neuronal antigens to the immune system. Moreover, the fact that antibodies reactive with gangliosides have been detected in some, but not all patients with autoimmune peripheral polyneuropathies makes it likely that humoral immunity to other antigens, or cell-mediated immunity and immune complexes are of pathogenic significance. This view raises a dilemma. Taking as an example the neuropathies with IgM monoclonal gammopathy, approximately half show binding to MAG and the others do not. Does this mean that MAG binding is an epiphenomenon and is extraneous to the pathogenesis of neuropathy? Maybe there are strong arguments on both sides of this question.

The overriding challenge for the future is to determine the role of anti-ganglioside antibodies in the underlying pathology of autoimmune human neuropathies and also to further investigate the precise site(s) of the responsible epitopes within the peripheral nervous system. Detailed clinical, neurophysiological and pathological studies are required to explain how antibody binding might lead to the various symptoms of patients with these disorders. For example, biopsy samples of motor point from patients with MFS might determine the anti-GQ1b antibody and complement binding to neuromuscular junctions and also the structural disruption of the integrity of neuromuscular junction in MFS patients. Also, future studies on the fine specificity of

anti-ganglioside antibodies in relationship to clinical features may explain the heterogeneity in the clinical manifestations of these disorders.

The role of T cell and its possible implication in the pathogenesis of GBS and related disorders is another opening field for investigation. Activated T cells that cross the blood-nerve barrier may recognize an antigen in the endoneurial space and produce cytokines and chemokines which open the blood-nerve barrier and allow the egress of antibodies. Then, antibodies can recognize cell surface molecules and activate the complement cascade or opsonize Schwann cells or the axolemma for the attachment of macrophages which then lead to the cascade of pathological events in AIDP and AMAN.

On the other hand, the report by Kusunoki et al. has established the first animal model for anti-ganglioside antibody-mediated neuropathy. This study provides clear and specific evidence that GD1b antibodies can mediate injury to sensory neurons in dorsal root ganglia and cause sensory ataxic neuropathy in rabbits (Kusunoki et al. 1996b). The existence of a reproducible specific animal model that could be studied in detail would give a new dimension in understanding basic aspects of *Campylobacter jejuni*-induced GBS. An animal disease model would be useful to broaden our knowledge about the implicated immune mechanisms and further explain differences between axonal and demyelinating GBS, antibody specificity, location of antigenic epitopes, location of complement deposition and cell infiltration. An animal would contribute to the ability to investigate in more detail other important aspects including electrophysiological effects, clinical illness and recovery. Overall, it may offer an opportunity to explore future therapies that remove or inactivate the pathogenic agent that causes human peripheral neuropathies.

In conclusion, the occurrence of antibodies to gangliosides is well recognised in various neurological diseases with a suspected autoimmune basis. However, neither the

clinical relevance, nor the mechanism which is responsible for this immune response has been fully elucidated. Further studies are needed to determine in more detail the antibody characteristics, the epitope specificity, and the antigen accessibility. Moreover, animal model studies have to be performed to understand the underling mechanism and answer the crucial question: are anti-ganglioside antibodies of diagnostic and clinical significance?

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