Chapter 1  Literature Review

1.1 Components and structure of peripheral nerve

In peripheral nerves the peripheral processes of neurons are formed into bundles by connective tissue (Kiernan and Barr, 2005a). The connective tissue is formed by fibroblasts and lies between axons as the endoneurium, as the wrapping around bundles of fibers - the perineurium, and surrounds the entire nerve as the epineurium. Fibrocytes, macrophages and mast cells are present in the endoneurium. Nerves are richly supplied by intraneural blood vessels, which form numerous anastomoses. Arteries pass into the epineurium, form arteriolar networks in the perineurium and give off capillaries to the endoenurium. Schwann cells (SCs) are able to divide in response to injury and become phagocytic. Injury to peripheral axons may be followed by regrowth in which axon sprouts are able to grow back through the connective tissue skeleton of the nerve.

1.1.1 Axon

Axons, the unique extensions from the neuronal cell body, are long cellular processes that make up the nerve (Kiernan and Barr, 2005b). The axon contains cytoplasm, microtubules and neurofilaments but no Nissl substance. Therefore, there is little ability in the axon to synthesize protein and the requirement for protein substrate must be met by axoplasmic transport from the cell body. Axons can be unmyelinated or myelinated by the SCs. The length of axons varies, ranging from a few millimeters to about a meter in human, depending on the type of neuron and the size of the species. Axons are specialized for the conduction of a particular type of electric impulse, an action potential which is a self-regenerating wave of electrical activity across the plasma membrane, outward, away from the cell body toward the axon terminus. Action potentials move rapidly, at speeds up to 100 meters per second. An action potential originates at the axonal hillock, the junction of the axon and cell body, and is actively conducted down the axon into the axon terminal, small branches of the axon that form the synapses, or connections, with other cells including other
nerve cells in the brain, spinal cord, and autonomic ganglia, and the cells of muscles and glands throughout the body (Lodish, 2000; Purves, 2004; Kiernan and Barr, 2005b).

The diameter and function of axons vary, along with impulse propagation velocity (Kiernan and Barr, 2005b). Regarding the functions of different nerve fibers, three types of peripheral nerve fibers can be distinguished: somatic motor fibers, somatic sensory fibers and autonomic fibers. Based on size, large diameter myelinated (A-alpha and A-beta), medium size myelinated (A-gamma), small diameter myelinated (A-delta) and unmyelinated (B and C) nerve fibers can be distinguished. A-alpha and A-beta nerve fibers carry motor functions, vibration sense and touch. A-gamma fibers carry motor function to muscle spindles. A-delta fibers, B-fibers and C-fibers carry temperature and pain sensation and autonomic functions (Hoitsma et al., 2004; Kiernan and Barr, 2005b). Conduction velocity in myelinated fibers is proportional to the diameter, while in unmyelinated fibers it is proportional to the square root of the diameter (Morell and Quarles, 1999).

1.1.2 Myelin

Peripheral nerve myelin (PNM) is a greatly extended and modified plasma membrane wrapped around the nerve axon in a spiral fashion which originates from and is a part of the SC. Each myelin-generating SC furnishes myelin for only one segment of any given axon. The periodic interruptions where short portions of the axon are left uncovered by myelin are the nodes of Ranvier (Morell and Quarles, 1999).

1.1.2.1 Characteristics of myelin

Myelin facilitates conduction. In myelinated axons, the excitable axonal membrane is exposed to the extracellular space only at the nodes of Ranvier where voltage-sensitive sodium (Na\(^+\)) channels are clustered at high density (Waxman and Ritchie, 1993). Active excitation of the axon membrane jumps from node to node (so-called saltatory conduction) because the low capacitance of the myelin results in
spreading of depolarization at an increased speed. Furthermore, $\text{Na}^+$ influx into the myelinated fibers during conduction is much less than in unmyelinated fibers because only the nodes of Ranvier are excited. Therefore, myelin facilitates conduction while conserving energy (Morell and Quarles, 1999).

Myelin has a characteristic ultrastructure. Myelin, as well as many of its morphological features, such as nodes of Ranvier and Schmidt-Lantermann clefts, can be seen readily with light microscopy (LM). When examined by polarized light and low-angle X-ray diffraction myelin exhibits a protein-lipid-protein-lipid-protein structure. Electro-microscopic (EM) studies visualize myelin as a series of protein layers appearing as alternating dark and less dark lines separated by lipid hydrocarbon chains which appear as unstained zones. Two adjacent segments of myelin on one axon are separated by a node of Ranvier. At the paranodal region and the Schmidt-Lantermann clefts, the cytoplasmic surfaces of myelin are not compacted and SCs or glial cell cytoplasm is included within the sheath (Hirano and Dembitzer, 1967).

Myelin is an extension of a cell membrane. Before myelination, the axon lies in an invagination of the SC. The plasmalemma of the cell then surrounds the axon and joins to form a double-membrane structure (mesaxon) that communicates with the cell surface and elongates around the axon in a spiral fashion. The cytoplasmic surfaces condense into a compact myelin sheath and form the major dense line. The two external surfaces form the myelin intraperiod line (Norton, 1977).

Myelin can be isolated in high yield and purity by conventional methods of subcellular fractionation. Myelin peels off the axons and reforms in vesicles of the size range of nuclei and mitochondria when the nerve tissue is homogenized in media of low ionic strength. These myelin vesicles have the lowest intrinsic density of any membrane fraction of the nervous system due to their high lipid content. Procedures for isolation of myelin take advantage of both the large vesicle size and the low density (Norton, 1984). Demonstration of purity includes EM appearance, characteristic markers and markers of contaminations.
1.1.2.2 Composition of myelin

Myelin consists of water, lipid and protein. One quarter of the dry weight of peripheral myelin is protein. Lipid make up about 76% of total nerve dry weight (Hartung and Kieseier, 1999). Carbohydrates are found conjugated in either glycolipids or glycoproteins. The Forssman antigen, also known as globopentosylceramide, is a ceramide containing five carbohydrate residues which is found abundantly in infectious agents as well as higher organisms. There is evidence that anti-Forssman antibodies are not consistently present in humans and that when generated, may contribute to the pathogenesis of the Guillain-Barré Syndrome (GBS) by binding to glycolipid components of PNM (Lowe and Marth, 1999).

I. Myelin proteins
i. Peripheral myelin protein zero (P0)

P0 is a 30-kilo dalton (kDa) glycoprotein that accounts for more than half of the total protein in compact peripheral nervous system (PNS) myelin and is confined to PNS myelin. Its amino-acid sequence is highly conserved across species and it is considered to have an important role in forming and maintaining the structure of compact myelin. It is organized into cytoplasmic, transmembrane and extracellular domains, the latest is responsible for the homophilic adhesion properties of the molecule and mediates cell-cell interactions (D'Urso et al., 1990). P0 protein is the major adhesive component of peripheral myelin, where it mediates self-adhesion of the SC plasma membrane and induces strong intercellular adhesion (Spiryda, 1998). The cytoplasmic domain which is positively charged also plays an important role in stabilizing the major dense line of compact PNS myelin (Lemke and Axel, 1985).

As the major protein of PNS myelin, P0 is generally reduced in a wide variety of neurological disorders with decreased myelin in peripheral nerve regardless of the cause (Quarles, 2002). In addition to some inherited and acquired human neurological diseases, P0 may also be involved in immune-mediated neuropathies, because the protein or peptides derived from it can cause experimental autoimmune neuritis (EAN), an animal model for GBS (Milner et al., 1987; Lington et al., 1992; Zhu et al., 2001). Furthermore, antibodies or T cells sensitized to P0 have been detected in
some patients with inflammatory demyelinating neuropathies (Khalili-Shirazi et al., 1993; Dahle et al., 1997; Yan et al., 2000). IgG antibodies against P0 protein from patients with chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) have been shown pathogenic to PNS myelinated nerve fibers (Yan et al., 2001).

ii. Myelin basic protein (MBP) and peripheral myelin protein 2 (P2)

MBP in PNS comprises a class of proteins ranging in molecular weight (MW) from 12 to 22 kDa. As a group, the MBPs are positively charged soluble proteins that reside in the intracellular portion of compact myelin. It has been estimated to comprise up to 15-20% of peripheral myelin protein (Lees and Brostoff, 1984). The 18-kDa component which is referred to as the P1 protein in PNS is the major MBP in central nervous system (CNS) myelin but not in PNS myelin.

P2 protein is another basic protein in PNS myelin with a MW of 17 kDa. It is largely confined to PNS myelin but small quantities are found in CNS myelin, especially myelin from the spinal cord rather than brain. It is present in some but not all myelin sheaths (Trapp et al., 1979) and is not accessible at the external surface of the SC. The amount of P2 protein is highly variable from species to species and within a species, and P2 is more prominent in the thicker myelin sheaths. P2 is unrelated in sequence to P1 but shows strong homology to a family of cytoplasmic lipid-binding proteins, whose functions appear to involve solubilization and transport of fatty acids and retinoids, suggesting that it might function similarly in myelination (Morell and Quarles, 1999). P2 is a neuritogen for EAN (Kadlubowski and Hughes, 1979; Rostami et al., 1985).

iii. Peripheral myelin protein 22 (PMP22)

PMP22, a 22 kDa transmembrane myelin glycoprotein, is synthesized by SCs and constitutes 2-10% of total PNM protein (Koehler et al., 1996). It is not present in the CNS. The function of PMP22 in SC differentiation and myelin formation remains poorly understood, but it may have a more dynamic function rather than a major structural role like P0 because it is quantitatively minor (Quarles, 2002). It may be involved in the control of the SC growth cycle (Zoidl et al., 1997), adhesion between SCs and axons (Snipes et al., 1992), intercellular signaling (Snipes et al., 1993) or the
maintenance of the structural integrity of myelin (Gabriel \textit{et al.}, 1998).

In addition to Charcot-Marie-Tooth disease type 1A and a milder hereditary neuropathy with liability to pressure palsies, PMP22 may also be important as an antigen in immune-mediated neuropathies. Immunization of Lewis rats with PMP22 causes EAN (Gabriel \textit{et al.}, 1998). Furthermore, antibodies to PMP22 have been detected in a high proportion of patients with acute and chronic inflammatory neuropathies (Gabriel \textit{et al.}, 2000; Ritz \textit{et al.}, 2000). Also, because PMP22 contains the human natural killer (HNK)-1 epitope, it could be a target in the human autoimmune demyelinating disorder associated with anti-myelin-associated glycoprotein (MAG) antibodies (Ritz \textit{et al.}, 2000).

\textbf{iv. Myelin-associated glycoprotein (MAG)}

MAG is a transmembrane glycoprotein and a member of the immunoglobulin (Ig) supergene family. It is a minor component of both CNS and PNS myelin and the polypeptide exists in two isoforms of 67 and 72 kDa. In the peripheral nerve it is located at the margins of the myelin sheaths, at paranodes and at Schmidt-Lantermann incisures, where the myelin has not been compacted. MAG shares antigenic carbohydrate epitopes with P0 and other myelin glycoproteins, with a glycoprotein on HNK cells recognized by monoclonal antibody (mAb) HNK-1, and with myelin glycolipids (McGinnis \textit{et al.}, 1988; Burger \textit{et al.}, 1990; Brouet \textit{et al.}, 1992). MAG is not essential for myelination. The studies on the knockout mice have indicated that the most important functions of MAG are different in the CNS and PNS. In the CNS its primary role appears to be in signaling from axons to oligodendrocytes to promote myelin formation and oligodendroglial health, whereas in the PNS it is essential for the signaling from SCs to axons that is needed for the normal maintenance of myelinated axons (Quarles, 2002).

MAG has been implicated more definitively in some acquired neurological disorders thought to be caused by autoimmune mechanisms. The best documented example of this is patients with a demyelinating sensorimotor neuropathy occurring in association with immunoglobulin M (IgM) gammopathy (Nobile-Orazio and Carpo, 2001; Vital, 2001). MAG has also been suggested to have a key role in the molecular
pathogenesis of multiple sclerosis (MS), but it seems unlikely that it is a primary target antigen (Andersson et al., 2002).

II. Myelin lipids

Gangliosides are sialic acid-containing glycosphingolipids highly enriched in the nervous system and are predominantly expressed on cell surface membranes (Ando, 1983). Gangliosides are embedded in neural plasma membranes to provide cell surface recognition sites with negative charges. A ganglioside molecule is composed of a hydrophilic sialosyloligosaccharide headgroup and a hydrophobic ceramide portion which consists of sphingosine and fatty acid. Gangliosides are classified according to their backbone structure consisting of neutral oligosaccharides, including galactocerebroside (Gal-C), cerebroside sulfate (Gal-S), monosialoganglioside-GM1(GM1), sialosylneolactotetraosycleramide (LM1), disialoganglioside-GD1a (GD1a), disialoganglioside-GD1b (GD1b), asialo-GM1, tetrasialoganglioside-GQ1b (GQ1b), trisialoganglioside-GT1a (GT1a), acidic glycosphingolipids sialosyl paragloboside (SPG), sialosyl lactosaminyl paragloboside (SLPG), sulphoglucoronyl paragloboside (SGPG), sulphoglucuronyl lactosaminosyl paragloboside (SGLPG) and Forssman antigen (Hughes, 1994; Svennerholm et al., 1994). Most gangliosides in the nervous system belong to the gangliotetraose series. Heterogeneity of ganglioside expression patterns in the peripheral nervous tissue has been observed; GM1 and GD1a are predominantly expressed in motor nerves and axons, GQ1b was found to be enriched in cranial nerves and GD1b is highly expressed in sensory nerves (van Sorge et al., 2004).

Gangliosides are thought to be primarily responsible for specific cellular reactions. It is now generally recognized that exogenous ligands such as bacterial toxins, antigens, calcium ions and so on, bind to specific gangliosides to induce sequential activation of membrane-associated enzymic reactions and cellular metabolism (Fishman and Brady, 1976). It has been speculated that gangliosides play a role in synaptic transmission and memory formation. They may regulate cell growth and nerve sprouting, suggesting potential therapeutic value for some neurological disorders (Ando, 1983).
1.2 Guillain-Barré syndrome (GBS)

Guillain-Barré syndrome (GBS) is a clinically defined syndrome of acute weakness of the limbs attributable to disorder of the peripheral nerves not due to systemic disease (Hughes, 1995). The syndrome was first described by Guillain, Barré and Strohl in 1916 with symmetrical, rapidly evolving flaccid paralysis and areflexia (Guillain et al., 1916). Most patients will have an acute monophasic sensory and motor polyradiculopathy reaching a peak within 4 weeks. The classic syndrome is usually due to the multifocal mononuclear cell infiltration throughout the PNS in which the distribution of inflammation corresponds to the clinical deficit. Immune responses against myelin glycolipids and proteins have been discovered which could explain the pathogenesis of some of these syndromes. In advances of a full understanding of the aetiology, empirical trials have shown that immunomodulatory treatment is helpful (Hughes and Cornblath, 2005).

1.2.1 Epidemiology and clinical features

The annual incidence of GBS is fairly uniform at between one and four cases per population of 100,000 throughout the world (Hughes et al., 1999). The disease affects males more than females (1.25:1) (Hughes and Rees, 1997). The incidence increases steadily with advancing age with a possible minor hump in the age distribution in young adults. Most cases are sporadic, but small clusters have been associated with outbreaks of bacterial enteritis caused by contaminated water (Roman, 1995), and summer epidemics occur in northern China, probably caused by Campylobacter jejuni (C. jejuni) infection (McKhann et al., 1991).

Diagnostic criteria for GBS were devised in 1978 and redefined in the light of advances in the electrophysiology of GBS. Required criteria include progressive weakness of more than two limbs, areflexia, and progression for no more than four weeks. Other causes of an acute neuropathy such as lead poisoning, vasculitis, botulism, and porphyria require exclusion. Supportive criteria include mild sensory symptoms or signs, relative symmetry of symptoms, cranial nerve involvement, autonomic dysfunction, absence of fever at onset, raised protein in the cerebrospinal
fluid (CSF) with a relatively normal cell count, and electrophysiological evidence of conduction block (Asbury and Cornblath, 1990).

Typically GBS develops over the course of a few days or, at the most, 4 weeks and reaches a plateau (Hughes, 1995). Paresthesias and numbness of the hands and feet are frequent and appear at the same time as limb weakness. The symptoms are usually approximately symmetrical and more marked at first in the lower limbs so that the paralysis appears to ascend. Pain may occur in the limb and back muscles and can be a serious problem. The advancing disease may involve the cranial nerves, especially the facial and bulbar nerves, and sometimes the nerves to the external ocular muscles (Hughes, 1995). In about 25% of cases the innervation of the respiratory muscles is so severely affected as to require the use of artificial ventilation (Hughes et al., 2004). Between 5-10% die of complications during the acute stage (Kuwabara, 2004). After a brief plateau phase, in most patients improvement begins with a gradual resolution of the paralysis that lasts from weeks to months. Many patients have persistent fatigue, 12% of patients still require aid to walk one year after the onset (Rees et al., 1998; Merkies et al., 1999) and 62% of patients still notice its effect on their or their carer’s lives three to six years later (Bernsen et al., 1999).

1.2.2 Subtypes

During recent years, it has been recognized that GBS comprises several subtypes with specific clinical, electrophysiological, and pathological features, rather than being a single disease entity (van Doorn, 2005). GBS can be subdivided into the classic acute inflammatory demyelinating polyradiculoneuropathy (AIDP), representing the great majority of cases in Europe and North America (Asbury et al., 1969; Hadden et al., 1998); acute motor axonal neuropathy (AMAN), the most prevalent form in China (McKhann et al., 1993); acute motor sensory axonal neuropathy (AMSAN) (Feasby et al., 1986; Griffin et al., 1996) and Miller Fisher syndrome (MFS) (Fisher, 1956).
1.2.2.1 Acute inflammatory demyelinating polyradiculoneuropathy (AIDP)

AIDP is the most common form of GBS in Western countries and accounts for 85-90% of the patients with GBS (Hadden et al., 1998). Electrodiagnostical and pathological studies show typical demyelination suggesting that the immune target is within the SC surface membrane or the myelin (Kuwabara, 2004). In addition to limb weakness and sensory symptoms (numbness and pain), AIDP is often associated with a variety of autonomic involvement, including fluctuating heart rate and blood pressure, gastroparesis, constipation, and urinary retention (Asahina et al., 2002). Therefore, the management of cardiovascular dysfunction is particularly important in the treatment of patients with AIDP because the dysfunction may cause sudden death. The characteristic electrophysiological abnormalities are slowing of nerve conduction velocities (CVs), prolongation of distal motor and F-wave latencies, and conduction block (Hahn, 1998).

The pathological substrate of AIDP is a multifocal inflammatory process throughout the PNS in which early lymphocytic infiltrates in spinal roots and peripheral nerves and macrophage-mediated segmental stripping of myelin are the hallmarks (Asbury et al., 1969; Prineas, 1981). Binding of complement and Ig to SC plasmalemma has been demonstrated in nerve roots from autopsy cases in the early stages of AIDP (Hafer-Macko et al., 1996b), suggesting that the immune attack is directed at an antigenic target located on the SC plasmalemma. Once the immune reactions come to a halt, repair and remyelination set in promptly, which correlates with a quick and, in most cases, complete recovery from the flaccid paralysis (Hahn, 1998). In many patients, however, particularly those with severe disease, the axons as well as the myelin sheaths are destroyed which makes the distinction between AIDP and axonal forms of GBS difficult (Hughes et al., 1999). Breakdown of axons in this setting is thought to be a secondary “bystander” event, caused possibly by intense inflammation, oedema, and swelling of nerves (Powell and Myers, 1996; Berciano et al., 1997). The degree of complicating axonal loss in AIDP is an important determinant of the speed of recovery, the lasting deficits, and the ultimate prognosis (Cornblath et al., 1988).
1.2.2.2 Acute motor axonal neuropathy (AMAN)

In China (McKhann et al., 1993; Ho et al., 1995) and Japan (Ogawara et al., 2000), a considerable number of patients with GBS have the AMAN type of GBS. The clinical and electrophysiological findings in AMAN show a purely motor disorder with a reduction in distally evoked compound muscle action potential (CMAP) amplitudes, relatively preserved motor conduction velocities (MCVs), normal or slightly prolonged distal motor and F-wave latencies, absence or paucity of temporal dispersion, and normal sensory studies (Hughes et al., 1999; Donofrio, 2003). Autonomic dysfunction is rarely observed and is mild if present. Pathological studies revealed a selective, noninflammatory, axonal degeneration of motor axons, with little evidence of demyelination (McKhann et al., 1993). The binding of immunoglobulin G (IgG) and activated complement components to the axolemma at nodes of Ranvier in large motor fibers can be detected (Hafer-Macko et al., 1996b). These appearances suggest the presence of targeting antibodies directed to axonal antigens. AMAN is often triggered by enteric infection by C. jejuni and is frequently associated with antiganglioside antibodies (GM1, GM1b, GD1a or ganglioside N-acetylgalactosaminyl GD1a (GalNAc-GD1a)) (Ogawara et al., 2000).

1.2.2.3 Acute motor sensory axonal neuropathy (AMSAN)

AMSAN is another axonal subtype of GBS with low incidence (Ogawara et al., 2000) but tends to be associated with severe illness and poor recovery (Kuwabara, 2004). Repeated electrophysiological studies early in the disease show the diminution of muscle and sensory action potentials (Feasby et al., 1986). The pathological pattern in AMSAN very closely resembles that in AMAN, including damage and degeneration of axons observed in initial disease stages, except that sensory nerves are affected in addition to motor nerves (Griffin et al., 1996).

1.2.2.4 Miller Fisher syndrome (MFS)

MFS, usually regarded as a variant of GBS, was first described by Fisher with the distinctive clinical triad of ophthalmoplegia, ataxia and areflexia (Fisher, 1956). Paralysis in MFS is restricted to certain cranial nerves, predominantly the ocular
motor (oculomotor, trochlear, and abducens) nerves. Therefore, conduction in limb motor nerves is not slowed or blocked and the striking finding is the disappearance of the sensory action potentials, which could either be due to selective demyelination of sensory nerve fibers or to a sensory axonopathy (Jamal and MacLeod, 1984; Fross and Daube, 1987). Due to the benign disease course, histopathological studies are rare, however, multifocal inflammation and segmental demyelination in full-blown MFS patients resembling AIDP have been described (Phillips et al., 1984; Dehaene et al., 1986). MFS is closely associated with antibodies against ganglioside GQ1b which most likely plays a key role in the pathogenesis of MFS (Willison and O'Hanlon, 1999).

1.2.3 Antecedent infections and molecular mimicry

Two-thirds of patients with GBS have an antecedent acute infectious illness, most commonly an upper respiratory tract infection or gastroenteritis that has resolved by the time of the onset of neurological symptoms. The interval between the preceding infection and the onset of GBS ranges from 1 to 3 weeks (mean 11 days) (Winer et al., 1988). In many patients, the pathogen responsible for the prodromal illness is unidentified, but a number of studies have shown that there is frequently serological evidence for the presence of a pathogen that could potentially be responsible for eliciting the onset of GBS (Kuwabara, 2004). The most common antecedent infection recognized before the onset of GBS is *C. jejuni* enteritis, which has been associated with electrophysiologic criteria of axonal neuropathy, antibodies to ganglioside GM1, pure motor GBS, and a less elevated CSF protein concentration (Griffin et al., 1995; Rees et al., 1995a; Hadden et al., 2001). The next most common infection preceding GBS is *Cytomegalovirus (CMV)* (Winer et al., 1988; Jacobs et al., 1998), which has been associated with prominent sensory involvement and severe disease (Visser et al., 1996) and raised concentrations of molecules associated with T cell activation and migration (Hadden et al., 2001). Other infectious pathogens preceding GBS identified include *Epstein-Barr virus (EBV)*, *Mycoplasma pneumoniae, Haemophilus influenzae (H. influenzae)*, and *Varicella-zoster virus (VZV)* (Jacobs et al., 1998; Ogawara et al., 2000). Many other identified pathogens such as
Human immunodeficiency virus (HIV) have been reported as antecedent infections of GBS but there is a lack of statistically valid evidence. Detailed studies of the molecular structure of antecedent pathogens, particular C. jejuni, are now providing new insights into putative immunological mechanisms of nerve damage in patients with GBS (Kuwabara, 2004).

The cause of GBS is still under investigation. The typical features of GBS histopathology, inflammation and demyelination of peripheral nerves, are considered to reflect an autoimmune attack directed against antigens of the peripheral nerves, such as the myelin-forming SC, resulting in damage to myelin sheaths, and/or the neuronal axon, leading to axonal degeneration (Schwerer, 2002; Hughes et al., 2004). The immune response is triggered by a preceding bacterial or viral infection. The triggering mechanism is not understood but might be the consequence of molecular mimicry. Molecular mimicry is one mechanism by which infectious agents (or other exogenous substances) may trigger an immune response against autoantigens (Albert and Inman, 1999). According to this hypothesis a susceptible host acquires an infection with an agent that has antigens that are immunologically similar to the host antigens but differ sufficiently to induce an immune response when presented to T cells. As a result, the tolerance to autoantigens breaks down, and the pathogen-specific immune response that is generated cross-reacts with host structures to cause tissue damage and disease. Molecular mimicry has been proposed as a pathogenic link between infections and GBS in that such infections drive a humoral immune response cross reactive with neural antigens (Hartung et al., 2001). In GBS, at least in some of its forms, shared epitopes between the bacterial species (such as C. jejuni, H. influenzae, or CMV) and nerve fibers have been identified as targets for aberrant cross-reactive immune responses (Yuki, 2001a).

1.2.4 Treatments

The key issues in effective treatment of GBS are excellent intensive care unit management including respiratory care, monitoring and early treatment of autonomic dysfunction, anticoagulation, and good nursing care (Hahn, 1998). Two disease-altering therapies, plasma exchange (PE) and high-dose intravenous immunoglobulin
(IVIg), have shown efficacy in speeding motor recovery and are indicated for patients who are unable to walk independently (Hartung et al., 2001; Kuwabara, 2004). Improvement following treatment can be determined at various levels, including impairment, disability, handicap and quality of life. The Hughes functional grading scale is widely used to evaluate clinical disability and the functional endpoint (van Doorn, 2005).

1.2.4.1 Supportive treatment

The advent of respiratory assistance with improved care has significantly improved the outcome of patients with GBS. Care for severely affected patients is best provided in tertiary centres with intensive care facilities and a team of medical professionals familiar with the special needs of patients with GBS (Ropper, 2001). Admission to an intensive care unit and ventilatory support is needed in 33% of these patients, who will often also show haemodynamic instability and autonomic dysfunction (Hahn, 1998). Mechanical ventilation should be considered when maximal expiratory vital capacity falls to 15 mL/kg body weight. Patients with oropharyngeal weakness may require intubation earlier to prevent aspiration, but mechanical ventilation is not always required at the same time. Continuous monitoring of blood pressure and heart rate is useful to prevent death from arrhythmia and haemodynamic instability related to autonomic involvement. Hypotension is managed by intravenous volume infusion and short-acting vasopressor agents and hypertension by rapidly titratable antihypertensive medication. Prevention of thromboembolic complications such as deep vein thrombosis and pulmonary embolism with heparin has become routine for high-risk patients. Tracheostomy is performed when effective clearance of the tracheobronchial airways fails and/or prolonged mechanical ventilation is required. Pain can be properly controlled with analgesics and ameliorated by early initiation of rehabilitation techniques such as frequent passive limb movement which is also useful to prevent contractures (Ropper, 2001).
1.2.4.2 Plasma exchange (PE)

PE was the first treatment proven to be beneficial and superior to supportive treatment alone in GBS (The Guillain-Barré syndrome Study Group, 1985; McKhann et al., 1988). The Cochrane review identified six eligible trials involving 649 patients comparing PE to supportive treatment (Raphaël et al., 2001). The review showed significant benefit from PE on all the available outcome measures. In the 585 patients for whom the outcome was available, patients undergoing PE have a significantly more rapid onset of motor recovery, shortened time to recover walking with aid, lower percentage of patients requiring artificial ventilation, shorter duration of mechanical ventilation and more frequent full muscle strength recovery after 1 year. However, there were more infectious events and cardiac arrhythmias in the PE than in the control group. In this review, two trials have been designed to evaluate the appropriate number of PEs. In mild GBS two sessions of PE were significantly superior to none. In moderate and severe GBS four sessions were significantly superior to two. Further exchanges did not generate any additional benefit. PE should preferably be started seven days or less after the onset of neuropathy. Nevertheless, patients with GBS may benefit from PE up to 30 days after disease onset. Approximately 10% of the patients develop secondary worsening within 1-2 weeks after PE, in which additional treatment by PE has been shown to lead to renewed improvement (Kleyweg and van der Meché, 1991). Still, at least 20% of patients treated with PE remain unable to walk independently at six months, and the mortality of the disease (3-5%) does not seem to be affected even when applied early in the disease. In addition, this treatment is not feasible in patients with severe autonomic impairment, electrolytic unbalance, difficult venous access, and in patients admitted in intensive care units where a mobile PE apparatus is not available. These considerations as well as the high cost prompted the search for other effective treatments in this disease (Hahn, 1998; Nobile-Orazio, 2005).

1.2.4.3 High-dose intravenous immunoglobulin (IVIg)

In 1992 the Dutch reported a trial suggesting that IVIg was slightly superior to PE for patients with severe GBS within 2 weeks from the onset of their disease (van der Meché and Schmitz, 1992). The comparable efficacy of PE and IVIg was also
highlighted in a Cochrane review in which 273 patients had been treated with IVIg and 263 with PE (Hughes et al., 2004). There were no significant differences between these treatments in improvement in disability grade after 4 weeks, time to walk unaided, mortality or proportion of patients dead or unable to walk without aid after a year. However, there is no directly relevant evidence to guide treatment of mild GBS, children, MFS and other variants, and disease which presents more than 2 weeks after onset. The standard dose in the trials has been 0.4 g/kg body weight daily for 5 days. The combined use of PE followed by IVIg is no better than PE alone in one large trial (Plasma Exchange/Sandoglobulin Guillain-Barré syndrome Trial Group, 1997). The incidence of relapse after initial treatment was similar for the groups receiving IVIg and PE. There is no trial evidence concerning the efficacy of a second dose of IVIg. The practical advantage of IVIg over PE, including the fact that it can be administered in any department without transferring the patient to an appropriate unit and to patients with the above-mentioned contraindications for PE, together with the infrequent contraindications for IVIg therapy (immunoglobulin A (IgA) deficiency, renal failure, congestive heart failure or increased risk of thromboembolism) make IVIg the preferred treatment for patients with GBS (Nobile-Orazio, 2005). For reasons of convenience and safety, and particularly in children and in patients with autonomic instability, IVIg is used as a standard treatment in most centres (van Doorn, 2005).

1.2.4.4 Potentially interesting treatments

Despite PE and IVIg treatment, GBS remains a severe disease. Severe weakness, complications, and residual deficit (approximately 40%) are important reasons to search for better treatment (Kieseier and Hartung, 2003; van Doorn, 2005).

CSF filtration was recently investigated in a small prospective study of 37 GBS patients (Wollinsky et al., 2001). The repetitive removal of small volumes of CSF through a lumbar catheter followed by filtration through a millipore filter and reinfusion through the same catheter was well tolerated, found to be equally safe, and claimed to be equally effective compared with conventional PE. The rationale of this therapeutic approach rests in the concept that the nerve roots are prominently affected
in some GBS cases; therefore, filtration of CSF rather than whole plasma might be more efficient. The possible therapeutic mechanism of CSF filtration is that removal of elevated concentrations of a variety of inflammatory mediators, such as the sodium channel-blocking pentapeptide, may play a role. However, the pathology of GBS is not restricted to the CSF compartment, raising questions about the utility of this treatment (Kieseier et al., 2004).

A therapeutic benefit from interferon (IFN)-β was suggested partly because IFN-β was found to inhibit in vitro lymphocyte adhesion to recombinant vascular cell adhesion molecule (VCAM)-1 and recombinant intercellular adhesion molecule (ICAM)-1 (Créange et al., 2001). A small placebo-controlled pilot study investigated the safety of IFN-β-1a when added to IVIg. It appears that the drug was safe, but it did not contribute to any significant further improvement (Pritchard et al., 2003).

In EAN, two new cyclo-oxygenase-2 inhibitors were found to inhibit clinical and histological features of the disease, suggesting that these might be useful as additional therapeutic agents in GBS (Miyamoto et al., 2002). New approaches include the use of more effective immunomodulators such as Linomide (Bai et al., 1997a), Fusidin (Di Marco et al., 1999), Leflunomide (Korn et al., 2001), Clomipramine (Zhu et al., 1998a), and Rolipram (Zou et al., 2000) or compounds that target the transmigration of immunocompetent cells across the blood-nerve-barrier (BNB) by blocking specific chemokine receptors (Kieseier et al., 2000), adhesion molecules (AMs) (Archelos et al., 1997; Archelos et al., 1999), or proteases (Kieseier et al., 1999). However, whether these encouraging approaches, effective in the animal model, will translate into efficacious clinical therapy still needs to be evaluated.

All the therapeutic approaches mentioned are aimed to modulate the immune system to reduce the amount of damage to the myelin sheath and/or the axon. Several lines of research are trying to identify strategies that promote remyelination and axonal regeneration (Kieseier and Hartung, 2003). Neurotrophic factors, at least in theory, could represent a group of proteins that might promote regeneration. In a small, placebo-controlled pilot trial of 10 GBS patients, a potential role for subcutaneous brain-derived neurotrophic factor was examined, but no differences in
outcome between the groups were observed (Bensa et al., 2000).

Through advances in epidemiology, immunology and microbiology, our understanding of the pathophysiology of the clinical syndrome of GBS is rapidly growing. The recent recognition of different pathological patterns of disease expression, suggesting a heterogeneous pathogenesis of this disorder, may lead to a search for and evaluation of more specific causative therapy (Kieseier and Hartung, 2003; Kuwabara, 2004).
1.3 Immunopathogenesis in Guillain-Barré Syndrome

The etiology and pathogenesis of GBS remain incompletely understood. Molecular mimicry has been proposed as a pathogenic link between infections and GBS in that self-tolerance to autoantigens breaks down, resulting in pathogen-specific immune response cross reactive with neural antigens (Hartung et al., 2001; Yuki, 2001a). However, the clinical spectrum and laboratory findings of this disorder cannot be explained solely by the molecular mimicry hypothesis. Apparently, both humoral- and cellular-mediated immunity are disturbed but the relative involvement is continually debated (Willison, 2005). Abnormal cellular responses to P2 or P0 proteins have been reported in some patients with GBS (Khalili-Shirazi et al., 1992). Antibodies to PNS myelin have repeatedly been demonstrated in GBS patients’ sera (Schwerer, 2002). Understanding the immunological mechanisms underlying any pathological responses is clearly crucial to selecting the correct strategies for novel therapeutic intervention.

1.3.1 Experimental autoimmune neuritis (EAN)

In many clinical, electrophysiological, histological and immunological aspects, EAN resembles human GBS and as such has been widely used as a model to study disease mechanisms in inflammatory demyelinating diseases of the PNS (Kieseier et al., 2004).

1.3.1.1 Induction of EAN

Since Waksman and Adams (Waksman and Adams, 1955) first described EAN in 1955 by inoculating rabbits with homogenized PNS tissue, investigators have studied EAN in various species. Early experiments showed that the antigen was present in PNM and biochemical dissection showed that neuritogenic activity was most easily recovered with the readily soluble P2 protein (Kadlubowski and Hughes, 1979). Since then P2 protein has been shown to be neuritogenic in rats, mice, guinea-pigs and rabbits (Hughes et al., 1999) and in rats EAN can be actively induced by bovine P2 protein, recombinant human P2 protein, or with peptides spanning the neuritogenic epitope (amino acid sequences 53-78) of P2 protein (Mäurer and Gold,
Subsequent experiments have shown that the less easily purified major myelin protein P0 and PMP22 are also neuritogenic in rats (Milner et al., 1987; Linington et al., 1992; Gabriel et al., 1998). Other antigens, which are shared by the CNS and PNS, such as MBP (Abromson-Leeman et al., 1995) and MAG (Weerth et al., 1999), induce inflammation predominantly in the spinal cord in the rat but focal cellular infiltration is also present in the spinal roots and sciatic nerves. Theoretically it is conceivable that a variety of other antigens of the PNS may also become a target of immune attack, similar to the situation in the central nervous system (Schmidt, 1999). Immunization efficacy can be enhanced by adding cerebroside, but not gangliosides, to the inoculum, although both are potential antigens of the PNS. EAN can also be produced by adoptive transfer of P2, P2 peptide-specific, P0, or P0 peptide-specific CD4+ T-cell lines (Hartung et al., 1995). In addition to the classic EAN models with PNS antigens, it is also possible to elicit an autoimmune nerve injury by activated, non-neural-specific T cells (Pollard et al., 1995; Harvey et al., 1995a).

1.3.1.2 Clinical features of rat EAN

The clinical picture of EAN is one of ataxia and weakness, leading in some instances to frank paralysis (Arnason and Soliven, 1993). High stepping and misplacement of feet may be prominent early. In the rat, weakness begins in the tail and moves proximally, hind limbs being involved next. Forelimbs are involved later and to a lesser extent. Symptoms begin about 12-16 days after immunization. Usually there is an abrupt loss of weight at onset of disease, and the extent of weight loss parallels disease severity. Progression to paralysis and death can be relatively rapid, over a day or two, or there may be a slower progression over a week or more. In nonfatal cases, after a week of progression or sometimes longer, there is a brief period of arrest followed by gradually clinical recovery. The sooner after immunization the symptoms begin, the greater the probability of severe disease.

1.3.1.3 Neuropathology in EAN

Microscopic lesions in EAN consist of perivenular cellular infiltration and segmental demyelination, mostly present in dorsal root ganglia, root nerves, cauda
equina and less frequently in peripheral nerves (Ballin and Thomas, 1969; Lampert, 1969). By LM early lesions, seen in animals 12-16 days after immunization, show perivenular infiltrates of mononuclear cells with little or no demyelination. Cells are attached to or within the walls of vessels. A few red blood cells, polymorphonuclear leukocytes, and fibrinous exudates are noted among the mononuclear cells in some lesions. The endoneurial spaces beyond the cellular infiltrate tend to be expanded and edematous. Late lesions, seen in animals 3 weeks after immunization, show many completely demyelinated axons and macrophages filled with myelin debris. Some of the large demyelinated axons are surrounded by very thin myelin sheaths, suggestive of beginning remyelination.

By EM early lesions show that the majority of the myelinated axons appeared normal (Ballin and Thomas, 1969; Lampert, 1969). Mononuclear cells are found between, below, and within endothelial cells. Plasma proteins also leaked through the vessels that are traversed by these cells. The early changes at the nodes of Ranvier consist of the separation of the terminal myelin loops from the axon and the loss of the SC nodal processes. The SCs around the otherwise normal sheaths often show an increased amount of granular endoplasmic reticulum and ribosomes. Usually the neurolemma surrounding such “activated” SCs appears intact but occasionally it is penetrated by a cytoplasmic process of a mononuclear cell. Cytoplasmic tongues from the invading cell project beneath the lamina and invade the mesaxon. After further penetration into the space between the outermost myelin and the outer tongue of SC cytoplasm, the invading mononuclear cell completely surrounds the myelin sheath, separating it from its supporting SC. While in contact with invading mononuclear cells, the myelin sheaths undergo a bubbly dissolution, beginning with the outer lamellae. After the focal dissolution, phagocytes pass through such myelin defects and remove the remaining intact remnants of the sheaths. Individual lamellae, pairs, or thick strata of compactly layered myelin are peeled from the sheaths (so-called macrophage-mediated demyelination). In this way the entire segment of a damaged sheath is removed from one node of Ranvier to the next (Lampert, 1969). SCs survive the destruction of their myelin and appear activated, expressing class I major histocompatibility complex (MHC) molecules on their surface. The lesions seen by
EM in EAN are exactly comparable to those observed in AIDP in humans (Arnason and Soliven, 1993).

Most of the neuropathological changes associated with EAN in the Lewis rat occur in the nerve roots and dorsal ganglia. Studies that have examined EAN with a moderate to severe course, or have sampled PNS tissues at or near the height of disease, have clearly established the predominance of primary segmental demyelination and inflammation in the nerve roots and dorsal root ganglia (Saida et al., 1983; McCombe et al., 1990; Ponzin et al., 1991). It has been consistently observed that the sciatic nerve becomes involved after the onset of EAN and is most affected during recovery from disease (Hughes and Powell, 1984; Ponzin et al., 1991). Demyelination and conduction block are greatest in the sciatic nerve during recovery from disease and correlate with the CV of sciatic nerve (Strigärd et al., 1987). Significant axonal degeneration also occurs in the sciatic nerve during recovery from EAN (Hughes and Powell, 1984; Hahn et al., 1988; Feasby et al., 1994).

There have been several studies focusing on the initial lesion in the peripheral nerves of Lewis rat with peripheral nerve homogenate induced EAN (Powell et al., 1983; Hahn et al., 1985; Rosen et al., 1990). These studies demonstrated that, during the onset of EAN, the early changes in BNB permeability in the nerve roots and sciatic nerve, and the concurrent leakage of rat Ig into the nerve endoneurium, may be initiated by infiltrating activated lymphocytes and was often coincident with the appearance of inflammatory macrophages. In a combined immunohistochemical, teased fiber and electrophysiological study of bovine peripheral nerve homogenate induced EAN in the Lewis rat (Stevens et al., 1989), it was noted that edema and paranodal demyelination in sciatic nerve teased fibers were the earliest pathological change but no systematic observations on the course of demyelination in teased fiber preparations were described.
1.3.1.4 Immune responses in EAN

The precise sequence of immunological and cellular events involved in the course of the development of the EAN is still uncertain. In general, it can be dissected into an induction and an effector phase (Gold et al., 1999).

I. Induction phase

i. Antigen presentation and activation of T helper (Th) cell

In the induction phase the injected autoantigen is presented to “ naïve” T cells by a class II MHC-positive antigen-presenting cells (APCs) such as macrophages, dendritic cells or B cells resulting in CD4\(^+\) Th cell activation (Gold et al., 1999). Two external signals are crucially required for an effective T cell activation by antigen presentation: the antigen-specific signal provided by the cell surface complex of immunogenic peptide and MHC molecule on APC through the T-cell receptor (TCR)-CD3 complex, and the antigen-independent signal called costimulation mediated by various costimulatory molecules such as B7-1, B7-2, and AMs such as ICAM-1 (Delves and Roitt, 2000). Activated T cells then differentiate to secrete cytokines.

CD4\(^+\) T cells can be divided into two major types: Th1 and Th2 cells (Delves and Roitt, 2000). Th1 cells are thought to mediate cellular immunity by activating cytotoxic CD8\(^+\) T cells and macrophages through IFN-\(\gamma\) and interleukin (IL)-2. Th2 cells are thought to mediate humoral immunity by stimulating B cells to differentiate and become antibody-producing plasma cells through IL-4, IL-5, IL-6, IL-10, IL-13, and transforming growth factor (TGF)-\(\beta\).

Resident endoneurial macrophages constitutively express MHC class II molecules which are strongly upregulated in EAN underlining their potential role in antigen presentation during EAN (Kiefer et al., 2001). However, MHC expression is not specific for inflammatory disease but also occurs during non-inflammatory activation of macrophages in genetically determined degenerative polyneuropathies (Stoll et al., 1998). In contrast, the costimulatory B7 molecules required for effective antigen presentation appear to be more specifically expressed in inflammatory neuropathy (Greenfield et al., 1998). They are undetectable by immunocytochemistry in normal nerves and nerves with degenerative neuropathies but may be induced on
endoneurial as well as epineurial macrophages during infectious or autoimmune inflammatory neuropathies (Kiefer et al., 2000). PNS endo- and epineurial macrophages thus fulfill another molecular requirement for effective antigen presentation by expressing costimulatory B7 molecules, particularly B7-1. As a further requirement for antigen presentation, PNS macrophages express the adhesion molecule ICAM-1, allowing cell adhesion with its counter-receptor lymphocyte function associated antigen (LFA)-1 on T-cells (Stoll et al., 1993a). Macrophages within the PNS, presumably both resident endoneurial macrophages and those of hematogenous origin, are thus equipped with several tools enabling them to present antigen.

ii. Release of cytokines

The antigen-specific interaction of the T cell and APC triggers a variety of cellular functions in both cell types. Most importantly, the local production of cytokines will be stimulated which determines the course of the subsequent inflammatory response (Linington, 1994). There is evidence for a relation between clinical activity of EAN and IL-1\(\beta\), IL-2, IFN-\(\gamma\), IL-6, IL-12, tumor necrosis factor (TNF)-\(\alpha\) and TNF-\(\beta\), consistent with a disease-promoting role for these cytokines. Elevation of TGF-\(\beta\)1, IL-4, IL-10 and cytolysin relates to recovery from EAN (Zhu et al., 1998b).

Among these cytokines, IFN-\(\gamma\) and TNF-\(\alpha\) appear to be particularly important cytokines (Kieseier et al., 2004). Both have multiple immunoregulatory effects including promoting T cell homing to the PNS through enhancing the BNB permeability, mediating myelin damage through activation of macrophages to enhance phagocytosis, release of noxious molecules such as reactive oxygen and nitrogen oxide metabolites, complement, and proteases. IFN-\(\gamma\), released by Th1 cells, is strongly upregulated during EAN (Schmidt et al., 1992) and markedly augments MHC class II antigen expression on macrophages when systemically applied in vivo (Vass and Lassmann, 1990). Systemic administration of recombinant rat IFN-\(\gamma\) worsens EAN in rats, while anti-IFN-\(\gamma\) mAb treatment before and after onset of the disease as well as pharmacologic blockade of IFN-\(\gamma\) synthesis ameliorates EAN and reduces MHC class II expression on macrophages and T cells in a dose-dependent
manner (Hartung et al., 1990; Tsai et al., 1991). Intraneural injection of TNF-α was associated with demyelination and conduction block in the presence of circulating antimyelin antibodies (Spies et al., 1995a), but high doses of TNF-α alone did not cause demyelination. There is some evidence of a role for TNF-α in central and peripheral demyelination, although it is unlikely that TNF-α alone is the major myelin toxin in inflammation (Selmay and Raine, 1988; Stoll et al., 1993b; Klinkert et al., 1997). However, the strong therapeutic effect of the TNF-α inhibitors raises hope as to TNF-α directed therapy of GBS (Klinkert et al., 1997).

iii. Lymphocytes homing, adhesion, and migration

To generate an inflammatory lesion in the nerve activated T cells circulating in blood need to enter the PNS, by a complex process of homing, adhesion, and migration involving AMs, matrix metalloproteinases (MMP), and chemokines (Kieseier et al., 2004). During inflammation, cytokines such as IFN-γ from CD4⁺ Th1 cells and endoneurial natural killer (NK) cells, or TNF-α, IL-1, IL-2, IL-4, and IL-6, which are liberated by resident macrophages, mast cells, and SCs, induce expression of AMs on the endothelial cells that form the BNB (Ho et al., 1998). These mechanisms recruit passing leukocytes, which bind to, and then insinuate their way through, the endothelium.

Three families of AMs are involved in leukocyte-endothelial interaction and in the regulation of leukocyte migration (Butcher and Picker, 1996): selectins, integrins, and proteins that are members of the Ig superfamily. Selectins mediate leukocyte rolling along endothelial cell walls, which slows down passing leukocytes. Sialic acid-containing carbohydrates apparently serve as ligands for selectins. The leukocyte rolling is followed by strong adhesion to endothelium mediated by interaction between the heterodimeric integrins and members of the Ig superfamily. Once strong adhesion is induced, leukocytes are attracted to cross the endothelium if appropriate chemoattractants, such as macrophage inflammatory protein (MIP)-1β for a subset of T cells and monocyte chemoattractant protein (MCP)-1 for macrophages, are present (Ho et al., 1998).

The presence of a variety of AMs on mononuclear and endothelial cells and the
upregulation of their ligands during active disease point to a common pathogenetic role of AM in the initiation of tissue inflammation in EAN (Gold et al., 1999). During the acute phase of EAN, upregulation of AMs such as VCAM-1 and ICAM-1 on lesion associated blood vessels parallels clinical disease and parenchymal infiltration (Stoll et al., 1993a; Enders et al., 1998). This notion was reinforced by therapeutic manipulation with mAbs in vivo. Archelos and colleagues demonstrated that EAN was attenuated by the treatment of mAbs to ICAM-1 (Archelos et al., 1993a), LFA-1 (Archelos et al., 1994), and L-selectin (Archelos et al., 1997). Enders et al. extended their findings on the very late antigen (VLA)-4 and its ligand VCAM-1 showing that blockade of VLA-4 (α4β1) by mAb was effective in EAN (Enders et al., 1998) and this integrin seems to be the most important AM in transendothelial migration of T cells in rodent EAN and hence a promising target candidate for therapeutic intervention in GBS.

II. Effector phase

Once the BNB breaks down, activated T cells penetrate the BNB. This transendothelial migration gives rise to the effector phase of the immune response in EAN. At least three mechanisms have been invoked to cause dysfunction and damage in the PNS (Ho et al., 1998): cytotoxic T cell lysis, antibody-mediated complement-dependent attack, and direct nonspecific effects of cytokines. These three mechanisms are not exclusive of one another, but can work in concert.

i. Cytotoxic T cell-mediated attack

The central role of T cells in disease pathogenesis was established when Linington et al (Linington et al., 1984) transferred EAN with a permanent T cell line (Th) specific for P2 protein. Subsequently T-cell lines specific for P2 peptides or P0 and its component neuritogenic peptides have been shown to transfer disease (Rostami et al., 1990; Linington et al., 1992). The importance of T cells is further supported by the inability of T-cell deficient rats to develop EAN upon immunization (Brosnan et al., 1987) and by the effective treatment and prevention with antibodies directed at the CD4+ T cell subset (Strigärd et al., 1988), the α/β TCR (Jung et al., 1992), or the IL-2 receptor (Hartung et al., 1989). Early histopathological studies
established that lesions begin with perivascular infiltration of mononuclear cells (Lampert, 1969) and that the degree of cellular infiltration subsequently correlates well with the degree of nerve function deficit (Strigärd et al., 1987).

Before onset of clinical signs most T-cells express the CD4^+ helper/inducer phenotype, whereas CD8^+ T cells prevail after the peak of the disease and during recovery (Brosnan et al., 1985). Once T cells have traversed the BNB, CD4^+ T cells recognize antigen in the context of MHC class II antigens, and the presence of CD4^+ T cells in close proximity to MHC class II (Ia) antigen-positive macrophages in the endoneurium in EAN satisfies the requirements for local T cell activation (Olsson et al., 1983). Evidence that this interaction is central to the pathogenesis of EAN is provided by the finding that mAbs to Ia antigens administered before onset of clinical disease reduce the severity of EAN in the rat (Strigärd et al., 1988). However, the precise mechanism of T cell action in EAN remains unclear. CD4^+ P2-specific T-cell lines capable of transferring EAN are cytotoxic to cultured SCs (Argall et al., 1992), but little in vivo evidence supports direct T cell–mediated damage to SCs or PNM. Focal demyelination has been demonstrated after intraneural injection of lymph node cells from animals with EAN (Hodgkinson et al., 1994); however, intraneural injection of neuritogenic P2-specific T-cell lines does not result in electrophysiological or histological evidence of demyelination (Spies et al., 1995a).

ii. Antibody-mediated attack

Antibodies against myelin components have been detected in the serum of EAN animals (Archelos et al., 1993b). Rat EAN serum does not transfer disease but rabbit EAN serum does. Systemic transfer of rabbit EAN serum only transfers disease when given with activated T cells to cause BNB breakdown (Spies et al., 1995a). If injected directly into nerve then rabbit EAN serum containing high levels of anti-Gal-C antibodies do induce disease (K Saida et al., 1978; T Saida et al., 1978). It was not possible to induce demyelination by PNS-specific antibodies. However, systemic administration of antibodies to Gal-C enhances the demyelination in adoptive transfer EAN (AT-EAN) (Hahn et al., 1993). Moreover, it has been shown that peripheral nerves of rabbits sensitized with GM1 gangliosides exhibited a predominant axonal neuropathy and may thus serve as an animal model for axonal GBS (Yuki et al.,
The possibility of an autoimmune nerve injury mediated by activated non-neural-specific T cells was studied by systemic transfer of ovalbumin-specific CD4\(^+\) T cells, followed by intraneural injection of ovalbumin representing a model of damage from bystander reactions (Pollard et al., 1995; Harvey et al., 1995a). On the side injected with ovalbumin a rapid endoneurial infiltration of T cells and macrophages occurred, which was associated with a marked increase in BNB permeability. Primary demyelination or axonal degeneration was demonstrable by electrophysiological studies when given in combination with antimyelin antibodies, thus replicating typical features of EAN.

Antibodies can conceivably induce myelin damage by three mechanisms. First, upon binding to the Fc receptor (FcR) of macrophages they can direct these cells to the putative (auto-) antigenic structures and induce so-called antibody-dependent cellular cytotoxicity (ADCC). Second, by opsonizing target structures, they can promote their internalization by macrophages. Finally, upon binding to the antigenic epitopes, they can activate the classic complement (C) pathway with subsequent assembly of the terminal complement complex (C5b-9). This results in pore formation allowing calcium influx, which triggers myelin-integral proteases that degrade the myelin sheath (Kieseier et al., 2004).

An important role of complement in the pathogenesis of inflammatory demyelination is underlined by the observation that decomplementation of animals with cobra venom factor which depletes the C3 component or inactivation by soluble complement receptor (CR)1 partly suppressed EAN (Feasby et al., 1987; Vriesendorp et al., 1995; Vriesendorp et al., 1998). In addition, complement involvement has been shown by deposition of the membranolytic attack complex (MAC; C5b-9) on SCs along the myelin sheath before myelin degradation in EAN (Stoll et al., 1991). Current knowledge suggests that complement may be important in recruiting macrophages into the endoneurium via Fc mediated mechanisms, in opsonizing myelin for phagocytosis, in amplifying ongoing inflammatory reactions, and in disintegrating the myelin sheath (Kieseier et al., 2004).
iii. Direct nonspecific effects of macrophages

Macrophages play a crucial role in the pathogenesis of EAN. They are the predominant cell population in the infiltrates of EAN nerve and are found early on in large numbers (Hartung et al., 1988a). They can be localized in spinal roots as well as in more distal segments of nerves. Histopathologically macrophages invade the SC basal lamina, penetrate myelin lamellae, strip myelin lamellae and phagocytose both damaged and apparently intact myelin (Ballin and Thomas, 1969; Lampert, 1969). The fundamental need for macrophages in the pathogenesis of the disease was demonstrated by depletion experiments in which macrophage elimination prevented all clinical, electrophysiological, and histological signs of EAN (Craggs et al., 1984; Jung et al., 1993). Activation of macrophages by pro-inflammatory cytokines like IFN-γ causes greatly enhanced disease severity (Hartung et al., 1990).

Apart from the direct phagocytotic attack, other mechanisms beside macrophage activation are also of relevance. Macrophages retrieved from animals with EAN generate and release increased amounts of arachidonic acid metabolites and toxic oxygen species (Mäurer et al., 2002). Animals are protected from the development of EAN or the disease severity is attenuated after selectively blocking macrophage function with cyclo-oxygenase inhibitors (Heininger et al., 1988) and oxygen radical scavengers (Hartung et al., 1988b), demonstrating that macrophage-derived reactive oxygen intermediates contribute to myelin damage.

III. Termination of the immune action: apoptosis

One crucial mechanism to terminate nervous system inflammation is apoptosis of autoreactive T cells (Gold et al., 1997). In EAN, T cells undergo apoptosis within the peripheral nerve early with peak apoptotic activity at the time of maximal T-cell infiltration (Zettl et al., 1994). SCs are a source of several proinflammatory cytokines but they can also express molecules that can terminate T cell inflammation and downregulate immune functions (Mäurer et al., 2002). Fas and its ligand are central molecules of a family of death factors that regulate T cell survival in the immune system (Nagata and Golstein, 1995). Expression of membrane-bound or secreted FasL molecules on SCs may lead to T cell apoptosis in EAN through crosslink with Fas
molecules on invading T cells while expression of Fas on SC membranes could render them susceptible to T-cell attack (Zettl et al., 1995). The ultimate functional aspect of the expression of Fas/FasL on SCs is not yet understood.

Induction of apoptosis may be used as an effective model of therapy in EAN. It was shown that high dose glucocorticoids could induce T-cell apoptosis in situ (Zettl et al., 1995). In addition intravenous treatment with recombinant P2 protein caused a profound increase in T-cell apoptosis in the sciatic nerve from AT-EAN and active EAN rats. With regard to induction of apoptosis in AT-EAN antigen therapy was at least as effective as steroids (Weishaupt et al., 1997). Moreover, therapeutic blockade of VLA-4/VCAM-1 was shown to activate T-cell apoptosis (Leussink et al., 2002).

1.3.2 Humoral immunity

The concept that aberrant B-cell responses to glycolipids and related conjugates are key to the pathogenesis of GBS is the focus of many studies (Hartung et al., 2001). Various observations suggest that humoral factors are paramount in the pathogenesis of GBS (Kieseier et al., 2004): (1) PE and IVIg result in clinical improvement; (2) circulating antibodies targeting structures on peripheral nerve tissue are detectable in sera from GBS patients; and (3) deposition of Ig and complement has been demonstrated on myelinated fibers in nerve biopsies as well as CSF samples from affected patients. In a large number of reports antibodies (mainly IgG) to a variety of different glycolipids, including GM1, asialo-GM1, GM1b, GalNAc-GD1a, GD1b, 9-O-acetyl-GD1b, GD3, GT1a, GT1b, GQ1b and LM1, have been described (Yuki, 2001a). Research has been driven by the hypothesis that the different syndromes underlying GBS could be explained by the different patterns of nerve damage caused by antibodies directed against different glycoconjugates shared by infective organisms and the nervous system (Hughes et al., 1999). In addition, peripheral nerve demyelination may be the result of serum constituents other than myelin-specific antibodies, such as cytokines, complement, or other inflammatory mediators and it remains possible that GBS sera contain substrates that might produce conduction block without necessarily causing demyelination or evoking failure of neuromuscular transmission (Kieseier et al., 2004).
1.3.2.1 Relationship between anti-ganglioside antibodies and different types of GBS

I. Acute inflammatory demyelinating polyradiculoneuropathy (AIDP)

No characteristic pattern of anti-ganglioside antibodies has been discovered in AIDP. The commonest associated antibody is an IgG1 antibody against ganglioside GM1, especially in those cases with a preceding \textit{C. jejuni} infection (Rees \textit{et al.}, 1995b). In addition, a link between IgM anti-GM2 antibodies and preceding CMV infection has been demonstrated in association with AIDP (Visser \textit{et al.}, 1996). A study of early human AIDP lesions has demonstrated lymphocytic infiltration and deposition of complement activation products along the outer surface of SCs in myelinated (motor and sensory) peripheral nerves (Hafer-Macko \textit{et al.}, 1996b). This observation suggests that binding of antibody (with as yet unknown epitope specificity) to the SC membrane as a primary target may initiate the demyelinating process through activation of the complement cascade.

II. Acute motor axonal neuropathy (AMAN)

Anti-GM1 IgG antibodies have been shown strongly linked to AMAN in European patients (Jacobs \textit{et al.}, 1996; Hadden \textit{et al.}, 1998). The incidence of anti-GM1 IgG may be found in up to 80\% of AMAN cases compared to 20\% in AIDP cases (Hadden \textit{et al.}, 1998). In addition to GM1, GD1a could be another ganglioside antigen for AMAN. High IgG antibody titers against GD1a have been found to be significantly more common in AMAN than in AIDP among Chinese patients (Ho \textit{et al.}, 1999). Furthermore, a link between anti-GalNAc-GD1a antibodies (Ang \textit{et al.}, 1999; Hao \textit{et al.}, 1999) or anti-GM1b antibodies (Yuki \textit{et al.}, 2000) and positive \textit{C. jejuni} serology has been observed in association with AMAN in GBS patients from Europe, Japan, and China.

III. Acute motor sensory axonal neuropathy (AMSAN)

The pathological pattern in AMSAN very closely resembles that in AMAN. Therefore, it is highly probable that AMSAN also represents a manifestation of antibody-mediated attack primarily targeting an axonal membrane antigen. Whether AMSAN and AMAN share the same target antigen and differ with respect to immune
response severity, or whether the difference between AMSAN and AMAN can be linked to distinct antigens, remains an open question (Schwerer, 2002).

IV. Miller Fisher syndrome (MFS)

Anti-GQ1b antibodies have the closest association with a GBS type. In MFS, a nearly 100% incidence of anti-GQ1b has been demonstrated, compared to around 5% in other types of GBS (Willison and O'Hanlon, 1999). In some patients with MFS, there has been a preceding *C. jejuni* infection and antibody titers decrease within weeks after MFS onset, thus paralleling the disease course (Chiba *et al.*, 1992). IgG anti-GQ1b/GT1a have also been detected in GBS patients presenting with other cranial nerve deficits, such as oropharyngeal paralysis (O'Leary *et al.*, 1996). The unique association between antibodies against GQ1b and MFS strongly suggests that GQ1b plays a role as a target antigen in immune injury to cranial, particularly ocular motor nerves (Schwerer, 2002).

1.3.2.2 Pathogenic mechanisms of anti-ganglioside antibodies

The abundant clinical, pathological, and serological evidence for a strong link between antibodies against gangliosides and the AMAN and MFS types of GBS supports a pathogenic role for these antibodies. However, the extent of their contribution to GBS pathogenesis and the mechanisms involved are less clear. GM1 and other gangliosides are widely distributed in neural membranes of the CNS and PNS. Taken as a whole, studies on the ganglioside composition of peripheral nerves do not reveal an increased content of GM1 in motor nerves that would account for the association of anti-GM1 antibodies with motor nerve disorders (Quarles and Weiss, 1999). Immunocytochemical studies have suggested that much of the GM1 and cross-reacting glycoconjugates are not readily accessible to antibodies, but do indicate that these antibodies bind to the surface of isolated bovine motor neurons (Corbo *et al.*, 1992), to nodes of Ranvier, and to neuromuscular junctions (NMJs) (O'Hanlon *et al.*, 1998). However, binding of human anti-GM1 antibodies is widespread, including sensory structures, and extends beyond those sites conventionally regarded as pathologically affected in motor nerve syndromes. This suggests that factors other than accessibility of antigen affect pathological outcome.
I. **Pathogenicity of ganglioside auto-antibodies**

There is increasing evidence for the pathogenic role of antiglycolipid antibodies in GBS (O'Leary and Willison, 2000). In terms of immunolocalization, GM1 moieties on the nodal and internodal axolemmal surfaces of mature myelinated nerve fibers and on SCs in human, rat, and mouse tissue was demonstrated (Sheikh et al., 1999). GT1b moieties were restricted to the axolemma. Anti-GM1 antibody-positive GBS patients were found to have a more rapid onset to peak disability with quicker resolution, indicating that a functional disturbance of axonal membrane at the node of Ranvier may be responsible, rather than frank demyelination (Kuwabara et al., 1999). In addition, immunisation of rabbits with bovine brain gangliosides or purified GM1 has been demonstrated to produce AMAN associated with high titers of anti-GM1 IgG, suggesting the development of an antibody-mediated animal disease model closely resembling the AMAN type of GBS (Yuki et al., 2001b). Further support for the pathogenic role of GQ1b is evidenced by the demonstration that ganglioside GQ1b is particularly abundant in the ocular motor nerves (Chiba et al., 1993). In addition, passive transfer of anti-GD1b antibodies to rabbits resulted in macrophage infiltration and vacuolation of dorsal column axons, lending further support to the role of these antibodies in the pathogenesis of sensory ataxic neuropathy (Kusunoki et al., 1999).

II. **Molecular mimicry**

Many studies investigated the role of carbohydrate mimicry between gangliosides and microbial structures. The presence of anti-GM1 antibody in GBS has been shown to be significantly associated with preceding *C. jejuni* infection and it may be induced by epitopes common to *C. jejuni* lipopolysaccharide (LPS) and GM1 (Yuki et al., 1993; Jacobs et al., 1996). The terminal structure of *C. jejuni* LPS is identical to that of the terminal tetrasaccharide of the GM1 ganglioside in nerve. In addition, it was shown that patients developed GBS after receiving bovine brain ganglioside treatment (including GM1, GD1a, GD1b, and GT1b) and in these patients anti-GM1 antibodies were shown bound to nodes of Ranvier (Illa et al., 1995). Furthermore, rabbits immunized with *C. jejuni* LPS produced high anti-GM1 IgG antibody titers (Ang et al., 2000), which strengthens the role of molecular mimicry in
the induction of anti-GM1 IgG antibody in GBS. Serologic evidences of *C. jejuni* infection suggest that three phenomena — axonal dysfunction, IgG antibodies against GM1, GD1a, or GalNAc-GD1a, and *C. jejuni* infection — are closely associated (Yuki, 2001a).

Antibodies against GQ1b associated with *C. jejuni* infection preceding MFS reacted with LPS, evidence that LPS of *C. jejuni* bear the GQ1b epitopes (Yuki *et al.*, 1994). The oligosaccharide structure of the *C jejuni* LPS isolated from a patient with MFS was shown to be identical to the terminal trisaccharide of GD3 (Salloway *et al.*, 1996). Some patients with MFS have anti-GD3 IgG antibody that cross-reacts with GQ1b, suggestive that infection by *C. jejuni* bearing a GD3-like LPS could induce anti-GQ1b IgG antibody. Almost invariably, anti-GQ1b also reacts with GT1a which is relatively enriched in the lower cranial nerves, producing the characteristic antibody pattern in MFS, and suggesting recognition of the terminal disialosyl group linked to galactose (Neu5Acα2–8Neu5Acα2–3Gal) as the epitope common to GQ1b and GT1a (Schwerer, 2002).

### III. Anti-ganglioside antibody-mediated pathomechanisms

Antibodies can mediate demyelination and impair nerve conduction by ADCC, in which they bind to functionally relevant epitopes on the outermost surface of the SC or axolemma and activate the complement system by the classic pathway yielding proinflammatory mediators and the lytic C5b-9 terminal complex. This results in the entry of calcium, which in turn activates calcium-sensitive enzymes capable of degrading myelin and axonal proteins (Kieseier *et al.*, 2004). The specific target of such antibodies remains elusive. Whether gangliosides and other glycolipids represent autoantigens in AIDP is uncertain at present.

Anti-GM1 antibodies have been observed to produce demyelination and conduction block following intraneural injection in vivo or application to peripheral nerve in vitro (Hughes *et al.*, 1999). Nevertheless, neither of these effects has been confirmed in more recent electrophysiological studies using anti-GM1 injected into peripheral nerve or applied to nerve roots in vivo. Furthermore, no conduction abnormalities have been detected in isolated nerve preparations incubated with anti-
GM1 in vitro; however, immunohistological evaluation has revealed binding of antibody and activation of complement at the nodes of Ranvier (O'Hanlon et al., 1998). Antibody deposition at myelinated fibre nodes or on axons has also been shown in rabbits immunised with GM1 (Yuki et al., 2001b), in GBS patients after administration of gangliosides (Illa et al., 1995), and following intraneural transfer of anti-GM1 (Harvey et al., 1995b). In addition, binding of IgG and activated complement components to the axonal membrane at the nodes of Ranvier has been seen in the AMAN type of GBS (Hafer-Macko et al., 1996b). These results suggest that activation of the complement cascade by bound anti-GM1 as demonstrated in vitro may cause damage to axons and thus might explain the close association between antibodies against GM1 and axonal involvement in GBS (Schwerer, 2002).

Antibodies against GQ1b have been shown to interfere with neuromuscular transmission using mouse phrenic nerve/hemidiaphragm preparations as an in vitro model (Willison and O'Hanlon, 1999). Following incubation with polyclonal anti-GQ1b IgG from MFS patients or mouse monoclonal anti-GQ1b IgM, complement-dependent electrophysiological changes leading to a blockade of nerve–muscle transmission have been demonstrated; moreover, deposition of antibody and complement activation products at motor end plates has been shown in these experiments (Goodyear et al., 1999). In addition, structural breakdown of motor nerve terminals with loss of cytoskeletal proteins has been reported to be linked to the disruption of neuromuscular transmission mediated by anti-GQ1b in vitro (O'Hanlon et al., 2001). These findings point to the NMJ as a major site of action for anti-GQ1b antibodies causing interference with motor nerve function in MFS.

Anti-ganglioside antibodies could potentially bind any ganglioside-containing membranes (Willison, 2005), including the ganglioside-rich, pre-synaptic component of the NMJ, exposed axolemna at nodes of Ranvier, and paranodal myelin. Studies using in vitro mouse hemidiaphragm preparations showed that anti-ganglioside antibodies (with different specificities for GQ1b, GT1a, GD3, GM1, and GD1a) can cause nerve terminal axonal degeneration culminating in synaptic necrosis, selectively ablate the perisynaptic SC that envelops the pre-synaptic region, or destroy both nerve terminal and perisynaptic SC (Willison, 2005).
1.3.3 Cellular immunity

Histopathological studies on nerve biopsy and autopsy material, studies in the animal model EAN, and immunological studies in GBS patients indicate that cell-mediated immune responses to peripheral nerve play an important role in the pathogenesis of GBS (Hartung et al., 1995). Demyelination and mononuclear cellular infiltration is the pathological hallmark of classical GBS (Prineas, 1981). Lymphocytes and macrophages accumulate in a focal and perivenular distribution throughout the PNS from root to motor terminals (Hall et al., 1992). Macrophages are the major mononuclear cell type located in close proximity to myelin. They strip off myelin lamellae and phagocytose myelin debris (Prineas, 1981; Hall et al., 1992). Macrophages and activated T cells express MHC class II antigens. However, the spectrum of pathological changes can range from focal or extensive demyelination in the presence or absence of cellular infiltration, to axonal degeneration with or without demyelination or inflammatory infiltrates. The varied histopathological features mirror the clinical diversity of GBS. Whereas AIDP is characterized by macrophage-mediated demyelination and intense T-cell infiltration, AMAN and AMSAN exhibit signs of a macrophage-mediated axonal neuropathy, and lymphocytic infiltrates are scarce (Griffin et al., 1996).

1.3.3.1 The role of T cells

I. Observations in EAN

Observations in EAN formed the basis for the implication of T-cell-mediated immune responses in the pathogenesis of GBS (Hartung et al., 1988c; Arnason and Soliven, 1993). In rats, the decisive role of T lymphocytes in initiating nerve damage was proven by demonstrating that the transfer of autoreactive P2, P0, or P2/P0 peptide-specific T-cell lines can induce the clinical, electrophysiological, and morphological features of classical EAN in naive recipient rats (Hartung et al., 1995). The pivotal role of T cells in EAN is also underscored by the effects of manipulations that eliminate or silence T lymphocytes (Hartung et al., 1989; Hartung and Toyka, 1990; Jung et al., 1992).
II.  Evidence for T-cell activation

T-cell immune responses are initiated by the trimolecular interaction between APCs that expose processed antigen fragments (epitopes, mostly peptides, 10-20 amino acids in length), MHC class II gene products, and a specific TCR. This interaction is enhanced by the presence of AMs such as CD2 and LFA-1 on T cells and LFA-2 and ICAM-1 on APCs, as well as costimulatory signals such as IL-1 (Hartung et al., 1995). The activation of T cells in GBS is evident from the presence of increased numbers of circulating T cells bearing activation markers and of increased concentrations of soluble IL-2 receptor (IL-2R) (Taylor and Hughes, 1989). In addition, higher concentrations of soluble IL-2R shed from activated T cells were measured in the serum of patients with GBS and correlated with disease activity (Bansil et al., 1991).

III.  T-cell responses in GBS

Various attempts to identify nerve antigens to which aberrant T-cell response is mounted, as well as attempts to establish specific T-cell lines from affected patients, have yielded controversial results. In GBS, an increased usage of V\(\beta\)15 TCR chains has been demonstrated, pointing to a potentially restricted T-cell response to a common antigen, or a role for an as-yet undetermined superantigen in the pathogenesis of GBS (Khalili-Shirazi et al., 1997). Furthermore, the observation of \(\gamma\delta\) T cells in nerve biopsies from affected patients points to potential involvement of this cell population in the inflamed PNS (Khalili-Shirazi et al., 1998). The predominant T cells in GBS are \(\alpha\beta\) T cells, while small numbers of \(\gamma\delta\) T cells are also found in the endoneurium in AIDP and are potentially important because they recognized non-protein antigens including glycolipids. CD1 molecules on endoneurial macrophages and possibly SCs are the antigen presenting molecules for \(\gamma\delta\) T cells and are up-regulated in GBS. The \(\gamma\delta\) T cells and CD1 molecules might play a role in the prominent anti-glycolipid antibody response in many patients with GBS, supporting the classical hypothesis that the pathological process in most cases of AIDP is due to T cell mediated immunity to myelin antigens directing macrophage-mediated demyelination as portrayed in EAN by Lampert (1969) and in GBS by Prineas (Prineas, 1972; Prineas, 1981; Hughes et al., 1999). Moreover, an unusual V\(\gamma\)8/\(\delta\)1
TCR chain usage was defined in a γδ T-cell line obtained from a sural nerve biopsy of a patient with AIDP, suggesting that gut-associated lymphocytes are critically involved in the pathogenesis of this disorder (Cooper et al., 2000). Others suggest a nonresponsiveness of γδ T cells inducing a defective regulation of antibody production and promoting an immune response against ganglioside-like epitopes on peripheral nerve (Van Rhijn et al., 2003).

IV. Mechanisms of T cell-mediated nerve damage

T-cell activation could contribute to the pathogenesis of GBS in several ways (Hartung et al., 1995). First, CD4+ T cells of the Th2 phenotype (synthesizing IL-4, IL-5, and IL-6) may cause B-cell proliferation and transformation into plasma cells that manufacture antibodies against peripheral myelin components. Second, CD4+ T cells of the Th1 inflammatory phenotype (synthesizing IFN-γ, IL-2, TNF-α, and TNF-β = lymphotoxin) could damage myelin by secreting proinflammatory and myelinotoxic cytokines and operate by recruiting macrophages to exert nerve damage. Third, activated CD8+, or perhaps a subset of CD4+ T lymphocytes, may be directly cytotoxic to SCs.

The pathogenetic sequence of T-cell-mediated responses to nerve antigens comprises a number of distinct steps: homing to and crossing of the BNB, endoneurial activation, clonal expansion, release of injurious molecules, and recruitment of other inflammatory effector cells. The selective recruitment of activated T cells into the peripheral nerve tissue is a crucial step, and is mediated by the differential expression of leukocyte and endothelial adhesion receptors, chemokines, and chemokine receptors (Kieseier et al., 2004). The complex process of T-cell homing, adhesion, and transmigration has been studied in EAN in great detail. In GBS patients, increased serum or CSF levels of soluble AMs (Previtali et al., 1998), chemokines (Press et al., 2003), and MMP (Hartung and Kieseier, 1999) can be detected, reflecting active T-cell migration across the BNB.

After autoreactive T cells leave the bloodstream and enter the PNS, they interact with macrophages displaying the appropriate neuritogenic epitope on its surface in concert with MHC class II antigen, resulting in T-cell proliferation and local
production of cytokines. This immune activation in situ initiates a cascade of inflammatory responses encompassing further cytokines, additional effector cells, and a host of inflammatory effector molecules both inside and outside the BNB (Hartung et al., 1995). Among the cytokines that orchestrate cellular interactions during immune responses, IFN-γ is of major importance. In a study on archival autopsy tissues including spinal roots, dorsal root ganglia, and peripheral nerve, the ratio of CD8+ to CD3+ T cells was significantly increased in cases of GBS with a subacute course, pointing to the role of a cytotoxic T-cell response in subacute stages of GBS (Wanschitz et al., 2003). Studies in the peripheral venous blood of GBS patients demonstrated an early elevation of T cells with an anti-inflammatory cytokine pattern, suggesting that a shift in the systemic distribution of the T-cell pattern toward Th2 cells may account for the self-limiting course of the disease (Press et al., 2002).

V. Synergy of T cells and antibody

In EAN the combination of antibodies to antigenic epitopes expressed on the surface of SCs and adoptive transfer of T cells elicits more severe disease with more florid demyelination than adoptive transfer of T cells alone (Hahn et al., 1993; Spies et al., 1995b). A favored scenario is that activated T cells cross the BNB, regardless of their specificity, and T cells which recognize an antigen in the endoneurial compartment are further activated, producing cytokines and chemokines which open the BNB and allow the egress of antibodies. Antibodies which recognize cell surface molecules then fix and activate the complement cascade or opsonize SCs or the axolemma for the attachment of macrophages via FcRs which then lead to the cascade of pathological events recognized in AIDP and AMAN. The production of some antibodies and in particular the switch from IgM antibody production early in the immune response to IgG1 and IgG3 subclasses requires direct T cell help. The antibodies to gangliosides discovered in the serum of GBS patients generally belong to these subclasses (Hughes et al., 1999).

1.3.3.2 The role of macrophages

Macrophages play a crucial role in GBS before and beyond the final act of macrophage-mediated demyelination. Macrophages are putatively involved in
virtually all steps of the pathogenetic process, from early immune surveillance, antigen presentation and activation of the cellular immune cascade throughout the disease to antigen-specific demyelination and axonal damage, non-specific secondary tissue destruction, removal of debris and regeneration (Kiefer et al., 2001).

I Macrophage recruitment into the inflamed peripheral nerve

Normal nerves contain macrophages in a perivascular distribution at the blood-nerve interface, making them uniquely suited to act as APCs in the PNS. Work carried out in EAN suggests that macrophages act as professional antigen presenters (Hartung et al., 1995). Macrophages feature predominantly in the nerve lesion of GBS. They may be derived from circulating monocytes that invade the peripheral nerve (hematogenous) or from endoneurial macrophages (resident). Recruitment of monocytes into the PNS requires several consecutive and coactive steps (Kiefer et al., 2001). As a first step, blood monocytes roll along the vascular endothelium and form loose reversible connections mediated by selectins (Tedder et al., 1995). The role of L-selectin and E-selectin have been demonstrated in EAN and GBS (Hartung et al., 1994; Archelos et al., 1997). In the second step, firm irreversible connections between monocytes and endothelial cells mediated by integrins and their ligands are established (Archelos et al., 1999). ICAM-1, a ligand for the CR3 (Mβ2 integrin), was upregulated on endothelial cells in EAN (Stoll et al., 1993a) and raised serum levels of soluble ICAM-1 were found in GBS patients (Trojano et al., 1998). Once adhesion has been established, chemotactic signals are perceived that guide the adhering monocytes towards the interior of the nerve. Among many chemotactic cytokines, chemokines are of particular relevance (Luster, 1998). In EAN, an upregulation of mRNA expression for MIP1α and MIP1β with a peak prior to maximum clinical disease severity suggested their role in macrophage recruitment (Kieseier et al., 2000). To penetrate endothelial cells and the underlying basal lamina, the action of protease is required. There is good experimental evidence that MMP are involved in this process (Woessner, 1994). In EAN and GBS, mRNA expressions for several MMPs were shown to be upregulated, together with the localization to blood vessels and infiltrating inflammatory cells and functional studies, suggesting that MMPs play a key role in macrophage recruitment and penetration through the BNB in PNS.
autoimmunity (Hartung and Kieseier, 1999; Kiefer et al., 2001).

II. Macrophages as sources of pro-inflammatory cytokines

Macrophages within inflammatory nerve lesions express and elaborate a multitude of regulatory molecules that may modulate the course of the inflammatory neuropathy. They, thus, not only act as chief effector cells in demyelination and tissue destruction but are also intimately involved in the control of the pathogenetic process. Pro-inflammatory mediators derived from macrophages that propagate inflammation include IL-12, TNF-α and possibly IL-6 whereas TGF-β and IL-10 have predominantly anti-inflammatory effects (Kiefer et al., 2001). IL-12 is crucial early in the development of an immune response, particularly by promoting T cell differentiation into the Th1 phenotype with prominent production of IFN-γ (Trinchieri, 1995). IL-12 augments the cytotoxic activity of both resting and cultured NK cells, and induces IFN-γ production by T cells. In EAN, IL-12 mRNA is expressed in cells within the inflamed nerve with a time course parallel to disease activity (Zhu et al., 1997). Elevated serum and/or CSF levels of TNF-α were detected in patients with GBS during active disease in most studies and were correlated with disease activity and electrophysiological abnormalities (Créange et al., 1996; Sharief et al., 1997). The pleiotropic effects of IL-6 include B cell stimulation and induction of acute phase response. High IL-6 concentrations were found in sera and CSF from patients with active GBS and correlated with clinical signs of GBS, suggesting a disease-promoting effect (Maimone et al., 1993).

III. Effector functions of macrophages

Macrophage-mediated segmental demyelination is the pathological hallmark of AIDP (Prineas, 1981). It is considered an active immunological process, where macrophages attack intact myelin sheaths wrapped around healthy axons. The immunological mechanisms have only partially been explored. There is good evidence that antibodies may direct macrophages towards their myelin or axonal targets and that, macrophages attack sites of antigen binding in a complement dependent manner, through a receptor-mediated type of interaction (Kiefer et al., 2001). Receptor-ligand pairs involved are FcR/Igs and CR/complement. In vitro,
myelin can be opsonized by antibody causing enhanced phagocytosis by macrophages which is mediated by FcRs (Trotter et al., 1986; Mosley and Cuzner, 1996).

However, antibody-dependent macrophage cytotoxicity is not the only mechanism by which segmental demyelination may occur. Demyelination may occur by antigen non-specific mechanisms (Pollard et al., 1995; Harvey et al., 1995a). Massive inflammation may lead to secondary axonal loss and tissue destruction beyond the actual immunological targets (Berciano et al., 1997; Massaro et al., 1998). Such changes are mediated by toxic mediators released from macrophages that may be intended to destroy the target of the macrophage attack but may leak into the surrounding tissues, particularly if there is a vigorous inflammatory response. Toxic attacks upon myelin and other structures in vitro are mediated at least in part by reactive oxygen species (van der Goes et al., 1998) and are associated with the secretion of nitric oxide (NO) and TNF-\(\alpha\) into culture supernatants (van der Laan et al., 1996). NO synthase may be expressed by macrophages upon stimulation by encephalitogenic T cells through the action of IFN-\(\gamma\) (Misko et al., 1995). Reactive oxygen species may damage myelin (Konat and Wiggins, 1985) and SCs, rendering them susceptible to attacks by macrophages (Brück et al., 1994), as does TNF-\(\alpha\) (Selmaj and Raine, 1988). Neither of these mediators is specific for any target, and the full-blown destructive effect of macrophages can only be affected by the concerted action of the entire armamentarium of these cells. It should be noted that hematogenous macrophages rather than resident macrophages are critically involved in myelin phagocytosis and thus pave the way for subsequent successful regeneration/remyelination (Kiefer et al., 2001).

IV. Role of macrophages during recovery

Macrophages might participate in the induction of apoptosis by secreting pro-apoptotic mediators including NO, reactive oxygen intermediates and TNF-\(\alpha\), and possibly direct cell contact (Wu et al., 1995; Zettl et al., 1997; Weishaupt et al., 2000). It was further demonstrated that macrophage-derived reactive oxygen intermediates may exert their action only when close contact between macrophages and T cells was allowed (Mix et al., 1999). In addition, macrophages elaborate anti-inflammatory
cytokines such as IL-10 and TGF-β. IL-10 was shown to inhibit EAN when given before or after onset of the disease (Bai et al., 1997b). TGF-β1 is expressed in patients with GBS (Sindern et al., 1996; Créange et al., 1998) and in EAN (Kiefer et al., 1993; Gregorian et al., 1994) with a time course suggesting an association with recovery.

Macrophages are tightly involved in the repair of peripheral nerve by promoting SC proliferation and survival, remyelination, and axonal regeneration, once inflammation has stopped (Kiefer et al., 2001). SCs proliferate following a lesion, a process thought to be triggered by macrophages although SC proliferation is not entirely dependent on macrophage infiltration. Macrophages secrete numerous growth and differentiation factors for SCs and promote remyelination. In addition, they facilitate axonal regeneration by secreting growth factors or cytokines that in turn stimulate growth factor secretion by SCs. They also contribute to the modulation of extracellular matrix components allowing axonal regeneration. IL-6, expressed by macrophages, SCs and neurons, acts as a neurotrophic agent on certain neurons (Gadient and Otten, 1997) and supports nerve regeneration (Zhong et al., 1999) in addition to its disease-promoting effect. Moreover, it supports SC differentiation towards myelinating cells (Haggiag et al., 1999). IL-6 is thus a molecule with both beneficial and destructive potentials in GBS.

1.3.4 Conclusions

GBS and related disorders are considered as a spectrum of disorders affecting different portions of the PNS and having different temporal courses. The underlying pathology of AIDP resembles EAN histologically and a T-cell mediated pathogenesis is likely although a complement dependent antibody targeted attack on SC surface antigens has also been proposed and may coexist (Hughes et al., 1999). No responsible antigens have been identified so far. The accumulating evidence implicates the role of antiglycolipid antibodies in the pathogenesis of GBS and their association with particular clinical phenotypes. Anti-GM1 and anti-GD1a IgG antibodies have been shown strongly linked to AMAN but antibodies to gangliosides do not appear to be directly responsible for most cases of AIDP. Several bacteria, but especially C. jejuni, contain ganglioside-like glycoconjugates in their membranes. The
presence of antibodies to GM1 in patients with GBS and not in patients with uncomplicated *C. jejuni* enteritis does indicate that there is something special about the anti-glycolipid immune response in patients with GBS which is worthy of further exploration. The strongest association is between antibodies to ganglioside GQ1b and MFS; the underlying pathology and precise sites of the responsible epitopes within the PNS have been reviewed by Willison (Willison, 2005).

Observations in the animal model of EAN provided profound insights into the role of the cellular immune response in the pathogenesis of AIDP (Hartung *et al.*, 2002). The selective recruitment of inflammatory cells into the inflamed peripheral nerve tissue is apparently a crucial step, and is mediated by the differential expression of leukocyte and endothelial adhesion receptors, chemokines, and chemokine receptors. Working in concert, these components permit cells to localize selectively to inflammatory sites. The role of T lymphocytes that are activated in GBS and represent an important component of the nerve lesion in AIDP is still largely unknown. Macrophages are putatively involved in virtually all steps of the pathogenetic process of AIDP, resulting in the pathological hallmark of macrophage-mediated segmental demyelination.
Figure 1.1 Schematic illustration of the immune responses in the inflamed PNS. Basic principles of the cellular immune responses: autoreactive T-cells (T) recognize a specific autoantigen presented by MHC class II molecules and the simultaneous delivery of costimulatory signals on the cell surface of antigen-presenting cells, such as macrophages (M), in the systemic immune compartment. Activated T-lymphocytes can cross the BNB in order to enter the PNS. Within the PNS, T-cells activate macrophages that enhance phagocytic activity, production of cytokines, and the release of toxic mediators, such as NO, MMPs, and proinflammatory cytokines, propagating demyelination and axonal loss. The termination of the inflammatory response is mediated, in part, by macrophages by the induction of T-cell apoptosis and the release of anti-inflammatory Th2/Th3 cytokines, such as IL-10 and TGF-β.

Figure 1.2 Schematic illustration of the immune responses in the inflamed PNS. Principles of the humoral immune response: autoantibodies (Abs), crossing the BNB or locally produced by B-cells, contribute to the process of demyelination and axonal damage. Antibodies can mediate demyelination by ADCC; they can block functionally relevant epitopes for nerve conduction, and can activate the complement system by the classic pathway yielding proinflammatory mediators and the lytic C5b-9 terminal complex.

1.4 Intravenous immunoglobulins (IVIg)

The generic appellation ‘intravenous immunoglobulins’ (IVIg) refers to a therapeutic biological product containing human IgG that is prepared by large-scale industrial fractionation of human plasma. In the last 15 years, the importance of IVIg among the three main plasma-derived fractionation products (Factor VIII, albumin, and IVIg) has dramatically increased (Lemieux et al., 2005). There are an ever-increasing number of indications for which IVIg is proposed. This expanded use is accompanied by significant increases in costs of care. Further increases in IVIg use may result in product shortages and increased market prices, which may impact on the continuous treatment of some patients such as those suffering from humoral immunodeficiency for which there are no alternative therapies (Lemieux et al., 2005). The objective is not only to rationalize utilization and enhance control of the amount of IVIg currently available but also to better understand its mechanisms of therapeutic action in the most frequent indications. Such a better understanding will permit to define the composition of biotechnology-derived substitutes and eventually stabilize and even reduce the use of plasma-derived IVIg thereby securing its supply for immunodeficient patients (Lemieux et al., 2005).

1.4.1 Immunoglobulin G (IgG)

1.4.1.1 Structure

IgG, one of the antigen recognition molecules of B cells, is the protein produced by B cells in a vast range of antigen specificities. It can be produced as a stationary molecule which serves as the B-cell receptor (BCR) or secreted into the circulation as antibodies. IgG antibodies are large symmetrical multi-chain glycoproteins, having a MW of approximately 150 kDa, composed of two identical heavy (H) chains (MW 50 kDa) and two identical light (L) chains (MW 25 kDa) that are held together by disulfide bonds (Janeway et al., 2001). The amino (N) terminal of each chain possesses a variable (V) domain that binds antigen through three hypervariable complementarity-determining regions (CDRs). The variability of antibody molecules allows each antibody to bind a different specific antigen, and the total repertoire of antibodies made by a single individual is large enough to ensure that virtually any
structure can be recognized. The carboxyl (C) terminal domains of the H and L chains form the constant (C) domains which define the class and subclass of the antibody and govern whether the L chain is of the \( \kappa \) or \( \lambda \) type. (Delves and Roitt, 2000). The C domains are far less variable and are the part that interacts with effector cells and molecules.

IgG antibodies have a distorted Y shape with two arms (the Fab fragments) containing at their tips identical antigen binding sites and with the stem (the Fc fragment) joined to the Fabs by a flexible hinge. The antibody molecules are flexible at the hinge region, which allows independent movement of the two Fab arms. Some flexibility is also found at the junction between the V and C domains, allowing bending and rotation of the V domain relative to the C domain. Flexibility at both the hinge and V-C junction enables the binding of both arms of an antibody molecule to sites that are various distances apart. Flexibility at the hinge also enables the antibodies to interact with the antibody-binding proteins that mediate immune effector mechanisms (Janeway et al., 2001).

### 1.4.1.2 Protease digestion

Proteolytic enzymes (proteases) that cleave polypeptide sequences have been used to dissect the structure of antibody molecules and to determine which parts of the molecule are responsible for its various functions (Janeway et al., 2001). The protein fragments obtained after proteolysis are determined by where the protease cuts the antibody molecule in relation to the disulfide bonds that link the two H chains. These lie in the hinge region between the CH1 and CH2 domains. Papain cleaves the antibody molecule on the N terminal side of the disulfide bonds. Another protease, pepsin, cuts in the same general region of the antibody molecule as papain but on the C terminal side of the disulfide bonds (Janeway et al., 2001).

Limited digestion with the protease papain cleaves antibody molecules into three fragments: the two Fab fragments and the Fc fragment. The Fab fragments, termed for fragment antigen binding, are identical and contain the antigen-binding activity. Each of Fab fragments contains the L chain and the first two domains of the H chain. The
Fc fragment, termed for fragment crystallizable because it was originally observed to crystallize readily, contains no antigen-binding activity but is the part of the antibody molecule that interacts with effector molecules and cells. The Fc fragment contains the C terminal of C domains of the two H chains. The functional differences between H-chain isotypes lie mainly in the Fc fragment (Padlan, 1994; Janeway et al., 2001).

Digestion of the antibody molecules with the protease pepsin produces the F(ab')2 fragment in which the two antigen-binding arms remain linked. The remaining part of the H chain is cut into several small fragments, the largest of which is called the pFc' fragment. The F(ab')2 fragment has exactly the same antigen-binding characteristics as the original antibody but is unable to interact with any effector molecule (Janeway et al., 2001).

1.4.2 Composition, pharmacokinetics and administration of IVIg preparations

1.4.2.1 Composition of IVIg

Batches of IVIg are prepared by cold ethanol fractionation (Cohn’s process) of human plasma derived from pools of 3,000 to 10,000 healthy blood donors (Dwyer, 1992). The products are purified by enzymatic treatment at low pH, followed by fractionation and chromatography. Subsequent treatment with solvents and detergents ensures the inactivation of hepatitis C and other viruses. In the final product, the purified Ig is stabilized with glucose, maltose, glycine, sucrose, mannitol or albumin to prevent aggregation formation (Dalakas, 2004). The large donor pool ensures a wide spectrum of natural and induced antibody activities in the preparation, including antibodies directed against external antigens, autoantibodies and anti-idiotypes. This diverse repertoire which contains a broad range of immune antibodies against pathogens and foreign antigens is likely important for the therapeutic effect (Dwyer, 1992). Careful donor screening for HIV, human T-cell lymphotropic virus (HTLV)-1, syphilis, and for hepatitis A, B and C minimizes the potential risk of transmission of infections. The differences in basic fractionation methods and the addition of various modifications for purification, stabilization, and virus inactivation and removal
resulted in products that were significantly different from each other with respect to chemical structure, antibody content, subclass distribution, and electrophoretic profile (Lemm, 2002). Some attempts at standardization, e.g., by a WHO Expert Committee on Biologic Standardization (WHO, 1982), have mandated that Ig must be as unmodified as possible, must maintain its biological function (opsonic activity, complement fixation, FcR binding), must contain certain levels of specific antibody, and must meet accepted safety standards.

The commercial IVIg contains greater than 95% IgG with a distribution of IgG subclasses corresponding to that in normal serum, less than 2.5% IgA, a negligible amount of IgM, trace amounts of soluble CD4, CD8, human leukocyte antigen (HLA) and certain cytokines (Kazatchkine and Kaveri, 2001). Not all the IVIg preparations are the same in reference to osmolality, stabilizing agents, content of IgA, subclasses of IgG, pH, formulation (lyophilized or liquid forms), and the proportion of IgG monomers, dimers, and polymers; however, there is no evidence that the biological action is different among the various products (Dalakas, 2004). In general, currently marketed IVIg products are safe with regards to the transmission of known viruses and infections. The theoretical risk remains for the transmission of unexpected infectious agents such as prion proteins, yet no instance of spongiform encephalopathy after IVIg has ever been reported (Hahn, 2000).

1.4.2.2 Pharmacokinetics of IVIg

Following the infusion of high-dose IVIg (2 g/kg body weight) the serum IgG level increases 5-fold, but it declines by 50% in 72 hr and returns to the pretreatment level in 21–28 days (Sekul et al., 1994). The marked initial decrease reflects extravascular redistribution. The half-life of IVIg is approximately 18–32 days, which is similar to that of native Ig. The IgG in the infusion easily enters the CSF. During the first 48 hr of the infusion when the serum IgG level is high, the concentration of IgG in the CSF increases as much as 2-fold, but returns to normal within one week (Sekul et al., 1994). The infused IVIg may enter freely the end-plate region and bathe the nerve roots that lack blood-CSF barrier; it is unknown, however, whether it also exerts a local effect on those areas. Its beneficial effect is most likely exerted in the
periphery by immunomodulating the immune network and the immune effector cells. However, when IVIg is in contact with neuromuscular tissues, it may also exert a direct action on the FcRs of muscle, nerve, endothelial cells, or on the myelin sheath (Dalakas, 1999).

1.4.2.3 Administration of IVIg

The therapeutic dose of IVIg is empirically set at 2 g/kg body weight which is divided into five daily doses of 400 mg/kg body weight each, based on the dose that is used in other autoimmune diseases. However, it has been advocated to divide the total dose into two daily doses of 1 g/kg body weight each, especially in younger patients who have normal renal and cardiovascular function (Dalakas, 1999). The 2-day infusion is not associated with more adverse reaction than the 5-day infusion. The rate of infusion should not exceed 200 mL/h or 0.08 mL/kg per minute.

1.4.3 Immunomodulatory action of IVIg for GBS

The precise mechanisms by which IVIg exerts its immunomodulatory and beneficial effects for GBS remains only incompletely understood. Taking into account evidence from multiple in-vivo and in-vitro experiments, several interactions with various steps of the amplification and effector phases of the immune responses are likely to occur and most probably they operate in concert (Hahn, 2000).

1.4.3.1 Effect on autoantibodies

Infused IVIg affects autoantibodies by three mechanisms (Dalakas, 2004). First, it supplies anti-idiotypic antibodies, which have the potential to bind to and neutralize pathogenic auto-antibodies, preventing them from binding to their target antigens. The F(ab’)_2, but not the Fc portion of the IgG in the IVIg preparations, inhibits the binding of anti-GM1 antibodies to GM1-coated enzyme-linked immunosorbent assay (ELISA) plates and the binding of cholera toxin to GM1 ganglioside, in a dose-dependent manner (Malik et al., 1996). If these antibodies are pathogenic, their inhibition by IVIg should suppress nerve damage and improve a patient’s symptoms. In addition, in an in vitro nerve-muscle preparation, the F(ab’)2 fragments of IVIg neutralized the
“blocking” effect on neuromuscular transmission exerted by the serum of acute GBS patients which contains various IgG glycolipid antibodies (Buchwald et al., 2002). In this study, the F(ab′)2 portion rather than Fc portion of IVIg was as effective as whole IVIg and the effect in reducing blockade was dose-dependent. Moreover, IVIg was shown to inhibit pathophysiological effects of anti-GQ1b-positive sera at motor nerve terminals through inhibition of antibody binding and to displace antibodies already bound to GQ1b in an ELISA (Jacobs et al., 2003). Second, the anti-idiotypic antibodies within IVIg may reduce antibody production through an inhibitory signal produced by cross-linking of Fc gamma receptor (FcγR) IIb to surface IgM or IgG molecules on B cells (Diegel et al., 1994) and through affecting the autoantibody-producing CD20+ (B1) subset of B cells by antibodies against CD5 molecules (Vassilev et al., 1993). Third, IVIg may accelerate the catabolism of serum IgG antibodies by saturating the protective transport receptors, neonatal FcR (FcRn), on endothelial cells and thus reduce the level of pathogenic auto-antibodies. Normally, IgG antibodies bind to FcRn to return intact in the circulation and avoid degradation in the lysosomes (Yu and Lennon, 1999). Saturation of FcRn with IVIg would inhibit the recycling and enhance catabolism of pathogenic auto-antibodies.

1.4.3.2 Inhibition of complement binding and prevention of MAC formation

IVIg has been shown to bind to complement factors C3, C4, C3a and C5a, and to inhibit the incorporation of C3b molecules into the C5 convertase assembly (Pollard, 2004). In dermatomyositis, IVIg inhibited the uptake of C3b and C4b fragments by formation of covalent or noncovalent complexes between C3 and specific receptor within the infused IgG molecules (Basta and Dalakas, 1994). Such an inhibition limits the available C3 molecules for further incorporation into the C5 convertase assembly, and prevents the formation and in situ deposition of MAC on the endomysial capillaries. These findings are pertinent to GBS since complement deposition has been shown on the SC surface in both AIDP and EAN (Stoll et al., 1991; Hafer-Macko et al., 1996b). In addition, IVIg has been shown to reduce complement binding by anti-GQ1b antibodies from MFS in vitro and complement activation in the mouse diaphragm model ex vivo (Jacobs et al., 2003).
1.4.3.3 Modulation or blockade of FcRs on macrophages

IVIg may induce blockade of the FcRs on phagocytic cells by saturating, altering, or down-regulating the affinity of the FcRs, a process that may render sensitized phagocytic cells unable to exert their action (Dalakas, 2004). There are specific FcRs for each class of Ig. IgG molecules bind through their Fc region to FcγR on hematopoietic cells, such as macrophages (Kazatchkine and Kaveri, 2001). Macrophages play a central role in demyelination in GBS and EAN. A blockade of the FcRs on the macrophages could inhibit the macrophage-mediated phagocytosis of antigen-bearing target cells and might prevent the macrophage-mediated demyelination. Although the FcγR blockade induced by IVIg has not been shown in situ or in the circulation in GBS, it may contribute to the efficacy of IVIg on demyelinating neuropathies (Dalakas, 1999).

1.4.3.4 Suppression of pathogenic cytokines and AMs

Modulation of the production of cytokines and cytokines antagonists is likely to be a major mechanism of the anti-inflammatory effects of IVIg (Kazatchkine and Kaveri, 2001). In vitro and in vivo studies have shown that IVIg causes a dose-dependent down-regulation of tissue expression or reduction in the circulating levels of cytokines, such as IL-1, IL-1β, TNF-α, IL-1β, TGF-β, and TGF-β mRNA (Abe et al., 1994; Aukrust et al., 1999; Amemiya et al., 2000). Because upregulation of cytokines is critical in patients with demyelinating neuropathies, the ability of IVIg to downregulate cytokine production is important and relevant to its action in GBS (Dalakas, 2002). In GBS patients, circulating levels of TNF-α and IL-1β decreased after treatment with IVIg, and the reduction was associated with the clinical improvement linked with IVIg treatment (Sharief et al., 1999). IVIg has been also shown to downregulate the expression of surface AMs such as ICAM-1 on the endothelial cells and LFA-1 on activated T cells, which are essential for the process of transmigration of T cells from the circulation into the endoneurium (Créange et al., 2003).
1.4.3.5 Modulation of T-cell function and antigen recognition

IVIg contains natural anti-CD4 antibodies which blocked T-cell proliferation in vitro and natural anti-MHC class I antibodies which blocked CD8\(^+\) T cell-mediated cytotoxicity in vitro (Simon and Spāth, 2003). It also contains anti-idiotypic antibodies against components of the TCR. In addition, there are a variable amount of solubilized CD4, CD8, HLA-I, HLA-II molecules, and TGF-\(\beta\) that may interfere with antigen recognition by T cells (Dalakas, 2004).

1.4.3.6 Interaction with APCs

IVIg has been shown to inhibit the differentiation and maturation of dentritic cells and down-regulate costimulatory molecules associated with cytokine secretion and antigen presentation (Bayry \textit{et al.}, 2003). Such an effect is relevant to all the autoimmune diseases in which IVIg exerts an immunomodulatory effect.

1.4.3.7 Effect of substances other than antibody within IVIg preparations

IVIg may contain cytokines and other molecules including soluble cytokine inhibitors, soluble CD4, and MHC class II. Stabilizing agents, mainly various sugars, exert an effect (Sewell and Jolles, 2002). Both maltose and sucrose, at concentrations present in commercial IVIg preparations, can inhibit PHA-induced, and to a lesser extent PMA-induced, mononuclear cell proliferation in vitro. Maltose, but not sucrose was able to inhibit an anti-CD3 induced response. Significant quantities of TGF-\(\beta\)1 and TGF-\(\beta\)2, which have been found in IVIg preparations, may have antiproliferative effects (Sewell and Jolles, 2002).

1.4.3.8 Possible effect on remyelination

Treatment with IVIg suppresses EAN (Gabriel \textit{et al.}, 1997) and GBS, probably via a combined effect on the immunoregulatory network mentioned above. Whether it also enhances remyelination by an effect directly on the myelin sheath remains to be determined (Dalakas, 2004).
1.5 IgG Fc receptors (FcγRs)

FcγR constitutes a family of cell-surface molecules expressed mainly on hematopoetic cell. In the PNS, FcγR has been demonstrated on SCs, perineurial cells, endothelial cells and scattered endoneurial macrophages (Vedeler et al., 2001). The receptors were found on the surface membrane, inner membrane or axolemma and on vesicles within the cytoplasm of SCs by EM (Vedeler and Fitzpatrick-Kløve, 1990). SCs in culture apparently lose their FcγR expression. FcγR has been recognized in fetal nerves at approximately 10 weeks of gestation, showing that the receptors are an innate component of the PNS (Vedeler and Fitzpatrick-Kløve, 1990).

1.5.1 FcγR polymorphisms and functions

Three subclasses of human leukocyte FcγR are currently distinguished, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), that differ in cell distribution, function and affinity for IgG isotypes (Takai, 2005). A total of 8 genes have been identified for the FcγR: 3 genes for FcγRI (FcγRIa, FcγRIb, and FcγRIc); 3 genes for FcγRII (FcγRIIa, FcγRIIb, and FcγRIIc); 2 genes for FcγRIII (FcγRIIIa and FcγRIIIb). In addition to leukocyte FcγR, endothelia, several mucosal epithelia, and placental trophoblasts express a structurally distinct IgG receptor class, FcRn, which consists of an α-chain, structurally related to MHC class I molecules and complexed with β2-microglobulin (van Sorge et al., 2003).

FcγR expression is regulated by cytokines or growth factors (van Sorge et al., 2003). FcγRI, which is constitutively expressed on the surfaces of mononuclear phagocytes including monocytes, macrophages and dendritic cells, binds monomeric and complexed IgG with high affinity. FcγRI can be induced on polymorphic neutrophils by granulocyte-colony-stimulating factor (G-CSF) or IFNγ. FcγRIIa is the most widely distributed subclass and is expressed on virtually all myeloid cells. FcγRIIb is expressed on phagocytes and B cells and the expression is increased by IFNγ and decreased by IL-4 (Pricop et al., 2001). FcγRIIc is expressed on NK cells. FcγRII binds monomeric IgG with low to undetectable affinity. FcγRIIIa is present on monocytes, macrophages, NK cells and γ/δ T cells, whereas FcγRIIIb is constitutively
expressed on neutrophils, and can be induced on eosinophils by IFNγ. Like FcγRII, FcγRIII is a low-affinity receptor for IgG.

Two general classes of FcγR are now recognized (Ravetch and Bolland, 2001): the activation receptors (FcγRI, FcγRIIa and FcγRIIIa), characterized by the presence of a cytoplasmic immune-receptor tyrosine-based activation motif (ITAM) sequence associated with the receptor, and the inhibitory receptor (FcγIIb), characterized by the presence of an immune-receptor tyrosine-based inhibitory motif (ITIM) sequence. Activation FcγRs are found on most effector cells of the immune system, notably monocytes, macrophages, NK cells, mast cells, eosinophils, neutrophils, and platelets, while absent from lymphoid cells (Ravetch and Bolland, 2001). Upon crosslinking, the activation FcγRs triggers an activating signaling cascade, resulting in a cell type-specific response such as ADCC by macrophages and monocytes (Daëron, 1997). The inhibitory FcγR, FcγRIIb, is capable of inhibiting activation by ITAM-bearing receptors. FcγRIIb may exert inhibitory effects on monocytes/macrophages effector functions and co-ligation of FcγRIIb with ITAM-containing receptors results in down-regulation of antibody responses in vitro as well as in vivo (van Sorge et al., 2003). These two classes of receptors function in concert and are usually co-expressed on the same cell surface. Because activation and inhibitory receptors bind IgG with comparable affinity and specificity, co-engagement of both signaling pathways is thus the rule, setting thresholds for and ultimately determining the magnitude of effector cell responses.

By linking humoral and cellular immune responses, FcγR plays a key role in host defence mechanisms through binding of the Fc domain of IgG to FcγRs (Takai, 2005). First, engagement of the activation FcγRs induces a plethora of cell type specific biological functions, including phagocytosis, endocytosis, degranulation, and the transcriptional activation of cytokine genes, leading to inflammatory cascades and ADCC while engagement of the inhibitory FcγIIb simultaneously downregulates these effector responses. The second function is the clearance of immune complexes (ICs) and enhancement of antigen presentation. FcγRs can internalize the captured ICs leading to homeostatic degradation of the complexes in macrophages as well as directing the degraded antigenic peptides to the antigen presentation pathway in
dendritic cells. The third function is protection of IgG from catabolism and IgG transport. IgG binds maximally to FcRn in the acidic conditions of the endosome in endocytotic vesicles and subsequently returns intact to the circulation (Yu and Lennon, 1999). Without this protective mechanism, IgG would pass to the lysosome and be degraded. Furthermore, FcRn can transport IgG across polarized epithelia, such as placenta and mucosa (van Sorge et al., 2003).

FcγR polymorphisms may influence the degree of the inflammatory response and may contribute to the differences in susceptibility to infectious and autoimmune diseases or the disease severity (van Sorge et al., 2003). Efficient FcγR-IgG interactions increase efficacy of leukocyte effector functions such as phagocytosis, cellular cytotoxicity, and cytokine production in vitro and specific FcγRII and FcγRIII genotypes have been found associated with autoimmune disease. Furthermore, FcγR polymorphisms influence effectiveness and side-effects of immunotherapy, which might provide a useful prognostic marker for treatment in the future.

1.5.2 FcγR polymorphisms and the pathogenesis in GBS

The association of FcγR genotypes with susceptibility to and severity of GBS has been investigated in three studies (van der Pol et al., 2000; Vedeler et al., 2000; van Sorge et al., 2005). The first study (van der Pol et al., 2000) documented the association between FcγRIIa-H/H131 genotype and susceptibility to GBS, as well as an increased risk for severe disease, whereas the second study (Vedeler et al., 2000) reported an association between FcγRIIIb-NA1/NA1 and mild disease. In the study by van Sorge et al (van Sorge et al., 2005), FcγRIIa, IIIa, and IIIb genotypes and allelic frequencies in two cohorts consisting of 192 Dutch and 91 British patients were determined and the data of two previous studies were subjected to meta-analysis. No association between FcγRIIa or other FcγR polymorphisms and the occurrence of GBS can be shown, suggesting that FcγR single nucleotide polymorphisms do not constitute significant risk markers for susceptibility to GBS. However, an association of FcγRIIIb genotypes, and a trend of FcγRIIIa genotypes, with GBS severity was shown, in which FcγRIIIb-NA1/NA1 genotype was significantly correlated with a lower mean maximal disability score and FcγRIIIa-F158 homozygotes suffered less
often from severe disease. Overall, FcγR genotype distributions did not differ between patients with GBS and control subjects. However, FcγRIIIa and FcγRIIIb genotypes were associated with severity of GBS, and may therefore represent mild disease-modifying factors in the pathogenesis of GBS.
1.6 Aims of this study

The work described in this thesis is primarily concerned with the mechanisms of action of IVIg in the treatment of EAN in Lewis rats in order to define the composition of biotechnology-derived substitutes in GBS and eventually stabilize and even reduce the use of plasma-derived IVIg thereby securing its supply for immunodeficient patients. The specific aims are as follows:

1. To test the efficacy of immunoglobulin and its Fab and Fc fragment in the treatment of EAN in Lewis rats.

2. To investigate which portion of immunoglobulin is operative in the effect of IVIg on EAN.

3. To clarify the possible mechanisms by which immunoglobulin exerts its action in the treatment of EAN in rats.
Chapter 2  Materials and Methods

2.1  Experimental design

EAN was induced by immunization with whole bovine PNM in adult inbred Lewis rats. In all groups of animals, clinical disease severity is measured by clinical score, weight and electrophysiological parameters monitored at regular intervals. Treatment of actively induced EAN was administered once daily for 2 days commencing from the onset of neurological deficit. Animals from each group were randomly chosen and sacrificed at predetermined intervals for histological analysis. The studies contained three sets of experiments: comparison of treatment efficacy between the normal saline and albumin, comparison of treatment efficacy between the albumin and IVIg, and comparison of treatment efficacy among the albumin, Fab fragments, Fc fragments and IVIg. The clinical and electrophysiological studies were conducted in a blinded fashion.

2.1.1  Experiment 1: comparison of treatment with normal saline and albumin

Immunized rats were randomised into two groups. Group 1 (n=9) received intravenous injection (i.v.) of 0.9% saline the same volume as albumin once daily for 2 days. Group 2 group (n=9) received i.v. 100 mg/100 g body weight albumin once daily for 2 days. All the 18 rats were weighed and examined for evidence of weakness before inoculation and daily for 30 days and received electrophysiological studies at predetermined intervals.

2.1.2  Experiment 2: comparison of treatment with albumin and IVIg

Immunized rats were randomised into two groups. Group 1 (n=25) received i.v. 100 mg/100 g body weight albumin once daily for 2 days. Group 2 (n=25) received 100 mg/100 g body weight IVIg once daily for 2 days. Of the 25 rats from each group, 17 rats were weighed and examined for evidence of weakness before inoculation and
daily for 30 days. Of these 17 rats, 12 rats received electrophysiologic studies at predetermined intervals. To evaluate the corresponding histopathological alterations of EAN, 12 animals from each group were randomly chosen and sacrificed on days 16 (n=2), 18 (n=2), 22 (n=2), 26 (n=2) and 30 (n=4).

2.1.3 Experiment 3: comparison of treatment with albumin, Fab fragments, Fc fragments, and IVIg

Immunized rats were randomised into four groups. Group 1 (n=8) received i.v. 100 mg/100 g body weight albumin once daily for 2 days. Group 2 (n=8) received i.v. 70 mg/100 g body weight Fab fragments once daily for 2 days. Group 3 (n=8) received i.v. 35 mg/100 g body weight Fc fragments once daily for 2 days. Group 4 (n=9) received 100 mg/100 g body weight IVIg once daily for 2 days. The doses of injections in group 2 and 3 were chosen based upon the molarity of the proteins. All treatments were given from the onset of neurological deficit. All the 33 rats received clinical evaluation daily and electrophysiologic studies at predetermined intervals followed by being sacrificed at the end of the observation period (day 30) for histopathological studies.
2.2 Experimental autoimmune neuritis (EAN)

2.2.1 Experimental animals

Eight to twelve weeks old male and female Lewis rats were bred and housed in a non-specific pathogen-free animal facility (Blackburn Building Animal House, University of Sydney, Australia). All procedures were conducted in accordance with protocols approved by the Animal Care and Ethics Committee of the University of Sydney.

2.2.2 Anesthesia

All invasive procedures, including immunization by foot pad inoculation, intravenous injection, and electrophysiological measurements, were performed under anaesthesia using halothane (Lyppard, Australia)/O2 (2% halothane, 0.5 litres/minute oxygen). The surgical procedures, including cardiac exsanguinations and perfusion for histology, were performed under pentobarbitone sodium (Nembutal®, Lyppard, Australia) anaesthesia. An intraperitoneal dose of 30 mg/kg was used for female rats and a dose of 60 mg/kg was used for male rats.

2.2.3 Preparation of myelin antigen

Myelin was isolated from bovine cauda equina according to the method described by Norton and Poduslo (Norton and Poduslo, 1973). Fresh bovine spinal cord and cauda equina were obtained from the abattoirs. In brief, the spinal roots were isolated from the spinal cord, stripped of fat and connective tissue and weighed. The remaining tissues were homogenized with 0.32 M sucrose (weight: volume ratio 1:20). The homogenate was centrifuged at 4000g for 15 minutes at 4°C and the top layer of fat material discarded. The remaining supernatant was layered over 0.85 M sucrose in 50 ml centrifuge tubes (Beckman®, USA) and centrifuged at 75,000g for 30 min at 4°C (L8-M Ultracentrifuge, Beckman®, USA). The crude myelin which formed at the interface between the two sucrose solutions was collected with a pipette and the remainder discarded. The crude myelin was suspended in water by homogenization and then submitted to the first and second osmotic shock with distilled water (myelin
to water volume ratio 1:4), centrifuged at 12,000g for 15min at 4°C each with supernatant fluid discarded. The myelin pellets were resuspended in 0.32 M sucrose. The suspension was layered over 0.85 M sucrose and again centrifuged at 75,000g for 30 min at 4°C. The purified myelin was collected from the interface with a pipette and washed with water for at least four times. The myelin sample was lyophilized in a freezing vacuum machine in a blue-topped plastic container (Greiner Labortechnik, Germany). The lyophilized sample was stored at -20°C for further use.

2.2.4 Induction of EAN by immunization with bovine PNM

Lyophilized bovine PNM was emulsified with *Mycobacterium tuberculosis* (strain H37Ra; BD Bioscience, Franklin Lakes, NJ, USA) in a 1:1 phosphate buffered saline (PBS): incomplete Freund’s adjuvant (Sigma, St. Louis, MO, USA) solution at a concentration of 50 mg/ml myelin and 5 mg/ml *Mycobacterium*. The emulsion was considered to be of the desired consistency if a drop placed in cold water did not disperse within 5 minutes. EAN was induced in male and female Lewis rats by immunization in each hind foot pad with 0.05 ml of emulsion (0.1 ml/rat).

2.2.5 Clinical score

Rats were examined for evidence of weakness before inoculation and daily for 30 days. Clinical disease severity was scored from 0 to 5 according to the following scale (Spies *et al.*, 1995b):

0 -- normal;
1 -- limp tail;
2 -- mild hind limb weakness, abnormal posture or waddling gait;
3 -- moderate hind limb weakness, difficulty walking;
4 -- paraplegia, useless hind limbs;
5 – moribund.

The weight of each rat is recorded throughout the illness and weight change from baseline used as another measure of disease severity.
2.2.6 Electrophysiologic studies

Electrophysiological studies were performed prior to footpad inoculation and on days 8, 10, 12, 14, 16, 18, 22, 26 and 30 after footpad inoculation. Following anaesthesia using halothane/O2, the nerve was stimulated through paired needle electrodes. Sciatic nerve motor conduction and spinal somatosensory evoked potentials (SSEPs) after tibial nerve stimulation were studied using a Neuromax™ electrophysiological machine (XLTEK, Ontario, Canada).

2.2.6.1 Sciatic nerve motor studies

The sciatic nerve was stimulated at the ankle and the sciatic notch. Supramaximal rectangular pulses of 0.1 ms duration were applied and the compound muscle action potential (CMAP) recorded from the dorsal foot muscles through stainless steel 30-gauge needle electrodes. The active recording electrode was inserted subcutaneously in the mid-dorsum of the foot and the reference electrode subcutaneously in the lateral toe. An earth electrode was placed on the lateral border of the foot proximal to the recording electrode.

For each study the amplitude of the CMAP evoked by stimulation at ankle and sciatic notch was measured. To compare the extent of conduction block from animal to animal the ratio of the amplitudes of CMAPs from proximal (hip, H) compared to distal (ankle, A) stimulation (H/A ratio) was measured. A reduction of greater than 20% in the H/A ratio was regarded as conduction block (Pollard et al., 1995). Motor nerve conduction velocity (MCV) was calculated over the hip to ankle segment as a measure of distal motor conduction.

2.2.6.2 Spinal somatosensory evoked potentials (SSEPs)

Rectangular pulses of 0.1 ms duration with stimulus intensities just above the motor threshold were applied to the tibial nerve at the ankle. The spinal evoked responses (S wave) were recorded with the active electrode placed between the spinous processes of T13 and L1 (Wiethölter and Hülser, 1985) and the reference electrode inserted subcutaneously just proximal to this. The earth electrode was inserted subcutaneously in the thigh. The latency of the S wave was used as a measure
of sensory conduction through the length of the peripheral nerve, the dorsal root ganglia, the dorsal root, and a small segment of the dorsal column. Two averaged traces, each containing at least 20 responses, were recorded for each nerve (Spies et al., 1995b).

### 2.2.7 Histological techniques

Under anaesthesia rats were perfused through the left ventricle with 150 ml of normal saline followed by 150 ml of 4% paraformaldehyde (Sigma, St. Louis, MO, USA) and 2.5% glutaraldehyde (Chem-Supply, Australia) in 0.1 M PBS. A midline dorsal incision was made from the cervical region to the base of the tail and the skin and subcutaneous tissues reflected. The paravertebral muscles were dissected off the spinal column and the posterior elements of the vertebral column removed to expose the spinal cord. The bony margins of the nerve root exit foramina were carefully removed to expose the exiting spinal roots. Further incisions were made from the sciatic notch to the knee bilaterally and the sciatic nerves exposed. The sciatic nerves and corresponding spinal motor roots were removed.

The nerves were fixed in 2.5% glutaraldehyde (Chem-Supply, Australia) solution overnight at 4°C, washed in 0.1 M PBS and post-fixed in Dalton’s chrome osmium solution containing 1% osmium (Johnson Matthey Chemicals, England) and 2% dichromate (BDH Laboratory Supplies, Poole, England) at 4°C for 2 hours. After fixation, they were dehydrated in increasing concentrations of ethanol (30% to 100%) followed by acetone and then embedded in Spurr’s resin (ProSciTech, Australia). Transverse sections of 0.5μm thick were cut on a microtome and stained with 0.5% toluidine blue for examination by LM.

### 2.2.8 Morphometric analysis

Transverse toluidine blue-stained epoxy sections were examined and photographed at x400 using an Olympus AH-2 microscope (Japan). Intraneural blood vessels were identified and the surrounding areas examined. Coded sections containing a total of approximately 100 such perivascular areas per animal were
evaluated and graded semiquantitatively according to the method described by Heininger and others using the following scale (Heininger et al., 1988):

0 -- normal perivascular area;
1 -- mild cellular infiltrate adjacent to a vessel;
2 -- cellular infiltrate plus demyelinating fibres adjacent to a vessel;
3 -- cellular infiltrate plus demyelination around a vessel and at more distant sites and minor axonal damage.

The percentage of perivascular areas with a given grade of inflammation and demyelination was calculated for each group.

2.2.9 Statistical analysis

In experiment 1, clinical scores were compared using the Mann-Whitney U test. Weight change and electrophysiological parameters were compared using unpaired t-tests. A p value less than 0.05 was considered significant. In experiment 2, clinical scores and histological findings were compared using the Mann-Whitney U test. Weight change and electrophysiological parameters were compared using unpaired t-tests. A p value less than 0.05 was considered significant. In experiment 3, the overall differences in the clinical scores and histological findings among the four groups were tested by the Kruskal-Wallis one-way analysis of variances (ANOVA). The Mann-Whitney U-test was applied to test the differences between pairs of groups. The overall differences in the weight changes and electrophysiological parameters among the four groups were compared using one-way ANOVA. The unpaired t-test was applied to determine the significance between pairs of groups. A p value less than 0.05 was considered significant. All statistical tests were performed with SPSS® 12.0 (Chicago, IL, USA).
2.3 IVIg fractions

2.3.1 Human IVIg and albumin

Human IVIg (Intragam® P) and albumin (20% NSA) were obtained from CSL Bioplasma, Hong Kong. Intragam® P, pH 4.25, is made by chromatographic fractionation of large pools of human plasma obtained from voluntary blood donors and contains 6 g of human protein and 10 g of maltose in each 100 mL. At least 98% of the protein has the electrophoretic mobility of IgG. Only trace amounts of IgA (nominally < 0.025 mg/mL) are found. At least 90% of the protein is IgG monomer and dimer. Based on data from three preclinical and four clinical batches, the IgG subclasses in Intragam® P are, on the average, 61% IgG1, 36% IgG2, 3% IgG3 and 1% IgG4. Intragam® P is intended for intravenous administration.

20% NSA is prepared from pooled human plasma obtained from voluntary donors. The composition of 20% NSA is as follows: human albumin 200 g/L; sodium 48 to 100 mmol/L; Octanoate 32 mmol/L. The manufacturing process of both Intragam® P and 20% NSA contains specific steps to reduce the possibility of virus transmission including pasteurisation (heating at 60°C for 10 hours) and incubation at low pH.

2.3.2 Preparation of Fab and Fc fragments

2.3.2.1 Equipment

Biologic LP Low Pressure Liquid Chromatography System
Bio-Rad Life Science Group, Hercules, CA, USA

Mini-PROTEAN® II Electrophoresis Cell
Bio-Rad Life Science Group, Hercules, CA, USA

Molecular Imager Gel Doc™ XR System
Bio-Rad Life Science Group, Hercules, CA, USA

PowerPac 300 Power Supply
Bio-Rad Life Science Group, Hercules, CA, USA

Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell
Bio-Rad Life Science Group, Hercules, CA, USA
### 2.3.2.2 Reagents

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<tr>
<th>Name of chemicals</th>
<th>Supplier information</th>
<th>Catalogue number</th>
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<td>Acrylamide/bis-acrylamide 30% solution (Mix ratio 37.5:1)</td>
<td>Sigma, St. Louis, MO, USA</td>
<td>A3699</td>
</tr>
<tr>
<td>Alkaline phosphatase (AP) conjugate substrate kit</td>
<td>Bio-Rad Life Science Group, Hercules, CA, USA</td>
<td>170-6432</td>
</tr>
<tr>
<td>Anti-human IgG (Fab specific) AP conjugate antibody</td>
<td>Sigma, St. Louis, MO, USA</td>
<td>A8542</td>
</tr>
<tr>
<td>Anti-human IgG (Fc specific) AP conjugate antibody</td>
<td>Sigma, St. Louis, MO, USA</td>
<td>A9544</td>
</tr>
<tr>
<td>Immobilized papain</td>
<td>Pierce, Rockford, IL, USA</td>
<td>20341</td>
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<tr>
<td>ImmunoPure® plus immobilized Protein A gel</td>
<td>Pierce, Rockford, IL, USA</td>
<td>22812</td>
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<td>L- cysteine monohydrochloride (Cysteine•HCl)</td>
<td>Fluka, Switzerland</td>
<td>30119</td>
</tr>
<tr>
<td>Micro BCA™ protein assay reagent kit</td>
<td>Pierce, Rockford, IL, USA</td>
<td>23235</td>
</tr>
<tr>
<td>SeeBlue® plus2 pre-stained standard</td>
<td>Invitrogen, Carlsbad, CA, USA</td>
<td>LC5925</td>
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<tr>
<td>Sepharcryl™ S-100HR</td>
<td>Amersham Biosciences AB, Uppsala, Sweden</td>
<td>17-0612-01</td>
</tr>
</tbody>
</table>
2.3.2.3 Buffers

**Antibody buffer**
1% (w/v) bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) in TTBS

**Binding buffer**
10 mM Tris (Sigma, St. Louis, MO, USA), pH 7.5

**Blocking solution**
5% (w/v) nonfat milk in Tris buffered saline (TBS)

**Dialyzing buffer**
20 mM sodium phosphate, 10 mM Ethylenediaminetetraacetic Acid Tetrasodium Salt (EDTA-Na₄) (Sigma, St. Louis, MO, USA), pH 7.0

**Digestion buffer**
20 mM sodium phosphate, 20 mM cysteine•HCl, and 10 mM EDTA-Na₄, pH 7.0

**Dulbecco’s PBS**
0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.3

**5x Electrode buffer (5x in stock)**
192 mM glycine (Fluka, Switzerland), 25 mM Tris, and 0.1% (v/v) sodium dodecyl sulfate (SDS)

**Elution buffer**
0.1 M glycine, pH 2.8

**Neutralization buffer**
1M Tris, pH 9.0

**10% resolving gel**
375 mM Tris, pH 8.8, 10% (v/v) acrylamide/bis-acrylamide, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate (Sigma, St. Louis, MO, USA), and 0.05% (v/v) N’, N’, N’, N’-tetramethylethyle-nediamine (TEMED) (Sigma, St. Louis, MO, USA)

**SDS sample buffer**
62 mM Tris, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol (Sigma, St. Louis, MO, USA), and 0.025% (v/v) bromophenol blue (Sigma, St. Louis, MO, USA)

**4% stacking gel**
125 mM Tris, pH 6.8, 4% (v/v) acrylamide/bis-acrylamide, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, and 0.05% (v/v) TEMED

**Tranfer buffer**
192 mM glycine, 25 mM Tris-HCl, and 20% (v/v) methanol

**Tris buffered saline (TBS)**
10 mM Tris and 250 mM NaCl, pH 7.5

**TTBS**
TBS with 0.05% (w/v) Tween-20

### 2.3.2.4 Procedures

**I. Cleavage of IgG molecule with papain**

IgG solution was dialyzed extensively against the dialyzing buffer overnight. The papain gel-glycerol-water slurry was mixed by inversion, washed twice with the digestion buffer and then resuspended in the digestion buffer in the polypropylene column (Pierce, Rockford, IL, USA). Dialyzed IgG solution was added to the column containing the papain gel at an enzyme: substrate ratio 1: 160 w/w and incubated overnight in the shaking water bath (Thermoline Scientific, Australia) at 37°C. The digests composed of solubilized Fab, Fc fragments and undigested IgG were separated from the immobilized papain gel by filtration using a polypropylene column and porous polyethylene disc (30 μm pore size, Pierce, Rockford, IL, USA). The recovered papain gel was regenerated by washing with digestion buffer twice and dialyzing buffer three times.

**II. Affinity chromatography**

The separation of Fab fragment from Fc and undigested IgG was carried out by affinity purification using a protein A column on the Bio-Rad biologic LP low pressure liquid chromatography system. The ImmunoPure® plus immobilized Protein A gel was packed in a C 16/20 column (Amersham Biosciences AB, Uppsala, Sweden). The protein A column was equilibrated with 5 column volumes of the binding buffer. The crude digest of less than 80% of column capacity was applied to the column followed by washing with the binding buffers, approximately 10 to 15 column volumes. The plateau of the flow-through containing Fab fragment was collected (Figure 2.1). The bound proteins were eluted with 3 column volumes of the elution buffer. The eluent containing Fc/IgG was immediately adjusted to a neutral
pH by addition of neutralization buffer. The column was regenerated by washing with 5 column volumes of elution buffer, followed by the binding buffer.

Figure 2.1 Separation of Fab fragments from Fc fragments and undigested IgG molecules by affinity chromatography. The Fab fragments were collected from the flow-through after the digested mixture was passed through the column and the Fc fragments/IgG molecules were collected from the second peak.

III. Dialysis and concentration of protein

Fab fragments and Fc/IgG solutions were dialyzed and concentrated against PBS using Vivaspin 20 apparatus (10,000 MWCO) following the Manufacture’s instructions.
IV. Size exclusion chromatography (gel filtration)

The separation of Fc from IgG was achieved by size exclusion. The gel filtration column packed with Sepharcryl™ S-100HR in an Econo-Column® chromatography column (2.5x75 cm, 344 ml, Bio-Rad Life Science Group, Hercules, CA, USA) was equilibrated with one-half column volume of distilled water at a flow rate of 1.3 ml/min followed by two column volumes of PBS at 2.6 ml/min. 3ml of concentrated Fc/IgG solution was loaded through a sample loop and the sample was run at a flow rate of 1.3 ml/min. The first peak was collected as undigested IgG and the second peak as Fc fragments (Figure 2.2). The column was regenerated after each run with one column volume of PBS at 2.2 ml/min. The collected Fc and IgG solutions were concentrated using Vivaspin 20.

![Figure 2.2 Separation of Fc fragments from undigested IgG molecules by gel filtration. IgG was collected from the first peak and Fc fragments from second peak.](image-url)
V. **Protein assay**

Micro BCA™ protein assay reagent kit containing the MA, MB, and MC reagents was used for the determination of final concentrations of purified Fab and Fc solutions. Albumin standards (2mg/ml) diluted into graded concentrations from 2.5 to 160 µg/ml, Fab and Fc solutions diluted 1 in 200, 1 in 400, and 1 in 800 with 0.9% saline were added to the 96-well microtitre plate (ICN Biomedicals, Irvine, CA, USA) in duplicate, 150 µl/well. The working reagent in which the MA, MB, and MC reagents were mixed in the ratio of 25: 24: 1 was added to the plate, 150µl/well. The plate was then covered and incubated for 2 hours at 37ºC and read with the Ultramark™ Microplate Reader at 550 nm wavelength. A linear standard curve of optical density (OD) readings was derived from the albumin standards. The protein concentrations of purified Fab and Fc solutions were then determined by referring to the standard curve.

VI. **SDS-PAGE (SDS-polyacrylamide gel electrophoresis)** (Laemmli, 1970)

All the procedures described below were performed using the Mini-PROTEAN® II electrophoresis system. A 10% SDS-polyacrylamide resolving gel was poured into the sandwich clamp assemblies on the casting stand of Mini-PROTEAN® II system leaving 4 cm space at the top. The gel was overlaid with isobutanol and left to polymerize followed by polymerizing a 4% stacking gel on the top. A plastic comb was pushed into the solution and removed after polymerization. The clamp assemblies were then snapped onto the inner cooling core to form the upper buffer chamber, which together with the lower chamber, were filled with the electrode buffer.

The purified Fab and Fc solutions were mixed with sample buffer and heated at 100ºC for 1 min. A SeeBlue® plus2 pre-stained standard ranging in MW from 4 to 250 kDa was loaded onto one well. 0.5 or 1 µg of Fab or Fc fragment was loaded onto each well and run at 100V using an electrophoresis constant power supplier (PowerPac 300 power supply) until the sample reached the base of the gel. The gel was then removed from the clamp assemblies and soaked in the transfer buffer for 15 min in readiness for transferring and immunoblotting.
VII.  Transfer and immunoblotting

Immun-Blot® polyvinylidene difluoride (PVDF) membrane (Bio-Rad Life Science Group, Hercules, CA, USA) was cut to sized and pre-wetted consecutively with methanol for 1 min, distilled water for 1 min three times and finally transfer buffer for 15 min. The filter papers (Amersham Biosciences, England) was cut to size and soaked in transfer buffer for 15 min before a transfer sandwich was assembled in the semi-dry transfer cell (Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell). The transfer was performed at 15 V, 250 mA for 30 min supplied by PowerPac 300 power supplier at room temperature.

Following transfer, the membrane was blocked in blocking solution for 1 hour with gentle agitation using an orbital shaker platform. The membrane was then washed with TTBS for 10 min and cut into two pieces, each containing one lane of SeeBlue® standard, one lane of Fab and one lane of Fc sample. One piece of the membrane was incubated with anti-Fab fragment antibody and the other with anti-Fc fragment antibody on the shaker platform for 1 hour at room temperature. After incubation, the membrane was washed with TTBS for 10 min three times followed by TBS for 10 min. The blots were then detected with AP conjugate substrate kit in which the developer was prepared just prior to use by mixing reagent A, B, and C in the ratio of 1: 1: 98 followed by analyzed and photographed using the Gel Doc™ XR system (Figure 2.3 and Figure 2.4).
Figure 2.3 Western blotting with anti-Fab fragment antibody. The standard molecular weight markers were shown in Lane A. Lanes B and D were loaded with Fab sample (Lane B: 0.5 μg; Lane D: 1 μg). Lanes C and E were loaded with Fc sample (Lane C: 0.5 μg; Lane E: 1 μg). There were traces of Fab in the Fc samples.
Figure 2.4 Western blotting with anti-Fc fragment antibody. The standard molecular weight markers were shown in Lane A. Lanes B and D were loaded with Fab sample (Lane B: 0.5 μg; Lane D: 1 μg). Lanes C and E were loaded with Fc sample (Lane C: 0.5 μg; Lane E: 1 μg). There was no Fc contamination in the Fab samples.

VIII. Sterile filtration

The purified Fab and Fc solutions were subjected to sterile filtration using a 20ml syringe and the Minisart® 0.2 μm syringe filter (Sartorius AG, Germany) prior to the injections into the animals.
Chapter 3  Results

3.1  Treatment of rat EAN with normal saline and albumin

3.1.1  Clinical scores and weight changes

Rats receiving the footpad inoculation of whole bovine PNM emulsion began to develop neurological symptoms 11 to 16 days after immunization, which was evidenced by increased clinical score and obvious weight loss. Of the 18 animals, 9 received normal saline injections and the other 9 received albumin injections. All the animals were observed and weighed throughout the 30-day period.

There was no significant difference in clinical scores between the normal saline and albumin groups throughout the 30-day observation period (Figure 3.1.1) (p > 0.1, Mann-Whitney U test). Peak clinical score was reached, on average, by day 19 in the normal saline group and day 21 in the albumin group. The maximal mean clinical score was 2.8 (± 1.3) in the normal saline group and 2.8 (± 1.6) in the albumin group. After approximately 10 days of progression, there was a brief period of arrest followed by gradually clinical recovery. By day 30, 1 out of 9 rats (11%) in the normal saline group and 2 out of 9 (22%) in the albumin group completely recovered from the clinical disease. The mean clinical score at day 30 was 1.9 (± 1.2) in the normal saline group and 2.3 (± 1.6) in the albumin group.

No significant difference in weight changes was noted between these two groups throughout the 30-day observation period (Figure 3.1.2) (p > 0.1, unpaired t test). After immunization, there was a temporary weight loss and then weight gain 2 to 3 days later. An abrupt weight loss was noted 1 to 2 days prior to the onset of symptoms followed by sustained weight loss as the disease progressed. The maximal mean weight loss was evident at day 26 in the normal saline group (86 ± 28.8 g) and day 22 in the albumin group (89 ± 33.1 g).
3.1.2 Electrophysiological changes

3.1.2.1 Somatosensory evoked potentials (SSEPs)

No significant difference was noted between the two groups in SSEP studies (Figure 3.1.3) (p > 0.1, unpaired t test). The mean latencies to peak of the average S waves began to increase at day 14 in both groups and then decreased after reaching the maximal latency (at day 26 in both groups) (Figure 3.1.3A). The magnitude of the maximal prolongation was dependent on peak of disease severity. The maximal mean S-wave latency was 6.2 (± 1.1) ms in the normal saline group (72% prolongation) and 6.1 (± 1.2) ms in the albumin group (65% prolongation). The corresponding mean amplitudes of S waves began to decrease at day 14 in both groups, and the magnitude of the maximal reduction was dependent on peak of disease severity (Figure 3.1.3B). The minimum mean amplitude of the S wave was evident at day 22 in both groups; 11.7 (± 8) μV in the normal saline group (59% reduction) and 12.2 (± 6.2) μV in the albumin group (56% reduction). During recovery from disease, the mean amplitudes of S waves gradually increased in both groups.

3.1.2.2 Motor conduction velocity (MCV) and compound muscle action potential (CMAP) amplitude

There was no significant difference between the two groups in the sciatic nerve MCV (Figure 3.1.4) (p > 0.1, unpaired t test). In both groups, the mean distal MCV began to decrease at day 14 and then increased from the minimum velocity. The maximal reduction of the mean MCVs was evident at day 26 in both groups. The minimum mean MCV was 24.7 (± 6.1) m/s in the normal saline group (37% reduction) and 25.2 (± 9.4) m/s in the albumin group (37% reduction).

No significant difference was noted in CMAP amplitudes between these two groups (Figure 3.1.5) (p > 0.1, unpaired t test). In both groups, the mean amplitudes of distal (stimulation at ankle) (Figure 3.1.5A) and proximal (stimulation at hip) (Figure 3.1.5B) decreased at day 8, slightly increased from day 8 to day 12, began to decrease at day 14, and then gradually increased after reaching the minimum amplitudes (at day 26 in the normal saline group and day 22 in the albumin group). The distal and proximal CMAP amplitudes exhibited a synchronous reduction. The minimum mean
amplitude of distal CMAP was 1.3 (± 2.0) mV in the normal saline group (88% reduction) and 1.6 (± 2.4) mV in the albumin group (85% reduction). The minimum mean amplitude of proximal CMAP was 1.0 (± 1.6) mV in the normal saline group (89% reduction) and 1.2 (± 1.8) mV in the albumin group (87% reduction).

When the H/A ratios were calculated, no significant difference was noted between the normal saline and albumin groups (Figure 3.1.6A, p > 0.1, unpaired t test). The mean H/A ratios decreased as disease progressed and then increased during recovery with the lowest at day 18 in both groups. A conduction block by definition (> 20 % reduction of mean H/A ratio compared with value on day of immunization) was evident at day 18 in both groups (Figure 3.1.6B).
Figure 3.1.1  Mean clinical score (± standard deviation, S.D.) of EAN after immunization with whole bovine PNM. The rats began to develop neurological symptoms 11 to 16 days after immunization. Normal saline (n=9) or albumin (n=9) was administered intravenously from the onset of neurological deficit once daily for two days. Peak clinical score was reached, on average, by day 19 in the normal saline group and day 21 in the albumin group. The acute period of progression was followed by a brief period of arrest and then gradually clinical recovery. No significant difference was noted throughout the 30-day observation period between these two groups (p > 0.1, Mann-Whitney U test).
Figure 3.1.2  Mean weight change of EAN (expressed as change from the weight on day of immunization) (± S.D.) after immunization with whole bovine PNM. Normal saline (n=9) or albumin (n=9) was administered intravenously from the onset of neurological deficit once daily for two days. After immunization, there was a temporary weight loss and then weight gain 2 to 3 days later. Sustained weight loss was noted as the disease progressed. The maximal weight loss was evident at day 26 in the normal saline group and day 22 in the albumin group. No significant difference was noted throughout the 30-day observation period between these two groups (p > 0.1, unpaired t test).
Figure 3.1.3  SSEPs (± S.D.) of EAN after immunization with whole bovine PNM (A: S wave latency; B: S wave amplitude). Normal saline (n=9) or albumin (n=9) was administered intravenously from the onset of neurological deficit once daily for two days. In both groups, the mean S wave latencies began to increase at day 14 and then decreased after day 26. The corresponding amplitudes of the S wave began to decrease at day 14 in both groups and then gradually increased after day 22. Both the latency and the amplitude of the S wave failed to show significant differences between these two groups (p > 0.1, unpaired t test).
Figure 3.1.4  The distal MCV (± S.D.) in EAN after immunization with whole bovine PNM. Normal saline (n=9) or albumin (n=9) was administered intravenously from the onset of neurological deficit once daily for two days. In both groups, the mean distal MCV began to decrease at day 14 and then increased after day 26. No significant difference was noted throughout the 30-day observation period between these two groups (p > 0.1, unpaired t test).
Figure 3.1.5  CMAP amplitudes (± S.D.) in EAN after immunization with whole bovine PNM (A: distal CMAP amplitudes; B: proximal CMAP amplitudes). Normal saline (n=9) or albumin (n=9) was administered intravenously from the onset of neurological deficit once daily for two days. In both groups, the mean amplitudes of distal and proximal CMAPs decreased at day 8, slightly increased from day 8 to day 12, began to decrease at day 14, and then gradually increased after reaching the minimum amplitudes. No significant difference was noted between these two groups (p > 0.1, unpaired t test).
Figure 3.1.6  Time course of mean H/A ratios of the CMAPs (A) and its change (expressed as the ratio to value on day of immunization) (B). The mean H/A ratios decreased as disease progressed and then increased during recovery with the lowest at day 18 in both groups. No significant difference was noted in the H/A ratios between the normal saline and albumin groups (p > 0.1, unpaired t test). However, a conduction block (> 20 % reduction of mean H/A ratio) was evident at day 18 in both groups.
3.2 Treatment of rat EAN with albumin and IVIg

3.2.1 Clinical scores and weight changes

Rats receiving the footpad inoculation with whole bovine PNM emulsion began to develop neurological symptoms 10 to 16 days after immunization except one that had the early onset of disease at day 7. Of the 50 animals, 25 received albumin injections and the other 25 received IVIg injections from the onset of neurologic deficit once daily for two days. Of the 17 rats from each group observed and weighed for 30 days, 12 rats received electrophysiological studies at predetermined intervals.

Rats receiving IVIg treatment developed less severe disease evidenced by lower clinical score (Figure 3.2.1). Peak clinical score was reached, on average, by day 16 in the albumin group and day 18 in the IVIg group. The maximal mean clinical score was 2.9 (± 0.8) in the albumin group and 2.2 (± 0.8) in the IVIg group. The clinical score was significantly different from day 14 to 20 and from day 25 to 30 (P <0.05, Mann-Whitney U test). After approximately 10 days of progression, there was a brief period of arrest followed by gradually clinical recovery. The recovery in rats in the IVIg group was significantly faster and more complete than rats in the albumin group. By day 30, 5 out of 17 rats (29%) in the albumin group and 12 out of 17 (71%) in the IVIg group completely recovered from the clinical disease. The sequelae (such as limp tail and waddling gait) were more severe in the albumin group. At day 30, the mean clinical score was 1.4 (± 1.2) in the albumin group and 0.5 (± 0.9) in the IVIg group.

After immunization, there was a temporary weight loss and then weight gain 2 to 3 days later (Figure 3.2.2). An abrupt weight loss was noted 1 to 2 days prior to the onset of symptoms followed by sustained weight loss as the disease progressed. The maximal mean weight loss was observed at day 19 in the albumin group and day 20 in the IVIg group. The maximal mean weight loss was 45 (± 23) g in the albumin group and 37 (± 21) g in the IVIg group and the difference in the weight changes of these two groups failed to reach statistical significance (P > 0.1, unpaired t test).
3.2.2 Electrophysiological changes

3.2.2.1 Somatosensory evoked potentials (SSEPs)

Latency to peak of the S wave was less prolonged in the IVIg group than in the albumin group (Figure 3.2.3A). The mean S wave latencies began to increase at day 12 in both groups and then decreased after reaching the maximal latency (at day 26 in both groups). The maximal mean S-wave latency was 6.3 (± 1.3) ms in the albumin group (75% prolongation) and 5.2 (± 1.3) ms in the IVIg group (41% prolongation). Significant differences in the S wave latency were evident at days 18, 22 and 26 (P < 0.05, unpaired t test).

The corresponding amplitudes of S wave were better maintained in the IVIg group than in the albumin group (Figure 3.2.3B). The mean amplitudes of the S waves began to decrease at day 14 in both groups, and then gradually increased during recovery from disease. The minimum mean amplitude of S wave was observed at day 22 in both groups; 12.1 (± 7.0) μV in the albumin group (55% reduction) and 14.4 (± 8.9) μV in the IVIg group (51% reduction). The differences in the S wave amplitude were evident at days 16, 18 and 26 (P < 0.05, unpaired t test).

3.2.2.2 Motor conduction velocity (MCV) and compound muscle action potential (CMAP) amplitude

The distal MCV was significantly higher in the IVIg group than in the albumin group (Figure 3.2.4). The mean distal MCV began to decrease at day 12 in both groups and then gradually recovered from the minimum velocity. The maximal reduction of the MCVs was observed at day 26 in the albumin group and day 18 in the IVIg group. The minimum mean MCV was 25.0 (± 11.0) m/s in the albumin group (38% reduction) and 33.8 (± 8.1) m/s in the IVIg group (15% reduction). The differences in the MCVs were evident from day 18 and persisted until day 30 (P < 0.05, unpaired t test).

The amplitudes of CMAPs after stimulation at ankle (distal) (Figure 3.2.5A) and hip (proximal) (Figure 3.2.5B) were better maintained in the IVIg group than in the albumin group. The mean amplitudes of distal and proximal CMAPs began to
decrease at day 8 in both groups and then gradually increased after reaching the minimum amplitudes (at day 26 in the albumin group and day 18 in the IVIg group). The distal and proximal CMAP amplitudes exhibited a synchronous reduction. The minimum mean amplitude of distal CMAP was 1.1 (± 1.9) mV in the albumin group (90% reduction) and 3 (± 2.6) mV in the IVIg group (75% reduction). The minimum mean amplitude of proximal CMAP was 0.8 (± 1.5) mV in the albumin group (90% reduction) and 2.0 (± 1.8) mV in the IVIg group (80% reduction). The differences in the distal and proximal amplitudes of CMAP were significant at day 26 and 30 (p < 0.05, unpaired t test).

When the H/A ratios were calculated, no significant difference was noted between the albumin and IVIg groups (Figure 3.2.6A, p > 0.1, unpaired t test). The mean H/A ratios decreased as disease progressed and then increased during recovery with the lowest at day 18 in the albumin group and at day 16 in the IVIg group. A conduction block by definition (> 20% reduction of mean H/A ratio compared with value on day of immunization) was evident at day 16 and 18 in the albumin group and at day 16 in the IVIg group (Figure 3.2.6B).

3.2.3 Histological changes

Some degree of inflammation and demyelination was evident in the spinal roots and sciatic nerves of EAN rats receiving either albumin or IVIg treatment. Toluidine blue stained transverse sections of the lumbosacral nerve roots and proximal sciatic nerves revealed areas of perivascular demyelination and axonal degeneration (Figure 3.2.7 & 3.2.8). Demyelinated axons and axonal loss were also frequently located subperineurally or endoneurally. The degree of demyelination and axonal degeneration varied considerably between different nerves and nerve roots in the same animal.

In the albumin group, the early lesions (Figure 3.2.7A) showed perivascular infiltrates of mononuclear cells and polymorphonuclear leukocytes with incomplete demyelination and some degree of axonal loss. Cells were also seen attached to or within the walls of vessels. Endoneurial edema and fibrinous exudates were visible among and beyond the cellular infiltration. The late lesions (Figure 3.2.8A) showed
many completely demyelinated axons, axonal loss, and macrophages filled with myelin debris (so-called ‘foamy’ macrophages). A few axons were surrounded by very thin myelin sheaths, suggestive of beginning remyelination. In the IVIg group, the early lesions (Figure 3.2.7B) showed less extensive cellular infiltration, less edema, and less axonal loss. The late lesions (Figure 3.2.8B) showed less ‘foamy’ macrophages with little demyelination and axonal degeneration. More prominent remyelination with thinly myelin sheaths and more small sprouts of regenerating axons in the IVIg groups than in the albumin group were also observed.

When the extent of perivascular inflammation and demyelination was graded, nerves from the albumin group were shown to have more extensive changes than those from the IVIg group evidenced by higher histological scores (Figure 3.2.9). The maximal mean histological score was seen at day 16 in both group; 2.82 (± 0.48) in the albumin group and 1.99 (± 1.03) in the IVIg group. At day 30, the mean histological score was 2.61 (± 0.66) in the albumin group and 1.87 (±1.17) in the IVIg group. When the percentage of perivascular areas with a given grade of inflammation and demyelination was calculated (Figure 3.2.10), nerves from the IVIg group had a higher percentage of grade 0 and lower percentage of grade 3 than the albumin group at day 16 (Figure 3.2.10A; 0 % of grade 0 and 86.1% of grade 3 in the albumin group; 9.4 % of grade 0 and 43.0 % of grade 3 in the IVIg group), day 18 (Figure 3.2.10B; 29.1 % of grade 0 and 41.1 % of grade 3 in the albumin group; 44.6 % of grade 0 and 38.5 % of grade 3 in the IVIg group), day 22 (Figure 3.2.10C; 10.8 % of grade 0 and 63.1 % of grade 3 in the albumin group; 39.2 % of grade 0 and 55.2 % of grade 3 in the IVIg group), day 26 (Figure 3.2.10D; 0.8 % of grade 0 and 77.5 % of grade 3 in the albumin group; 13.3 % of grade 0 and 60.0 % of grade 3 in the IVIg group), and day 30 (Figure 3.2.10E; 1.1 % of grade 0 and 69.2 % of grade 3 in the albumin group; 20.6 % of grade 0 and 41.5 % of grade 3 in the IVIg group). The difference between these two groups was significant from day 16 to day 30 (P < 0.05, Mann-Whitney U test).
Figure 3.2.1  Mean clinical score (± S.D.) after immunization with whole bovine PNM. The rats began to develop neurological symptoms 10 to 16 days after immunization. Albumin (n=17) or IVIg (n=17) was administered intravenously from the onset of neurological deficit once daily for two days. Rats receiving IVIg treatment developed less severe disease evidenced by lower clinical score. Peak clinical score was reached, on average, by day 16 in the albumin group and day 18 in the IVIg group. The acute period of progression was followed by a brief period of arrest and then gradually clinical recovery. The clinical score was significantly different from day 14 to 20 and from day 25 to 30 (P <0.05, Mann-Whitney U test).
Figure 3.2.2  Mean weight change (expressed as change from the weight on day of immunization) (± S.D.) after immunization with whole bovine PNM. Albumin (n=17) or IVIg (n=17) was administered intravenously from the onset of neurological deficit once daily for two days. After immunization, there was a temporary weight loss and then weight gain 2 to 3 days later. An abrupt weight loss was noted 1 to 2 days prior to the onset of symptoms followed by sustained weight loss as the disease progressed. The maximal mean weight loss was evident at day 19 in the albumin group and day 20 in the IVIg group and the weight changes of these two groups failed to reach statistical significance (P > 0.1, unpaired t test).
Figure 3.2.3  SSEPs (± SD) after immunization with whole bovine PNM (A: S wave latency; B: S wave amplitude). Albumin (n=12) or IVIg (n=12) was administered intravenously from the onset of neurological deficit once daily for two days. In both groups, the mean S wave latencies began to increase at day 12 and then decreased after day 26. The S wave latencies were lower in the IVIg group and significantly different at days 18, 22 and 26 (P < 0.05). The mean amplitudes of the S wave began to decrease at day 14 and then gradually increased after day 22. The amplitudes of the S waves were better maintained in the IVIg group and significantly different at days 16, 18 and 26 (P < 0.05, unpaired t test).
Figure 3.2.4  The distal MCV (± S.D.) after immunization with whole bovine PNM. Albumin (n=12) or IVIg (n=12) was administered intravenously from the onset of neurological deficit once daily for two days. The mean distal MCV began to decrease at day 12 in both groups and then gradually recovered after day 26 in the albumin group and day 18 in the IVIg group. The distal MCV was significantly higher in the IVIg group and these differences were evident from day 18 and persisted until day 30 (P < 0.05, unpaired t test).
Figure 3.2.5  CMAP amplitudes (± S.D.) after immunization with whole bovine PNM (A: distal CMAP amplitudes; B: proximal CMAP amplitudes). Albumin (n=12) or IVIg (n=12) was administered intravenously once daily for two days. In both groups, the mean amplitudes of distal and proximal CMAPs began to decrease at day 8 and then gradually increased after reaching the minimum amplitudes (at day 26 in the albumin group and day 18 in the IVIg group). The distal and proximal CMAP amplitudes exhibited a synchronous reduction. The amplitudes of distal and proximal CMAPs were better maintained in the IVIg group and these differences were significant at day 26 and 30 (p < 0.05, unpaired t test).
Figure 3.2.6  Time course of mean H/A ratios of the CMAPs (A) and its change (expressed as the ratio to value on day of immunization) (B). The mean H/A ratios decreased as disease progressed and then increased during recovery with the lowest at day 18 in the albumin group and at day 16 in the IVIg group. No significant difference was noted in the H/A ratios between the albumin and IVIg groups (A, p > 0.1, unpaired t test). However, a conduction block (> 20 % reduction of H/A ratio) was evident at day 16 and 18 in the albumin group and at day 16 in the IVIg group.
Figure 3.2.7  Toluidine blue stained transverse sections of proximal sciatic nerves taken from albumin (A) and IVIg (B) treated animals 16 days after induction of EAN. In both groups of animals the characteristic early histological features of EAN were present, with perivascular lymphocytic infiltrates, endoneurial oedema, incomplete demyelination, and axonal degeneration. However, nerves from IVIg treated animals showed less extensive cellular infiltration with little oedema and less active axonal degeneration (знак указывает на демиелинизацию; знак указывает на аксональную дегенерацию; знак указывает на тонко миелинированные волокна; только часть патологических изменений была отмечена).
Figure 3.2.8  Toluidine blue stained transverse sections of sciatic nerves taken from albumin (A) and IVIg (B) treated animals 30 days after induction of EAN. In both groups of animals the characteristic late histological features of EAN were present, with lymphocytic infiltrates, endoneurial oedema, thinly myelinated and demyelinated axons, macrophages full of myelin debris and axonal degeneration. However, nerves from IVIg treated animals showed less oedema, less extensive cellular infiltration and demyelination, and less axonal loss. Many small sprouts of regenerating axons can be seen in nerves from IVIg group (☞ indicated demyelination; ▶ indicated axonal degeneration; ♦ indicated thinly myelinated fibres; only a sample of pathological changes has been labelled).
Figure 3.2.9  Extent of perivascular inflammation and demyelination of nerves from the albumin and IVIg treated EAN rats. The maximal mean histological score was evident at day 16 in both groups and the difference between these two groups was significant from day 16 to day 30 (P < 0.05, Mann-Whitney U test).
Figure 3.2.10  Extent of perivascular inflammation and demyelination of nerves from the albumin and IVIg treated EAN rats. When the percentage of perivascular areas with a given grade was calculated, nerves from the IVIg group had a higher percentage of grade 0 and lower percentage of grade 3 than the albumin group at day 16 (A), day 18 (B), day 22 (C), day 26 (D), and day 30 (E).
3.3 Treatment of rat EAN with albumin, Fab fragments, Fc fragments, and IVIg

3.3.1 Clinical scores and weight changes

Rats receiving the footpad inoculation of whole bovine PNM emulsion began to develop neurological symptoms 9 to 15 days after immunization. Of the 33 animals, 8 received albumin injections, 8 received Fab fragment injections, 8 received Fc fragment injections and the other 9 received IVIg injections from the onset of neurologic deficit once daily for two days. All the rats from each group were observed and weighed throughout the 30-day period.

Treatment with different protein solutions resulted in significant differences in the clinical scores among the groups (Figure 3.3.1) (p < 0.05, Kruskal-Wallis one-way ANOVA). Rats receiving Fc and IVIg treatment developed less severe disease evidenced by both lower clinical scores and less weight loss. Peak clinical score was reached, on average, by day 16 in the albumin and Fc groups, day 15 in the Fab group, and day 17 in the IVIg group. The maximal mean clinical score was 3.3 (± 0.8) in the albumin group, 2.7 (± 0.8) in the Fab group, 2.4 (± 0.9) in the Fc group, and 2.3 (± 1) in the IVIg group. Significant differences (Table 1) were evident at day 13, 14, and from day 16 on when comparing the Fc group with the albumin group, from day 16 on between the albumin and IVIg groups, from day 20 on between the Fab and Fc groups, and from day 19 on between the Fab and IVIg groups (p < 0.05, Mann-Whitney U test). There were no significant differences between the albumin and Fab groups or Fc and IVIg groups throughout the observation period (p > 0.1, Mann-Whitney U test). After approximately one week of progression, there was a brief period of arrest followed by gradually clinical recovery. The recovery from illness in the Fc and IVIg groups was significantly faster and more complete than that in the albumin and Fab groups. By day 30, 0 out of 8 (0%) in the albumin group, 1 out of 8 (13%) in the Fab group, 4 out of 8 (50%) in the Fc group, and 6 out of 9 (67%) rats in the IVIg group completely recovered from the clinical disease. The sequelae (such as limp tail and waddling gait) were more severe in the albumin and Fab groups. At day
30, the mean clinical score was 1.9 (± 1.1) in the albumin group, 1.6 (± 1.1) in the Fab group, 0.6 (± 0.9) in the Fc group, and 0.4 (± 0.7) in the IVIg group.

There were significant differences in the weight changes among the groups (Figure 3.3.2) (p < 0.05, one-way ANOVA). After immunization, there was a temporary weight loss and then weight gain 2 to 3 days later. An abrupt weight loss was noted 1 to 2 days prior to the onset of symptoms followed by sustained weight loss as the disease progressed. The maximal mean weight loss was observed at day 22 in the albumin group, day 16 in the Fc group, and day 17 in the Fab and IVIg groups. The maximal mean weight loss was 22.5 (± 19) g in the albumin group, 13.5 (± 8.4) g in the Fab group, 9.25 (± 9.07) g in the Fc group, and 9.78 (± 7.1) g in the IVIg group. Significant differences (Table 2) were evident from day 17 on when comparing the Fc or IVIg group with the albumin group (p < 0.05, unpaired t test). Difference in the weight change between the albumin and Fab groups was observed from day 18 to 25 and day 28 to 29 but failed to reach statistical significance (0.1 > p > 0.05, unpaired t test). No significant difference was noted between the Fab and Fc groups, Fab and IVIg groups, or Fc and IVIg groups (p > 0.1, unpaired t test).

3.3.2 Electrophysiological changes

3.3.2.1 Somatosensory evoked potentials (SSEPs)

There were significant differences in the latencies to peak of the S wave among the groups (Figure 3.3.3A) (p < 0.05, one-way ANOVA). The S wave latency was less prolonged in the Fc and IVIg groups than in the albumin and Fab groups. The mean S wave latencies began to increase at day 12 in all the four groups and then decreased after reaching the maximal latency (at day 22 in the albumin and Fab groups, day 18 in the Fc group, and day 16 in the IVIg group). The maximal mean S wave latency was 6.5 (± 1.5) ms in the albumin group (76% prolongation), 5.8 (± 1.3) ms in the Fab group (57% prolongation), 4.8 (± 0.6) in the Fc group (30% prolongation), and 5.3 (± 2.3) ms in the IVIg group (43% prolongation). Significant differences (Table 3) were evident from day 12 on when comparing the Fc group with the albumin group, from day 18 on between the albumin and IVIg groups, from day 14 on between the Fab and Fc groups, and from day 22 on between the Fab and IVIg
groups (p < 0.05, unpaired t test). There were no significant differences between the albumin and Fab groups or Fc and IVIg groups throughout the observation period (p > 0.1, unpaired t test).

There were significant differences in the corresponding S wave amplitudes among the groups (Figure 3.3.3B) (p < 0.05, one-way ANOVA). The S wave amplitudes were better maintained in the Fc and IVIg groups than in the albumin and Fab groups. The mean amplitudes of the S waves began to decrease at day 14 in all the four groups and then gradually increased during recovery from disease. Maximal reduction of the mean S wave amplitudes was observed at day 22 in the albumin group, day 18 in the Fab and IVIg groups, and day 16 in the Fc group. The minimum mean amplitude of S waves was 20.2 (± 9.5) μV in the albumin group (57% reduction), 21.3 (± 9.5) μV in the Fab group (51% reduction), 28.4 (± 11.9) μV in the Fc group (43% reduction), and 32.9 (± 16.9) μV in the IVIg group (29% reduction).

Significant differences (Table 4) were evident from day 16 to day 26 when comparing the Fc group with the albumin group, from day 16 on between the albumin and IVIg groups, at day 18 between the Fab and Fc groups, and from day 18 on between the Fab and IVIg groups (p < 0.05, unpaired t test). Differences in the S wave amplitudes were observed between the albumin and Fab groups at day 22 and 26 and between the Fc and IVIg groups at day 22 but failed to reach statistical significance (0.1 > p > 0.05, unpaired t test).

3.3.2.2 Motor conduction velocity (MCV) and compound muscle action potential (CMAP) amplitude

There were significant differences in the distal MCV among the groups (Figure 3.3.4) (p < 0.05, one-way ANOVA). The distal MCV was significantly higher in the Fc and IVIg groups than in the albumin and Fab groups. The mean distal MCV began to decrease at day 14 in all the four groups and then gradually recovered from the minimum velocity (at day 26 in the albumin group, day 30 in the Fab group, day 16 in the Fc group, and day 22 in the IVIg group). The minimum mean MCV was 22.6 (± 9.9) m/s in the albumin group (34% reduction), 24.0 (± 9.3) m/s in the Fab group (32% reduction), 31.1 (± 3.9) m/s in the Fc group (12% reduction), and 31.3 (± 4.9)
m/s in the IVIg group (15% reduction). Significant differences (Table 5) were evident from day 22 on when comparing the Fc group with the albumin group, from day 16 on between the albumin and IVIg groups, the Fab and Fc groups, or the Fab and IVIg groups (p < 0.05, unpaired t test). There were no significant differences between the albumin and Fab groups or Fc and IVIg groups throughout the observation period (p > 0.1, unpaired t test).

There were significant differences in the CMAP amplitudes among the groups (Figure 3.3.5) (p < 0.05, one-way ANOVA). The amplitudes of CMAP after stimulation at ankle (distal) (Figure 3.3.5A) and hip (proximal) (Figure 3.3.5B) were better maintained in the Fc and IVIg groups than in the albumin and Fab groups. The mean amplitudes of distal and proximal CMAPs began to decrease at day 8 in all the four groups and the distal and proximal CMAP amplitudes exhibited a synchronous reduction. The maximal reduction of mean distal CMAP amplitude was observed at day 26 in the albumin group, day 30 in the Fab group, day 16 in the Fc group, and day 18 in the IVIg group. The minimum mean amplitude of distal CMAP was 2.3 (± 3.3) mV in the albumin group (79% reduction), 2.8 (± 2.7) mV in the Fab group (78% reduction), 5.2 (± 4.1) mV in the Fc group (57% reduction), and 6.4 (± 4.8) mV in the IVIg group (47% reduction). Significant differences (Table 6) were evident from day 14 on between the albumin and Fc groups, from day 16 on when the IVIg group was compared with the albumin or Fab group, and from day 22 on between the Fab and Fc groups (p < 0.05, unpaired t test). Differences in the distal CMAP amplitude were observed between the Fc and IVIg groups at day 26 and 30 but failed to reach statistical significance (0.1 > p > 0.05, unpaired t test). There was no significant difference between the albumin and Fab groups throughout the observation period (p > 0.1, unpaired t test). The maximal reduction of mean proximal CMAP amplitude was observed at day 18 in the albumin group, day 30 in the Fab group, day 16 in the Fc group, and day 18 in the IVIg group. The minimum mean amplitude of proximal CMAP was 1.9 (± 2.7) mV in the albumin group (81% reduction), 2.0 (± 1.7) mV in the Fab group (82% reduction), 3.3 (± 2.8) mV in the Fc group (67% reduction), and 5.0 (± 3.7) mV in the IVIg group (49% reduction). Significant differences (Table 7) were evident at day 14, 22, and 30 between the albumin and Fc groups, from day 14
on when the IVIg group was compared with the albumin or Fab groups, and at day 22 and 30 between the Fab and Fc groups (p < 0.05, unpaired t test). Differences in the proximal CMAP amplitude were observed between the Fc and IVIg groups at day 16, 26, and 30 but failed to reach statistical significance (0.1 > p > 0.05, unpaired t test). There was no significant difference between the albumin and Fab groups throughout the 30-day observation period (p > 0.1, unpaired t test).

When the H/A ratios were calculated, no significant difference was noted among the groups (Figure 3.3.6A, p > 0.1, one-way ANOVA). The mean H/A ratios decreased as disease progressed and then increased during recovery with the lowest at day 14 in the albumin group, day 22 in the Fab group, day 18 in the Fc group, and day 16 in the IVIg group. However, significant differences (Table 8) in the H/A ratios were evident at day 14 between the the albumin and IVIg groups, at day 10, 18 and 22 between the Fab and IVIg groups, and at day 18 between the Fc and IVIg groups (p < 0.05, unpaired t test). A conduction block by definition (> 20 % reduction of mean H/A ratio compared with value on day of immunization) (Figure 3.3.6B) was evident at day 12, day 14 and 16 in the albumin group, at day 16, 18 and 22 in the Fab group, and at day 16 and 18 in the Fc group. No conduction block was observed in the IVIg group throughout the 30-day observation period.

### 3.3.3 Histological changes

All the 33 rats in four groups were sacrificed on day 30 for histological analysis. Toluidine blue stained transverse sections of the lumbosacral nerve roots and proximal sciatic nerves revealed areas of perivascular demyelination and axonal degeneration (Figure 3.3.7). Demyelinated axons and axonal loss were also frequently located sub-perineurally or endoneurially. The degree of demyelination and axonal degeneration varied considerably between different nerves and nerve roots in the same animal.

In the albumin and Fab groups, the lesions (Figure 3.3.7A and 3.3.7B) showed perivascular infiltrates of mononuclear cells and polymorphonuclear leukocytes. Endoneurial edema and fibrinous exudates were visible among and beyond the
cellular infiltration, extensive perivascular demyelination and axonal degeneration, and macrophages filled with myelin debris (so-called ‘foamy’ macrophages). A few axons were surrounded by very thin myelin sheaths, suggestive of beginning remyelination. In the Fc and IVIg groups, the lesions (Figure 3.3.7C and 3.3.7D) showed less extensive cellular infiltration, less edema, less extensive perivascular demyelination and axonal degeneration, and less dominant ‘foamy macrophages’. More prominent remyelination with thinly myelin sheaths and more small sprouts of regenerating axons in nerves from the Fc and IVIg groups than the albumin and Fab groups were also observed.

When the extent of perivascular inflammation and demyelination was graded, nerves from the albumin and Fab groups were shown to have more extensive changes than those from the Fc and IVIg groups evidenced by higher histological scores (Figure 3.3.8). At day 30, the mean histological score was 2.37 (± 1.14) in the albumin group, 2.31 (± 1.13) in the Fab group, 1.67 (± 1.34) in the Fc group, and 1.50 (±1.30) in the IVIg group. When the percentage of perivascular areas with a given grade of inflammation and demyelination was calculated (Figure 3.3.9), nerves from the Fc and IVIg groups had a higher percentage of grade 0 and lower percentage of grade 3 than the albumin and Fab groups (15.4 % of grade 0 and 73.6 % of grade 3 in the albumin group; 15.1 % of grade 0 and 68.5 % of grade 3 in the Fab group; 33.4 % of grade 0 and 45.1 % of grade 3 in the Fc group; 35.4 % of grade 0 and 36.4 % of grade 3 in the IVIg group). The differences were significant (Table 9) between the albumin and Fc groups, albumin and IVIg groups, Fab and Fc groups, Fab and IVIg groups, and Fc and IVIg groups (P < 0.01, Mann-Whitney U test).
Figure 3.3.1 Mean clinical score (+ S.D. in the albumin and Fab groups and – S.D. in the Fc and IVIg groups) after immunization with whole bovine PNM. Rats began to develop neurological symptoms 9 to 15 days after immunization. Albumin (n=8), Fab fragments (n=8), Fc fragments (n=8) or IVIg (n=9) was administered intravenously from the onset of neurological deficit once daily for two days. Significant differences were noted among the groups (p < 0.05, Kruskal-Wallis one-way ANOVA). Rats receiving Fc and IVIg treatment developed less severe disease evidenced by lower clinical scores. Peak clinical score was reached, on average, by day 16 in the albumin group and Fc groups, day 15 in the Fab group, and day 17 in the IVIg group. The acute period of progression was followed by a brief period of arrest and then gradually clinical recovery. Significant differences were evident at day 13, 14 and from day 16 on between the albumin and Fc groups, from day 16 on between the albumin and IVIg groups, from day 20 on between the Fab and Fc groups, and from day 19 on between the Fab and IVIg group (p < 0.05, Mann-Whitney U test).
Figure 3.3.2  Mean weight change (expressed as change from the weight on day of immunization) (- S.D. in the albumin and Fab groups and + S.D. in the Fc and IVIg groups) after immunization with whole bovine PNM. Albumin (n=8), Fab fragments (n=8), Fc fragments (n=8) or IVIg (n=9) was administered intravenously from the onset of neurological deficit once daily for two days. Significant differences were noted among the groups (p < 0.05, one-way ANOVA). After immunization, there was a temporary weight loss and then weight gain 2 to 3 days later. An abrupt weight loss was noted 1 to 2 days prior to the onset of symptoms followed by sustained weight loss as the disease progressed. The maximal mean weight loss was observed at day 22 in the albumin group, day 16 in the Fc group, and day 17 in the Fab and IVIg groups. Significant differences were evident from day 17 on when comparing the Fc or IVIg group with the albumin group (p < 0.05, unpaired t test).
Figure 3.3.3  SSEPs after immunization with whole bovine PNM (A: S wave latency; B: S wave amplitude). Albumin (n=8), Fab fragments (n=8), Fc fragments (n=8) or IVIg (n=9) was administered intravenously from the onset of neurological deficit once daily for two days. Significant differences were noted among the groups (p < 0.05, one-way ANOVA). The mean S wave latencies (A) (+ S.D. in the albumin and Fab groups and - S.D. in the Fc and IVIg groups) began to increase at day 12 in all the four groups and then decreased after reaching the maximal latency (at day 22 in the albumin and Fab groups, day 18 in the Fc group, and day 16 in the IVIg group). The S wave latency was less prolonged in the Fc and IVIg groups. Significant differences were evident from day 12 on between the albumin and Fc groups, from day 18 on between the albumin and IVIg groups, from day 14 on between the Fab and Fc groups, and from day 22 on between the Fab and IVIg groups (p < 0.05, unpaired t test). The mean amplitudes of the S waves (B) (- S.D. in the albumin and Fab groups and + S.D. in the Fc and IVIg groups) began to decrease at day 14 in all the four groups and then gradually increased after day 22 in the albumin group, day 18 in the Fab and IVIg groups, and day 16 in the Fc group. Significant differences were evident from day 16 to day 26 between the albumin and Fc groups, from day 16 on between the albumin and IVIg groups, at day 18 between the Fab and Fc groups, and from day 18 on between the Fab and IVIg groups (p < 0.05, unpaired t test).
Figure 3.3.4  The distal MCV after immunization with whole bovine PNM (- S.D. in the albumin and Fab groups and + S.D. in the Fc and IVIg groups). Albumin (n=8), Fab fragments (n=8), Fc fragments (n=8) or IVIg (n=9) was administered intravenously from the onset of neurological deficit once daily for two days. Significant differences were noted among the groups (p < 0.05, one-way ANOVA). The mean distal MCV began to decrease at day 14 in all the four groups and then gradually recovered after day 26 in the albumin group, day 30 in the Fab group, day 16 in the Fc group, and day 22 in the IVIg group. The distal MCV was significantly higher in the Fc and IVIg groups. Significant differences were evident from day 22 on between the albumin and Fc groups, from day 16 on between the albumin and IVIg groups, the Fab and Fc groups, or the Fab and IVIg groups (p < 0.05, unpaired t test).
Figure 3.3.5  CMAP amplitudes after immunization with whole bovine PNM (- S.D. in the albumin and Fab groups and + S.D. in the Fc and IVIg groups) (A: distal CMAP amplitudes; B: proximal CMAP amplitudes). Albumin (n=8), Fab fragments (n=8), Fc fragments (n=8) or IVIg (n=9) was administered intravenously from the onset of neurological deficit once daily for two days. Significant differences were noted among the groups (p < 0.05, one-way ANOVA). In all the four groups, the mean amplitudes of distal and proximal CMAPs began to decrease at day 8 and the distal and proximal CMAP amplitudes exhibited a synchronous reduction. The maximal reduction of mean distal CMAP amplitude (A) was evident at day 26 in the albumin group, day 30 in the Fab group, day 16 in the Fc group, and day 18 in the IVIg group. The amplitudes of distal and proximal CMAPs were better maintained in the Fc and IVIg groups. Significant differences were evident from day 14 on between the albumin and Fc groups, from day 16 on when the IVIg group was compared with the albumin or Fab groups, and from day 22 on between the Fab and Fc groups (p < 0.05, unpaired t test). The maximal reduction of mean proximal CMAP amplitude (B) was observed at day 18 in the albumin group, day 30 in the Fab group, day 16 in the Fc group, and day 18 in the IVIg group. Significant differences were evident at day 14, 22, and 30 between the albumin and Fc groups, from day 14 on when the IVIg group was compared with the albumin or Fab groups, and at day 22 and 30 between the Fab and Fc groups (p < 0.05, unpaired t test).
Figure 3.3.6  Time course of mean H/A ratios of the CMAPs (A) and its change (expressed as the ratio to value on day of immunization) (B). No significant difference in the H/A ratios was noted among the groups (p > 0.1, one-way ANOVA). The mean H/A ratios (A) decreased as disease progressed and then increased during recovery with the lowest at day 14 in the albumin group, day 22 in the Fab group, day 18 in the Fc group, and day 16 in the IVIg group. Significant differences were evident at day 14 between the albumin and IVIg groups, at day 10, 18 and 22 between the Fab and IVIg groups, and at day 18 between the Fc and IVIg groups (p < 0.05, unpaired t test). A conduction block (> 20 % reduction of mean H/A ratio) (B) was evident at day 12, day 14 and 16 in the albumin group, at day 16, 18 and 22 in the Fab group, and at day 16 and 18 in the Fc group.
Figure 3.3.7  Toluidine blue stained transverse sections of spinal motor roots taken from albumin (A), Fab (B), Fc (C), and IVIg (D) treated animals 30 days after induction of EAN. In all the 4 groups of animals the characteristic early histological features of EAN were present, with perivascular lymphocytic infiltrates, endoneurial oedema, thinly myelinated and demyelinated axons, macrophages full of myelin debris (foamy macrophages) and axonal degeneration. However, nerves from Fc and IVIg treated animals showed less extensive cellular infiltration, less oedema, less extensive perivascular demyelination and axonal degeneration, and less dominant ‘foamy macrophages’. Many small sprouts of regenerating axons can be seen in nerves from Fc and IVIg treatment groups (🪐 indicated demyelination; ➡ indicated axonal degeneration; ✰ indicated thinly myelinated fibres; only a sample of pathological changes has been labelled).
Figure 3.3.8  Extent of perivascular inflammation and demyelination of nerves from the albumin, Fab, Fc and IVIg treated EAN rats. The inflammation and demyelination of the albumin and Fab groups is more extensive than the Fc and IVIg groups evidenced by higher histological scores. The differences were significant between the albumin and Fc groups, albumin and IVIg groups, Fab and Fc groups, Fab and IVIg groups, and Fc and IVIg groups (P < 0.01, Mann-Whitney U test).
Figure 3.3.9  Extent of perivascular inflammation and demyelination of nerves from the albumin, Fab, Fc and IVIg treated EAN rats. When the percentage of perivascular areas with a given grade of inflammation and demyelination was calculated, nerves from the Fc and IVIg groups had a higher percentage of grade 0 and lower percentage of grade 3 than the albumin and Fab groups.
## Table 1  
Statistical significance of Mann-Whitney U test in clinical scores between pairs of groups (N/S: non significance) (Alb: albumin)

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Table 5  Statistical significance of unpaired t test in distal MCV between pairs of groups (N/S: non significance)

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Table 6  Statistical significance of unpaired t test in distal CMAP amplitudes between pairs of groups (N/S: non significance)

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Table 7  Statistical significance of unpaired t test in proximal CMAP amplitudes between pairs of groups (N/S: non significance)

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<th>Day</th>
<th>proximal amplitude</th>
<th>Alb vs Fab</th>
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<th>Alb vs IVIg</th>
<th>Fab vs Fc</th>
<th>Fab vs IVIg</th>
<th>Fc vs IVIg</th>
</tr>
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</tr>
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Table 8  Statistical significance of unpaired t test in H/A ratios between pairs of groups (N/S: non significance)

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<th>Day</th>
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<th>Alb vs Fab</th>
<th>Alb vs Fc</th>
<th>Alb vs IVIg</th>
<th>Fab vs Fc</th>
<th>Fab vs IVIg</th>
<th>Fc vs IVIg</th>
</tr>
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</tr>
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<td>N/S</td>
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<tr>
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<td>p &lt; 0.05</td>
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Table 9  Statistical significance of Mann-Whitney U test in histological grades between pairs of groups (N/S: non significance)

<table>
<thead>
<tr>
<th>Day</th>
<th>Alb vs Fab</th>
<th>Alb vs Fc</th>
<th>Alb vs IVIg</th>
<th>Fab vs Fc</th>
<th>Fab vs IVIg</th>
<th>Fc vs IVIg</th>
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<tbody>
<tr>
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**Histology U test**
Chapter 4  Discussion

4.1  Treatment of rat EAN with normal saline and albumin

The aim of this study was to establish that albumin can be used as a control therapy in the subsequent treatment studies of EAN using IVIg, Fab fragments or Fc fragments, which are all proteins. The normal saline group served as the control group and the albumin group as the treatment group.

Albumin is an essential plasma protein, which makes up more than half of the total protein present in serum (Bush, 1990). It has been considered most important for its contribution to colloid osmotic pressure, accounting for ~80% of colloid osmotic pressure in humans (Martin, 2005). Albumin is synthesized in the liver and serves in the transport of bilirubin, hormones, metals, vitamins, and drugs. In its natural form, albumin has a free cysteine residue at position 34 (Cys34) that accounts for the majority of plasma free sulhydryl groups and makes albumin the majority extracellular antioxidant in human plasma (Martin, 2005). Albumin is degraded into amino acids that are utilized for energy requirements of the cell or secreted into the pool of extracellular amino acids (Bush, 1990). Clinically, albumin has been available for human administration since the 1940s, with millions of doses resulting in few reported adverse reactions (Martin, 2005).

In this study, it was shown that albumin and normal saline have a similar effect in the treatment of rat EAN. No significant difference between these two groups was observed in clinical scores, weight changes, S wave latencies and amplitudes, MCVs, and CMAP amplitudes after stimulation at ankle and hip, indicating that albumin, along with normal saline, is ineffective in the EAN treatment. This result confirmed albumin as an ideal reagent for the control group in animal studies evaluating the efficacy and mechanisms of action of IVIg in GBS.
4.2 Treatment of rat EAN with albumin and IVIg

The aim of this study was to establish that IVIg can modulate experimental inflammatory demyelination in the PNS to obtain a model that would be suitable for the delineation of mechanisms underlying the therapeutic effects of IVIg in GBS. The albumin group served as the control group and the IVIg group as treatment group.

In this study, IVIg administered at the onset of signs of disease proved effective in preventing further progression of disease and shortening disease duration as demonstrated by clinical, electrophysiological and histological parameters. The effect is relatively modest, but it is statistically significant. The clinical differences were evidenced by significantly lower clinical scores in the IVIg group from day 14 to 20 and day 25 to 30. The electrophysiological differences included significantly less prolongation of S wave latencies, better maintained S wave amplitudes, less reduction of distal MCVs, and better maintained distal and proximal CMAP amplitudes in the IVIg group. The histological differences were evidenced by significantly lower histological grades, lower percentage of grade 3 and higher percentage of grade 0 in the IVIg group.

4.2.1 Comparisons with other studies

Three previous studies examined the efficacy of human Ig in rat EAN with conflicting results. In the study by Enders et al. (Enders et al., 1997), IVIg (Sandoglobulin®, Sandoz, Nürnberg) was administered daily at 400 mg/kg body weight, which started on the day of immunization in active EAN or cell transfer in AT-EAN and continued until day 4 after the maximum of disease. Diseases were monitored by clinical score, immunochemistry and histological analysis. They found that IVIg treatment failed to demonstrate a beneficial effect in both active EAN and AT-EAN and thus concluded that IVIg was not effective in EAN. In contrast, Miyagi et al. (Miyagi et al., 1997) found that IVIg administration on days 0, 7, 14, 15, and 16 at 400 mg/kg body weight each prevented the paralysis in EAN. Diseases were monitored by clinical score. Moreover, Gabriel et al. (Gabriel et al., 1997) found that the administration of intraperitoneal human Ig (Sandoglobulin®, Sandoz, UK) at the
onset of signs of disease significantly accelerates recovery from EAN and less beneficial effect was observed with intravenous treatment. In their study, human Ig was administered at 400 mg/kg body weight once daily for 5 days. Diseases were monitored by clinical score, a terminal electrophysiological assessment, and serum and histological analysis. Although there was a significant difference in clinical scores, they failed to show any significant difference in the electrophysiological parameters (CMAP amplitude & MCV) and the histological scores. However, they observed that the proportion of demyelinated fibres which had started to remyelinate was greater in the Ig-treated rats, suggesting that administration of human Ig may enhance the down-regulation of the immune response, reduce the total amount of demyelination, or permit earlier recovery, and thus facilitate remyelination.

The methodological differences between this study and the other three studies described above may account for the different results generated. First, the clinical disease severity was assessed using a 5-score grading in this study instead of a 10-score grading in the study by Enders et al., a 3-score grading in the study by Miyagi et al., and an 18-score grading in the study by Gabriel et al.. The severity of histological change was analysed by evaluating the extent of perivascular inflammation and demyelination in this study rather than the density of infiltrating T cells and macrophages in the study by Enders et al. and the percentage of fibres affected in the study by Gabriel et al.. Second, the total dose of IVIg for infusion was divided into 2 daily doses of 1 g/kg body weight each in this study instead of 5 daily doses of 400 mg/kg body weight each in the other three studies. Considering the drug’s rapid diffusion to the extravascular space, achieving a high concentration of serum Ig quickly appears to be associated with better therapeutic effects (Dalakas, 1999). Experimental studies, both in vitro and in vivo, suggested a superior effect on cytokine neutralization (Svenson et al., 1993), FcR modulation (Kurlander and Hall, 1986), and inhibition of C3 fragments (Dalakas, 1995) when IVIg concentrations equivalent to 2 g/kg body weight were given in a bolus infusion rather than divided doses. The effect of bolus infusion was also documented in children with Kawasaki’s syndrome (Newburger et al., 1991) and with GBS (Kanra et al., 1997). Third, the clinical, electrophysiological and histological assessments of the animals were
performed at regular intervals throughout the 30-day period rather than a terminal examination or a shorter period of observation (ie. 20 days) in the other three studies. Some beneficial responses from IVIg treatment as in S wave latencies, MCVs, and amplitudes of CMAPs were particularly evident after day 20 in this study. In two large clinical controlled randomised trials (van der Meché and Schmitz, 1992; Plasma Exchange/Sandoglobulin Guillain-Barré syndrome Trial Group, 1997), the beneficial effect of IVIg in GBS was observed 6 and 12 months after randomisation respectively, indicating that the efficacy of IVIg in EAN may be evident in the recovery period in addition to the acute progressive period.

4.2.2 Comparisons with other treatments

Although the effect of IVIg was modest, the effect was clearly demonstrated in animals which had already shown signs of clinical disease. Many putative therapies given to EAN rats prior to the onset of clinical disease can show more marked efficacy. The monoamine reuptake inhibitory anti-depressants, including zimeldine and its metabolites norzimeldine and CPP 200 (Bengtsson et al., 1992), clomipramine, and imipramine (Zhu et al., 1998a), have been shown to effectively inhibit clinical manifestation of EAN when given from the day of immunization due to an action on myelin T and B cell autoreactivity. Moreover, the clinical signs of EAN have been significantly suppressed dose-dependently by the administration of linomide (roquinimex) (Bai et al., 1997a; Zhu et al., 1999) or ABR-215062 (Zou et al., 2002) from the day of immunization through regulation of Th1/Th2 cytokine balance. Furthermore, many other immunomodulatory agents, such as high dose cyclosporin A (Hartung et al., 1987; McCombe et al., 1990), sodium fusidate (fusidin) (Di Marco et al., 1999), phosphodiesterase inhibitor pentoxifylline (Constantinescu et al., 1996), cyclooxygenase-2 inhibitor (nimesulide) (Miyamoto et al., 1998), and rapamycin (Lin and Spies, 2002) have previously been shown to effectively inhibit clinical manifestations of EAN. The therapeutic effect of these reagents was more marked than that of IVIg as shown in this study; however, the administration was performed before the onset of clinical disease and thus such therapy is not clinically relevant since patients can only be treated once they present with clinical disease.
4.2.3 Activity of human Ig in animal models

This study could be criticised on the basis that human, not rat Ig was used. It is generally believed that the efficacy of human IVIg is dependent on the large donor pool (>10,000) from which each batch is composed. It would clearly not be possible to produce rat Ig with such diversity and to make sufficient IVIg for this study would in any event consume an unacceptable number of animals. Immunoglobulin molecules are highly conserved between species and there is no reason to believe that human Ig would not be active in rat. In the studies by Yan et al (Yan et al., 2000; Yan et al., 2001), Ig from patients with inflammatory demyelinating neuropathy was shown to be able to bind to the sciatic nerve from normal rat. Certainly no animals in this study showed any signs of anaphylactic reaction following the 2-day treatment. In addition to the study conducted by Miyagi et al. (1997) and Gabriel et al. (1997) as described above, human Ig has also been shown to be effective in other animal models of autoimmune disease, inhibiting the active induction of experimental autoimmune encephalomyelitis (EAE) and adjuvant arthritis (Achiron et al., 1994), preventing the occurrence of experimental autoimmune uveoretinitis (Saoudi et al., 1993), and ameliorating the progression of experimental autoimmune myocarditis (George et al., 2001).

4.2.4 Effectiveness of human IVIg in the treatment of rat EAN

IVIg is believed to play an immunomodulatory role through several mechanisms including an idiotype-antiidiotype effect on autoantibodies, inhibition of complement binding and prevention of MAC formation, FcR modulation on invaded macrophages, suppression of pathogenic cytokines and AMs, modulation of T-cell function, and possible effect on remyelination (Dalakas, 2004). This study has not attempted to define by what mechanism human IVIg is active in this situation but clearly it is having an effect not significantly dissimilar to the effect in human GBS; clinical, electrophysiological and histological parameters all showed that human IVIg is effective in the treatment of rats EAN, ameliorating the clinical symptoms and accelerating recovery from EAN.
In this study, the effect of human IVIg in the treatment of EAN in rats was first demonstrated by the significant difference in clinical scores between the control and treatment groups. The clinical difference was evidenced by less severe disease, shorter clinical course and better outcome in the IVIg group. The clinical benefit from IVIg treatment was evident during both the acute progressive period (day 14-20) and recovery phase (day 25-30). At peak of disease severity, the difference in the mean clinical scores (2.9 in the control group and 2.2 in the treatment group) was considerable since at 2.9 rats have difficulty in walking whereas 2.2 represents only mild hind limb weakness or abnormal posture. In addition, more rats completely recovered from the clinical illness in the treatment group (71%) than in the control group (29%) by day 30, suggesting that IVIg treatment may facilitate recovery and prevent functional disability.

No significant difference in weight change between the control and treatment groups can be observed in this study. This could be explained by the fact that disease severity is not the only element determining the weight change in the active EAN. There are at least two factors influencing the weight change; the disease severity and the degree of foot pad inflammation due to immunization. The time course of weight change corresponded to the disease course which showed more weight loss in more severely affected animals. The influence of foot pad inflammation on weight change was evidenced by a temporary weight loss after foot pad inoculation and the observation that animals with footpad ulceration exhibited decreased mobility in the absence of any other clinical symptom and sign.

The clinical differences were supported by significant electrophysiological differences. SSEPs test the integrity of sensory conduction through peripheral nerve and nerve root and are a very sensitive measure in inflammatory neuropathies since the dorsal roots are the major site of pathology (Spies et al., 1995b). Significant differences in S wave latency and amplitude were consistent with less severe disease (inflammation and demyelination) in the IVIg-treated animals and the difference lasted from the disease peak to day 26, suggesting that the recovery in the control group was evident later in the course and IVIg treatment facilitated the recovery of S wave latency and amplitude. The magnitudes of the maximal changes of S wave
latency and amplitude were dependent on peak of disease severity. However, the time
course of mean S wave latencies didn’t correspond to the course of disease, in which
the S wave latency continued to lengthen after disease peak and reached its maximum
during the recovery phase (day 26). This inconsistency of the time course could be
due to the later demyelination in the distal sciatic nerve trunk and tibial nerve. In
contrast, the time course of mean S wave amplitudes correlated with the disease
course and returned towards the baseline amplitude during recovery from disease,
indicating that the changes in S wave amplitude were consistent with demyelination
(reduction of amplitude via dispersion) and remyelination (recovery of amplitude) in
the lumbar dorsal roots and proximal sciatic nerves (Taylor and Pollard, 2003).

MCV measures the motor nerve conduction in distal nerve segments and is
another indicator of the extent of demyelination. Significant differences in MCV in
the sciatic motor nerves were consistent with less severe disease and reduced
demyelination in the IVIg-treated animals and the significance lasted from disease
peak (day 18) to the end of study period (day 30). The magnitude of the maximal
reduction of MCV was dependent on peak of disease severity, indicating that the more
severe course of disease may partly attribute to the greater involvement of the motor
nerves. The time course of MCV in the control group failed to correlate with the
disease course, suggesting that the sciatic nerve is most affected during recovery from
disease, as reviewed in Chapter 1. While in the treatment group, the time course of
MCV and disease severity was consistent, which may indicate that IVIg treatment in
rat EAN prevented further sciatic nerve damage and facilitated the recovery of MCV.

Conduction block, severe dispersion and synchronous reduction of the distal and
proximal CMAP amplitudes were also observed in both the control and treatment
groups. Conduction block is accepted as an important physiological correlate to
underlying demyelination (Sumner et al., 1982) and the H/A ratio was used as an
indicator of conduction block in this study. No significant difference in conduction
block between the two groups could be shown in this study. One of the possibilities is
that conduction block might be present in the proximal roots where the pathology is
greater than in the sciatic nerve. The difference in distal and proximal CMAP
amplitudes was only significant at day 26 and 30. This finding could be explained by
axonal degeneration, which may occur simultaneously with or subsequently to
demyelination. However, less reduced amplitudes of CMAP after stimulation at ankle
and hip in the treatment group was observed from day 14 to 22 although the
difference failed to reach statistical significance, suggesting IVIg treatment may
prevent axonal degeneration either by reducing inflammation and secondary
bystander axonal loss or by other mechanisms.

The treatment effect of IVIg in rat EAN was further confirmed by the results of
neuropathological studies in which significant difference was evident from day 16 on,
particularly at day 16 and 30. Axonal degeneration was a prominent pathological
change in the nerve roots and trunk in addition to demyelination in this study,
consistent with the low CMAP amplitudes. However, this finding was dissimilar to
the typical EAN lesions where primary segmental demyelination and inflammation
with the rare occurrence of axonal degeneration has been observed in the nerve roots
and dorsal root ganglia, as reviewed in Chapter 1. Although EAN is used as a model
of inflammatory demyelinating neuropathies, severely affected nerves also show
marked axonal degeneration (Hughes, 1994). Axonal degeneration is more marked
following immunization with a larger dose of immunogen (Hahn et al., 1988) or
transfer of a larger number of T cells in the cell transfer model (Hahn et al., 1991).
Indeed, axonal degeneration was the dominant pathological feature observed in the
roots and nerve trunks from EAN induced with high doses of P2-specific (Hahn et al.,
1991) or P0-specific (Linnington et al., 1992) T-cell lines. Axonal damage, which also
occurs in GBS, may be the secondary consequence of inflammatory mediators
released within the endoneurium or of a rise in endoneurial pressure associated with
oedema (Hughes, 1994). Of the inflammatory mediators, NO, secreted by SC during
inflammation, has been shown in vitro and in vivo to mediate axonal degeneration
(Smith et al., 2001; Kapoor et al., 2003), possibly by intra-axonal accumulation of
sodium and calcium (Bechtold et al., 2005). Axons are particularly vulnerable to
degeneration if they are stimulated during exposure to NO (Smith et al., 2001), as will
occur as axons conduct physiological impulse traffic through inflammatory lesions.
The prominent axonal degeneration seen in the animals in this study may have
resulted from a larger dose of immunogen.
4.3 Treatment of rat EAN with albumin, Fab fragments, Fc fragments, and IVIg

The aim of this study was to investigate which portion of IVIg is effective in the treatment of EAN. This may help to understand the mechanism of action of IVIg and to develop more efficacious treatment in the future. The albumin group served as the control group and the Fab, Fc and IVIg groups as the treatment groups.

In this study, there is concordance in the clinical, electrophysiological and histological evidence showing the efficacy of IVIg and its Fc component rather than the Fab portions in the treatment of EAN in the Lewis rat. The clinical differences were evidenced by significantly lower clinical scores and less prominent weight loss in the Fc and IVIg groups. The electrophysiological differences included significantly less prolongation of S wave latencies, better maintained S wave amplitudes, less reduction of distal MCVs, and better maintained distal and proximal CMAP amplitudes in the Fc and IVIg groups. The histological differences were evidenced by significantly lower histological grades, lower percentage of grade 3 and higher percentage of grade 0 in the Fc and IVIg groups.

4.3.1 Studies concerning the Fab and Fc-mediated mechanisms of IVIg

4.3.1.1 Studies supporting Fab-mediated mechanism of IVIg

This result was unexpected since published studies of the mechanism of IVIg in patients with GBS to date suggest that Fab interactions with anti-idiotypic antibodies is an important mechanism and that therapeutic IVIg inhibits the pathophysiological effects of GBS sera by neutralizing neuromuscular blocking antibodies, probably by idiotypic-antiidiotypic mechanisms (Buchwald et al., 2002; Jacobs et al., 2003; Zhang et al., 2004).

Buchwald et al. (Buchwald et al., 2002) studied the effect of sera from GBS and MFS patients on neuromuscular transmission using a macro-patch-clamp technique. They showed that preinfusion sera (sera from patients before IVIg treatment) induced
a severe blockade of neuromuscular transmission while postinfusion sera (sera from patients after IVIg treatment) or the mixture of preinfusion sera with the therapeutic IVIg preparations failed to display the blocking activity. When the purified GBS-IgG from preinfusion sera was coincubated with the therapeutic IVIg, the blocking activity of the GBS-IgG was diminished dose-dependently with increased IVIg concentration, indicating that the neutralizing effect was exerted by a direct interaction of therapeutic IVIg with the GBS-IgG rather than other GBS serum components such as complement or low-molecular-weight compounds. Furthermore, the neutralizing activity resided in the IgG, F(ab’)_2 and Fab fractions, but not the Fc component. They suggested the effect of IVIg was therefore through antiidiotype mechanisms.

In the study by Jacob et al. (Jacobs et al., 2003), the effects of IVIg on the interaction of anti-GQ1b antibodies with GQ1b in vitro and on anti-GQ1b antibody-mediated NMJ injury ex vivo were studied. Sera containing anti-GQ1b IgG antibodies were obtained from MFS and GBS patients before treatment. They showed that IVIg inhibited the binding of anti-GQ1b antibodies to GQ1b in ELISAs in a dose-dependent manner. In addition, IVIg prevented the α-latrotoxin-like effects of MFS/GBS sera at mouse NMJs in twitch bioassay and in ex vivo electrophysiological studies. Moreover, complement binding to anti-GQ1b antibodies in vitro and complement activation in the mouse diaphragm model ex vivo were reduced when IVIg preparation was coincubated with the complement source. They thus concluded that IVIg directly interferes with the pathogenic effects of anti-GQ1b antibodies at motor nerve terminals.

In the study by Zhang et al. (Zhang et al., 2004), an anti-ganglioside antibody-mediated cytotoxicity assay was used to study the role of ganglioside expression and complement, and whether human IVIg can protect neuronal cells from anti-ganglioside antibody-mediated injury and the mechanisms involved. GBS sera containing anti-ganglioside antibodies or mAbs with anti-ganglioside reactivity was shown to cause complement-dependent cytotoxicity resulting in neuronal cell lysis by targeting specific cell surface gangliosides. Moreover, IVIg was shown to provide significant protection from this anti-ganglioside antibody-mediated injury dose-dependently and the mechanisms of this protection included anti-idiotypic antibodies.
against anti-ganglioside antibodies and down regulation of complement activation, one of the Fc effector functions.

The studies described above involved the sera from GBS and MFS patients in which antibodies against gangliosides are present. However in AIDP, the most common form of GBS, no clear role for antibody has been established. In addition, one of the possibilities of the failure to identify the amelioration of EAN by Fab fragment is that the Fab mediated anti-idiotypic effects previously reported are very likely to be species specific, whereas Fc related effects might be expected to have a broader species specificity and thus a broader cross-species effect. Therefore, human Ig does not contain appropriate idiotypic antibodies to inhibit rat genotypes and the effect of Fab fragments might be less in the rat than in human. Studies concerning the neutralization effects of virus antigen with purified F(ab')2 or Fab fragments showed that neutralization was not sufficient for protection in vivo with either F(ab')2 or Fab fragment despite their ability to neutralize virus in vitro, suggesting that the in vitro neutralization does not necessarily correlate with in vivo protection (Baldridge and Buchmeier, 1992; Schlesinger and Chapman, 1995; Liang et al., 1996). A possible explanation for this is that in the absence of a Fc region, Fab fragment alone may not be able to participate in several humoral and cellular immune functions that are potentially important for the treatment effect of IVIg.

4.3.1.2 Studies supporting Fc-mediated mechanism of IVIg

The results of this study confirm the previous study of Miyagi et al (Miyagi et al., 1997), in which rat EAN was treated with intravenous F(ab')2 fragment and IVIg. They found that intact-type IVIg is superior to F(ab')2 in suppressing the clinical signs of EAN and suggested that the Fc portion plays an important role in the effect of IVIg.

There are a number of other studies which emphasize the importance of the Fc signalling in the modulation of immune responses by passive antibody. The Fc fragment has been shown to play an important role in the regulation of humoral immunity, cellular immunity, lymphocyte and monocyte activation, and immune
mediator secretion (Ling et al., 1990). Evidence supporting Fc signalling was reviewed by Sinclair (Sinclair, 2001). The ability of antigen-specific antibody to inhibit long-lasting IgG immunological priming was markedly reduced following removal of the Fc fragment, indicating that the Fc portion may be required in vivo and in vitro immunosuppression. The results of in vivo studies on the suppression of immune responses by antigen-specific antibody supported the coaggregation of BCR with FcγRIIb, suggesting that this effect was Fc-dependent. Intact antibody but not F(ab’)_2 antibody was able to terminate established immune responses and the F(ab’)_2 IgG antibody was shown to interfere with IgG antibody-mediated immunosuppression. An elaborate negative FcγRIIb signalling mechanism was defined in the B cells. Moreover, cross-linking of BCRs and FcRs by antigen-antibody complexes was demonstrated to be a blocking signal for B cells (FcR off-signal) and this FcR off-signal in the B cell led to inhibition of antigen-induced blastogenesis and proliferation and the induction of B cell apoptosis. Blockade of inhibitory Fc-signalling by anti-Fc antibody including rheumatoid factor augmented immune responses. Furthermore, the importance of FcγRIIb inhibitory signaling in regulating immune responses is evident in FcγRIIb-deficient mice, which exhibited enhanced antibody production, autoimmunity, anaphylactic responses, and antibody-dependent clearance of tumor metastases (Ott et al., 2001).

The results from this study suggest that FcR-mediated mechanisms which serve as a link between the humoral and cellular immune responses may play a more important role than that of anti-idiotypic antibody mechanisms in rat EAN. Acute EAN is an animal model for assisting in understanding the pathogenesis of human GBS. Numerous studies have shown that EAN is an autoimmune disorder involving both cellular and humoral immune mechanisms (Kieseier et al., 2004). T-cells play an essential role in the initiation of the EAN and the disease can be passively transferred by activated T cells alone (Linington et al., 1984; Heininger et al., 1986; Linington et al., 1992). Moreover, it has been demonstrated that administration of P2-specific T cells in passively transferred EAN induces BNB permeability (Spies et al., 1995b; Taylor and Pollard, 2001), illustrating the synergy between cellular and humoral mechanisms. Macrophages are the predominant mononuclear cell type in the PNS of
EAN rats and constitutively express the MHC class II antigen and CR3. They are an integral part of the disease process and are the final common pathway for myelin removal, as so called macrophage-mediated demyelination (Hartung and Toyka, 1990; Kiefer et al., 2001). Because of the central role for T cells and macrophages, FcR-mediated phagocytosis of myelin is a most prominent feature of the pathology of EAN and the modulation, down regulation and saturation of FcR by IVIg may well be expected to suppress the disease severity and alter the disease course.

4.3.2 IVIg and FcγR

The Fc portion of the infused IgG may exert its immunomodulatory action by interaction with FcγRs on target cells. The blockade of FcγR on macrophages is considered to underlie the mechanism of IVIg in idiopathic thrombocytopenic purpura (ITP) and other autoantibody-mediated cytopenias. Blockade of FcRs on phagocytic cells by IVIg may also cause inhibition of ADCC, providing the protective effect against demyelination in inflammatory neurologic disorders (Sewell and Jolles, 2002).

4.3.2.1 Inhibition of phagocytosis via blockade of FcγRs on macrophages and effector cells

IVIg may induce blockade of the FcRs on phagocytic cells such as macrophages by saturating, altering, or down-regulating the affinity of the FcRs, a process that may render sensitized phagocytic cells unable to exert their action (Dalakas, 2004). Since the concentration of IgG in normal blood is 12 mg/mL, and it increases rapidly upon IVIg, the IC binding abilities of FcγRI, FcγRII, and FcγRIII is hampered (Takai, 2005). Macrophages play a central role in demyelination in GBS and EAN. In IVIg treatment of GBS patients, inhibition of macrophage FcγRs binding to autoantigen-IgG IC may occur, leading to the inhibition of macrophage activation and the macrophage-mediated phagocytosis of antigen-bearing target cells therefore intercepting the macrophage-mediated demyelination (Dalakas, 2004).

4.3.2.2 Inhibition of phagocytosis via inhibitory FcγR

IVIg up-regulates the inhibitory FcγRII and modulates the FcγRII/FcγRIII ratio
on macrophages (Samuelsson et al., 2001). IgG monomers in IVIg predominantly bind to FcγRI, whereas polymeric IgG or IgG in the form of ICs interact with FcγRII and FcγRIII (Takai, 2005). FcγR with intracellular ITAM or ITIM mediate inflammation or immune effector functions via activation or inhibitory signaling. The ratio of expression of the inhibitory and activation FcγR determines the final immune response; overexpression of FcγRI and FcγRIII favors activation, whereas overexpression of FcγRII infers inhibition of phagocytosis and interception of ADCC (Samuelsson et al., 2001).

It is postulated that the beneficial effects of IVIg in the treatment of immune disorders may be attributable, at least in part, to engagement of FcγRIIb (Ott et al., 2001). Samuelsson et al. (Samuelsson et al., 2001) demonstrated that FcγRIIb is crucial for mediating the anti-inflammatory activity of IVIg and the cross-linking of FcγRIIb and FcγRIII by ICs abolishes the activating signal, resulting in abrogation of phagocytosis. The inhibitory FcγRIIb was required for protection, because disruption either by genetic deletion or with a blocking mAb reversed the therapeutic effect of IVIg. In addition, an increase in the number of monocytes bearing the FcγRIIb and an increase in the FcγRII/FcγRIII ratio on monocytes has been noted in GBS and CIDP patients who started to improve after IVIg, suggesting that such inhibitory signaling may be clinically relevant (Créange et al., 2003). In the study by Siragam et al. (Siragam et al., 2006), IVIg-primed dendritic cells from FcγRIIb-deficient mice only ameliorated mouse ITP when the recipient mouse expressed FcγRIIb whereas IVIg-primed dendritic cells from activating FcγR-deficient mice did not inhibit ITP, indicating that activating FcγRs on dendritic cells is the primary functional target of IVIg while inhibitory FcγRIIb has a role in a downstream phase of IVIg function.

4.3.2.3 Increased catabolism of IgG antibody

IVIg have been reported to accelerate autoantibody catabolism by binding to FcRn on endothelial cells (Yu and Lennon, 1999), which is an important mechanism of action of IVIg. Normally, the IgG is protected from lysosomal degradation when pinocytosed into cells from plasma by binding to FcRn present in the endocytotic vesicles. IVIg treatment may result in the saturation of FcRn and preventing
Pathogenic autoantibodies from binding through competition, with the consequence of their accelerated degradation (Simon and Späth, 2003). In fact, studies in knock-out mice lacking the gene encoding β2-microglobulin revealed that the low serum level of IgG present in these animals is attributed to the loss of FcRn. Moreover, in the presence of hypergammaglobulinemia, FcRn is saturated and the degradation of IgG is possible in proportion to its total concentration in plasma. The same effect could be achieved either by mAbs modifying FcRn covalently, by antisense nucleotides downregulating the expression of FcRn, or by synthetic ligands with a higher affinity than IgG for FcRn (Yu and Lennon, 1999).

4.3.3 Purification of Fab and Fc fragments

4.3.3.1 Affinity chromatography

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatography matrix (Pharmacia LKB Biotechnology, 2002). Affinity purification offers high selectivity, hence high resolution, and, usually, high capacity for the target protein(s). The target molecule is concentrated into a smaller volume and purity levels as high as 99% are achievable in one step. In a single step, affinity purification can offer immense time-saving over less selective multistep procedures such as diethylaminoethyl (DEAE)-ion exchange chromatography described in other studies (Toyka et al., 1980; Johnstone and Thorpe, 1996; Buchwald et al., 1998). The concentrating effect enables large volumes to be processed. Target molecules can be purified from complex biological mixtures, native forms can be separated from denatured forms of the same substance and small amounts of biological material can be purified from high levels of contaminating substances.

At the moment, affinity chromatography represents the most powerful fractionation technique for the purification of biotechnological products. When there is an immunospecific interaction affinity chromatography is often the first, and frequently the only, step required (Pharmacia LKB Biotechnology, 2002). Successful affinity purification requires a biospecific ligand that can be covalently attached to a chromatography matrix. The coupled ligand must retain its specific binding affinity
for the target molecules and, after washing away unbound material, the binding between the ligand and target molecule must be reversible to allow the target molecules to be removed in an active form. Any component can be used as a ligand to purify its respective binding partner.

Protein A and protein G affinity chromatography are the standard methods for purifying polyclonal and monoclonal antibodies, primarily because it can be performed easily and achieves high-purity levels (Aybay, 2003). Both protein A and G are bacterial cell wall proteins (from *Staphylococcus aureus* and *Streptococcus*, respectively) that have the ability to bind to the Fc domain of many types of antibodies (Eliasson et al., 1989). This binding is quite strong under physiological conditions but can be easily disrupted by decreasing the pH of the surrounding solution.

Although proteins A and G have very similar applications, there are differences in their binding properties (Eliasson et al., 1989). Protein G reacts with more IgG isotypes and reacts less with other Ig. Of human IgG, both proteins A and G bind well to IgG1, IgG2, and IgG4; however, IgG3 is considerably stronger bound by protein G as compared with protein A. Protein A was reported to be able to bind the Fab fragment of human IgM (Aybay, 2003). Moreover, protein G was shown to be able to bind both the Fab and Fc portions of IgG while protein A was totally non-reactive with the Fab portion (Eliasson et al., 1989; Aybay, 2003). Of the two bacterial receptors, the determinants on the Fc region of human polyclonal IgG are mutual or closely overlapping. Nevertheless, there exist determinants located within the Fab region of human IgG which are selectively identified by protein G. Furthermore, protein A showed an overall considerably higher avidity to the Fc region of human IgG than did protein G, which was demonstrated by competitive binding assay (Eliasson et al., 1989).

In this study, protein A chromatography was used to separate Fab from Fc portions based on the following reasons. First, protein G binds to the Fab domain in addition to Fc domain while protein A exclusively binds to the Fc domain of human IgG as described above. Since the separation and purification of Fab and Fc fragments
were essential for the study, protein G was practically inappropriate. Second, the higher avidity to the Fc region of human IgG of protein A ensured the maximum binding of Fc domain to protein A. Third, human IgG₃, to which protein A was shown not to bind, only accounts for less than 3% in human IVIg preparation (Intragam® P), thus the unbound Fc domains is negligible.

4.3.3.2 Purity of Fab and Fc fragments

In this study, the purity of purified Fab and Fc solutions was checked by SDS-PAGE and immunoblotting (Figure 2.3 and 2.4). Incomplete separation of Fab fragments from the Fc and undigested IgG mix was observed according to the result of the immunoblotting as shown in Figure 2.3. The purified Fc solution eluted from the gel filtration was contaminated with Fab fragment and undigested IgG molecule, in which IgG molecule accounted for approximately 4% of total protein when the quantity of each band was estimated using the Quantity One® software (Bio-Rad Life Science Group, Hercules, CA, USA). On the other hand, complete separation of Fc and undigested IgG mix from Fab fragment by affinity chromatography was demonstrated as in Figure 2.4. The purity of Fab solution was greater than 98% and no trace of Fc fragment can be observed.

When evaluating the purity of Fc solution, controversy over the component of Fc solution was aroused due to the two bands with the MW approximately 50 kDa shown in the Fc lane when immunoblotting with anti-Fab fragment antibody. To clarify which fragment the bands represent, a further western blotting with anti-Fab fragment antibody loaded with reducing and nonreducing IgG molecule was performed (Figure 4.1). This showed that the upper band of the Fc lane with MW 50 kDa lined up with the IgG H chain from IgG lanes created by the reducing agent (Mercaptoethanol) while the lower band lined up with the Fab fragment. Since the Fc solution contained 4% undigested IgG, it was possible that the upper band represented the H chain of contaminated IgG from natural degradation which accounted for approximately 3.5% of protein in Fc solution estimated by Quantity One® and the lower band the contaminated Fab fragment (1.5% of protein in Fc solution). Therefore, the purity of the Fc solution was approximately 90% in this study.
The results of this study were not affected by the fact that the Fc solution was contaminated with small amount of Fab and IgG. The reasons are as follows. First, Fab fragment only made up 1.5% of protein in the Fc solution and was demonstrated ineffective in the treatment of rat EAN in this study. Second, the amount of the intact IgG in Fc solution was not large enough to exert a treatment effect in rat EAN. Intact IgG accounted for only 4% of protein in Fc solution and thus the dose injected into rats was minimal. IVIg exerts an effective treatment during active phase of GBS only at high infusion dose (Lemieux et al., 2005).

![Western blotting with anti-Fab fragment antibody](image)

**Figure 4.1** Western blotting with anti-Fab fragment antibody. The standard molecular weight markers were shown in Lanes A and H. Lane B was loaded with Fab sample. Lane C was loaded with Fc sample. Lanes D and F were loaded with IgG solution eluted from the gel filtration. Lanes E and G were loaded with the original IgG solution (Intragam® P). All the samples loaded contained 0.5μg protein each. The samples for lanes D and E were treated with reducing agent (Mercaptoethanol) before loading. This showed that the upper band of the Fc lane with MW 50 kDa lined up with the IgG H chain from IgG lanes created by reducing agent while the lower band
lined up with the Fab fragment, indicating that the upper band was H chain of IgG while the lower band Fab fragment.

4.3.3.3 Change of Fab and Fc biological features after digestion and purification

The Fab fragment, F(ab’)_2 fragment and the intact IgG molecule share some common properties but differ in other immunoactivities (Sedlacek et al., 1983). For example, both Fab and F(ab’)_2 fragments retain their antigen-binding properties as in IgG molecule; although the F(ab’)_2 fragment is bivalent whereas the Fab fragment monovalent, they are both effective in their ability to neutralize and eliminate toxin.

The half life time of IgG, F(ab’)_2, and Fab in blood are 18 days, 2 days, and < 1 day, respectively. F(ab’)_2, Fab, and the whole IgG also initiate different pathways in complement activation: ICs containing F(ab’)_2 do not activate the classical pathway but only the alternative pathway, whereas ICs containing Fab fragments do not significantly active either pathway.

Controversy over the importance of bivalent binding of Fab fragment has been aroused. F(ab’)_2 fragment was shown superior to Fab fragment in its in vivo and in vitro antibacterial and antiviral effect and in its in vitro phagocytosis and exocytosis via C3b receptors (Sedlacek et al., 1983). In one study (Fjeld et al., 1992), the set of binding parameters of iodine-125 labelled intact IgG, F(ab’)_2 and Fab fragments were compared, including K_a (the association constant of the binding reaction between a ^125I-mAb and a binding site on the target polymer particle or target cell). It showed that the K_a for the F(ab’)_2 fragment was similar to that for IgG while the K_a for the Fab fragment was moderately lower (10-20%) than that for IgG. The possible explanation for the moderate difference rather than a 50% difference between the K_a-F(ab’)_2 and K_a-Fab was that the bivalent molecules were not bivalently attached to the particles. Moreover, bivalency was found not to be essential for neutralization or protection but enhanced these properties in a study by Lamarre et al. (Lamarre and Talbot, 1995). Furthermore, studies concerning the neutralization effects of virus antigen with F(ab’)_2 orFab fragments showed that neutralization was not sufficient for protection in vivo with either F(ab’)_2 or Fab fragment despite their ability to neutralize virus in vitro (Baldridge and Buchmeier, 1992; Schlesinger and Chapman, 1995;
Liang et al., 1996). The above observations have clearly shown that F(ab')2 and Fab fragment do not differ significantly in their biological activities in vivo, which provided the basis for our decision to use Fab fragment for the study.

There are certain inherent difficulties in making comparisons of the immunomodulatory effects of the Fc fragment and IVIg in vivo. Consideration must be given to possible differences in Fc-mediated immunomodulation between Fc portion of intact IgG and Fc fragments separated from IgG. The solubility of the Fc fragment after separation from the IgG molecule is very low and they easily aggregate, therefore, it is extremely difficult to produce a monomeric Fc fraction. The effector functions of such aggregates of Fc fragment are much more close to aggregated IgG than to monomeric and dimeric IgG that accounted for more than 90% of the protein in IVIg preparation (Intragam® P). In a study in which the capacity of IVIg, heat-aggregated IVIg, and Fc fragments to induce cytokine IL-6 was compared (Ling et al., 1990), it was shown that aggregated IVIg and Fc fragment but not monomeric IVIg were able to induce IL-6 secretion from monocytes, indicating the different immunomodulatory function between aggregated and nonaggregated Fc portions of IgG. An additional experiment using heat-aggregated IgG would help to clarify this however such an experiment is difficult to perform in animals. Further studies using an in vitro system would be an option to compare the effect of monomeric and aggregated IgG and Fc fragment.

4.3.4 Effectiveness of Fc fragment and IVIg in the treatment of rat EAN

In this study, an attempt was made to investigate the mechanisms of human IVIg for the treatment of rat EAN, by comparing the treatment effects of albumin, Fab fragment, Fc fragment, and IVIg. Human IVIg and its Fc fragment rather than Fab fragment and albumin administered at the onset of signs of disease proved effective in preventing further progression of disease and shortening disease duration as demonstrated by clinical, electrophysiological and histological parameters.
In this study, the effect of IVIg and Fc fragment was first demonstrated by the significant difference in the clinical scores among the four groups. The clinical difference was evidenced by less severe disease, shorter clinical course and better outcome in the Fc and IVIg groups, indicating that IVIg and Fc fragment ameliorated the clinical symptoms and accelerated recovery from EAN. The clinical benefit from IVIg and Fc treatment was shown from the acute progressive period and persisted until the recovery phase. At peak of disease severity, the mean clinical scores of the Fc and IVIg groups were similar (2.4 and 2.3, respectively) while in the albumin group the maximum mean clinical score of 3.3 was considerably different from those of the Fc and IVIg groups. Nevertheless, the maximum mean clinical score of 2.7 in the Fab group was not significantly different from those of the albumin, Fc, and IVIg groups, indicating that Fab fragment has a relatively modest effect of ameliorating the clinical symptoms. By day 30, more rats completely recovered from the clinical illness in the Fc (50%) and IVIg (67%) groups than in the Fab (13%) and albumin (0%) groups, suggesting that treatment with Fc fragment and IVIg may facilitate recovery and prevent functional disability.

The extent of mean weight loss was greatest in the albumin group followed by the Fab group and then the Fc and IVIg groups. Significant difference in weight changes was evident from day 17 on when the Fc or IVIg group was compared to the albumin group, suggesting that treatment with Fc and IVIg prevented EAN rats from considerable weight loss. The extent of weight loss in the Fab group was less severe than that of the albumin group and no difference was shown when the Fab group was compared to the Fc or IVIg group, indicating that Fab fragment prevented weight loss to a lesser extent.

The clinical differences were supported by significant differences in electrophysiological results. Significant differences in S wave latency and amplitude were consistent with less severe disease (inflammation and demyelination) in the Fc fragment and IVIg-treated animals. The magnitudes of the maximal changes of S wave latency and amplitude were dependent on peak of disease severity. The difference in S wave latency was more prominent when the Fc group was compared with the Fab or albumin group than when the IVIg group was compared, suggesting
that Fc fragment was superior to IVIg in preventing prolongation of S wave latency. The difference in S wave amplitude was more prominent when the IVIg group was compared with the Fab or albumin group than when the Fc group was compared. In addition, some differences were observed between the albumin and Fab groups or the Fc and IVIg groups but failed to reach statistical significance (0.1 > p > 0.05). Taken together, it indicated that IVIg was superior to the Fc fragment in facilitating the recovery of S wave amplitude and that treatment with Fab fragment diminished the reduction of S wave amplitude to a lesser extent.

MCV is another indicator of the extent of demyelination. Significant differences in MCV in the sciatic nerves were consistent with less severe disease and reduced demyelination in the Fc fragment and IVIg-treated animals. The magnitude of the maximal reduction of MCV was dependent on peak of disease severity, indicating that the more severe course of disease may partly attribute to the greater involvement of the motor nerves. The differences between the Fc or IVIg groups and albumin or Fab groups were evident from the acute progressive phase, suggesting that treatment with Fc fragment or IVIg effectively prevented further demyelination and facilitated the recovery of MCV.

Results from studies of the CMAP amplitudes supported the effect of Fc fragment and IVIg. Conduction block is accepted as an important physiological correlate to underlying demyelination (Sumner et al., 1982) and the H/A ratio was used as an indicator of conduction block. Conduction block was observed in the albumin, Fab and Fc groups during the acute progressive phase but not in the IVIg group, suggesting that IVIg treatment was more effective even than Fc fragment in suppressing the inflammation and demyelination. Severe dispersion and synchronous reduction of the distal and proximal CMAP amplitudes during acute phase were evident in all the four groups. The differences in the distal and proximal CMAP amplitudes in favour of Fc fragment and IVIg treatment were more prominent when the IVIg group was compared with the Fab or albumin group. These results again suggested that IVIg was superior to the Fc fragment in maintaining the amplitudes of both distal and proximal CMAPs and facilitating the recovery.
The treatment effect of the Fc fragment and IVIg in rat EAN was further confirmed by the results of neuropathological studies in which significant differences were evident on day 30 between the albumin and Fc groups, albumin and IVIg groups, Fab and Fc groups, Fab and IVIg groups and even Fc and IVIg groups. The histological difference between the Fc and IVIg groups was less prominent than the other compared groups but it indeed reached statistical significance, suggesting that IVIg was superior to the Fc fragment in preventing further inflammation and demyelination and facilitating recovery despite the treatment effect seen in Fc group. Axonal degeneration was a prominent pathological change in the nerve roots and trunk in addition to demyelination in this study, consistent with the low CMAP amplitudes. Again, this finding was dissimilar to the typical EAN lesions where primary segmental demyelination and inflammation with the rare occurrence of axonal degeneration has been observed in the nerve roots and dorsal root ganglia, as described in the preceding study (page 135).

More prominent remyelination in nerves from the Fc and IVIg groups suggested that treatment with Fc fragment and IVIg may be able to facilitate remyelination, possibly by enhanced downregulation of the immune response and permitting earlier recovery. Alternatively, an increase uptake of myelin debris and the simultaneous clearance of myelin-associated inhibitory molecules that blocked remyelination is another possible explanation (Kuhlmann et al., 2002). IVIg has been shown to increase the myelin uptake by macrophages in sciatic nerves and this improved myelin uptake was mediated by FcRs (Kuhlmann et al., 2002). The binding of IVIg to damaged SCs and myelin may induce an enhanced FcR mediated opsonisation of lesional debris finally resulting in remyelination. This view is strengthened by a cuprizone induced animal model of demyelination in which mRNA upregulation of myelin genes indicating the beginning of remyelination correlated with the number of infiltrating macrophages/microglia (Morell et al., 1998).
Conclusions

EAN was induced in adult inbred Lewis rats by immunization with whole bovine PNM prepared from fresh bovine spinal cord and cauda equina. The immunized rats were randomised into groups, monitored by the clinical score, weight and electrophysiological parameters (SSEPs, MCVs, and CMAPs) at regular intervals, and received intravenous injection of normal saline, albumin, human IVIg preparation, purified Fab fragment, or Fc fragment. Fab and Fc fragments were prepared from the human IVIg preparation in a large scale by papain digestion, affinity chromatography, and gel filtration. Animals from each group were randomly chosen and sacrificed at predetermined intervals for histological analysis. In the first study, the treatment efficacy of normal saline and albumin was compared to rationalize the use of albumin as the control therapy in the subsequent studies and to establish the basic database of EAN in Lewis rat. In the second study, the treatment efficacy of albumin and human IVIg preparation was compared to obtain an animal model for the delineation of mechanisms underlying the therapeutic effects of IVIg in GBS. In the third study, the treatment efficacy of albumin, Fab, Fc, and IVIg preparation were compared to investigate which portion of Ig is effective in the treatment of EAN in order to clarify how IVIg acts on GBS and to develop more efficacious treatment in the future.

The results of these three studies showed that there were significant differences in treatment efficacy between different treatment reagents. In the first study, no significant difference was noted between the normal saline and albumin groups. In the second study, significant differences in favour of IVIg treatment were evident in clinical, electrophysiological and histological parameters during the acute progressive and recovery phases. The differences included significantly lower clinical scores, significantly less prolongation of S wave latencies, better maintained S wave amplitudes, less reduction of distal MCVs, better maintained distal and proximal CMAP amplitudes, and significantly lower histological grades in the IVIg group. By day 30, more rats completely recovered from the clinical disease in the IVIg group than in the albumin group (29% in the albumin group and 71% in the IVIg group). In the third study, the differences were demonstrated by significantly lower clinical
scores, less prominent weight loss, significantly less prolongation of S wave latencies, better maintained S wave amplitudes, less reduction of distal MCVs, better maintained distal and proximal CMAP amplitudes, and significantly lower histological grades in the Fc and IVIg groups. By day 30, more rats completely recovered from the clinical disease in the Fc and IVIg groups (0% in the albumin group, 13% in the Fab group, 50% in the Fc group, and 67% in the IVIg group).

These finding suggested that human IVIg administered at the onset of signs of disease proved effective in preventing further progression of disease and shortening disease duration in rat EAN as demonstrated by clinical, electrophysiological and histological parameters. The effect is relatively modest, but it is statistically significant. Moreover, Fc fragment rather than Fab fragment was shown to have the similar therapeutic effect as IVIg in ameliorating the clinical symptoms and reducing the electrophysiological and pathological damages. Fab fragment played a part in maintaing the amplitudes of S wave and CMAP but it failed to show a statistical significance. In addition, more rats completely recovered from the clinical illness in the Fc and IVIg groups, indicating that Fc fragment and IVIg treatment may facilitate recovery and prevent functional disability. Other mechanisms in which IVIg exerts its immunomodulatory and beneficial effects are possible because the effect of Fc fragment was not exactly the same as IVIg. In addition, difficulties in making comparisons between the aggregated Fc fragment and monomeric IgG arose. Further studies using an in vitro system to compare the effect of monomeric and aggregated IgG and Fc fragment would help to clarify this.

Taken together, the current study provides direct evidence for the first time that the Fc fragments of IVIg are effective in the treatment of rat EAN. Since there are considered to be many similarities between EAN and the inflammatory demyelinating neuropathies, this may help us design more effective and less costly treatments for these neuropathies. Further experiments focusing on which region of the Fc fragment is important for the therapeutic effects and how Fc fragments mediate this effect and further studies clarifying the involvement of different FcγRs in IVIg treatment would be necessary to help develop a substitute treatment for IVIg.
Bibliography


