A RESONANT MIRROR BIOSENSOR APPROACH TO UNDERSTAND ANTIBODY-ANTIGEN INTERACTIONS IN THE GUILLAIN BARRé SYNDROME

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A resonant mirror biosensor approach to understand antibody-antigen interactions in Guillain Barré Syndrome

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AIDP	Acute inflammatory demyelinating neuropathy
AMAN	Acute motor axonal neuropathy
AMSAN	Acute motor-sensory axonal neuropathy
BBB	Blood brain barrier
BNB	Blood nerve barrier
BSA	Bovine serum albumin
C. jejuni	*Campylobacter jejuni*
CD	Cluster of differentiation
CIDP	Chronic inflammatory demyelinating polyradiculoneuropathy
CMV	Cytomegalo virus
EBV	Epstein-barr virus
EDC	N-ethyl-N-(3-diethylaminopropyl)carbodiimide
ELISA	Enzyme-linked immunosorbent assay
Gal	Galactose
GalNAc	N-acetylgalactosamine
GBS	Guillain-Barré syndrome
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HPTLC	High pressure thin layer chromatography
HRP	Horse radish persoxidase
IAsys	Interaction analysis system
IFN	Interferon
IgM/IgG	Immunoglobulin M/G
IL	Interleukin
INCAT	Inflammatory neuropathy cause and treatment
IVIg	Intravenous immunoglobulin
LPS	Lipopolysaccharide
LUV/SUV	Large/small unilamellar vesicles
MFS	Miller Fisher
MHC	Major histocompatibility complex
MLV	Multilamellar vesicles
MΦ	Macrophage
NeuAc	N-acetylneuraminic acid
NF-κB	Nucleura factor- κB
NHS	N-hydroxysuccinimide
NMJ	Neuromuscular junction

PBS	Phosphate buffered saline
PC	Phosphatidyl choline
PE	Plasma exchange
PEN	Of the PENNER serotype
PNS	Peripheral nervous system
scFv	Single chain variable fraction
SEM	Scanning electron microscopy
SGLPG	Sulfated glucoronyl lactosaminyl paragloboside
SGPC	Sulfated glucuronyl paragloboside
SNP	Single nuleotide polymorphism
TCR	T-cell receptor
TLC	Thin layer chromatography
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)aminomethane

Chapter 1
Introduction

1-1. The Guillain Barré Syndrome

Although the Guillain-Barré syndrome (GBS) was first described by Jean-Baptiste Octave Landry in 1859 the name was only born in 1916 when Georges Guillain, Jean-Alexandre Barré and André Strohl reported two soldiers who recovered from a severe ascending paralysis (Seneviratne 2000; Winer 2001; Pritchard and Hughes 2004; Hughes and Cornblath 2005). These soldiers showed reduced or delayed tendon reflexes, with a latency of almost twice normal, leading these physicians to deduce that nerve conduction was impaired or the central part of the reflex affected. A lumbar puncture was also performed on these soldiers and the results showed an increase in protein concentration but a normal cell count. This feature of the GBS was since then used as a tool to distinguish the syndrome from other neuropathies and poliomyelitis (Pritchard and Hughes 2004).

1-1.1. The clinical picture

Today, GBS is recognized as the most common diagnosis for acute flaccid paralysis. The Guillain Barré Syndrome affects about 1.2-1.9 per 100 000 people per year and is found more often in elderly than young people (Van Koningsveld, Van Doorn *et al.,* 2000; Govoni and Granieri 2001; Chio, Cocito *et al.,* 2003; Bogliun and Beghi 2004; Hughes and Cornblath 2005). The diagnostic criteria are based entirely on clinical features even though many other features are associated with the disease for instance location of neuron damage, specific antibodies present and preceding infections (Hartung, van der Meché *et al.,* 1998).

The disease usually starts with a tingling feeling in the peripheral limbs which rapidly progress to weakness and total paralysis in 20% of cases (Willison 2005). Acute GBS patients usually reach a nadir (peak of disease) 2-4 weeks after the onset of clinical symptoms. In 25% of GBS cases patients require artificial ventilation and 10% of patients are disabled while 5% of cases are fatal (Hughes, Swan *et al.,* 2006). Most patients recover satisfactory within 2-3 months. In chronic cases, like CIDP which is a chronic relapsing, motor and sensory polyradiculoneuropathy, nadir takes longer and

recovery is slower (Pritchard and Hughes 2004). The major criteria for diagnosis are mainly symmetrical weakness, impaired myotactic reflexis and nadir within 4 weeks (Hartung, van der Meché *et al.,* 1998).

Cerebrospinal fluid analysis usually reveals a rise in protein concentration over weeks, but is of limited value (Hartung, van der Meché *et al.,* 1998). On the contrary, electromyograms help to demonstrate polyneuropathic changes and also assist to determine if demyelination or axonal damage occurs, since the latter often has a pure prognosis. Electrophysiological studies revealed that 69% of GBS cases show demyelination, 3% axonal damage, 3% unexcitable nerves, 2% abnormal impulse conduction and 23% were inconclusive (Hadden, Cornblath *et al*., 1998).

Electromyograms and autopsy studies lead to the sub classification of GBS into mainly two groups namely demyelinating and axonal GBS. The acute inflammatory demyelinating neuropathy (AIDP) is the most common GBS subtype and constitutes 80 – 90% of GBS cases in the western world. In AIDP macrophages have been found to enter the Schwann cell basement membrane causing damage to the Schwann cell cytoplasm and subsequently leading to demyelination (Hughes and Cornblath 2005).

In the axonal subtypes of GBS, motor or sensory functions can be affected simultaneously or independently. The acute motor axonal neuropathy (AMAN) is most commonly found in China with a 50-60% occurrence in GBS cases, while it only makes out 10% in the western world. The sensory function might also be affected (AMSAN), but this occurs less often. Autopsy studies show that axonal degeneration could occur without inflammation in 5 out of 10 cases (Chowdhury and Arora 2001). Furthermore, electronmicroscope pictures show macrophages infiltrating neurons and dissecting into the axolemma as well the deposition of complement and immunoglobulin G while myelin remains intact (Hartung, van der Meché *et al.,* 1998). A variant of GBS is often found where mainly the eye muscles are affected causing ophthalmoplegia. In addition, loss of muscle control (ataxia) and reflexes (areflexia) were typical. This syndrome was considered a variant of GBS and subsequently named after Miller Fischer as the Miller Fisher Syndrome (MFS) (Hughes and Cornblath 2005). The disease is very rare and occurs in only about 5-10% of GBS patients in the western world but has a higher

incidence in Taiwan (20% of cases)(Hughes and Rees 1997; Hartung, van der Meché *et al.,* 1998).

Antibodies against gangliosides, glycolipid epitopes on especially nerves, drew particular attention in GBS due to its association with the disease and the deposition of antibodies at nerve fibres (Hartung, van der Meché *et al.,* 1998). Antibodies are readily formed against the gangliosides GM1, GM1b, GD1a, GalNAc-GD1a for the axonal neuropathies and antibodies against GQ1b for MFS and GBS cases with ocular involvement. About 20-40% of the AIDP cases of GBS were found to have pathogenic auto-antibodies. The antibodies present in AIDP are usually specific for the myelin protein P0 and the search for anti-ganglioside antibodies in AIDP has as yet not been fruitful. Table 1 summarizes the general clinical picture of GBS and its subtypes.

Table 1-1: A summary of the clinical picture of GBS.

Subtype	Frequency	Clinical Features	Electrophysiology	Antibodies
AIDP	80 - 90% in western world	Affecting myelinated limb, axial and lower cranial motor and sensory nerves	Primary demyelination and secondary axonal damage	Myelin protein P0.
AMAN	10% in western world 50 – 60% in China.	Motor impairment	Axonal degeneration	GM1, GM1b, GD1a, GalNAc-GD1a
AMSAN	Few cases	Motor and sensory impairment	Axonal damage on motor and sensory neurons	GM1, GM1b, GD1a, GalNAc-GD1a
MFS	5 – 10% in western world, 20% in Taiwan	Ophthalmoplegia, ataxia, and areflexia	Demyelination	GQ1b

1-1.2. Aetiology of GBS

Autopsy studies of GBS cases revealed that degenerated neurons are inflamed with intrusion of T-cells and macrophages (Prineas 1981; Griffin, Li *et al.,* 1996). Macrophages were concentrated at sites of nerve damage implicating a direct immune attack on epitopes on nerves. The finding of antibodies specific to gangliosides confirmed that a specific immune attack to nerves occured, but the cause of this attack was not as clear. Preceding infections in GBS cases are often found and anti-ganglioside antibodies cross-react with epitopes on infectious agents. From this the theory of molecular mimicry between lipid antigens on infectious agents and nerves as a cause of this disease became

appealing. The counter argument to this is that only 1 in 1000 enteritis infections leads to GBS, even though epitopes are similar in infections agents and neurons in non-GBS cases (Allos 1997). Figure 1-1 illustrates the theory of molecular mimicry for development of GBS.

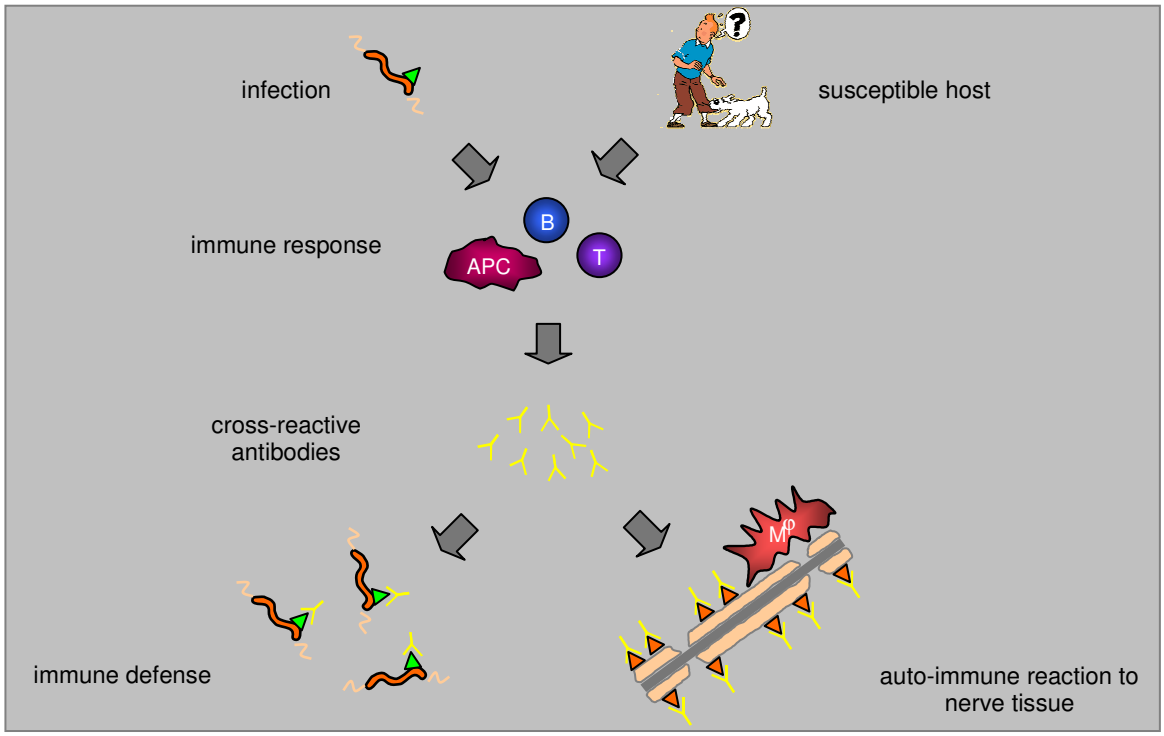


Figure 1-1: The theory of molecular mimicry for the pathogenesis of GBS. The disease is initiated by infection of a susceptible host. An immune response against the infection results in antibodies reacting to the infectious agent as well as epitopes on neurons. Macrophages are recruited and complement deposited causes demyelination and or axonal damage (Artwork by B.C. Jacobs, Department of Neurology, Erasmus MC Rotterdam, The Netherlands).

Infection and antibodies

Two thirds of GBS patients had an infectious illness, most commonly of the respiratory or gastrointestinal tract, 1-2 weeks prior to clinical symptoms (Hahn 1998; Yuki 1999). Several infectious agents have been identified for European countries including *Campylobacter jejuni* (30-40%) Epstein-Barr virus (EBV, 8%), *Mycoplasma pneumoniae* (5%) and cytomegalo virus infection (CMV, 15%), all of which were found to be closely associated with the disease (Schwerer 2002). Of special interest was the prevalence of antibodies against the gangliosides GM1 and GQ1b and their correlation with antecedent *C. jejuni* infections. Anti-GM1 IgG antibodies were found in 20-30% of GBS patients and often resulted in an AMAN clinical subtype, while in 5-10% of patients anti-GQ1b was found that more closely associated with MFS (Jacobs, van Doorn *et al.,* 1996; Ariga and Yu 2005). Anti-GM2 IgM and GD1a antibodies are often found in patients with

sensory impairment after CMV infections (Visser, van der Meché *et al.,* 1996; Ang, Jacobs *et al.,* 2000). Elevated titres of anti-galactocerebroside antibodies have frequently been found in patients that have had *M. pneumoniae* infection (Ariga and Yu 2005). A summary of the association between preceding infection, antibodies and clinical symptoms are shown in figure 1-2.

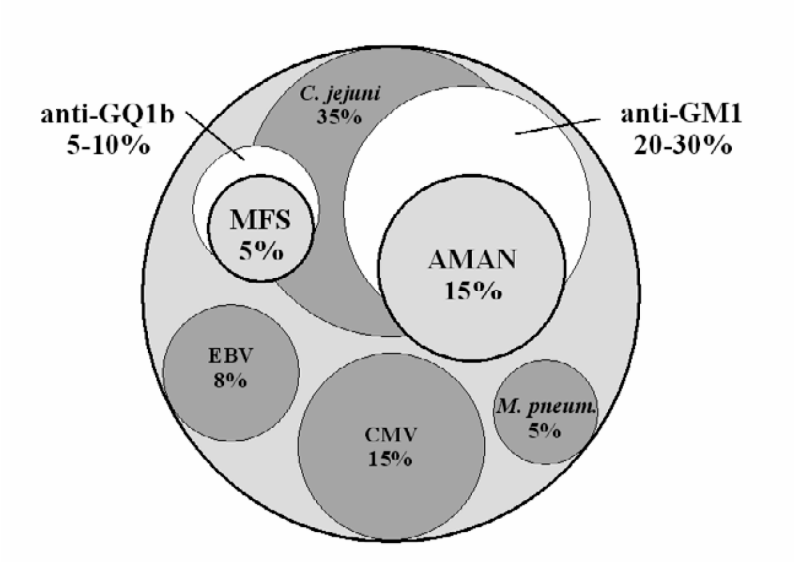


Figure 1-2: Antecedent infections and antibodies associated with GBS in Europe. Infections with *C. jejuni*, CMV, EBV, and *M. pneum* (*Mycoplasma pneumoniae*) are illustrated. Antibodies against gangliosidies GM1 and GQ1b, as well as clinical associations with AMAN and MFS are shown in % frequency (Schwerer 2002).

It is not clear how anti-ganglioside antibodies cause nerve dysfunction or injury, but three possible mechanisms have been proposed: 1) macrophages destructing axons at the Nodes of Ranvier after antibody binding, 2) complement mediated damage of nerve-terminals, and 3) reversible blocking of pre- and post synaptic neuromuscular transmission by IgG antibodies (Ariga and Yu 2005).

Molecular mimicry

Not all *C. jejuni* infections lead to GBS, in fact only 1 in 1000 infections do and therefore gave suspicion that the bacteria associated with GBS shared something that distinguished them from the bacteria not associated with GBS. Studies in Japan pursued this notion and found that 83% of isolates from GBS patients were of the PEN O:19 serotype. Given that PEN O:19 is found in only 2% of *C. jejuni* strains, it was thought that this strain of bacteria is a key player in triggering GBS (Kuroki, Saida *et al.,* 1993; Takahashi, Koga *et al.,* 2005).

The lipopolysaccharide (LPS) of the PEN 0:19 *C. jejuni* serotype isolates were found to bind cholera toxin (Yuki *et al.,* 1992; Yuki 1999). This was most interesting since cholera toxin is well known to bind strongly with the ganglioside GM1 thus implying that the PEN O:19 LPS mimics the structure of GM1. The LPS showing reactivity to cholera toxin was further characterized and was found to contain galactose (Gal), N-acetylgalactosamine (GalNAc), and N-acetylneuraminic acid (NeuAc), which are also sugar components of GM1 (Yuki, Taki *et al.,* 1993). ^{1}H nuclear magnetic resonance analysis showed that this sugar structure protrudes from the LPS core and that it is identical in structure to GM1. The mimicry and its immunological significance was further supported by studies that reported binding of anti-GM1 IgM and anti-GM1 IgG to the surface of *C. jejuni* (Wirguin, Suturkova-Milosevic *et al.,* 1994; Oomes, Jacobs *et al.,* 1995). These were the first studies in GBS to demonstrate the existence of molecular mimicry between epitopes on nerves and LPS (Yuki 1999). Many possible candidates for molecular mimicry were identified since then. The most interesting LPS is the Penner O:19 mimicking GM1 and GD1a, and the Penner O:10 LPS that mimics GQ1b and GD3. Their structures are depicted in figure 1-3 (Moran 1997; Ang, Endtz *et al.,* 2000).

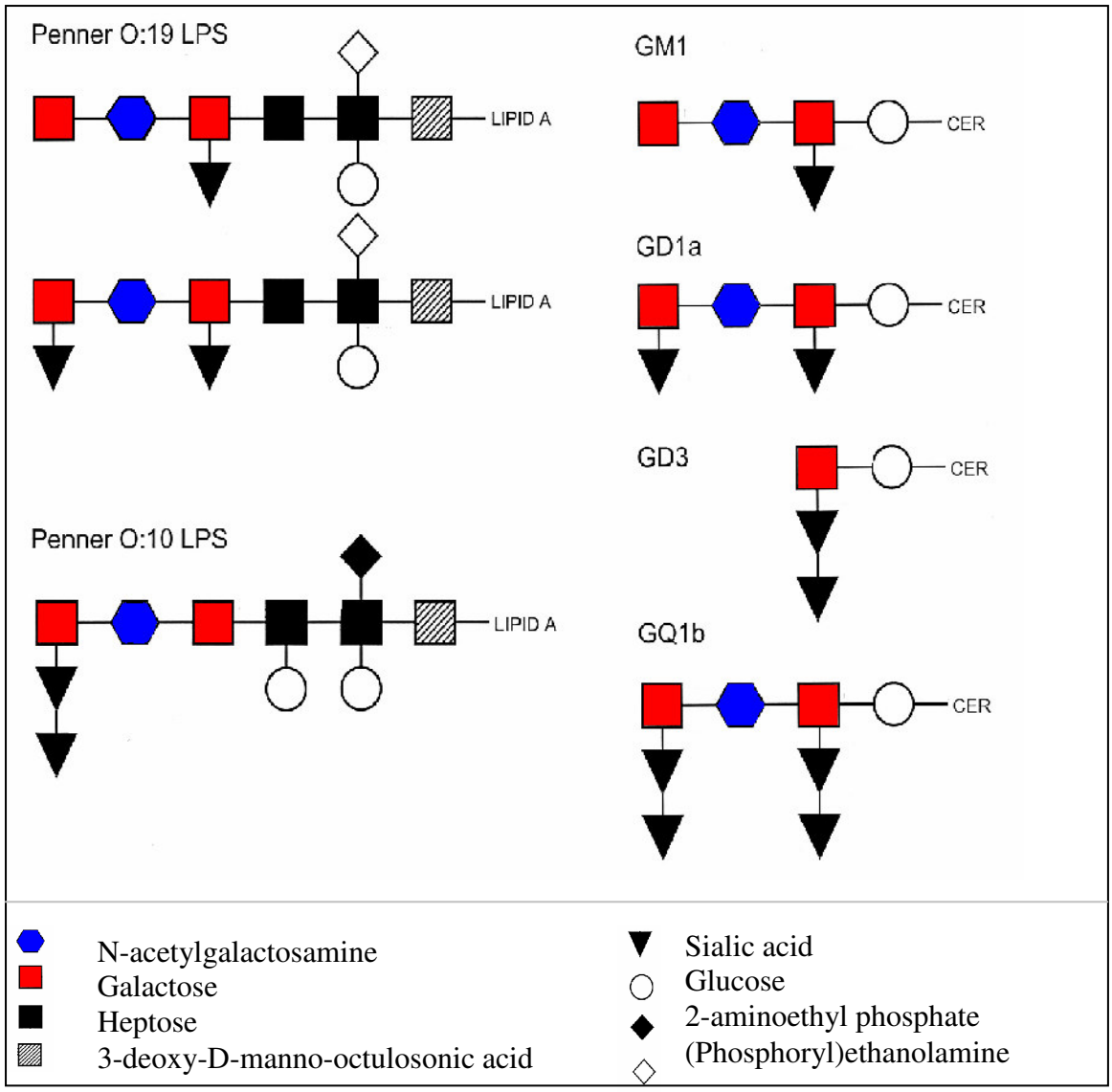


Figure 1-3: The structure of lipopolysaccharides and the mimicked gangliosides most commonly associated with GBS (Moran 1997; Ang, Laman *et al.,* 2002).

The Guillain Barré Syndrome is probably the only disease that closely adheres to the criteria for antigenic molecular mimicry to trigger the disease (Ang, Jacobs *et al.,* 2004). First, infectious agents are associated with the disease of which *C. jejuni* is the most prevalent. Second, clinical symptoms can be correlated to antibodies and the antecedent infections. Third, structures on LPS mimic gangliosides as was indicated by toxin and lectin binding studies as well as mass spectrometry analysis. A final step for molecular mimicry to be accepted as a trigger for GBS is the development of an animal model. Lipopolysaccharides mimicking the ganglioside GM1 were injected into rabbits and serological studies showed that anti-GM1 antibodies were produced and bound to the GM1 rich regions at the nodes of Ranvier, thus resembling the antibody attack in humans

(Moran, Annuk *et al.,* 2005). In another experiment in Japan, N. Yuki and colleagues have demonstrated that after a long immunisation of rabbits with LPS from *C. jejuni,* rabbits developed anti-GM1 antibodies, flaccid limb weakness and pathological changes in peripheral nerves (Yuki, Susuki *et al.,* 2004). The perfect animal model would probably require the development of clinical GBS and anti-GM1 antibodies after colonization of *C. jejuni* in the respiratory or gastrointestinal tract, but such a model does not exist yet. Nonetheless, these studies on rabbits provided evidence that antigenic molecular mimicry may be the initiator of GBS.

The antigenic molecular mimicry plays a key role in the induction of anti-ganglioside antibodies as suggested by the finding that bacterial isolates from GBS patients more frequently expressed ganglioside mimics than isolates from uncomplicated enteritis patients (Ang, Laman *et al.,* 2002). The reason why enteritis patients do not develop GBS when infected with bacteria that contains ganglioside mimics is not clear, and therefore host factors are expected to play a role.

Host factors

In one study where a family outbreak of the same *C. jejuni* infections occurred, only one of the 3 enteritis patients contracted GBS (Ang, Endtz *et al.,* 2000). What then are the host factors that determine the susceptibility for developing GBS? Several immunological polymorphisms have been investigated for possible association with disease susceptibility, disease severity and auto-immune antibody production. Table 1-2 summarizes some of the genetic studies associated with GBS.

Table 1-2. Genetic studies to correlate host factors with disease susceptibility.

Host factor	Genetic entity	Study and outcome
LPS receptors	CD 14, TLR 4 Polymorphism	No correlation with disease susceptibility (Geleijns, Jacobs *et al.,* 2004).
	HLA class II alleles	No correlation with severity of disease. (Geleijns, Schreuder *et al.,* 2005).
	CD1 polymorphisms	Correlate with susceptibility (Caporale, Papola *et al.,* 2006).
Removal of auto-reactive B- and T-cells (apoptosis)	Fas and sFas (SNP in promoter)	Possible correlation with prevalence of autoantibodies (Geleijns, Laman *et al.,* 2005).
Immunoglobulin	Light chain allotypes	Certain allotype (KM3/KM3) frequently found in GBS and correlated with anti-GD1a antibodies (Pandey and Vedeler 2003; Pandey, Koga *et al.,* 2005).
	Fcγ receptors	No correlation with disease susceptibility, but possible correlation with severity of disease (Vedeler, Raknes *et al.,* 2000).
Cytokines	TNF allele and IL-10 SNP	Possible correlation with susceptibility after *C. jejuni* infection (Ma, Nishimura *et al.,* 1998; Myhr, Vagnes *et al.,* 2003).
Lipid transport	Apolipoprotein	Unlike Alzheimer's disease, no correlation in GBS was found (Pritchard, Hughes *et al.,* 2003).

These results and other studies provided evidende in correlating disease severity and outcome with required host factors, but identifying candidate genes are very difficult due to the numerous possibilities. Nevertheless, the family case described above gives an argument for host factors being involved. Furthermore, there is an estimated recurrence rate of 1 – 5% in GBS, one patient even had GBS 4 times, and therefore argues for host factors to be important (Geleijns, Brouwer *et al.,* 2004).

Miscellaneous cases and causes

Bovine ganglioside administration was frequently used in Italy since 1975 for its neuroprotective role and its ability to repair neurons. The gangliosides GM1, GD1a, GD1b and GT1b were common in these mixtures. However, many GBS cases were reported after treatment started and many cases had antibodies to ganglioside. It was consequently decided that this treatment posed a threat and ganglioside therapy was withdrawn in 1993. The incidence of GBS has not changed after 1993, even though ganglioside therapy was used extensively. Therefore, the contribution of ganglioside therapy to the occurrence of GBS remains controversial even though a causal effect in minor GBS cases is likely (Govoni, Granieri *et al.,* 2003). A similar situation was found when anti-GD2 antibodies were administered in a therapeutic trial for cancer. Subsequent

to treatment some patients developed sensorimotor polyneuropathy and others the syndrome of inappropriate antidiuretic hormone secretion. Further studies found that these antibodies with complement were cytotoxic to cancer cells, but antibodies also bound to sites on peripheral nerves and the pituitary gland. This could therefore explain the neuropathy symptoms and loss of antidiuretic hormone secretion (Willison and Yuki 2002).

The occurrences of GBS followed by vaccination were found in case reports or small series of studies, but still lack clear causal associations. Population surveillance and case control studies rejected measles vaccine, tetanus toxoid and oral polio from being causal for GBS, while a possible association of rabies vaccine containing murine myelin could exist. The incidence of GBS was slightly increased in 1976 following the "swine flu" vaccination in the USA and a marginally significant but small risk of 1 in 1 million was estimated to precipitate GBS in the early 1990s (Hughes, Hadden *et al.,* 1999).

The cellular and humoral immunity are considered to be both important in the development of GBS, but the occurrence of GBS has also been found in immunosuppressed patients. Examples of these are GBS cases found in HIV patients before treatment was started, as well as in the immunocompromised Hodgkin's (a disease with the lack of T-cell proliferation to common antigens). GBS cases were also found among pharmacological immunosuppresed patients who had transplants, including renal, cardiac, bone marrow and orthotropic liver and stem cell transplants. Common immunosuppressants used in these listed cases were prednisolone, cyclosporine, corticosteroid, aziathioprine and other steroids (Qureshi, Cook *et al.,* 1997).

The co-occurrence of GBS and solid tumours are uncommon but has been documented and include lymphomas, leukemias, breast, colon and endometrial cancers (Tho, O'Leary *et al.,* 2006). A population based study suggested that the relationship between GBS and cancer might not be coincidental. A postulate for the cause of GBS with cancer is the immune response to gangliosides on tumour cells that cross-react with neurons. Myeloma cells for example express GM3, GD3, GM2 and GD2 that are also expressed on Schwann cells. No serum or cerebrospinal fluid markers exist though to support this hypothesis (Tho, O'Leary *et al.,* 2006).

1-1.3. Treatment

Corticosteroids are used to reduce inflammation and therefore its therapeutic ability in GBS was investigated. Corticosteroids, and more specifically methylprednisolone, were found to have no effect on recovery when administered orally but a non-significant benefit trend was found when administered intravenously (Hughes, Swan *et al.,* 2006). In general corticosteroids alone did not show any benefit or harm, but when co-administered with intravenous immunoglobulin recovery was hastened but the long term effect remained insignificant. Corticosteroids are valuable as treatment in other autoimmune diseases, but the question of why it is not beneficial in GBS remains unanswered.

Plasma exchange (PE) is more promising and the main logic for its use is the removal of immune factors involved in causing neuronal damage. This method involves connecting the patient's blood circulation to a machine that substitutes the plasma for an alternative solution, usually albumin (Raphaël, Chevret *et al.,* 2002). Plasma exchange has been shown to play an important role in the therapy of polyneuropathies, including GBS, for a long time. In addition to removal of pathogenic immune factors, PE has also been found to affect the immune system by activating the complement system, altering cellular components and even removing tissue bound autoantibodies (Kiprov and Hofmann 2003).

An alternative and very effective treatment is the intravenous administration of pooled immunoglobulin from about 3000-10000 blood donors commonly called intravenous immunoglobulin (IVIg/IGIV) (Dalakas 1997). Intravenous immunoglobulin could gain access to the site of injury induced by antibodies and therefore is a good therapeutic candidate. A thorough review in 'The Cochrane Collaboration' on the use of IVIg in GBS has also compared its use to PE (Hughes, Raphaël *et al.,* 2006). The authors concluded from randomised trials that administration of IVIg hastens recovery as much as PE. It was not clear whether adverse effects are more common in either treatments, although some reports state the PE induce more adverse effects compared to IVIg (Shahar 2006).

1-2. Anti-ganglioside antibodies

Methods have been developed for the detection of anti-glycolipid antibodies for diagnostic and research purposes. The most common way to detect antibodies is with ELISA where relevant glycolipids are immobilized onto a solid support and bound antibodies detected after serum has been added. Alternatively, the high performance thin layer chromatography (HPTLC) method is also used in special laboratories (Willison and Yuki 2002). The disadvantage of these current techniques is that they measure binding to glycolipids that are not presented in their physiological state, since glycolipids *in vivo* are imbedded in lipid membranes. The effect that this has on antibody-antigen interaction is not clear. Nevertheless, with these methods high titres of anti-glycolipid antibodies were found in 5 – 60 % of GBS patients (Yuki 2001; Willison and Yuki 2002).

Gangliosides are glycosphingolipids that consist of sialic acids that are linked to an oligosachharide core by sialyltransferases. The ceramide of gangliosides are imbedded into the plasma membrane while the carbohydrate moiety protrudes externally from the cell and are therefore free to interact with immune components. Gangliosides are in abundance in the nervous system and are usually well protected by the blood brain barrier (BBB), protecting nerve gangliosides from immune attack and interaction with toxins (Willison and Yuki 2002). This is not the case in the neuromuscular junction (NMJ). The binding of antibodies to GM1, GD1b and polysialated gangliosides in the NMJ confirms that this site is susceptable for immune attack (Willison and Yuki 2002). The physiological function of gangliosides is not clear, but its involvement in cell-cell interactions and regulation of cell signaling has been suggested (Kolter, Proia *et al.,* 2002).

1-2.1. Correlation with clinical symptoms

Gangliosides are distributed to different locations in the nervous system that could correlate with sensory or motor nerve impairments if antibodies bind to them. For example, the preferential distribution of GM1 and GD1a at the ventral root axons of the peripheral nervous system (PNS) can explain the motor paralysis encountered with anti-GM1/GD1a antibodies, while GD1b, found preferentially at the dorsal roots, could explain the sensory impairments should anti-GD1b antibodies bind there. The best example is ocular impairment that correlates with antibodies found against GQ1b which

is preferentially located at the nodes of Ranvier of ocular nerves. The preferential location of gangliosides do not always correlate with clinical symptoms. An example is with GM1 that is also found at the dorsal root ganglion and which should affect sensory functions, yet does not manifest like that (Gong, Tagawa *et al.,* 2002).

The correlation between MFS and the presence of anti-GQ1b and anti-GT1a has motivated many researchers to pursue correlations between anti-ganglioside antibodies and variants of GBS or other diseases. These studies have shed light on the importance of anti-ganglioside antibodies and their possible pathogenicity as already discussed for studies on AMAN, where anti-GM1 plays an important role, but the same antibody can also be found in not so common neuropathies like the multifocal motor neuropathy, distinguished from GBS by the asymmetrical progression of paralysis affecting mainly the upper limbs (Willison and Yuki 2002; Leger and Behin 2005). Antibodies against GQ1b have been found in several oculomotor diseases and MFS related conditions and are worth mentioning (Table 1-3). The first MFS related condition to mention is the *Bickerstaff's brainstem encephalitis* where in one study 66% of patients had anti-GQ1b antibodies and axonal degeneration (Odaka, Yuki *et al.,* 2003). In *acute ophthalmoparesis*, which resembles MFS but where ataxia or areflexia is absent, anti-GQ1b antibodies were found (Chiba, Kusunoki *et al.,* 1993); in *ataxic GBS* patients, anti-GQ1b antibodies were found and the importance of GD1b IgG in sensory ataxia was emphasized in another study (Yuki, Susuki *et al.,* 2000; Miyazaki, Kusunoki *et al.,* 2001). Anti-GT1a antibodies, some of which can also cross-react with GQ1b, are also found in *pharyngeal-cervical-brachial weakness* (Kashihara, Shiro *et al.,* 1998; Koga, Yuki *et al.,* 1998).

Table 1-3: Clinical syndromes associated with specific anti-glycolipid antibodies (Willison and Yuki 2002).

Clinical Syndrome	Antibody against	Antibody Isotype
Chronic sensory-demyelinating neuropathy	SGPG, SGLPG	IgM (monoclonal)
Chronic ataxic neuropathy	GD1b, GD2, GD3 GT1b, GQ1b	IgM (monoclonal)
Multifocal motor neuropathy	GM1 GD1b, asialo-GM1	IgM (polyclonal or monoclonal)
Acute motor axonal neuropathy (AMAN/AMSAN)	GM1, GD1b, GD1a, GalNAc-GD1a	IgG
Miller Fisher syndrome (MFS)	GQ1b, GT1a	IgG
Bickerstaff's brainstem encephalitis	GQ1b, GT1a	IgG
Acute ophtalmoparesis	GQ1b, GT1a	IgG
Ataxic Guillain-Barrè Syndrome	GQ1b, GT1a	IgG
Pharyngeal-cervical-brachial weakness	GT1a (GQ1b)	IgG

Paraproteinaemic neuropathies have also received a lot of attention due to the specificity of anti-ganglioside antibodies correlating with disease severity and specific clinical outcomes. These neuropathies are caused by monoclonal B-cells producing monoclonal antibodies directed to specific carbohydrate structures. Two of the common clinical subtypes are the chronic sensori-motor demyelinating neuropathy and the chronic ataxic neuropathy (Table 1-3). The first type normally has antibodies directed to the myelin associated glycoprotein (MAG) but these monoclonal antibodies also cross-react with the acidic glycolipids called SGPG and SGLPC (Chassande, Leger *et al.,* 1998). The second subtype usually shows antibodies directed toward the disialylated gangliosides, like GD1b and GQ1b, which are normally associated with sensory neurons (Serrano-Munuera, Rojas-Garcia *et al.,* 2002).

1-2.2. Immunological manifestation of antibodies in disease

Antibodies are generally found with high titres at the onset of GBS, which decreases as the disease progresses (Willison and Yuki 2002). It was also found that axonal degeneration is more likely to occur with high affinity anti-GM1 antibodies than in patients without or with low affinity antibodies (Deisenhammer, Kier *et al*., 1996; Ariga

and Yu 2005). The specificity of antibodies to gangliosides may vary between patients; in some patients antibodies against GM1 are specific for the gangliosides while antibodies specific for GM1 from other patients may cross-react with ganglioside sharing similar epitopes, for instance the Gal(β1-3)GalNAc moiety shared by GA1, GM1, GD1b and peripheral glycoproteins (Ariga and Yu 2005). A study in 2001 also claimed that the three dimensional structure is very important for cross-reactivity since anti-GM1 antibodies cross-reacted with gangliosides that did not have a similar carbohydrate moiety i.e. GM1b and GalNAc-GD1a (Koga, Tatsumoto *et al.,* 2001).

The importance of the three dimensional structure of gangliosides for binding was also supported when noticed that some antibodies could not bind to gangliosides immobilized on a solid support, but bound to gangliosides in solution (Lopez, Comin *et al.,* 2006). To complicate the matter further, it was found that some antibodies in the antibody repertoire of GBS and MFS patients recognized complexes of gangliosides while recognition of single gangliosides was absent or limited (Kaida, Morita *et al.,* 2004; Kaida, Kanzaki *et al.,* 2006). These antibodies against complexes of gangliosides are considered to be associated with severe disability in GBS (Kaida, Morita *et al.,* 2006). The importance of membrane constituents on the antigenicity of gangliosides was emphasised by studies showing that antibody binding to gangliosides changes when gangliosides are surrounded by different phospholipids (Hirakawa, Morita *et al.,* 2005). This also raises the question if the density of gangliosides in the membrane could also be important for its antigenicity. The results clearly shed light on the importance of not only the combination of gangliosides for antibody recognition, but also on their presentation. This surely needs to be investigated in more detail when a suitable system is found.

All isotypes of antibodies are found in GBS. With regard to anti-GM1 antibodies it was found that patients with high titres of the IgA isotype had a poor clinical outcome (Koga, Yuki *et al.,* 1999; Ariga and Yu 2005). Another study showed that IgM is usually associated with the chronic form of the disease while IgG and IgA with the acute form (Lopez, Lardone *et al.,* 2002). Sub-classification of the IgG isotype revealed that antibodies specific to GM1 were predominantly of the isotype IgG1 and IgG3 (Willison and Veitch 1994). In one study *C. jejuni* infections were found more often in patients with IgG1 (76%) isotype and recovery was worse than for patients with an IgG3 (36% of patients) isotype. The latter normally had preceding respiratory infections (Koga, Yuki *et*

al., 2003). The prolonged serum half life of IgG1, compared to IgG3, and its effector characteristic to induce antibody-mediated cytotoxicity could explain the severe outcome in the patients with high IgG1 isotype titres. Immune responses against bacterial LPS are normally of the IgG2 isotype and are usually T-cell independent with B1-cells involved. Therefore, in GBS the immune response to LPS is different to the one found in individuals where autoimmunity does not follow infection.

1-3. Intravenous Immunoglobulin

In the past ten years the use of intravenous immunoglobulin (IVIg) has gained popularity in treating autoimmune neuromuscular disorders. Even though the treatment is as effective as plasma exchange or steroids in some disorders, the IVIg treatment is much easier to administer and safer. This treatment is expensive though and because the target and mechanism of action of IVIg is still speculative, insurance carriers, healthcare organizations, and government agencies express considerable skepticism and scrutiny of its use (Dalakas 2004).

1-3.1. Composition and preparation of intravenous immunoglobulin

Using cold ethanol fractionation (Cohn's process), IVIg is prepared from pooled human plasma obtained from 3 000 to 10 000 donors (Dalakas 2004). Purification is performed using enzymatic treatment at low pH, followed by fractionation and chromatography. Several methods, like adding caprilate and nanofiltration, are applied and detergents added to eliminate viruses and prions. The final product is then stabilized with glucose, maltose, glycine, sucrose, mannitol, or albumin (Dalakas 2004).

The final product of IVIg contains 95% IgG, less than 2.5% IgA, and negligible amounts of IgM. The IgG subclass consists of between 55-77% IgG_1, 0-6% IgG_2, and 0.7-2.6 subclass IgG_4. The standard dose of treatment of a neurological disorder is 2 g/kg IVIg (Pyne, Ehrestein *et al.,* 2002). Kinetic studies have shown that the serum level of IgG increases 5 fold, but it declines by 50% in 72 hours and returns to pre-treatment levels after 21-28 days (Dalakas 1997; Dalakas 2002). The half-life of IVIg is similar to that of native immunoglubulin, which is about 18-32 days. During the first 48 hours after infusion of IVIg, the IgG in cerebrospinal fluid increases 2 fold but returns to normal concentration levels within a week (Dalakas 1997; Dalakas 2002).

1-3.2. Natural auto-antibodies and immunoglobulin dimers

Interestingly, it was found that about 40% of the immunoglobulins in IVIg are in the dimeric form i.e. antibodies bound to each other through their $F(ab)_2$ regions (Roux and Tandersley 1990; Vassilev, Bineva *et al.,* 1995). These antibodies are thought to bind to each other by recognizing specific epitopes called idiotopes on the $F(ab)_2$ regions.

Antibodies recognizing idiotopes are also called anti-idiotypic antibodies. Further investigation found that these dimers consisted of mainly natural auto-antibodies which were bound to their anti-idiotypic antibodies (Djoumerska, Tchorbanov *et al.,* 2005). Anti-ganglioside antibodies are natural auto-antibodies and therefore their anti-idiotypic antibodies might also be present in the dimer population. Interestingly, recovery from disease was improved with increased immunoglobulin dimers in the autoimmune disease thrombocytopenia purpura, which could be due to idiotypic interactions (Teeling, Jansen-Hendriks *et al.,* 2001). The $F(ab)_2$ regions are often responsible for the therapeutic effect of IVIg in GBS (Buchwald, Ahangari *et al.,* 2002). Anti-idiotypic antibodies in IVIg are therefore suggested to be beneficial in treating autoimmune diseases (Dietrich and Kazatchkine 1990).

1-3.3. Possible immunopathogenic targets for IVIg

Immunoglobulins from IVIg can target various components of serum, extra-cellular fluid and cell surfaces in order to facilitate recovery. The main targets of IVIg in neuromuscular diseases preceded with infections are illustrated in Figure 1-4 (Dalakas 2004).

The various therapeutic effects of IVIg's have been suggested to reside in its numerous immunoregulating effects, but only some have been proven. Some suggested mechanisms that could be applicable to GBS include Fc receptor blockade; idiotypic and anti-idiotypic antibody interactions neutralizing pathogenic antibodies; interference in the Fas apoptotic pathway through agonistic and antagonistic anti-Fas antibodies; regulation of complement components; modulation of cytokine secretion; interference with natural T cell function; inhibition of metalloproteinase-9 activity and subsequent inflammation; suppression of NF-κB activation; G1 cell cycle arrest; decrease in leukocyte recruitment; reduction of T cell stimulation; modification of antibody kinetics and effects on dentritic cells (Sapir and Shoenfeld 2005).

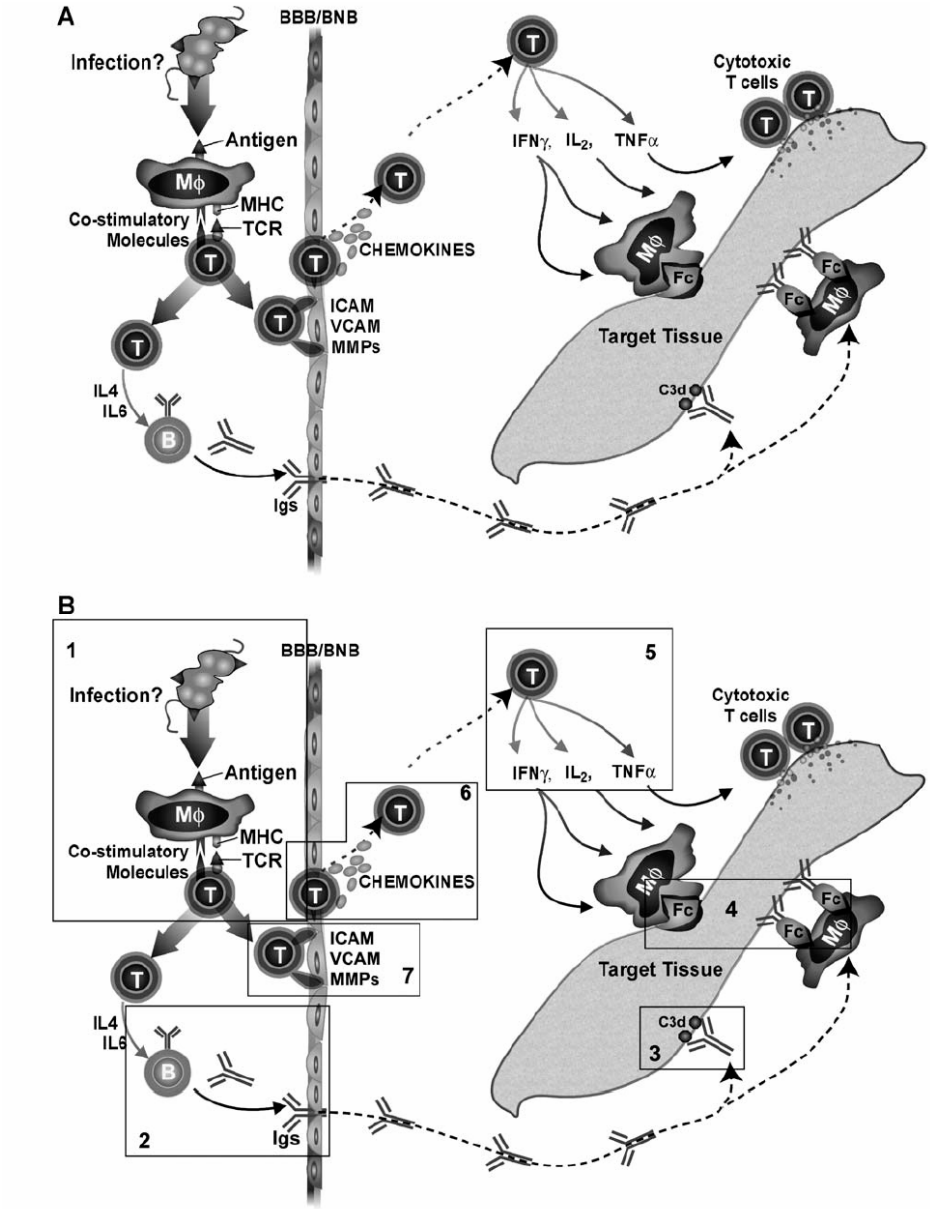


Figure 1-4: Pathogenic networks involved in GBS and possible therapeutic targets by IVIg. (A) Several immune mediated diseases, especially GBS, are preceded by infection and are thought to be the initiator of autoimmunity. Pathogen infection leads to antigen presentation to antigen presenting cells, like macrophages, which present processed antigen to lymphocytes. Tolerance is broken and via co-stimulatory molecules T cells undergo clonal expansion and cytokines and chemokines are released. Adhesion molecules (ICAM, VCAM and MMP) on endothelial cells are up-regulated and T-cells migrate to targeted tissues. T-cells also assist in the production of antibodies by stimulating B-cells through IL-4 and -6. Antibodies bind to targeted tissues causing damage by activating complement and assembling MAC (membrane attack complex). Cytokine activated macrophages invade tissue (example myelin or muscle) through their Fc receptors or release injurious molecules (e.g. IFN-y, IL-2, TNF-α). Macrophages cause damage after binding to antibodies on target cells via their Fc receptors. (B) Possible targets of IVIg: (1) interference with co-stimulatory molecules; (2) provision of anti-idiotypic antibodies or suppression of antibody production; (3) interference with the activation of complement and interception of MAC formation; (4) modulation of the expression and function of Fc receptors on macrophages; (5) suppression of cytokines (6) chemokines, and (7) adhesion molecules; and alterations of the activation, differentiation and effector functions of T-cells. B = B-cell; BBB = blood-brain-barrier; BNB = blood-nerve barrier; C3 = complement 3; ICAM = intracellular adhesion molecule; IFN-y = Interferon gamma; Igs = immunogloublins; IL = interleukin; MΦ = macrophages; MHC = major histocompatibility complex; MMP = matrix metalloproteinase; T = T cell; TNF- α = tumour necrosis factor alpha; VCAM = vascular adhesion molecule (Dalakas 2004).

1-3.4. Idiotypic antibodies in IVIg interacting with anti-ganglioside antibodies

In GBS the prevalence of anti-ganglioside antibodies are gaining much focus due to accumulating evidence on their involvement in the disease. Intravenous immunoglobulin has been found to be very effective in treating GBS and several studies investigated the effect of IVIg on the effects of anti-ganglioside antibodies. The binding of anti-GQ1b antibodies to gangliosides, as well as its α-latrotoxin-like effects at mouse neuromuscular junctions were inhibited with antibodies in IVIg (Jacobs, O'Hanlon *et al.,* 2003). Furthermore, the binding of anti-ganglioside antibodies to their target antigen was prevented and their blocking effect on conduction ameliorated in mice. In that study it was found that the $F(ab)_2$ isolate of IVIg was responsible for this effect possibly through idiotypic antibodies (Malik, Oleksowicz *et al.,* 1996; Buchwald, Ahangari *et al.,* 2002). It was later confirmed that anti-idiotypic antibodies from IVIg bind to the anti-ganglioside antibodies, subsequently inhibiting their binding (Lopez, Irazoqui *et al.,* 2000). An early study found that 1% of IVIg antibodies have an inhibitory effect on neuron binding of GBS patient serum through possible idiotypic interactions (Lundkvist, van Doorn *et al.,* 1993). Therefore, antibodies in IVIg, also called idiotypic antibodies, are binding to anti-ganglioiside antibodies and this interaction is suggested to play an important role in the efficacy of treatment (Zhang, Lopez *et al.,* 2004).

1-3.5. Problems to solve

The reliability of treatment with IVIg requires that differences between batches are small or absent in relation to effectivity of treatment. Intravenous immunoglobulin batches vary in antibody activity against various antigens and are therefore subject to possible variability in therapeutic efficacy (Lemm 2002; Gelfand 2006). Limited studies have been performed to evaluate efficacy between various IVIg brands. In one study no difference between 2 IVIg brands were found for preventing antibody binding to the neuromuscular junction and to gangliosides, while another study detected differences between 2 brands when testing their therapeutic effect on neuromuscular transmission (Buchwald, Ahangari *et al.,* 2002; Jacobs, O'Hanlon *et al.,* 2003). A recent study tested 8 IVIg brands for efficacy in reducing the cytolytic effects induced by anti-GM1 and anti-GD1a in GBS serum but no differences between batches were found (Zhang, Lopez *et al.,* 2006). It therefore remains inconclusive if variation between IVIg batches is a serious problem. It would be useful if a standard protocol were available to evaluate the therapeutic value of IVIg batches.

Even though intravenous immunoglobulin administration is the preferred treatment of GBS by some physicians, IVIg treatment only reduces the severity of the disease (Willison 2005). Furthermore, all patients do not respond and recover equally well with IVIg treatment, which therefore creates scope for improvement. Lack of insight into the mechanism of IVIg in treatment hinders progress in finding ways to improve treatment with IVIg.

1-4. The IAsys biosensor

Biosensors are good candidates for measuring molecular interactions due to their sensitivity and ability to measure molecular interactions without the need to label molecules. Biosensors have been developed that can be classified into 4 basic types based on the transducer system used, namely electrochemical, optical, mass sensitive and thermometric (Table 1-4).

The use of optical biosensor technology allows the study of molecular interactions in real time between proteins, nucleic acids, carbohydrates and peptides (Buckle, Davies *et al.,* 1993). Surface plasmon resonance and waveguiding produce evanescent fields that are used to convert molecular interactions into binding signals, thereby avoiding the need for labelling of ligates and ligands. Biosensors can be used to determine the association and dissociation rates of interacting molecules due to real time measurements (Buckle, Davies *et al.,* 1993). It therefore allows quantitative as well as qualitative assessment of binding and dissociation reactions that can be applied to epitope mapping and affinity determinations (Rich and Myszka 2005).

Table 1-4: The transduction systems used in different classes of biosensors (Marazuela and Moreno-Bondi 2002; Gustafson, 2003a).

Biosensor Type	Example of Transducer
Electrochemical	Ion-selective electrode (ISE)
Amperometric	Glass electrode
Potentiometric	Metal electrode
Conductivity	Ion-sensitive field-effect transistor (ISFET)
Optical	
Absorbance	Surface plasmon resonance (SPR)
Reflectance	Resonant mirror (RM)
Refractive Index	Total internal reflection of fluorescence (TIRF)
Luminescence	
Light Scattering	
Mass sensitive	Quartz crystal microbalance (QCM)
Thermal	
Calorimetry	Thermistor

The IAsys biosensor, a typical optical biosensor, has been used before to detect auto-antibodies in serum from patients with Goodpasture's disease, a disease caused by antibodies attacking the glomerular basement membrane of the kidneys (Dougan, Levy *et al.,* 2002). In this assay the presence of antibodies in serum was detected directly by measuring binding to the antigen, which is immobilized onto the resonant mirror surface. Results obtained from the biosensor correlated well with ELISA, were consistent, reproducible and quantifiable. The biosensor assay was much quicker than the ELISA. It therefore seemed possible to measure the interactions between gangliosides and antibodies from GBS patient serum using the biosensor.

Gangliosides have been immobilized before onto the IAsys biosensor surface using liposomes (Vrey 2003). Liposomes offer a lipid environment to gangliosides that better resembles their natural environment, in contrast to the presentation of ganglioside antigens in ELISA. The three dimensional structure of gangliosides are disturbed in ELISA, as was shown in an antibody study (Lopez, Comin *et al.,* 2006). Liposomes may be able to retain the three dimensional structure of ganglioside antigens The effect that these two ganglioside presentation environments have on antibody binding has not been compared before. It was hoped that the biosensor could provide the means to further characterize anti-ganglioside antibodies binding to gangliosides and their idiotypic antibodies, a feat that could not be achieved with ELISA and related technologies.

1-5. Hypothesis

Auto-antibody activity against gangliosides in GBS can be investigated by immobilizing the antigens in liposomes onto a solid surface and measuring antigen-antibody interactions in an evanescent field biosensor. The scope of this approach is to improve IVIg treatment of the disease by enabling the selection of healthy serum donors for their efficiency as immunoglobulin source.

1-6. Aims of this study

The current study aimed to achieve an outcome where the knowledge of the idiotypic antibody network involvement in the manifestation of GBS in humans could be best exploited to the benefit of the diagnosis, treatment and management of the disease.

The first aim was to develop a method to demonstrate and evaluate the therapeutic potential of IVIg and healthy human sera for GBS patients. In this way different IVIg formulations can be prepared from selected human sera that offer maximal treatment potential for patients with particular GBS phenotypes.

The second aim was to design and optimize a protocol for measuring anti-ganglioside antibodies directly from patient serum using the IAsys biosensor and liposomes to present the ganglioside antigens. The scope of this aim is to eventually be able to analyze idiotypic interactions found in GBS patients in order to further the understanding of the manifestation of the disease and how to prevent and treat it.

Chapter 2

The potential of healthy human sera for treatment of GBS

2-1 Introduction

Our immune system consists of different components of which T-lymphocytes and antibody producing B-lymphocytes play an integral part/role in the production of antibodies (Jerne 1985). The immune system's main function is to patrol the body and guard it from harm by means of intruding antigens (Ags). Antibodies generated by B-cells have the unique capability to specifically recognize foreign antigens and trigger immune responses through Fc receptors that will result in the elimination of intruding pathogens. In an effort to understand how the immune system works, immunologists proposed a few theories that tried to explain for example how the immune system maintains memory of antigen after infection, how it generates the ability to recognize such a big variety of different antigens, and how the immune system is modulated. In 1957, the clonal selection theory answered many of these questions. This theory describes the immune system as clonal cells, B-and T-cells, which are genetically programmed to produce antibodies of a defined specificity. Upon exposure to antigen these cells undergo somatic mutation to broaden the specificity of antibodies being produced (Wilson, Wilson *et al.,* 2000; Nayak, Mitra-Kaushik *et al.,* 2001). These immune cells clonally produce more cells like themselves.

In the 1970s Niels Jerne saw the possible existence of an integrative, unknown dense network, which he termed the idiotypic network (Jerne 1973). The essence of this network is that immune components can recognize epitopes, called idiotopes, and also present epitopes themselves, which can be recognized by other immune components. These immune components include antibodies, B-cells and T-cells and therefore the possibility of a dense network can be anticipated. For example, antibodies specific for an antigen also present idiotopes that might be recognized by other antibodies and corresponding B-cells that will subsequently be recognized and regulated by T-cells. Each antibody may present several idiotopes causing many antibodies and immune components to recognize these and present idiotopes themselves, which subsequently allow the integration of immune components thus forming a dense network. Anti-

idiotypic antibodies binding to idiotopes on autoimmune antibodies may prevent auto-antibodies from binding and therefore have therapeutic potential.

2-1.1. Immunogenic epitopes on immunoglobulins

Antibodies, under a variety of circumstances, can present many epitopes (immunogentic determinants) that can be immunogenic, i.e. they have the ability to elicit the production of antibodies specific to these epitopes. These epitopes can be classified and categorised according to their location on the antibody molecule and their occurrence on antibodies of different classes, from different individuals, or different species. The three main categories are idiotypic, allotypic, and isotypic epitopes (Novotny, Handschumacher *et al.,* 1986; Bona and Bonilla 1990).

Isotypic epitopes: Isotypic epitopes are characteristic to the constant regions of the heavy or light chains of antibodies and differ between species (Figure 2-1). Antibodies specific for isotypes are obtained by immunization with antibodies from different species, also called heterogeneic immunization (Bona and Bonilla 1990).

Allotypic determinants: Within species, there exist genes that are polymorphic; implying that several different alleles exist for genes encoding for certain immunoglobulin epitopes. These are called allotypic epitopes, for example rheumatoid factors found in humans. Allotypes can be obtained by immunizing an individual of the same species that does not express the particular allotype, i.e allogeneic immunization (Bona and Bonilla 1990). Specific allotypes are inherited from parents and are located on the heavy and light chains (Figure 2-1) (Benner, van Dongen *et al.,* 1996).

Idiotypic determinants: The number and diversity of idiotypes found in an individual is much greater than any of the other types of immunogenic epitopes. Idiotypes can also be classified according to their location on the variable region of the antibody. Their diversity is mainly due to the fact that there are many more genes encoding for the variable region than those encoding for the constant region. In addition to this diversity caused by the gene make-up, further diversity is induced by somatic hypermutation. Even though idiotypes are so diverse, it has been found that idiotopes may be shared between

antibodies that have the same or different specificities, i.e. recognizing the same or different antigen (Reale, Manheimer *et al.,* 1986; Lemke and Lange 2002). Immunizing an individual with its own idiotypic antibody may produce antibodies raised against those idiotypes. Natural anti-idiotypic antibodies have been found in various responses to infection or antigen administration (Lundkvist, van Doorn *et al.,* 1993). The production of antibodies and their anti-idiotypic antibodies forms the basis for the idiotype network theory (Jerne 1973; Bona and Bonilla 1990). Figure 2-1 shows the location of idiotopes on the variable regions of the light and heavy chains.

Idiotopes may be present on the variable region of antibodies, as well as the variable region on antigen receptors on both T- and B-lymphocytes. Idiotopes may be divided into public or private idiotopes. Public idiotopes are the phenotypic markers of variable region germ line genes and are inherited in a Mendelian fashion. Private idiotopes are rare structures that are the markers of random somatic mutational events that occur in a single clone of T-or B-cells of a single individual. These idiotopes are not inherited and are produced during somatic mutation of genes, before or after having encountered antigen (Somme, Roth *et al.,* 1983).

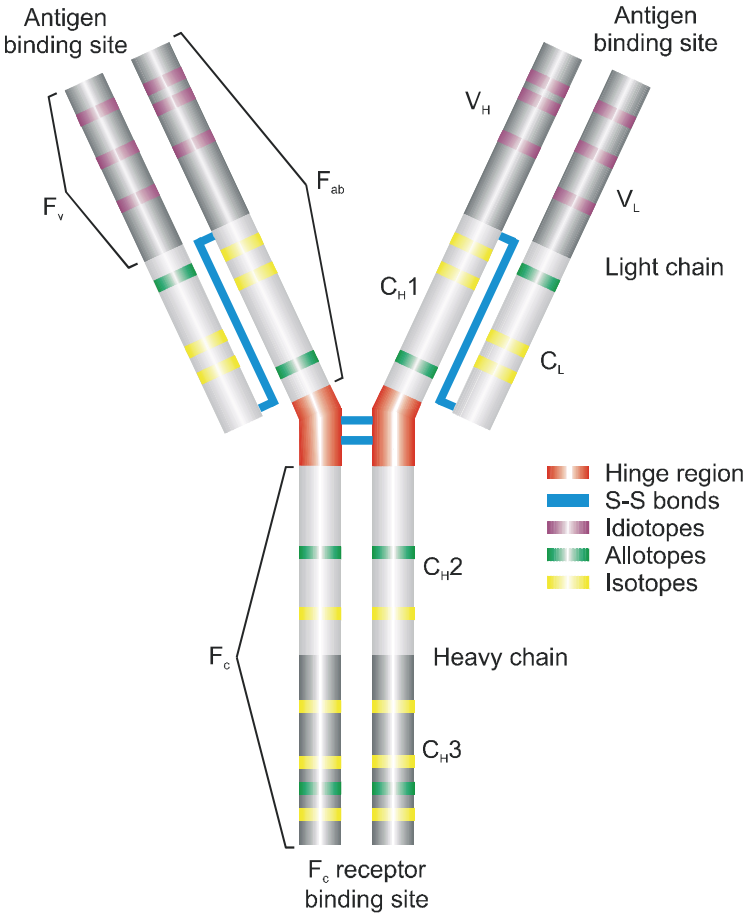


Figure 2-1: A Structure of an antibody indicating the locations of isotopes, allotopes and idiotopes (Benner, van Dongen *et al.,* 1996).

2-1.2. Production and location of idiotopes on antibodies

When an idiotope is recognised by an antibody, it is said to be antigenic. When an idiotope can elicit specific antibody in the same or different animal, it is said to be immunogenic. The region of the antibodies' receptors binding to the antigen is called the paratope. Idiotopes may be located within or outside the paratope region. Therefore, idiotopes can be categorized according to their ability to be antigenic when antigen is present. The result indicates the general localization of the idiotope, i.e. idiotopes lacking the ability to be antigenic in the presence of antigen are situated within the paratope and will not be recognised by anti-idiotypic antibodies if the corresponding antigen is present. Idiotopes maintaining their antigenicity and immunogenicity in the presence of antigen are situated on the surface of the variable regions outside the paratope and will be recognised by anti-idiotypic antibodies irrespective of the presence or absence of antigen (Greenspan and Davie 1985).

Idiotopes may also be antigenic only when antigen is bound to the antibody. This is possible due to the conformational change that antibodies undergo while bound to antigen and the formation of an immunogenic and antigenic idiotope (Meuer, Hussey *et al.,* 1984; Strickland, Gleason *et al.,* 1987).

2-1.3. Classifying anti-idiotypic antibodies

The first antibody (Ab) produced against an antigen is termed Ab1; the second antibody raised against an idiotope on Ab1 is called Ab2. The antibody produced against Ab2 is Ab3. The end-point of this antibody cascade has not been determined, but a dense idiotypic network can be anticipated (Frodin, Faxas *et al.,* 1991) that may include closed networks (eg. when Ab3 = Ab1) and open networks (eg. when Ab3 is different from Ab1, etc.).

When looking at the antibody-idiotope interaction, four different classes of anti-idiotypic antibodies can be distinguished that are also used to classify anti-idiotypic antibodies. The classification of these antibodies is an indication of the topographical location of the targeted idiotopes.

Antibody 2α (Ab2α) are anti-idiotypic antibodies specific for idiotopes located outside the paratope. In certain instances these antibodies have been found to broaden the specificity of Ab1 for different antigens. Such anti-idiotypic antibodies might be useful for the identification and neutralization of natural bacterial and viral variants arising by antigenic drift (Anders, Kapaklis-Deliyannis *et al.,* 1989; Pan, Yuhasz *et al.,* 1995).

The Ab2γ antibodies recognize idiotopes located within or close to the paratope and cannot be produced when Ab1 is bound to antigen during immunization. The paratopes of Ab2γ antibodies are not internal images of the antigen though. Antibody 2β antibodies, also called homobodies, are similar to Ab2γ, but carry the internal image of the antigen. Antibody 2β is therefore an internal image of the Ab1 paratope. Ab2γ and Ab2β antibodies could be distinguished by their ability to bind idiotopes in xenogeneic antisera specific for the antigen recognized by the Ab1. The largest class of anti-idiotypic antibodies studied to date are Ab2β and received considerable attention since the origin of the concept of the idiotype network (Jerne 1973). Figure 2-2 illustrates the differences in binding of Ab2α and Ab2β to Ab1.

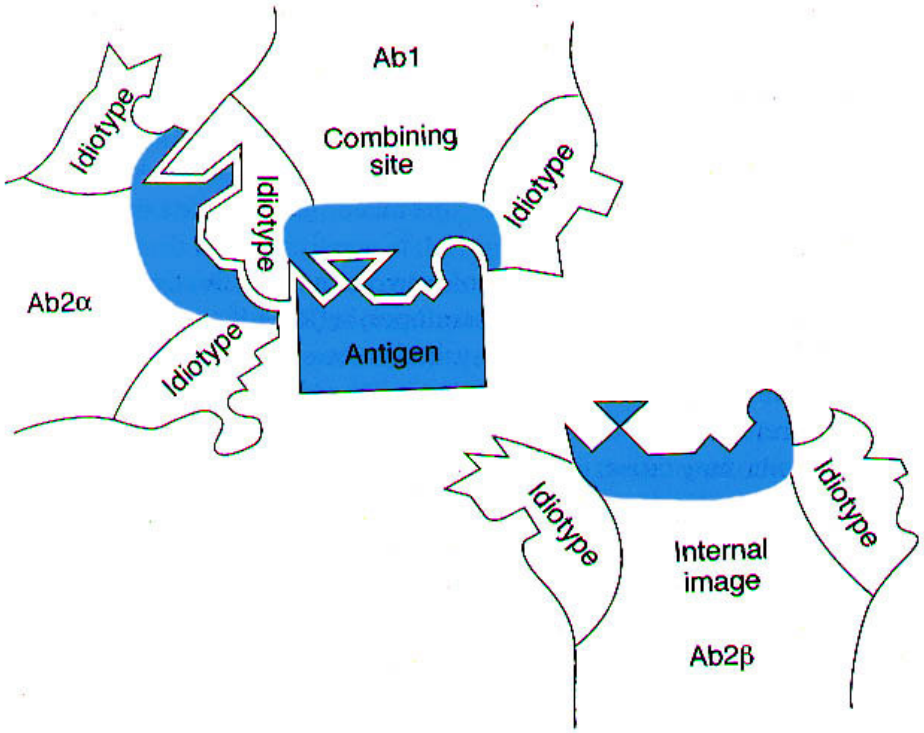


Figure 2-2. The binding of Ab2α to Ab1. Interaction of Ab2β with Ab1 is inhibited due to the presence of antigen. Ab2β is an internal image of the antigen (Bona and Bonilla 1990).

The fourth type of anti-idiotypic antibody is Ab2ε, also called epibodies, that are antibodies recognizing an idiotope on the immunoglobulin, T-or B-cell receptors as well as the epitope for which the paratope of that receptor has specificity for (Poskitt, Jean-Francois *et al.,* 1991). Epibodies may be peculiarities of particular immunizations, eg. against nitrophenyl haptens, that do not perform a particular function in the idiotypic network in general (Muhumuza *et. al.,* 1998).

2-1.4. The idiotypic antibodies in the Guillain-Barré Syndrome (GBS)

The general method for detecting anti-idiotypic antibodies in serum is by determining the inhibition of antibodies in serum to antigen (Lopez, Irazoqui *et al.,* 2000). Guillain Barrè Syndrome patients that spontaneously recovered from the disease contained anti-idiotypic antibodies that inhibited the binding of serum anti-neuroblastoma antibodies to neuroblastoma cells of which the targeted antigen was unknown (Lundkvist, van Doorn *et al.,* 1993). These anti-neuroblastoma antibodies probably contained idiotopes that allowed anti-idiotypic antibodies in IVIg to inhibit anti-neuroblastoma antibodies from binding. These specific anti-idiotypic antibodies inhibiting anti-neuroblastoma antibodies from binding constitute about 1% of the total IgG of IVIg. Intravenous immunoglobulin is pooled immunoglobulin from a large pool of healthy individuals and therefore implies

that healthy control serum may contain idiotypic antibodies. This was confirmed in 2000 when pooled and purified human immunoglobulins from 10 healthy individuals were reported to inhibit anti-GM1 antibodies from binding to GM1 on a TLC plate (HPTLC). (Lopez, Irazoqui *et al.,* 2000). These inhibitory antibodies were rather termed as 'blocking' antibodies instead of anti-idiotypic antibodies since the idiotope on the variable region of the antibody remains undetermined. These 'blocking' antibodies were not internal images of the antigen binding site (GM1 paratope), but were still inhibiting anti-GM1 antibody binding, making them likely to be Ab2γs.

These reports detecting anti-idiotypic antibodies in GBS are few and the presence of the idiotypic network in GBS is not thoroughly investigated. The report by Lundkvist and coworkers suggested that the idiotypic network is involved in GBS patients, but the specific antigen was not defined and the dysfunction of idiotypic network as cause for GBS remains far from proven (Lundkvist, van Doorn *et al.,* 1993). Furthermore, IVIg is used as treatment in GBS, but the responses of patients to treatment vary (Dalakas 2004). Pooled immunoglobulin (IVIg) is used as treatment, but there is scope for improvement, since IVIg is not equally successful in different GBS patients. The success of IVIg as treatment might depend on the healthy sera pooled, since sera might contain different therapeutic potentials. This has not been formally investigated yet and no system has been prepared evaluate therapeutic potentials of healthy sera.

2-1.5. Hypothesis

The potential to inhibit anti-GM1 antibodies in GBS patient blood from binding to their auto-immune GM1 in nervous tissue differs among healthy sera and can be evaluated utilizing the ELISA system.

2-1.6. Aims

- To demonstrate the inhibition of anti-GM1 antibodies from binding to GM1 by recovered and healthy control sera using ELISA.
- Comparing the capacity for displacement of bound pathogenic anti-GM1 auto-antibodies among various healthy control sera to determine their therapeutic potential.

2-2. Materials and Methods

2-2.1. Materials

Nunc 96-well plates (96 MicroWell™ plates-maxiSorp™, from Maxisorb, Roskilde, Denmark) were used for ELISA experiments. The monosialoganglioside GM1 was purchased from Sigma and dissolved in a 1:1, choloroform: methanol, solution for storage (2mM) and diluted with EtOH to a GM1 concentration of 3 µM prior to use. The GM1 was placed in an ultrasonic water-bath before dilution and immobilization onto the ELISA plates. The phosphate buffer pH 7.8 (PBS) and bovine serum albumin (BSA fraction V 96-99%) were purchased from Sigma while the peroxidase conjugated rabbit anti-human IgG was purchased from Jackson Immuno Research, Pennsylvania, USA. The reagents for the substrate solution were from Sigma and include the citric acid solution, O-phenylenediamine dihydrochloride tablets (5 mg) and hydrochloric acid. IVIg was purchased from Gammagard, Baxter Care, USA. Sigma products were supplied by Sigma-Aldrich Corp., St. Louis, Missouri, U.S.A.

Sera tested: Two GBS patients sera sample taken at peak of disease were used as a source of anti-GM1 antibodies namely B017 (called B017A) and B003 (called B003A), both with ELISA titres of 3200 for GM1. A convalescent serum sample from patient B017 was taken after recovery and was labeled B017D (titre < 100). This recovered serum sample was used for the inhibition study. The healthy control sera used for the inhibition and displacement experiments had titers of less than 100 and were used in the standard INCAT ELISA as a negative control in testing for anti-GM1 antibodies. It was therefore assumed that these healthy control sera did not contain significant amounts of anti-GM1 antibodies compared to the patient sera. Serum samples were obtained from the out-clinic of the Erasmus MC, Rotterdam, The Netherlands. Serum samples from healthy controls were obtained from healthy male volunteers of the GBS research group at the Erasmus MC, Rotterdam, The Netherlands, who agreed to their sera being used for research purposes. All samples were stored at -20 ^{o}C or -80 ^{o}C prior to testing.

2-2.2. ELISA protocol for the inhibition of serum antibodies binding to GM1

The ganglioside GM1 was immobilized onto a 96-well ELISA plate by adding 100 µl of a 3000 pmol/ml GM1 solution to each of 2 wells for duplicate experiments, and 100 µl of EtOH to another 2 wells as control for background binding to the plate. The EtOH was then evaporated until the wells were completely dry by using a hair dryer. Two hundred µl of 1% BSA dissolved in PBS pH 7.8 was then added and allowed to block the plate for 2 hours at room temperature followed by 2 hours at 4^{o}C. Plates were then dried after BSA incubation by vigorously slamming out the contents onto several layers of filter paper. Diluted patient serum was pre-incubated with diluted control serum for 2 hours at room temperature and 100µl of this solution was added to the GM1 coated and control wells. Serum was allowed to incubate in the wells overnight at 4^{o}C. The following day the plates were washed 6 times with PBS and slammed out on filter paper. One hundred µl of 1:2500 diluted peroxidase conjugated rabbit anti-human IgG, diluted in 1% BSA/PBS, was added to the wells and incubated at room temperature (± 20^{o}C) for 90 minutes. One hundred µl of the substrate solution was then added to the wells and left to develop for 10 minutes before reading the absorbancies (extinctions) at 492 nm. The substrate solution was prepared as follows: citric acid solution (6 ml, 102mM), sodium hydrogen phosphate solution (6.5 ml, 202mM), milli-Q water (12.5 ml), one o-phenylenediamine dihydrochloride tablet, and 30% H_2O_2 (12.5 µl) were mixed. This solution was sufficient

for 2 plates. The pH of the substrate solution was always checked to be between pH 4.95 and 5.2. An indication of the amount of antibody bound, also called antibody activity for GM1, was obtained by subtracting extinctions found for the GM1 negative wells (wells not covered with GM1) from that obtained from GM1 positive wells. Percentage inhibition was calculated using equation 1.

Equation 1

$$Percentage\ Inhibtion = \frac{(Positive\ serum\ binding) - (Bound\ serum\ after\ pre-incubation)}{(Positive\ serum\ binding)}\ X\ \frac{100}{1}$$

2-2.3. ELISA protocol for the displacement of serum antibodies binding to GM1

The coating of the wells with GM1, and EtOH as control, was performed in the same way as was done for the inhibition experiments. For the displacement experiments 2% BSA in PBS pH 7.8 was used to block the GM1-coated wells. One hundred µl of patient serum (100X diluted) was added to the GM1-coated wells (and non-GM1 coated control wells) and allowed to incubate overnight at 4 °C. The next day the wells were washed 6 times with PBS before adding diluted control sera which was diluted in 2% BSA/PBS. As a positive control for no displacement occurring, 2% BSA/PBS was added instead of control serum. The healthy control sera were allowed to incubate on the plates for 1 hour at room temperature. Wells were thereafter washed first with PBS followed by 5 times washing with 0.005% Tween 20/PBS. One hundred µl conjugate was then added, prepared as for the inhibition experiments, but diluted with 2% BSA/PBS and incubated on the wells for 90 minutes. The substrate solution was added and bound antibody measured as described for the inhibition assay. When the displacement of anti-GM1 antibodies by various control sera was tested, 0.05% instead of 0.005% Tween 20/PBS was used to wash the wells after control serum incubation. This was used due to the high amount of background binding for some control sera when used at a 25 times dilution. The first four wells as well as the last four wells on the ELISA plate were used to determine the antibody activity to GM1 without displacement and the average of these two results were used for further calculations for antibody binding to GM1. The amount of antibody activity for GM1 measured after displacement, as well as positive serum binding (no displacement) was then used to calculate the percentage displacement using the following formula:

Equation 2

$$Percentage\ Displacement\ by\ Control\ Serum = \frac{(Postive\ serum\ binding) - (reading\ after\ displacement)}{(Postive\ serum\ binding)}\ X\ \frac{100}{1}$$

2-3. Results

Exploratory experiments for determining the presence of possible idiotypic antibodies are usually performed by inhibition experiments where a serum or antiserum inhibits autoantibodies in the test serum from binding to their auto-antigen. In GBS, this found practical application for therapy of patients by administration of IVIg to inhibit pathogenic anti-ganglioside auto-antibodies from binding. The inhibition of anti-GM1 antibodies binding to GM1 by healthy and recovered patient serum has not been determined with ELISA before. To demonstrate how humoral factors, probably Ab2 antibodies, can contribute to the protection of an individual against the auto-immune effects of anti-GM1 antibodies, GBS patient serum, containing anti- GM1 antibodies, was pre-incubated with serum from either the convalescent patient or from a healthy individual and binding against GM1 compared with ELISA.

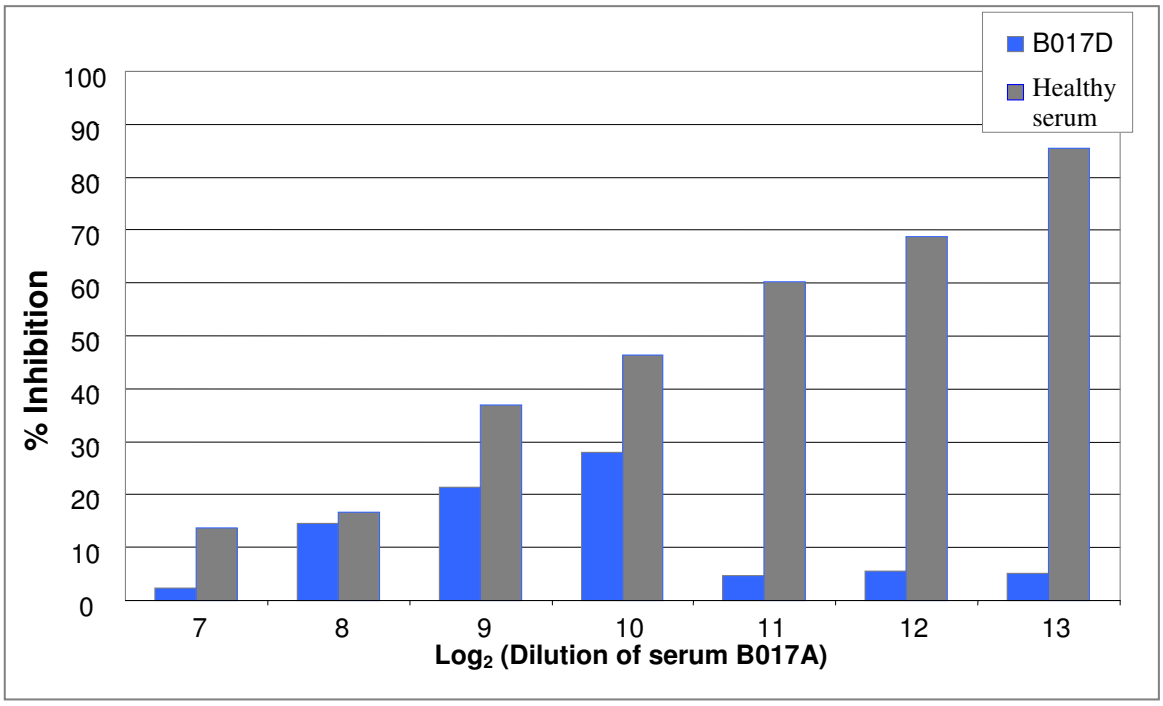


Figure 2-3: Demonstration of inhibition of anti-GM1 antibody binding by pre-incubation of GBS patient serum with serum from either the convalescing patient or from a healthy individual with an ELISA inhibition assay. Inhibiting sera (100X diluted, anti-GM1 titre < 100) were pre-incubated with various dilutions of patient serum (B017A, anti-GM1 titre = 3200) and thereafter tested for anti-GM1 binding activity in ELISA. The percentage inhibition was calculated by expressing the difference in anti-GM1 binding activity between serum (B017A) at a specific dilution and inhibited serum as a percentage of patient serum GM1 binding activity at the particular dilution. The sera B017A and B017D, were taken from patient B017, at peak of disease and after recovery, respectively.

For simplification of the discussion the inhibiting factor will be assumed to be Ab2. In the experiment, anti-GM1 antibodies are allowed to bind to Ab2 in serum from the recovered patient or from the healthy individual and unbound anti-GM1 antibodies will

bind to GM1 immobilized in the ELISA wells. Figure 2-3 illustrates that the capacity for inhibition of the serum of the healthy individual was bigger than that from the convalescent individual, as it gave an inhibition titre greater than $\log_2$ 13 (dilution of 6400), which is more than 8 times stronger than that obtained with the serum from the convalescent patient (dilution of 800). This could be explained by the ratio of Ab2 over anti-GM1 antibody activity that is higher in the healthy individual than in the convalescent patient. This could be an indication of lower Ab2 concentration during convalescence than in healthy individuals, if the affinities of Ab2 for anti-GM1 in both inhibitory sera are the same.

This illustrative result suggests the presence of Ab2 antibodies that demonstrates the therapeutic potential of normal serum for GBS patients, represented in practice by the immunoglobulin fraction (IVIg) of donated serum. The therapeutic potential of these Ab2 antibodies becomes significant once anti-GM1 antibodies can be displaced since it is the bound anti-GM1 antibodies to neuronal cells that are pathogenic. The ELISA assay for displacement of these antibodies could also be used to compare the therapeutic potentials of IVIg or sera from healthy individuals. Displacement of anti-GM1 antibodies in ELISA by serum from a healthy individual has not been documented before and positive results will support investigating idiotypic interactions further.

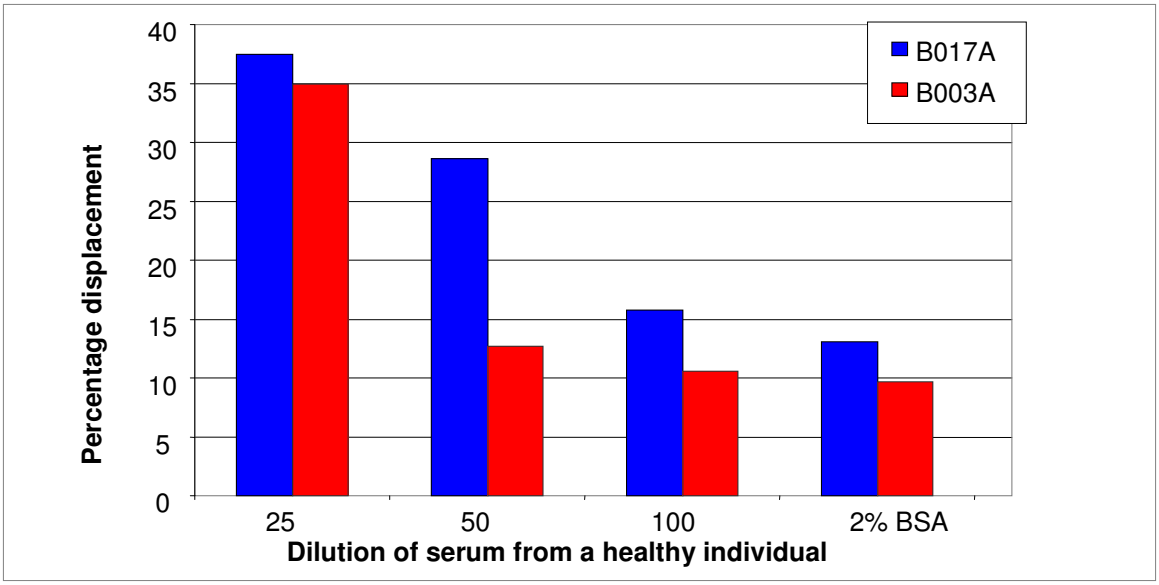


Figure 2-4: The displacement of anti-GM1 antibodies from two GBS patient sera by various dilutions of healthy serum. Antibodies from two patients, B017 and B003, taken at peak of disease (denoted by 'A') were adsorbed from sera (IgG titre=3200 for both) to immobilized GM1. Serum from a healthy individual at various dilutions were added after binding and the relative amount of antibodies removed is expressed as a percentage of the amount of antibodies bound without displacement (equation 2). All samples contained 2% BSA.

In figure 2-4 the results of displacing patient anti-GM1 antibodies by serum from a healthy individual are depicted. GM1-bound anti-GM1 antibodies were challenged with a displacement factor within healthy serum, assumed to be Ab2 antibodies. The immobilized patient anti-GM1 antibodies were displaced depending on the concentration of Ab2, as well as the affinity and specificity of anti-GM1 for Ab2 compared to the GM1. The results in figure 2-4 show that the displacement of anti-GM1 antibodies decreased concurrent with decreasing concentration of healthy serum. The percentage displacement was higher for antibodies from B017A than for B003A, which implies that serum from healthy individuals is more efficient in displacing antibodies from patient B017 than from B003, even though both patient sera have the same ELISA titres against GM1. The preferential displacement of antibodies from B017A might be due to either anti-GM1 antibody concentration, affinity for GM1, or the type of idiotope presented on Ab1.

The 2% BSA appears to displace as effectively as the 100X diluted healthy control serum, but it is important to note that all samples contain 2% BSA, therefore the final BSA concentration for 2% BSA, as indicated in figure 2-4, is actually 4%. Healthy control serum's protein concentrations vary between individuals and it is therefore difficult to predict the exact protein concentration in this control serum in order to compare it with that of BSA. Assuming a relatively high protein concentration of 14 % for healthy control serum, the effective 2% BSA is about 14.3 times more concentrated than the protein concentration in 100X diluted serum (the default 2% BSA concentration excluded). The displacement by healthy serum does not appear to be due to non-specific protein interaction, but due to a specific interaction like Ab2 with anti-GM1 antibodies.

The results in figure 2-4 support the concept of using the ELISA displacement assay to evaluate the therapeutic potential of sera from healthy individuals or different batches of IVIg. Only one healthy control serum was tested in figure 2-4. In order to learn what can be expected from different donated batches of serum in terms of their capacity to treat GBS, the displacement assay for anti-GM1 antibodies was applied to a number of different sera from healthy donors.

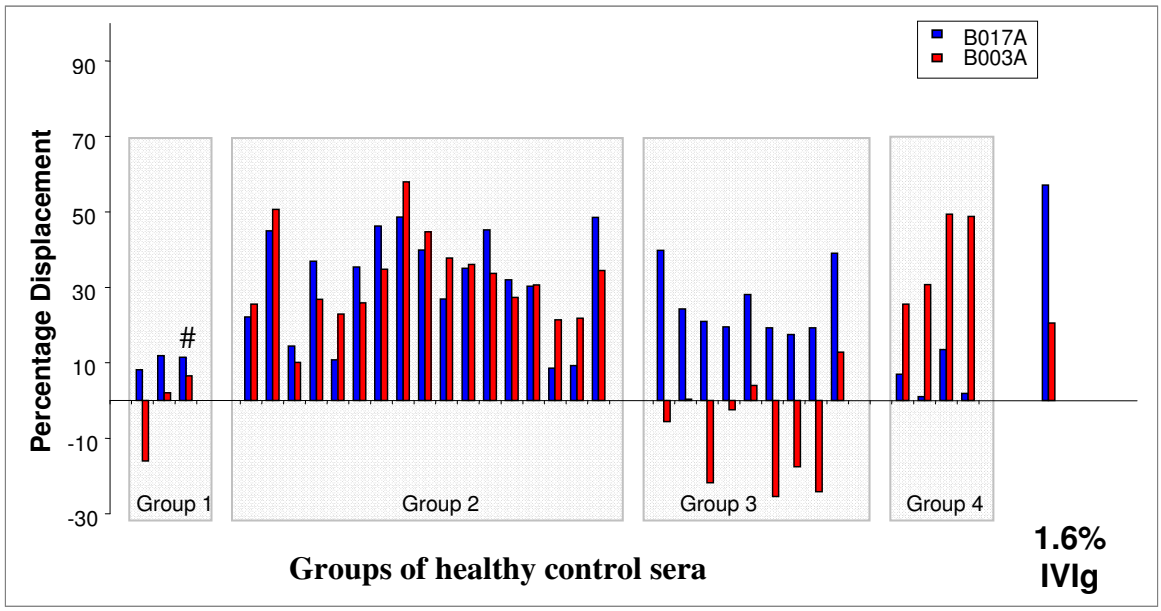


Figure 2-5: Differentiating groups of healthy sera manifesting similar displacement abilities of bound anti-GM1 antibodies. Antibodies from two patient sera, B017A and B003A (100 times diluted) were displaced with 25 times diluted sera from different healthy individuals. Group 1 represents the control sera that do not displace antibodies significantly. The healthy control serum that was used also in figure 2-4 is indicated with #. Group 2 clusters the healthy control sera that displace antibodies from both patients similarly. Group 3 groups the healthy control sera that appear to show a preferential displacement of antibodies from patient B017, while group 4 represents those displacing antibodies from B003A more effectively than from B017A. The displacement by 1.6% IVIg is also illustrated.

Figure 2-5 depicts the displacement of bound anti-GM1 antibodies by numerous healthy control sera and intravenous immunoglobulin. The first group of healthy sera shows weak or no displacement of anti-GM1 antibodies from either of the two patient sera. Positive displacement of bound anti-GM1 antibodies were assumed only when displacement was more than 14% for patient B017 and 12% for B003 and is based on variation across the ELISA plate. Included in this group is the healthy control serum used in the previous experiments and is pointed out with a hash (#). Results in figure 2-5 indicated that this healthy control serum does display inhibition and displacement of anti-GM1 antibodies, therefore this group of control serum can now be classified as being a weak displacer of anti-GM1 antibodies.

Group 2 represents those control sera that were considered to be displacing antibodies from B017A and B003A similarly. This was once again based on the 14 and 12% variation across the ELISA plates and the difference in percentage displacement between the two patient sera not being more than 14%. Group 3 and 4 represent the control sera showing a significant preferential displacement of antibodies from either of the two patient sera, i.e. group 3 with sera displacing antibodies from B017A and group 4 with

sera displacing antibodies from B003A more effectively. All the control sera contain about 0.04% immunoglobulin when considering that the average immunoglobulin concentration in humans is 1% (10mg/ml) and the sera tested were diluted 25 times. The IVIg (1.6% IVIg) could displace bound antibodies from patient B017 more effectively than antibodies bound from B003. This was also true for the healthy control serum that was used to displace antibodies in figure 2-4.

The results in figure 2-5 illustrate that healthy control sera differ in their ability to displace anti-GM1 antibodies. Furthermore, some healthy control sera may displace anti-GM1 antibodies from one patient more effectively than those from another patient (figure 2-5, groups 3 and 4); while other healthy control sera seem to have the same ability for displacing anti-GM1 antibodies (group 2). Another group of healthy sera displaces anti-GM1 antibodies very weakly or not at all (group 1). The IVIg also appeared to be more effective in displacing anti-GM1 antibodies from one patient (B017) than from the other. It is quite clear from this that the nature of Ab2 differs from one healthy control serum to the next and that this may influence the donated serum's potential to be useful for therapy of GBS.

The current ELISA displacement setup could discriminate between the different displacement potentials of various sera from healthy individuals. This assay might therefore be employed to discriminate between different IVIg sources. These results also suggest that one could test and prior select healthy sera with the aim of improving the therapeutic potential of IVIg. It is also evident that sera donated by healthy individuals are not to be classified simply as of good or less good general potential for treating GBS. The nature of the anti-ganglioside auto-antibody of the patient also plays a role and needs to be assessed against the particular therapeutic batch of donated serum or IVIg. This is illustrated in figure 2-5 where anti-GM1 antibodies from two patients are displaced differently by healthy control sera, some of which are better inhibitors for one patient than the other and vice versa.

2-4. Discussion

The approach used in this study to demonstrate a possible idiotypic network by performing antibody inhibition assays with heterologous sera only focuses on the Ab2β and Ab2γ antibodies since they are the only anti-idiotypic antibodies considered to be able to inhibit antibody (Ab1) from binding to its target antigen (Lopez, Irazoqui *et al.,* 2000). Lundkvist *et al.* (1993) reported that recovered GBS patient sera contain anti-idiotypic antibodies that inhibit anti-neuroblastoma antibodies from binding to neuroblastoma cells. Zhang *et al.* (2004) showed that IVIg was able to inhibit GBS patient anti-GM1 and anti-GD1a antibodies from binding to their respective auto-antigens using the HPTLC (high pressure thin layer chromatography) system. The current study corroborates this finding using the ELISA system, using GM1 as one target antigen. It is expected that the ELISA system will also be applicable to assay idiotypic auto-antibodies that are pathogenic in causing GBS where other gangliosides are targeted.

The current study is the first to suggest that the idiotypic antibodies from a recovered patient's serum are of lower concentration than those in healthy control serum. Furthermore, only one recovered GBS patient was tested. Testing more recovered patient sera in a system that measures affinities more directly and sensitively, like the IAsys biosensor, should give valuable information regarding the activity of pathogenic antibodies in GBS patients.

Investigation into possible idiotypic network interplay related to anti-GM1 antibodies and healthy sera were initiated by Lopez *et al.* (2000). Using the HPTLC, the latter study reported that pooled sera from a small group (10 people) were able to inhibit anti-GM1 antibodies from binding and that the inhibition was caused by the $F(ab)_2$ region of the pooled antibodies. The possibility of carbohydrate epitopes in serum (glycosylated proteins) or even on the $F(ab)_2$ region of antibodies was ruled out by the same study showing that the binding of rabbit anti-GM1 antibodies could not be inhibited. The fact that rabbit anti-GM1 antibodies could not be inhibited suggests that the idiotypic antibodies involved with inhibition do not mimic the antigen in their paratopes; therefore Ab2γ idiotypic antibodies are probably involved instead of Ab2β. That study only showed that anti-GM1 antibodies could be inhibited, but the pooled healthy control sera could not displace bound anti-GM1 antibodies. Only in a later study could anti-GM1

antibodies be displaced when using a larger pool of immunoglobulins (IVIg) and the HPTLC technique (Zhang, Lopez *et al.,* 2004). Pooled intravenous immunoglobulin is a common treatment for GBS and the displacement of bound antibodies is considered to be an important contribution to its therapeutic efficacy. This was confirmed in a study, which showed that IVIg displaces bound anti-GQ1b (related to Miller Fischer Syndrome) antibodies in the ELISA system (Jacobs, O'Hanlon *et al.,* 2003).

The current study confirms that IVIg can displace anti-GM1 in the ELISA system, but also suggest that various individual healthy sera, not pooled, can also displace anti-GM1 antibodies in the ELISA system. The various individual control sera tested were found to have different inhibition potentials and were clustered into 4 groups accordingly: the first group of healthy sera, also the smallest group, appeared not to be able to inhibit anti-GM1 antibodies from two GBS patients from binding. Those in the second group, the largest group, appeared to be equally sufficient in inhibiting antibodies from both patient serum samples from binding. Those in the third and fourth group appeared to be able to be more efficient in inhibiting anti-GM1 antibodies from one of the two GBS patients. This variation between control sera for inhibiting anti-GM1 antibodies could be a determining factor in the efficiency of IVIg, thus requiring a large pool of donors for effective general treatment of GBS, as suggested recently (Zhang, Lopez *et al.,* 2004).

Strong evidence in other studies suggests that the inhibition of anti-GM1 antibody binding, and the displacement of the antibodies bound, is due to the $F(ab)_2$ region on isolated antibodies from IVIg (Jacobs, O'Hanlon *et al.,* 2003; Zhang, Lopez *et al.,* 2004). The current study only shows that anti-GM1 antibodies can be inhibited and displaced, but the exact idiotypic antibody (Ab2β or Ab2γ) is not certain. To follow up this investigation, isolation of the inhibitory antibodies from the healthy serum would be needed to confirm $F(ab)_2$ related inhibition. Further characterization of these antibodies should be pursued to determine the basis of intra-species variation of Ab2 antibodies to control GBS, as well as the nature of the idiotypic pathogenic auto-antibodies that cause the various manifestations of GBS in patients.

Lastly, different displacement potentials of healthy sera have been illustrated using the ELISA displacement system. This is the first study that compares the abilities of various healthy sera to displace bound anti-GM1 antibodies and could be employed to select sera

to be pooled in order to improve the therapeutic efficiency of IVIg. This assay might also be used to compare the IVIg sources for their therapeutic potential. This study has also shown that the same healthy control serum might displace anti-GM1 antibodies from one patient more efficiently than in another patient. Therefore, healthy control serum will not displace all Ab1 with the same specificities similarly; therefore other attributes of Ab1 also influence the therapeutic efficiency of healthy serum. Antibodies specific to gangliosides therefore still need further characterization in order to correlate therapeutic potential of healthy sera with the particular Ab2 antibodies with specific attributes.

The current ELISA system presents gangliosides on a solid surface and measures antibody-antigen interactions at a non-physiological temperature (4 ^{o}C). The ELISA system can only give an indication of the relative affinity of antibodies. The biosensor on the other hand can present gangliosides in a more physiological way using liposomes, which allows gangliosides to move freely within the liposome layer, thus mimicking the physiological membrane. Interactions are also measured at room temperature which is much closer to the physiological temperature compared to the ELISA setup. The biosensor is very sensitive and can give direct indication of antibody affinities. The biosensor is therefore a good candidate for characterizing anti-ganglioside antibodies and might reveal antibody characteristics that the ELISA system cannot see.

Chapter 3
Optimizing antibody binding in IAsys

3-1. Introduction

From the previous chapter the observation was made that healthy patient sera inhibit antibodies from binding to GM1, which is suggestive of possible idiotypic network interplay. This was investigated further using the IAsys biosensor. The IAsys biosensor allows the detection of binding of molecules with each other as well as the affinity of interaction. Affinity determination is made possible through real time analysis that reveals the rate of association and dissociation between molecules (Buckle, Davies *et al.,* 1993). ELISA is not suitable for the detection of idiotypic interactions, because it cannot discriminate antibody 2 from antibody 1 when using conjugated anti-human immunoglobulin to produce an antibody binding signal. The IAsys overcomes this challenge by measuring mass accumulation to produce a binding response signal and might therefore be able to detect antibody 2 binding to antibody 1 when appropriate controls are used. In addition, the IAsys biosensor can measure interactions at conditions closer to the physiological, i.e. where the gangliosides' antigens are presented in liposomes, rather than as fat adsorbed onto plastic, as is the case in ELISA. By means of liposome presentation of lipid antigens, the effects of cholesterol, sphingolipids and glycolipids on antibody reactivity can all be tested with the biosensor, thereby giving an indication of the role of membrane structures on antibody reactivity, which is anticipated to be a disease factor in GBS.

The IAsys biosensor offers a solid support of HfO_2 on a glass prism onto which liposomes can be immobilized and interactions measured in the evanescent field. The immobilization of liposomes onto solid supports was not done by a standard published procedure, but by means of a method that was developed in the supervisor's laboratory and that was not fully optimized yet at the start of this study. The immobilization of liposomes and antibody interaction was therefore further optimized and investigated. Measurement of interaction of antibodies with antigens presented in liposomes on the biosensor is not commonly used for analyses, requiring proper investigation with appropriate controls to determine the validity of the results.

3-1.1. The IAsys biosensor

The use of optical biosensor technology allows the study of molecular interaction between proteins, nucleic acids, carbohydrates and peptides in real time without labelling of molecules (Buckle, Davies *et al.,* 1993). One of the interacting molecules (ligand) is immobilized onto a solid support while the second one (ligate) in solution is allowed to interact freely with the ligand. Various surfaces are available to immobilize various ligands and include non-derivatised surfaces, dextran, aminosilane and hydrophobic surfaces. Ligands containing amino and thiol groups are usually immobilized onto a dextran surface using EDC/NHS chemistry. Furthermore, biotinylated compounds can also be immobilized onto dextran surfaces after capturing streptavidin onto the surface using EDC/NHS chemistry. Biomolecules, particulates or cells may be immobilized onto aminosilane surfaces using a bis sulfosuccinimidyl suberate (BS_3) cross-linker. Hydrophobic molecules like liposomes can be immobilized onto the hydrophobic surface. Non-derivatised cuvettes can be employed if the above surfaces do not seem favourable for immobilizing a ligand. A very attractive new way of immobilizing and presenting ligands are through liposomes (Affinity Sensors, Cambridge, UK). Liposomes form artificial membranes on solid supports and can host amphiphatic molecules within the membrane, thus allowing interactions with molecules in solution. Ligands originally extracted from membranes are ideally presented in liposomes to antibody ligates in order to mimic more closely the system in nature where antibodies interact with ligands in membranes.

The interaction between ligand and ligate in the IAsys biosensor is measured in an evanescent field created by a waveguide underneath the ligand coated surface. Incident light at a continuously varying angle is passed through dielectric layers of a prism, and propagated in the waveguide from where it is internally reflected to leave the prism and is detected in a photodetector. The angle of incident light that effects total internal reflection is sensitive to the refractive index of the solution present on the surface of the biosensor (figure 3.1). Perpendicular to the waveguiding, an evanescence field is produced that dissipates from the dielectric surface. Molecules in solution that enter or exit the evanescent field cause a change in the refractive index. Once this occurs the angle of the incident light needed to effect total internal reflection changes. The angular change of the incident light is subsequently converted into a binding response (Cush, Cronin *et al*., 1993).

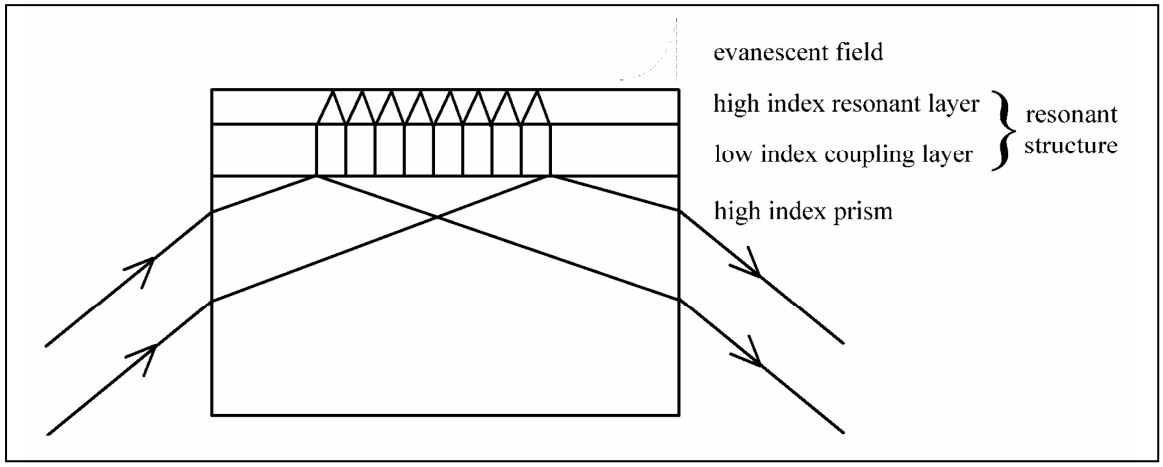


Figure 3-1: Schematic representation of the operating principle of the IAsys sensor device. Light is directed at changing angles to a glass prism that homes the resonant structure. This structure consists of a low refractive index coupling layer, and a high refractive index resonant layer. At critical angle light is reflected, and a series of internal reflection takes place at the boundary between the high and low index layers subsequently forming an evanescent field. This evanescent field is sensitive to the refractive index of the surface above the high index resonant layer. Biomolecules binding to immobilized ligand in this field change the refractive index at the surface. The angle of incident light changes to accommodate the new refractive index and this angle change is converted into a binding signal (Cush, Cronin *et al*., 1993; Gustafson, 2003a).

The solid supports presented in figure 3-1 are provided in a cuvette format that can be easily inserted and removed from the instrument. These cuvettes usually contain one or two chambers each containing a solid support. Cuvettes hosting two chambers, also called 'cells', may be used to compare results obtained from the separate cells, or may be used as duplicate experiments. The limited number of solid support cells in a cuvette also limits the number of experiments that can be performed at once. Multiple cells with solid supports available for simultaneous analysis is a good future prospect, but such systems are not known to date.

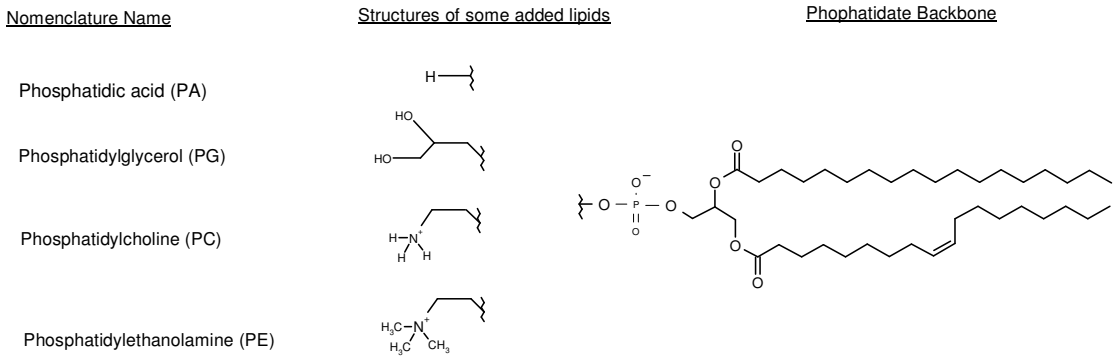
3-1.2. Gangliosides

A ganglioside is a glycosphingolipid consisting of a sialic-acid containing carbohydrate chain and a lipid tail consisting of a ceramide (aminoalcohol sphingosine with a fatty acid) (Thiesen, Rosenfeld *et al.,* 2006). These gangliosides are amphiphatic membrane constituents found mostly in brain and nerves tissues. An important feature of gangliosides is the branched oligosaccharide chain built up by glycosyltransferases onto the primary hydroxyl group of the sphingosine backbone (Kolter, Proia *et al.,* 2002). Sialyltransferases transfer various amounts of sialic acid residues onto the third position of the galactose moieties, which contribute a negatively charge ganglioside for each sialic acid residue added (Kolter, Proia *et al.,* 2002). Gangliosides that were used in this

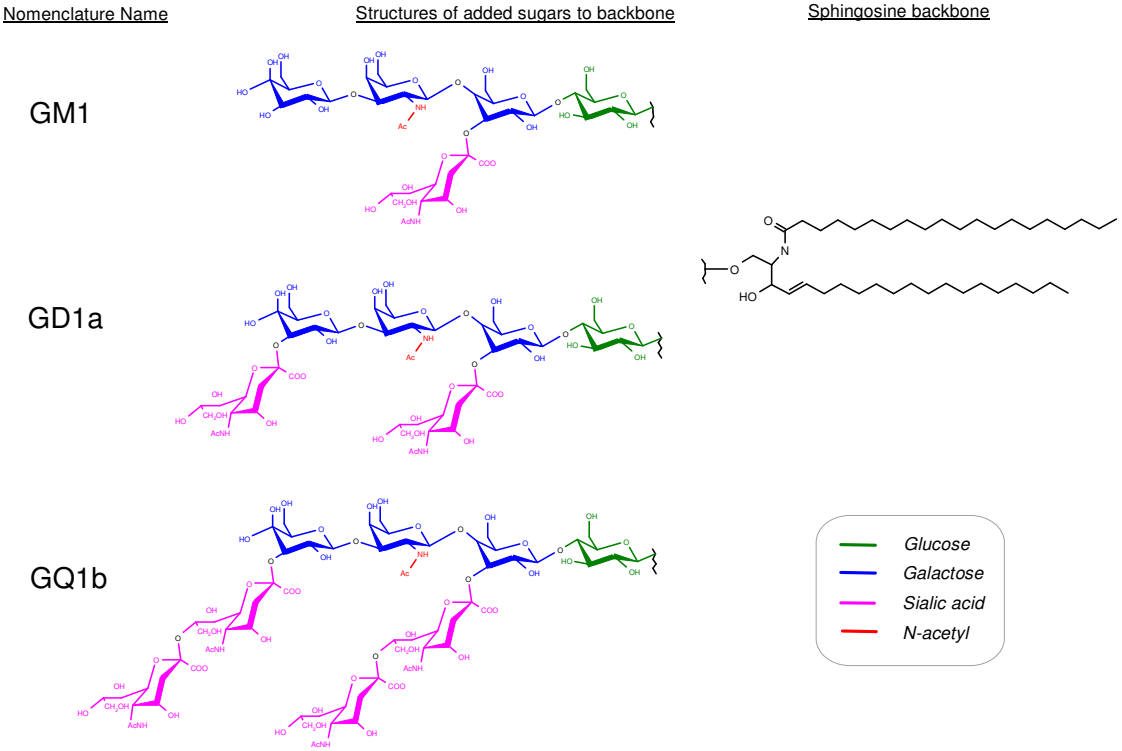
study are presented in figure 3-2, as well as some common phospholipids of the membrane. The amount and position of sialic acids on the carbohydrate chain produces more than 100 different combinations and are responsible for the diverse variety of gangliosides. The typical biological function of gangliosides is still unclear, but its importance in cell-cell interaction and cell signaling has been recognised before (Hakomori 2000). An aberrant role of gangliosides becomes apparent when they act as receptor molecules for antibodies in GBS and for bacterial toxins like in cholera. Gangliosides are not homogenously distributed within the lipid membrane, but segregate with membrane components to form microdomains called lipid-rafts (Wang and Silvius 2003; Yokoyama, Ohta *et al.,* 2004). Membrane structures known to segregate with gangliosides are glycosylphosphatidylinositol-anchored proteins, sphingomyelin and cholesterol (Skwarek 2004; Wang and Paller 2006).

Figure 3-2: Structures of some commonly found membrane phospholipids and gangliosides used in this study. Template for gangliosides obtained from Thiesen, Rosenfeld *et.al.* (2006).

Membrane phospholipids



Gangliosides



3-1.3. Liposomes

Phospholipids are abundant in all biological membranes and are also used for creating artificial membranes. When phospholipids are placed in water they spontaneously assemble into lipid vesicles called liposomes (Lasic 1998). These vesicles capture liquid in the process, may consist of several layers and assume various sizes, which are also used to classify liposomes as large multilamellar vesicles (MLVs), or large and small unilamellar vesicles (LUVs and SUVs) (Steinem, Janshoff *et al.,* 1997). The size of unilamellar vesicles may vary from 20nm to 500nm and the thickness of one bilayer is about 4 nm (Gustafson, 2003a). Liposomes allow the encapsulation of drugs and are used as drug delivery vehicles that increase the half-life of drugs, eg. anti-cancer drugs (Dass and Choong 2006) (figure 3-3). Drugs could also possibly be made to target specific organs by incorporating organ targeting compounds onto the encapsulating liposomes. Another application that is relevant to this study is the insertion of amphiphatic structures into liposomes for interaction analysis. This approach has been used by some researchers to present gangliosides in a way that mimics the *in vivo* situation. A number of authors have reported examples where GM1-liposomes were used in this way (MacKenzie, Hirama *et al.,* 1997; Mitzutamari, Kremer *et al.,* 1998; Fabani, Gargini *et al.,* 2002; Gustafson, 2003b; Thiesen, Rosenfeld *et al.,* 2006).

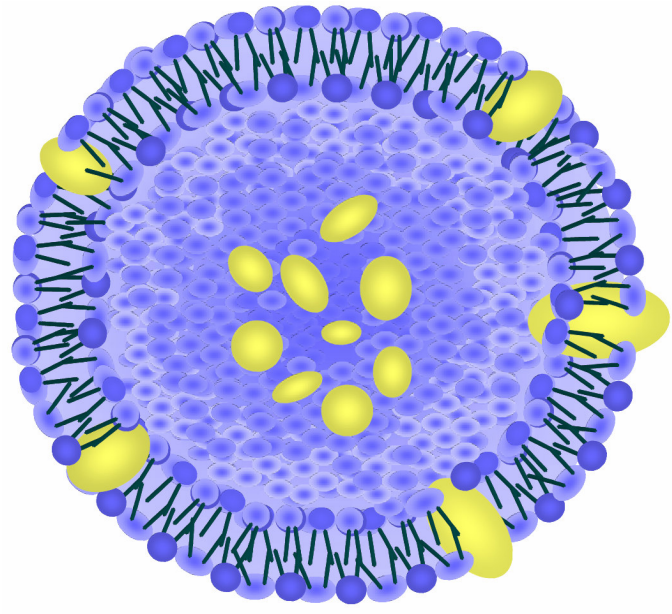


Figure 3-3: A schematic representation of a unilamellar liposome. The encapsulation of drugs (yellow spheres) is also illustrated (Gustafson, 2003a).

There are many ways of preparing liposomes and each method may result in liposomes with different properties, even when the same lipids were used, eg. size, stability and number of layers. The most common method used is the evaporation of organic solvent containing lipids, and the subsequent hydration with aqueous media. This method usually produces large multilamellar liposomes after manual shaking and sonication (Fabani, Gargini *et al.,* 2002). Unilamellar liposomes with generally defined sizes can be produced by extruding large multilamellar liposomes through a filter. Amphiphilic molecules can be incorporated into the liposome when drying the organic solvent and drugs can be encapsulated through hydration with the aqueous phase containing the drugs (Lasic 1998).

3-1.4. Formation of lipid membranes on supports

Artificial membranes can be formed in various ways including free suspended membranes, and membranes on solid supports. The free suspended membranes, also called black liposomes, are formed over a small hole in Teflon but are vary unstable for routine analysis (Gustafson, 2003a). For more stable membranes, liposomes are normally assembled directly on surfaces such as stainless steel, gold, platinum, and silicon. The solid supported membranes can be formed using the Langmuir-blodget technique where a monolayer of amphiphilic molecules oriented in a specific way on water are compressed and transferred onto a solid support (Menke, Kunneke *et al*., 2002). These lipids spread across the water surface may also be deposited onto a solid support by vertically dipping followed by horizontal dipping of the support (Gustafson, 2003a).

The most common method for creating membranes on solid supports is through direct fusion of lipid vesicles or liposomes onto the surface. This method is favoured due to its experimental simplicity and reproducibility leading to almost defect-free membrane formation on solid surfaces. Not all surfaces result in the formation of a bilayer membrane and many supports have rather produced surfaces covered with intact vesicles. Examples of such surfaces onto which bilayers were successfully prepared are silica, glass, Si_3N_4, mica, platinum, gold and TiO_2. Spontaneous bilayer formation does not occur on all surfaces, for example TiO_2 and oxidized gold (Reimhult, Höök *et al.,* 2003; Janshoff and Steinem 2006). Supported membranes are much more stable than intact vesicles and therefore research into the mechanism of bilayer formation and the factors involved are still under investigation and only partially understood.

Many models regarding the formation of bilayers from liposomes have been proposed and many factors identified. These models all identify three important phases in bilayer formation on hydrophilic and hydrophobic surfaces, namely adhesion, rupture and spreading (Gustafson, 2003a). The surface-vesicle interaction plays a critical role in bilayer formation and is influenced by van der Waals, electrostatic, hydration, and steric forces (Cha, Guo *et al.,* 2006). This has led many researchers to investigate the mechanism of membrane formation by varying factors that might be involved in membrane formation on solid supports (Table 3-1).

In the current study the biosensor will be used to measure interaction between immobilized liposomes. The liposomes will be immobilized onto a HfO_2 surface, but formation of a bilayer has not been proven for this solid surface. A previous study by Pieter Vrey has shown that the uncertainty of bilayer formation onto HfO_2 raises no problem for analysis. In his study the interaction of the cholera toxin to gangliosides was measured using liposomes to immobilized and present gangliosides. The affinity constant for cholera toxin obtained compared well with the constants retrieved from literature (Vrey 2003). In the thesis of Inga Gustafson, it was stated that intact liposomes on solid surfaces have a refractive index similar to that of the surrounding medium (Gustafson, 2003a). Once liposomes flatten and rupture, a change in refractive index occurs and could therefore support a hypothesis that bilayer formation occurs on HfO_2 (Very, 2003).

Table 3-1: Factors influencing liposome immobilization and bilayer formation.

Varying factor	Favourable effect	Possible mechanism	Ref.
pH	Lower pH favours vesicle adsorption and membrane spreading for neutral and charged lipids on glass	Enhances Van der Waals and electrostatic interactions between surfaces	(Cremer and Boxer 1999).
Ionic strength	High ionic strength causes faster vesicle adsorption and membrane spreading.	Lowering the barrier to rupture by changing the sizes of liposomes on the surface	(Cremer and Boxer 1999; Reimhult, Höök *et al.*, 2003).
Surface charges of lipids and solid supports	For zwitterionic lipids: Favourable bilayer formation in presence of small counterions of opposite charge to surface. Charged lipids: Solid surface and lipids of opposite charge favour adsorption of lipids	Enhanced electrostatic interactions between surfaces.	(Cha, Guo *et al.*, 2006).
Lipid sizes	Larger liposomes increase bilayer formation	Larger vesicles are easier deformed by surface interaction	(Reimhult, Höök *et al.*, 2003).
Temperature	Increased temperature favours bilayer formation	Vesicle rupture is activated thermodynamically	(Reimhult, Höök *et al.*, 2003).
Bivalent ions	Increase bilayer formation	Facilitate rupture of vesicles, increase interaction with surface	(Ekeroth, Konradsson *et al.*, 2002).

3-1.5. Alum to assist in immobilization

In a preliminary experiment serum from a GBS patient resulted in a higher amount of binding to GM1 liposomes compared to healthy control serum (Vrey 2003). The GM1 liposomes were immobilized by priming the surface with 20mM hydrochloric acid prior to liposome addition. This method of immobilization proved sufficient for the determination of the affinity of binding of cholera toxin B and the results also compared well with results found in literature where other methods were used for determination of the affinity of binding of Cholera toxin B to GM1. Unfortunately when other patients with high anti-GM1 antibody titres (determined by ELISA) were tested, no patients showed such high interactions with the liposomes in the biosensor. Another observation is the excessive amount of background binding in these experiments, which might be due to non-specific binding of serum components to the HFO_2 surfaces and liposomes. In the authors study it was decided to address the binding to the HfO_2 surface by introducing a different immobilization method using potash alum.

Potash alum is an aluminium salt ($KAl(SO_4)_2$) that is commonly used as an extremely versatile coagulant in the treatment of polluted water (Van Benschoten and Edzwald 1990). It serves mainly for the removal of suspended solids and natural organic material through coagulation or flocculation (Zouboulis and Traskas 2005). This process is very complex and still not fully understood, but two theories exist namely destabilizing the colloidal system through charge neutralization, or by adsorption to the precipitate ("sweep floc") formed by the alum (Zouboulis and Traskas 2005). This is better understood when one looks at the chemistry of alum in water. When alum is dissolved in water, it ionizes to form free aluminium ions. The aluminium ions, like most metals, then hydrate to form a complex, metastable combination of monomeric and polymeric hydroxide complexes (Gregory and Duan 2001). These species are influenced by the pH, the effect of which is illustrated in figure 3-4.

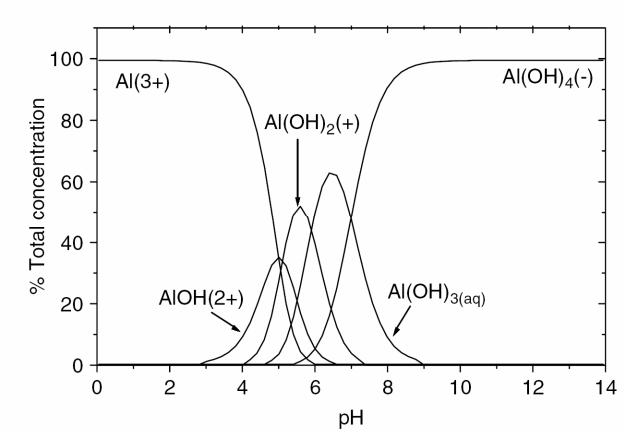


Figure 3-4: A predicted diagram illustrating the aluminium species (produced by MineQL+, a chemical equilibrium modelling system) without crystalline solids and ionic strength corrections. The speciation occurred at conditions of 25 °C, 101,325 kPa and 2mg/l initial concentration (Zouboulis and Traskas 2005).

These species should therefore play a very important role in charge neutralization during coagulation. At pH values higher than 3, Al^{3+} hydrolysis of alum begins and with a OH/Al ratio of over 2.5 and the total aluminium concentration higher than 10^{-2} mol.ℓ^{-1}(in water), the precipitate $Al(OH)_3$ forms (Zhang, Hahn *et al.,* 2004). The $Al(OH)_3$ is highly insoluble with a solubility product of 1.26 x 10^{-33}. This precipitate is responsible for "sweep floc", eg. in swimming pools.

The combination of charge neutralization and adsorption to the precipitate could therefore favour faster immobilization of liposomes carrying a negative charge. The

charged aluminium species might also serve as counter ions improving the interaction between the liposomes and the solid support.

3-1.6. Hypothesis

Biomolecular interaction of anti-ganglioside antibodies and idiotypic immunoglobulins present in GBS patient sera can be measured using an evanescent field biosensor after immobilizing ganglioside antigens in liposomes on the cuvette surface using alum.

3-1.7. Aims

- Optimize the immobilization of liposomes containing gangliosides onto a non-derivatised solid support (cuvette) using alum.
- Measure the binding of purified anti-ganglioside antibodies to the immobilized liposomes containing various gangliosides.
- Measure the binding of anti-GM1 antibodies directly from serum to immobilized GM1-liposomes in the IAsys biosensor, and subsequently inhibit its binding and displacing bound antibodies using healthy serum.

3-2. Materials and Methods

3-2.1. General reagents

The gangliosides GM1, GD1a GQ1b from bovine brain, and L-α-phosphatidylcholine (DPPC also called PC in this study) were purchased from Merck. Bovine serum albumin for biochemical analysis, sodium chloride (analytical grade), sodium azide (analytical grade), potassium aluminum sulphate (analytical grade), EDTA (analytical grade) and alum were from Merck while Tris (hydroxymethyl)aminomethane (analytical grade) was from Saarchem. From BDH absolute ethanol, KOH, and HCl were bought with analytical grade specification, while $CHCl_3$ was chemically pure and NaOH was a general purpose reagent. Cover glass slides for microscopy were obtained from Merck and used for electron microscopy analysis while the liquid nitrogen was provided by Afrox South Africa. Goat anti-human IgG (horseradish peroxidase) against whole immunoglobulin was from Sigma, while goat anti-rat IgG (horseradish peroxidase) against whole antibody was from ICN biochemicals. Saarchem, BDH and Merck products were supplied by Merck NT Laboratories, Darmstadt, Germany. Sigma products were supplied by Sigma-Aldrich Corp., St. Louis, Missouri, U.S.A.

3-2.2. Sera and antibodies

Serum samples labelled F102A, F229A, F226A, F152A and F183A from Guillain Barré patients tested positive for anti-GM1 antibodies using the standard INCAT-ELISA method, also described in the previous chapter but without inhibition or displacement of antibodies, and were used for the serum analyses (Willison, Veitch *et al.,* 1999). Serum samples were obtained from the out-clinic of the Erasmus MC, Rotterdam, The Netherlands and patients agreed to the use of serum for research purposes. All samples were stored at -20 ^{o}C or -80 ^{o}C prior to testing. Antibodies specific for GM1 from one GBS patient, not listed above, was isolated using protein A affinity chromatography and was labelled SM-1. Control serum tested was taken from the author of this study. Neither he nor any family member ever suffered from GBS. The monoclonal antibodies used were a kind gift from Dr Hugh Willison, Glasgow, Scotland UK. The mouse monoclonal antibodies specific for GM1 were purified from hybridoma cultures using Protein A chromatography. The immunization of mice and preparation of the hybridomas are described elsewhere (Boffey, Odaka *et al.,* 2005).

3-2.3. Instruments

IAsys biosensors at the Department of Biochemistry, University of Pretoria, South Africa, and Department of Immunology, Erasmus MC, Rotterdam, The Netherlands, were used for biomolecular interaction analysis. The instruments and non-derivatised cuvettes were supplied by IAsys Affinity Sensors, Cambridge, U.K. Electron microscopy experiments were performed with a JEOL 840SEM microscope, Tokyo, Japan at the Laboratory for Microscopy and Micro-analysis, University of Pretoria. For sonification of liposomes, a MSE 150 sonificator was used in the Netherlands while the Virsonic 600 (Virtis, USA) was used in South Africa.

3-2.4. Electron microscopy

A cover glass slide, generally used for microscopy, was washed using chloroform and ethanol as well as 0.05 M Tris buffer (containing 3mM sodium azide and 1mM EDTA) between washing steps. Two glass plates were then treated with 10 mM Alum, pH 7, for 5 minutes incubation while two other glass plates were treated with 0.05 M Tris buffer at pH 7.5 for 5 minutes. For each treatment one glass plate was incubated with GM1 liposomes and the other with PC liposomes. All liposomes were incubated for 25 minutes to allow binding and glass slides were rinsed with Tris buffer before microscopy analysis. Glass slides with immobilized liposomes were then frozen using cryo treatment (liquid nitrogen). Analyses of the glass slides were performed after about 25-35 minutes of sublimation which was sufficient to sublimate ice present on the glass.

3-2.5. Liposome preparation

A stock solution of 1 mg PC dissolved in 1ml chloroform was prepared and 878 µl of this solution were added to a vial containing 100 µg of GM1. The chloroform was evaporated on a heat block under a steady stream of N_2 gas, or by leaving the vial in a flow cabinet till all chloroform had been evaporated. The different methods of evaporating chloroform did not affect the outcome of results. The mixtures were then reconstituted with 1956 µl 0.05 M Tris buffer pH 7.5 (containing 3mM sodium azide and 1mM EDTA), vortexed for 1 minute and the milky solution was then sonified using a sonifier probe. The mild sonification was achieved with settings of output 2, pulsed for 2 minutes. The milky solution of liposomes was used for the alum optimization experiments and serum analyses. The harsher sonication to prepare liposomes was achieved with settings of output 6, continuous for 10 minutes. The clear solution of liposomes thus obtained was used in the isolated antibody assays. For calculating the molarity of liposomes, GM1 was assumed to have a molecular weight of 1588 Da and PC 734 Da.

3-2.6. Biosensor analysis

The general sequence of events performed on the IAsys biosensor is depicted in the biosensorgram in figure 3-5. The first step was to obtain a Tris buffer baseline with 75 µl of 50 mM Tris buffer pH 7.5 for 5 minutes. The 0.05 M Tris buffer pH 7.5 was then aspirated and 25 µl of 10 mM alum pH 7 added and allowed to incubate for 40 seconds, unless indicated otherwise, before adding 50 µl of liposomes devoid of, or with gangliosides. For the optimization experiments alum at concentrations ranging from 0.01 to 200 mM were tested by adding liposomes 40 seconds after alum was added and subsequently measuring the amount of liposomes immobilized. After 10 minutes of liposome binding the surface was washed 5 times with 100 µl of the same Tris buffer followed by washing 4 times with 10 mM NaOH. The surface was then washed again 5 times with 100 µl Tris buffer before adding 25 µl of the same buffer. After obtaining a stable baseline (liposomes baseline) of 5 minutes, 25 µl of blocking agent was added. The blocking agent used most commonly was 1000 times diluted healthy (control) serum in Tris buffer, but 0.05% (w/v) BSA dissolved in Tris buffer was also used occasionally as indicated. Blocking lasted 10 minutes before 25 µl of serum or antibody at an indicated concentration was added and allowed to bind for 10 minutes. Serum and antibodies were dissolved and diluted in Tris buffer when diluted control serum was used as blocking, while 0.05% BSA in Tris buffer was used to dilute samples if the blocking was with 0.05% BSA/buffer. Dissociation after binding was then initiated by washing the surface 5 times with 75 µl Tris buffer or 0.05% BSA/Tris buffer depending on the type of

blocking used. Dissociation time of 5 minutes was allowed. In certain analyses the buffer was aspirated after dissociation and 75 µl of goat-anti-human or goat-anti-rat IgG (160 µg/ml) was added and allowed to bind for 5 minutes. Dissociation was then initiated by washing the surface 5 times with 75 µl Tris buffer. The surface was then regenerated and the next binding analysis performed once a 5 minute stable Tris buffer baseline was obtained.

3-2.7. Regeneration of biosensor cuvette surface

The regeneration of the cuvette surface entailed mainly 4 treatments that were separated by washing the surface 10 times with 100 µl double distilled deionised water. All steps used 80 µl of solution. The first step was to treat the surface 5 times with 20 mM HCl, the second step 5 times with 10 mM NaOH, the third step 4 times with absolute EtOH and the final step 3 times with 12.5M KOH followed by 2 minutes incubation. This regeneration procedure was adequate when analyzing only liposome binding. For serum analysis the surface sometimes required repeating of this procedure before an adequate liposome baseline could be obtained. After regeneration 75 µl of 0.05 M Tris buffer was added and a 5 minute baseline obtained before proceeding with the next analysis.

3-2.8. Analysis of biosensor data

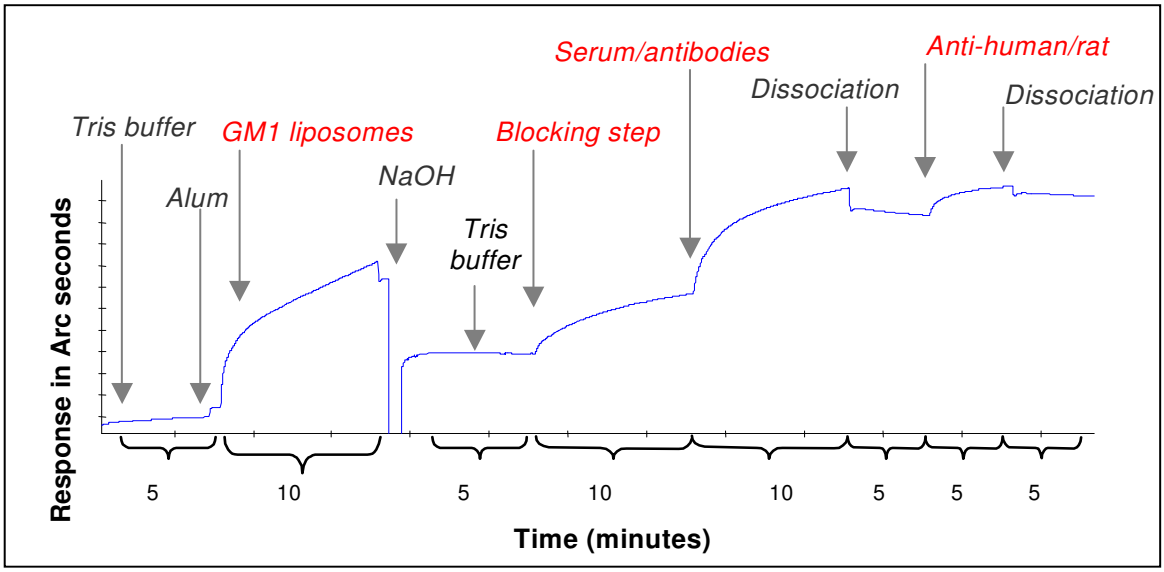


Figure 3-5: A biosensorgram obtained for the sequential steps for one binding analysis of serum or antibodies to ganglioside containing liposomes. The duration of each step is indicated on the x-axis and the binding response (in arc.seconds) on the y-axis. Steps that were investigated in this study are indicated in red. Dissociation events are initiated by aspiration and substitution with Tris buffer. This also preceded the NaOH treatment after liposome coating.

Amount of binding: The IAsys biosensor measures real-time mass accumulation as a binding response measured in arc seconds. The binding capacity is expressed as the difference in arc seconds at the time of addition (serum added) and the end of the dissociation event. Liposome binding is the difference in arc seconds between the liposome baseline after Tris buffer substitution of the liposome solution, before NaOH treatment commences and the first Tris buffer baseline prior to liposome addition.

Data handling: The biosensor cuvette consists of two cells, i.e. two surfaces that can be used simultaneously to perform two experiments. The signal strength in the two cells were often not the same, such that comparative values could not be obtained by comparing the signals of the two cells in parallel. Experiments were performed sequentially and were separated by regeneration steps which removed all bound material. Once the surfaces were regenerated, liposomes were immobilized and the next experiment performed. The binding results between sequential experiments in the same cell were compared to inform optimization conditions such as alum concentration, alum pH, alum incubation time, liposome concentration and antibody binding. Sequential comparison of results was also performed for comparing sonication procedures and antibody specificities. When the results of sequential analyses in one cell were compared to that in the other cell, results were normalized to get comparable results. Parallel comparison of results between the two cells were only done for goat-antibody binding following serum binding, due to the acceptable reproducibility between the two cells that was obtained in only that particular experiment.

3-3 Results

3-3.1. Alum optimization

The first aim was to optimize the immobilization of liposomes containing gangliosides onto an IAsys non-derivatised solid support using alum. Alum has not been used before to immobilize liposomes onto solid supports. It was therefore important to first determine whether alum could improve liposome immobilization compared to previous attempts.

Cuvette pre-treatment for liposome coating

The mere addition of liposomes onto a solid support has been used extensively by other researchers for immobilizing liposomes. Furthermore, the immobilization of liposomes has also been attempted in our group using hydrochloric acid. In an explorative experiment, the improved liposomes immobilization using alum (Figure 3-6) warranted further optimization for the use of alum.

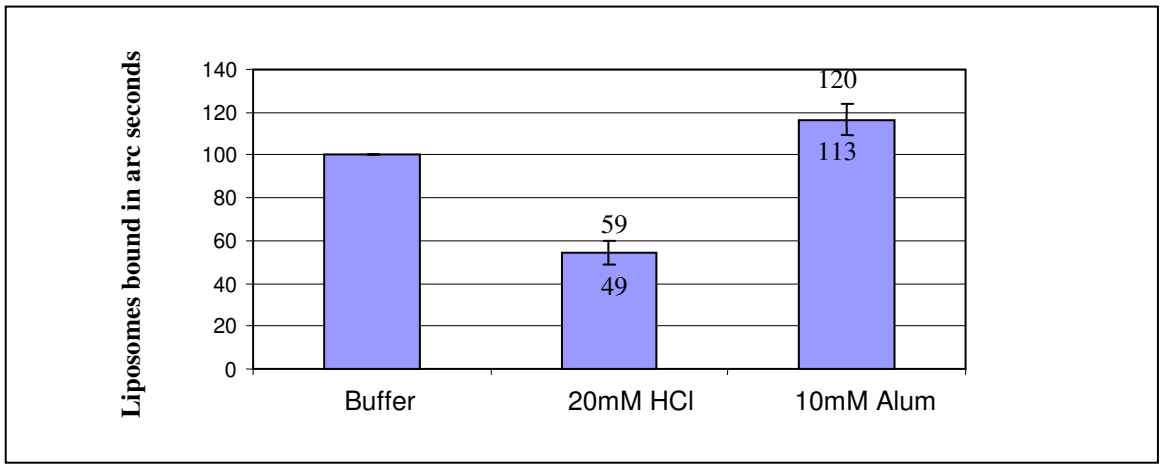


Figure 3-6: Coating efficiency of 5% GM1-PC liposomes (640 µM) in the biosensor cuvette after liposomes were added to aspirated cuvette cells that contained either Tris buffer; HCl (20 mM) or alum (10 mM) before aspiration. Experiments were duplicated and normalized to the amount of liposome binding where Tris buffer was used to fill the cells before aspiration and addition of liposome suspensions. Bars indicate the average of the duplicated experiments, while the values of binding are iserted and indicated through error bars.

Figure 3-6 depicts the amount of 5% GM1-PC liposomes immobilized (in arc seconds) onto an IAsys solid support (non derivatised HfO2) in a cuvette setup using different pre-treatments of the surface, i.e. either Tris buffer, HCl (20 mM), or alum (10 mM). Hydrochloric acid pre-treatment of the surface was found to reduce GM1-liposome immobilization when compared to using buffer only. The alum on the other hand showed improved liposome immobilization of almost 20% compared to Tris buffer pre-treatment. Experiments were only duplicated, but these initial results indicated that alum pre-

treatment of the non-derivatised IAsys solid support could potentially enhance liposome immobilization in comparison to HCl or Tris buffer treatment, but further optimization is required.

Optimum alum concentration for cuvette pre-treatment

Optimization of alum conditions are required to exploit the potential of alum to immobilize liposomes. The first optimizing condition to be investigated is alum concentration.

The optimization of the alum concentration for pre-treatment of cuvette surfaces before coating with 5% GM1-PC liposomes is depicted in figure 3-7. The amount of liposomes bound increased as the concentration of alum increased to 10 mM. Alum concentrations above 10 mM did not show a substantial improvement on increasing the amount of liposomes immobilized as can be seen in figure 3-7a. It was also observed that the amount of liposomes bound after treatment with 10 mM alum was almost 900 arc seconds. This amount of liposome immobilization was not obtained when repeated in the right hand graph of figure 3-7a; only 450 arc seconds of liposome were immobilized using 10 mM alum. These experiments were performed in the same IAsys cuvette cell (surface), but on different days. Even though this difference occurred, relative profiles were identical when the experiment was repeated i.e. 10 mM alum immobilized more liposomes compared to the lower alum concentrations and similar to the higher concentrations. Furthermore, when looking at the binding profiles in figure 3-7b, more liposomes initially adhered to the surface for higher alum concentrations, but most liposomes were washed off resulting in end liposome immobilization values that were similar to when 10 mM alum was used.

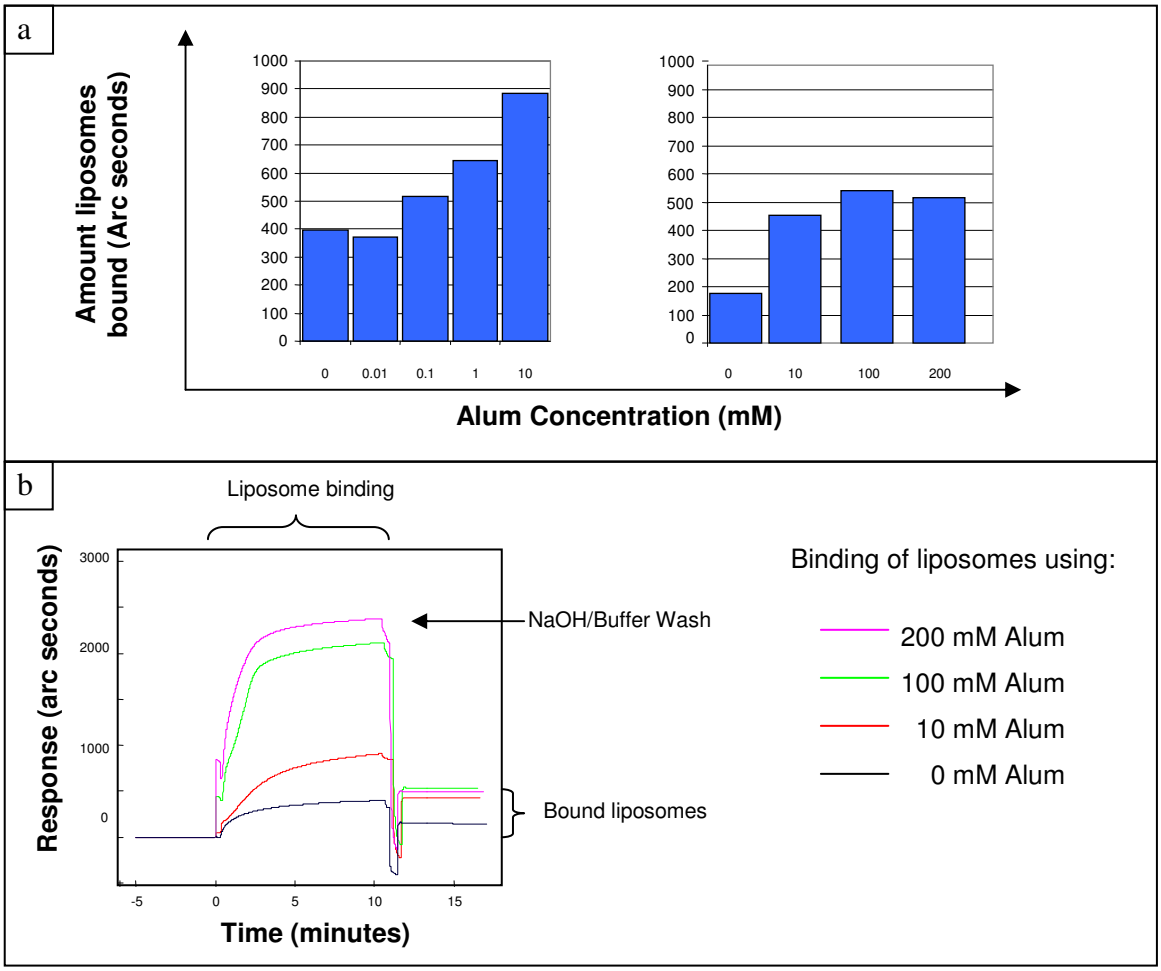


Figure 3-7: Alum dilution experiments for optimizing the immobilization of 5% GM1-PC liposomes. a: Amount of liposomes immobilized after 20 seconds alum pre-treatment of the cuvette at different concentrations and subsequent adding of GM1 liposomes . The left graph illustrates alum concentrations below 10 mM that were tested on one day, and the graph on the right alum concentrations higher than 10 mM tested on another day. b: The liposome binding profiles obtained on the IAsys biosensor for the right hand graph in figure a. Graphs were overlaid using the FASTplot program. Experiments were repeated once and results showed similar profiles.

Optimum pH of alum for cuvette pre-treatment.

Dissolved alum is known to form various aluminium hydroxide species carrying different charges. The formation of these aluminium hydroxide species are dependent on the pH of the surrounding solution and could effect the immobilization of liposomes. The effect of pH on the immobilization was therefore investigated.

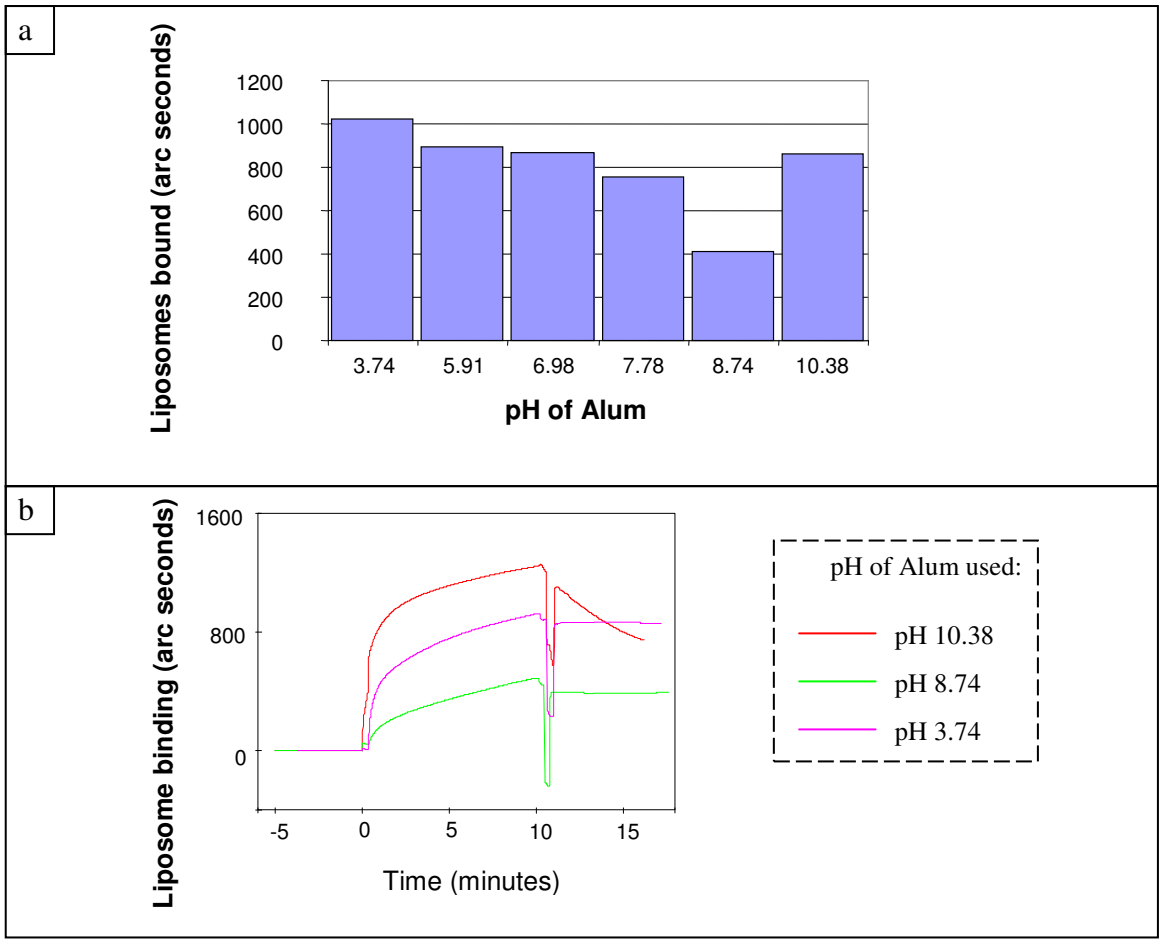


Figure 3-8: Different pH conditions for immobilization of 5% GM1-PC liposomes onto an IAsys solid support (non-derivatised,) using 10 mM alum.a: The amount of liposomes bound, after NaOH wash, using alum at different pHs. b: Overlaid IAsys graphs for the binding of liposomes using different pHs.

Extreme pHs of dissolved alum seem to enhance the amount of liposomes being immobilized onto the IAsys solid support as depicted in figure 3-8. The amount of liposomes immobilized decreased as the pH of 10 mM alum increased from pH 3.74 to 8.74, from there it doubled towards pH 10.38. Even though the amount of liposomes immobilized was high at pH 10.38, the stability seemed to be impaired as illustrated with figure 3-8b where a stable liposome baseline could not be obtained after liposome immobilization, 10 mM NaOH and buffer wash. An aluminium hydroxide precipitate was present in all experiments except when a very low concentration of alum was used,

and at very high pH values (greater than 10.38, results not shown). Interestingly, very high concentrations of alum allowed larger amounts of liposomes to adhere to the surface, but was easily washed off resulting in a lower liposome baseline. These liposomes' baselines were similar to the liposomes' baselines after immobilizing liposomes using a lower alum concentration. This might be due to the increase in flocculation occurring at high alum concentrations due to the higher concentration of $Al(OH)_3$ present. Lower alum concentrations are therefore preferable for maximum immobilization of liposomes. The instability of the liposome layer for alum used at pH 10.38 is observed as a continued decrease in the IAsys binding signal in buffer after washing, which can be understood as liposomes escaping from the solid support. It was decided to continue experiments using 10 mM alum at pH 7 due to these results and the common use of alum at this pH as coagulation agent in water treatment (McCurdy, Carlson *et al.,* 2004).

Optimized method for treating the cuvette surface with alum

Two alternative methods of using alum for immobilizing liposomes were investigated and compared.

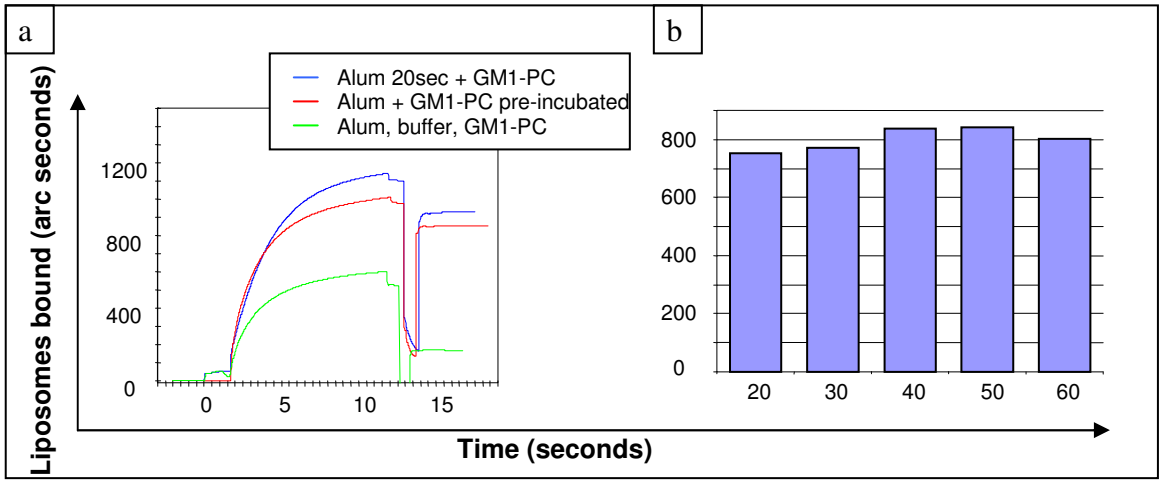


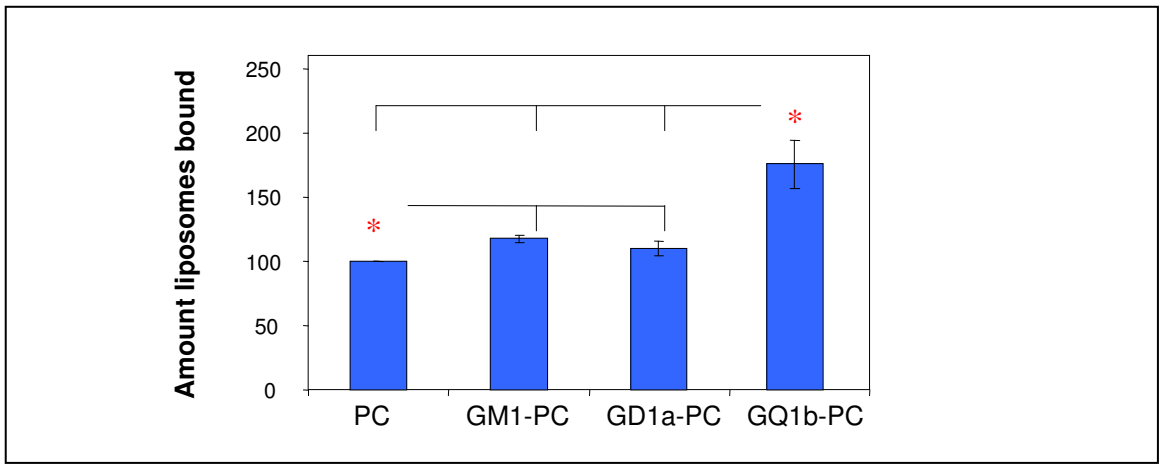
Figure 3-9: A comparison of different strategies for immobilizing 5% GM1-PC liposomes using 10 mM alum pH 7. a: Liposome binding profiles obtained by either adding liposomes after incubating alum in the cuvette for 20 seconds; pre-incubating liposomes with alum prior to adding to the cuvette; allowing alum to interact with the cuvette surface for 20 seconds, aspiration, adding buffer and finally adding liposomes. b: Amount of liposomes bound after incubating alum in the IAsys cuvette for various time spans prior to liposome addition according to the first strategy.

The outcome of the three strategies of using 10 mM alum pH 7 to immobilize 5% GM1-PC liposomes are shown in figure 3-9. The first strategy allows alum to interact with the IAsys solid support prior to liposome addition, while in the second alum was pre-

incubated with liposomes and then added to the surface. The first method appeared to be more efficient than the second (Fig 3-9a). Both these strategies were superior to the third, where alum was contacted with the surface for 20 seconds, aspirated, buffer added and eventually liposomes added. The latter strategy removes unbound alum from the surface and this clearly has a negative effect on liposome immobilization. It was decided to use the first immobilization method that is based on the principle of allowing alum to interact with the IAsys solid support for a specific time and subsequently adding 5% GM1-PC liposomes for coating. Figure 3-9b illustrates that the time of 10 mM alum pre-contact with the IAsys solid support prior to liposomes addition does not significantly influence the amount of GM1 liposomes immobilized. It was decided to perform subsequent experiments using an alum incubation time of 40 seconds without aspiration prior to liposome addition due to its practicality.

The immobilization of different ganglioside-liposomes

The optimal concentration of 10 mM and pH 7 has been found to be the optimal conditions of alum for the immobilization of GM1-liposomes. The immobilization of liposomes containing different liposomes as well as PC liposomes would be beneficial for determining the specificity of anti-ganglioside antibodies and patient serum. This might be very interesting also for idiotypic antibody analyses where a correlation between inhibition potential of idiotypic antibodies and specificity of anti-ganglioside antibodies might exist. In the IAsys system the immobilization of different ganglioside-liposomes using alum has not been attempted before.



*: p≥ 0.05 when correlated as indicated

Figure 3-10: Comparing different ganglioside liposomes immobilized using 10 mM alum pH 7. Experiments were repeated 4 times and the amount of liposomes bound was expressed as a percentage of the amount of PC liposomes bound. The mean value of PC was significantly lower than all the other liposomes immobilized, and the mean of GQ1b-PC was significantly higher than all the other liposomes, Significance was tested using the student t-test. The difference of coating between the GM1- and GD1a -PC liposomes was significant at a 90% confidence level.

The incorporation of gangliosides into PC liposomes facilitates the efficiency of immobilization, as depicted in figure 3-10. The liposomes containing GM1, GD1a and GQ1b significantly coated better than empty liposomes (PC liposomes). The ganglioside GQ1b, containing four sialic acid residues, created the most negatively charged liposome that coated the best with a 76% additional liposome binding compared to PC. GM1-PC and GD1a-PC liposomes showed 18 and 10% enhanced coating efficiency respectively, compared to PC liposomes. The difference between the GM1-PC and GD1a-PC was not significant. It was therefore concluded that the different liposomes immobilized differently on the IAsys cuvette, with a preferential binding of liposomes containing a higher negative charge (GQ1b>GM1; GD1a>PC).

Scanning electron microscopy of liposomes

The more efficient immobilization of GM1-PC liposomes using alum compared to buffer, as suggested in the biosensor results, was investigated using scanning electron microscopy (SEM). For the SEM analyses glass was substituted for HfO_2 as the medium to which liposomes were immobilized.

The SEM electron micrographs of 5% GM1-PC liposomes immobilized onto glass using 10 mM alum pH 7 and 0.05 M Tris buffer are shown in figure 3-11. The sediment formed in 10 mM alum, pH7, can clearly be seen in figure 3-11a where the glass surface

was left unwashed for SEM analysis after alum contact. In figure 3-11b, small sediments formed by 10 mM alum pH 7 might be present, but none was detected. The immobilized liposomes appear as small vesicles on the glass surface that may stick together as seen at the bottom left corner of figure 3-11b. It therefore appears as if alum sediments do not play a role in the interaction of liposomes with the solid support, but it might still accelerate immobilization by flocculating liposomes out of solution, thus accelerating liposome-solid support interaction.

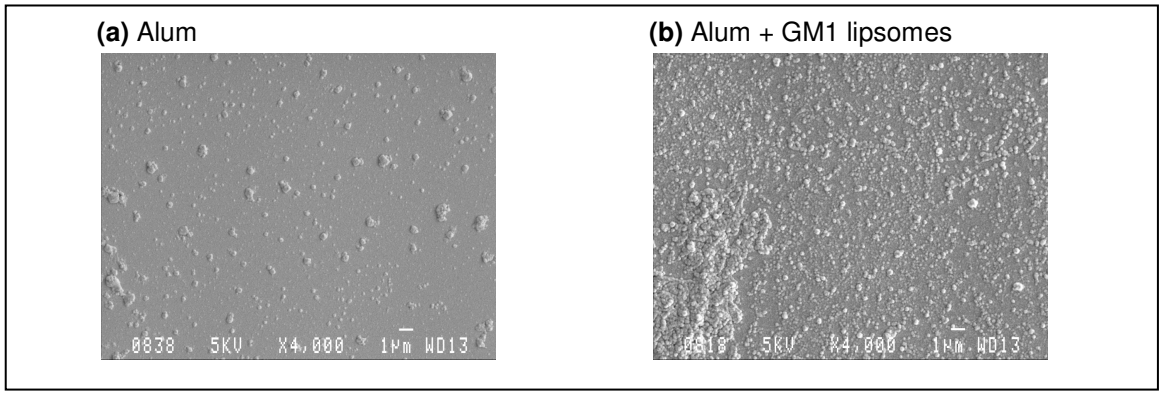


Figure 3-11: SEM electron micrographs of sediments from 10 mM alum pH 7, and 5% GM1-PC liposomes immobilized on a glass slide using 10 mM alum pH 7 (a) A glass plate incubated with alum of which the glass was not rinsed before analysis. (b) A glass plate incubated with 10 mM alum pH 7, GM1-PC liposomes added and the glass rinsed prior to SEM analysis.

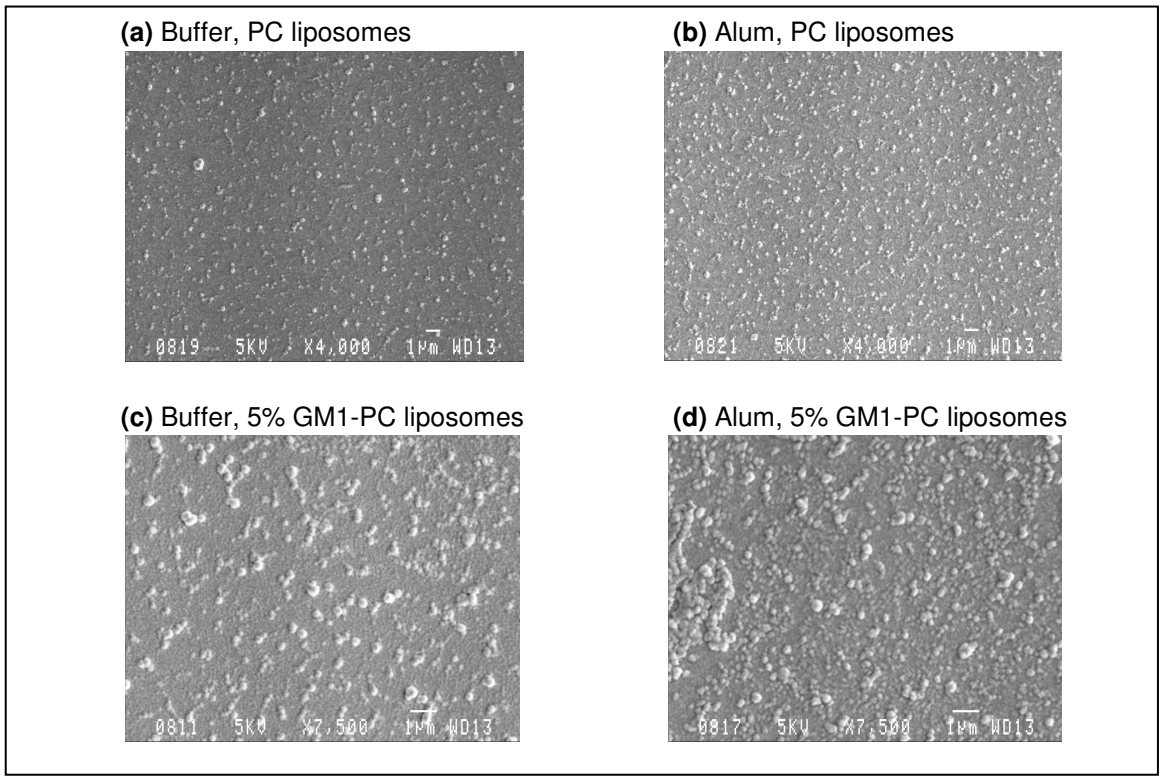


Figure 3-12: Scanning electron micrographs of PC liposomes and 5% GM1-PC liposomes immobilized onto glass plates using 10 mM alum pH 7 or buffer. (a) and (b): PC liposomes immobilized on a glass plate (4000X magnification) after buffer treatment or alum treatment of the glass slide before liposome addition;(c) and (d): GM1 liposomes immobilized using buffer or alum (7500X magnification).

Figure 3-12 compares the effect of 10 mM alum pH 7 and buffer on immobilizing 5% GM1-PC. Figure 3-12a and c show that liposomes were immobilized without the use of alum. When 10 mM alum pH 7 was added to the PC liposomes a higher number of liposomes were immobilized (Figure 3-12a vs. b). The 10 mM alum pH 7 used also increased the number of 5% GM1-PC liposomes being immobilized as depicted by the increase in the amount of smaller vesicles present on the glass surface (figure 3-12c vs. d). Furthermore, the glass surface that is exposed between immobilized vesicles appeared rougher for the surface where the alum was used to immobilize GM1 liposomes (figure 3-12c and d) compared to when buffer was used as treatment (figure 3-12a and b). The liposomes appear to be immobilized as intact vesicles, or as fused vesicles (figure 3-12c and d). This feature is especially visible for the surface treated with 10 mM alum pH 7 and immobilization of 5% GM1-PC liposomes. These results suggest that alum assists in the immobilization of liposomes on glass surfaces, and by extrapolation also to the HfO_2 surface.

3-3.2. Binding of isolated antibodies to immobilized liposomes

The previous section in this study optimized the immobilization of liposomes onto an IAsys cuvette using alum. The functionality of these immobilized liposomes for monitoring antigen-antibody interactions was addressed next.

Optimized liposome immobilization evaluated for antibody analyses

With the optimal alum conditions for the immobilization of GM1-liposomes determined, it was still not clear if the cuvette surface was sufficiently covered with liposomes to be applied in functional antibody analysis. This question was addressed by determining the effect of different liposome concentrations for coating, the amount of mass accumulation during blocking of the liposome coated surface, and the amount of subsequent mouse monoclonal antibody binding (figure 3-13).

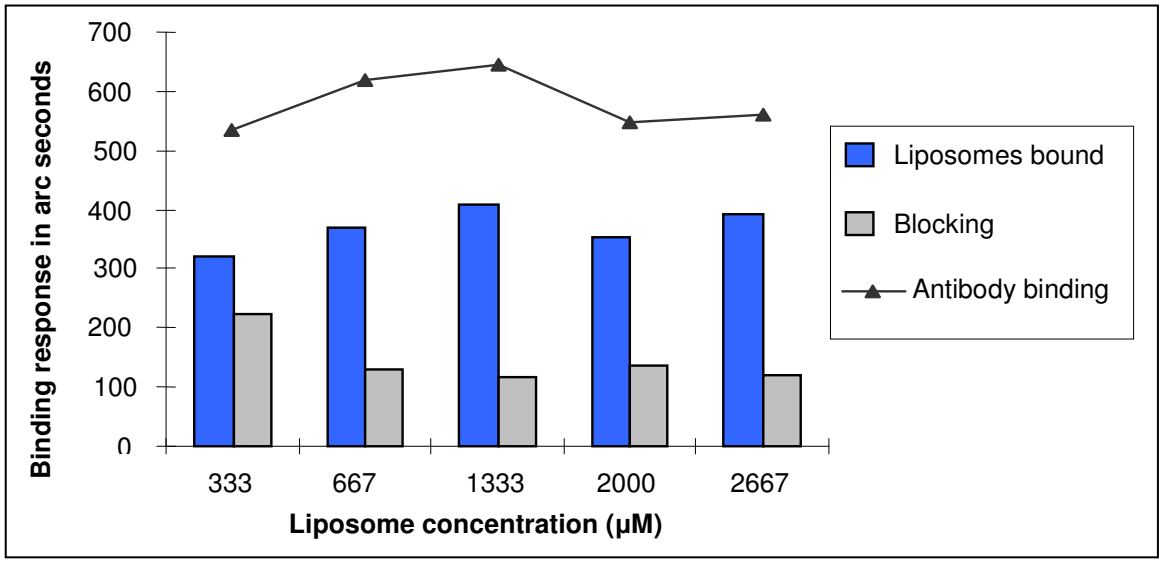


Figure 3-13: The effect of liposome concentration on coating, mass accumulation during blocking, and mouse monoclonal antibody (anti-GM1) binding. Ten mM alum pH 7 was used to immobilized 5% GM1-liposomes, 2000 times diluted healthy serum was used as blocking agent, and a monoclonal anti-GM1 antibody concentration of 67 µg/ml was tested. The final liposome concentrations in the cuvette is given.

The amount of coating was the least at 333 µM liposome concentration (lipid concentration) and increased to a maximum at 1333 µM from where a plateau was reached up to 2667 µM. The mass accumulation during blocking with diluted healthy serum decreased with an increase in liposome coating concentration reaching a minimum at 1333 µM. This confirmed that the surface at 1333 µM liposome coating concentration was adequately covered, since increases in liposomes concentration did not significantly affect non-specific mass accumulation. The amount of mouse monoclonal antibody

binding to the GM1 liposomes increased with liposome coating concentration to a maximum at 1333 µM,

From the exploratory experiment in figure 3-13 it was concluded that the cuvette surface is already adequately covered with liposomes at a coating concentration of 333 µM, since a further increase did not significantly affect liposome immobilization, amount of mass accumulation during blocking, or antibody binding. Nevertheless, the liposome concentration of 1333 µM did appear to be optimal. In this experiment, it was assumed that mouse monoclonal antibodies would bind specifically to the immobilized liposomes presenting the GM1 on the cuvette surface.

Large liposome sizes facilitate bilayer formation (refer to introduction) onto solid supports, but the effect of various liposome sizes on immobilization using alum in the IAsys system has not been investigated. Therefore, the sizes of liposomes were changed by applying a harsher sonication procedure during the preparation of liposomes. The mild sonication procedure entailed a pulsed sonication of 1 minute and produced a milky liposome solution. A much harsher sonication, continuous for 10 minutes at output 10, was performed that produced a clear liposome solution. The mild sonication was assumed to have produced larger liposomes, because the solution was milky.

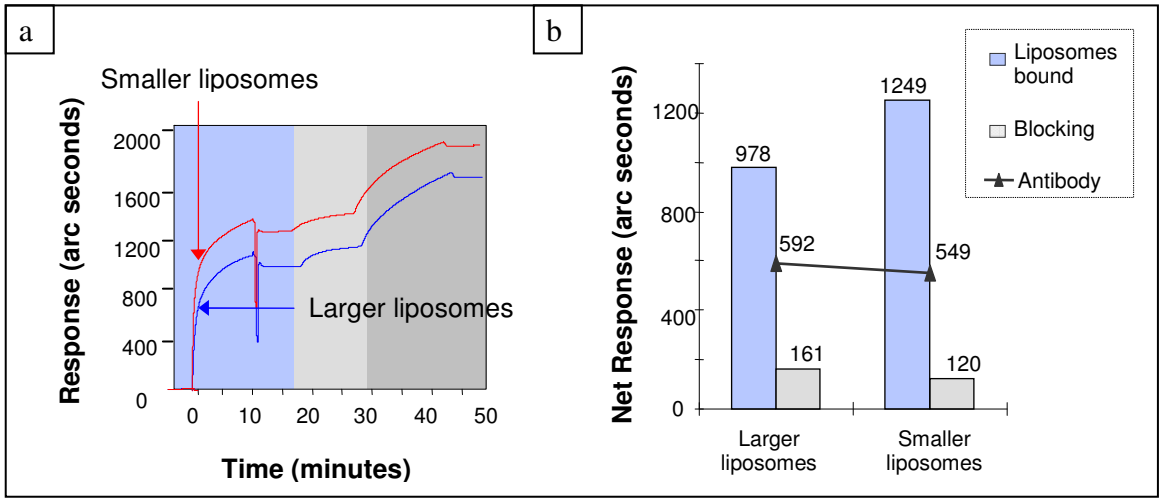


Figure 3-14: The effect of liposome size on coating, mass accumulation during blocking, and mouse monoclonal antibody (anti-GM1) binding. Eighteen percent GM1-PC liposomes at final concentration of 1333 µM were immobilized using 10 mM alum pH 7 and monoclonal antibody concentration of 67 ug/ml was tested. a. An overlayed biosensorgram of the liposomes immobilized, blocking and antibody binding comparing the effect of liposomes sizes. The FASTplot program was used. b: The results from the biosensorgram in bar diagramme.

The exploratory experiment in figure 3-14 shows that the smaller liposomes appear to favour liposome immobilization. The effect of liposome size on the amount of mass accumulation during blocking and antibody binding is not significant. Both liposome preparations appeared sufficient for antibody analyses.

Antibody specificity determined with immobilized liposomes

The binding of anti-GM1 antibodies to the immobilized GM1-containing liposomes was assumed, but the preference of binding of these antibodies to GM1 instead of PC was not determined. Determining the specificity of isolated antibodies for gangliosides presented in liposomes and confirming the specificity with ELISA would prove that the introduced protocol on the IAsys biosensor is reliable.

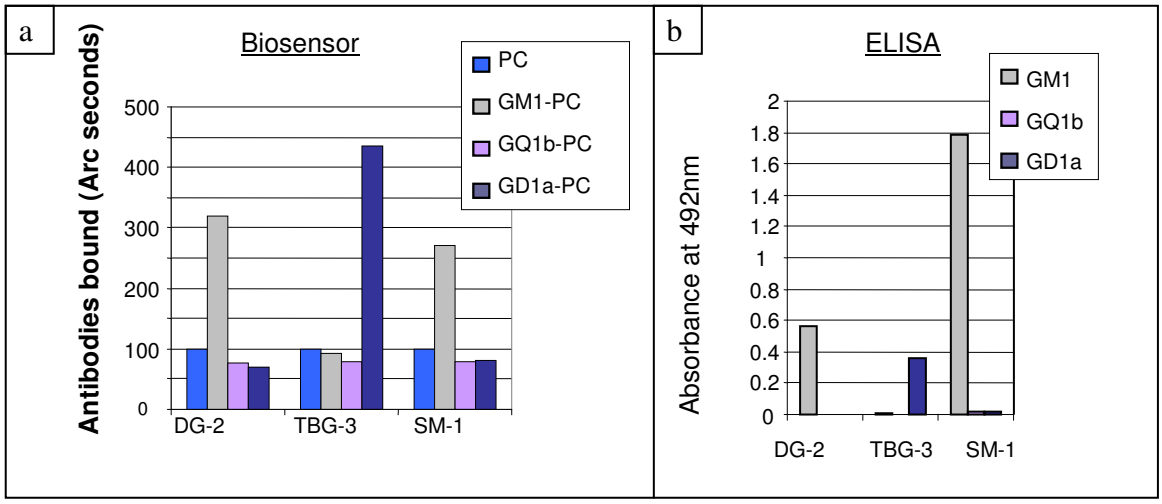


Figure 3-15: Comparing the specificity of isolated antibodies determined by either the IAsys biosensor or ELISA. Antibodies DG-2 and TBG-3 are monoclonal antibodies against GM1 and GD1a respectively, while SM-1 is protein A purified antibodies against GM1 isolated from a GBS patient. a: Specificity for gangliosides in liposomes (5% ganglioside PC liposomes) of antibodies tested after normalizing results to binding to PC. A 0.05% BSA solution in Tris was used as blocking agent. Effective antibody concentration in the cuvette for DG-2 and TBG-3 is 67 ug/ml and 5ug/ml for SM-1. b: The binding of antibodies to gangliosides in ELISA at a concentration of 20 ug/ml.

The specificities of the antibodies determined by the IAsys biosensor and ELISA are depicted in figure 3-15. The monoclonal antibody specific for GM1 according to ELISA (DG-2) also showed high specificity for GM1 on the IAsys biosensor. The same correlation with ELISA was obtained for the monoclonal antibody specific for GD1a namely TBG-3. The antibody isolated from human (SM-1) showed high specificity for GM1 in both the biosensor and ELISA. These results therefore confirm that the gangliosides presented in PC liposomes are functional for antibody binding analysis and that the binding of anti-ganglioside antibodies, monoclonal and polyclonal (from human

serum) to these ganglioside liposomes are specific. Furthermore, coating concentrations between 333 and 2667 µM of ganglioside liposomes (refer to figure 3-10) was not critical for the monitoring of antibody binding.

Sensitivity range of antibody concentration in biosensor analysis

The final question that was addressed with isolated antibodies is the sensitivity of the optimized protocol in the IAsys biosensor for measuring different antibody concentrations. This will especially be important when determining the inhibition potential of anti-idiotypic antibodies on anti-ganglioside antibodies from binding. Two antibody sets were analyzed which are DG-2 (mouse monoclonal anti-GM1), and SM-1 (protein A purified anti-GM1 antibodies from a GBS patient).

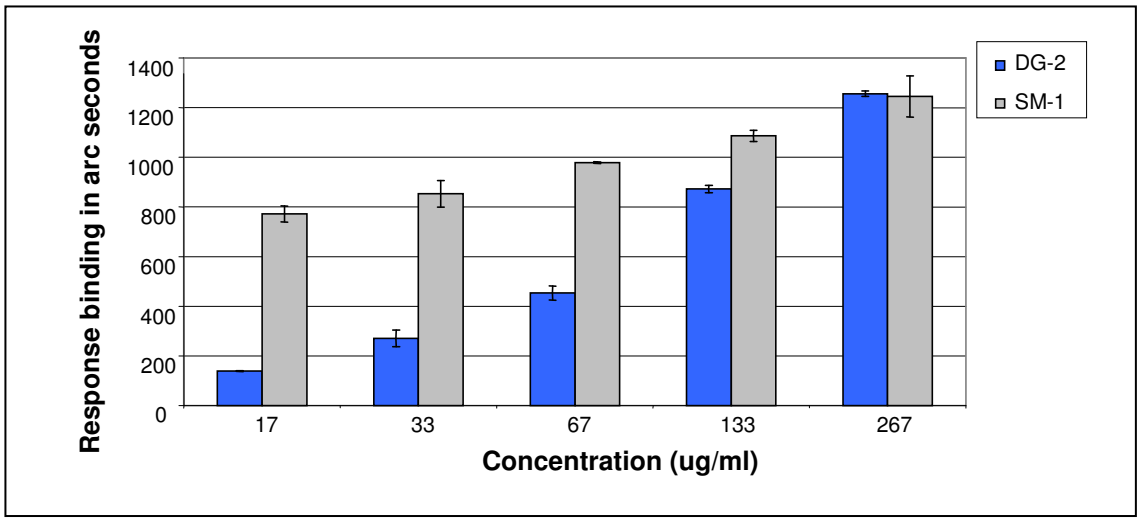


Figure 3-16: Concentration dependent binding of mouse monoclonal (DG-2) and human isolated (SM-1) antibodies to 1% GM1 liposomes (2000 µM). The final antibody concentration in the cuvette is indicated. Solid bar values are the average of two experiments. Errors represent the two results for the duplicate experiments.

The change in mouse monoclonal and human anti GM1 antibodies binding within a range of antibody concentration was found to be highly reproducible for the duplicate experiment (figure 3-16), but the amount of binding of SM-1 was found to be much higher than DG-2 at the same concentrations tested except for the highest concentration. The current experimental setup therefore is sensitive to antibody concentration in monoclonal and polyclonal antibodies and could therefore potentially be used to measure idiotypic antibody inhibition of anti-ganglioside antibody binding directly from serum. It is evident that the sensitivity range of binding will have to be determined for every new antibody or serum sample that is to be measured on the biosensor.

3-3.3. Serum antibody binding to GM1-PC liposomes

The optimized alum procedure for immobilizing 5% GM1-PC liposomes onto the IAsys solid support was found to be sufficient for measuring purified anti-ganglioside antibody binding. This procedure was then utilized for the detection of anti-GM1 antibodies directly from serum and to distinguish serum reactivity to GM1-liposomes between GBS patient and healthy serum. The immobilized 5% GM1-PC liposomes were first blocked using 2000X diluted healthy human serum (end concentration in cuvette). Guillain Barré Syndrome patient and healthy human serum were then added at higher concentration (300X diluted) and allowed to bind to the immobilized GM1-liposomes.

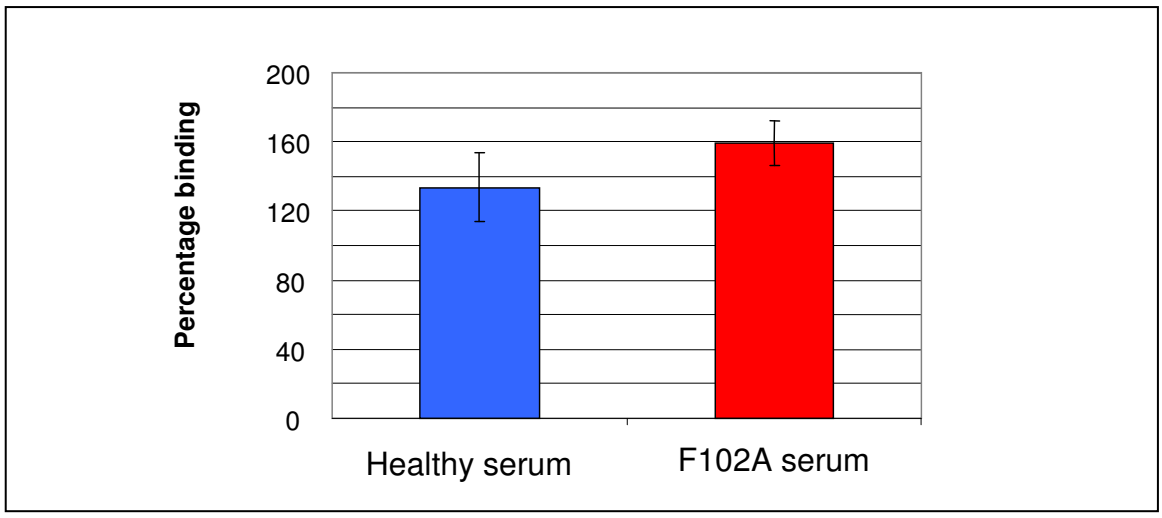


Figure 3-17: Comparing GBS patient (F102A) serum with healthy human serum binding to immobilized 5% GM1-PC liposomes. Sera were diluted 300 times for analysis and the amount of patient serum binding was expressed as a percentage of healthy serum binding. Control serum binding was repeated 6 times and patient serum 8 times. Error bars are the standard deviation.

The difference in the means of serum binding between healthy and GBS patient serum binding (F102A) was significant with a 94% confidence level (figure 3-17). Conversely, the difference between the means (160 vs. 133) does not reflect the high amount of anti-GM1 antibodies that are present according to the high titre value of the patient serum determined earlier by ELISA by other researchers (3200 for IgM, 6400 for IgG). These results were unexpected since the isolated antibody analyses already indicated that anti-GM1 antibodies are detectable with the biosensor. A dilution range of these sera were tested to obtain optimum differences between the sera, but attempt were unsuccesfull (result not shown).

The inability to distinguish patient from control serum binding in figure 3-17 might be due to the binding of other components to the liposome surface, for example lipoproteins,

and therefore conceal antibody binding. Goat anti-human IgG (HRP conjugated) was added after serum binding to determine if antibodies from serum did bind. The concentration of the anti-human antibodies exceeded the expected concentration of total antibodies in the serum. The IAsys binding profiles of a healthy human control serum (GBS negative), a GBS patient serum (F102A), and goat anti-human immunoglobulin binding are depicted in figure 3-18.

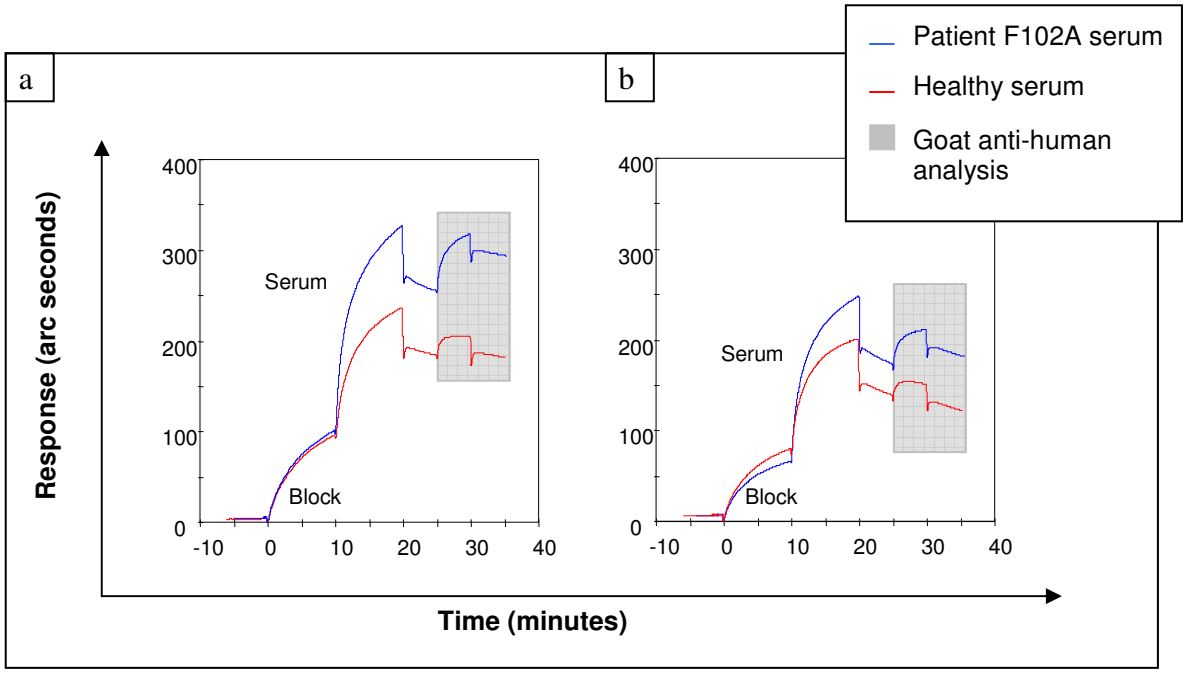


Figure 3-18: The binding of goat anti-human IgG (HRP conjugated, 160 µg/ml) after the binding of GBS patient serum (F102A) and healthy human serum to 5% GM1-PC liposomes in a twin-channel biosensor cuvette. a: The experiment performed in channel 1. b: The experiment repeated in channel 2.

In figure 3-18 the difference between GBS patient (F102A) and healthy serum binding is illustrated as well as the subsequent anti-human IgG binding. The left graph showed a superior binding of patient serum over healthy serum as already mentioned in figure 3-17. The subsequent binding of goat anti-human IgG showed superior binding after patient serum binding compared to when control serum was bound. The goat anti-human IgG only recognized bound antibodies (IgG and IgM) and therefore it appears as if the accumulated mass from the GBS patient serum contained more anti-GM1 antibodies than in healthy control serum. The same experiment was repeated in the other cell of the cuvette and was called channel 2 in figure 3-18, right hand graph. Results were similar, showing more anti-GM1 antibodies from GBS patient serum bound than from healthy control serum, although the differences in serum and goat anti-human IgG binding between the two sera were smaller than in the first experiment (channel 1). The binding

signal in the two channels was therefore not identical for the same experimental procedure, thus preventing the use of the twin cuvette to measure parallel differences simultaneously. This is probably due to a reduced responsiveness caused by slight differences in the two HfO2 surfaces on which the experiments were performed.

In summary, the results in figure 3-18 illustrate that antibodies from both GBS patient and healthy serum bind to the immobilized liposomes. Furthermore, anti-human immunoglobulin bound more after GBS patient serum binding; therefore might be used to assist in distinguishing patient and healthy serum binding. Unfortunately the reproducibility of experiments between the two cells was poor and might be problematic in distinguishing patient from healthy serum with repeat experiments. It was therefore decided that comparison of binding results of experiments performed sequentially in one cuvette cell might reduce experimental variation.

Only one GBS patient (F102A) was tested thus far for anti-GM1 antibodies and therefore more GBS patient sera need to be tested to generalise the conclusion above. The possibility of distinguishing GBS patient from healthy control sera was explored further using both serum binding and anti-human antibody binding. Guillain Barré patient sera taken at peak of disease and tested to have high titres for anti-GM1 antibodies were chosen and tested. The results are summarized in figure 3-19.

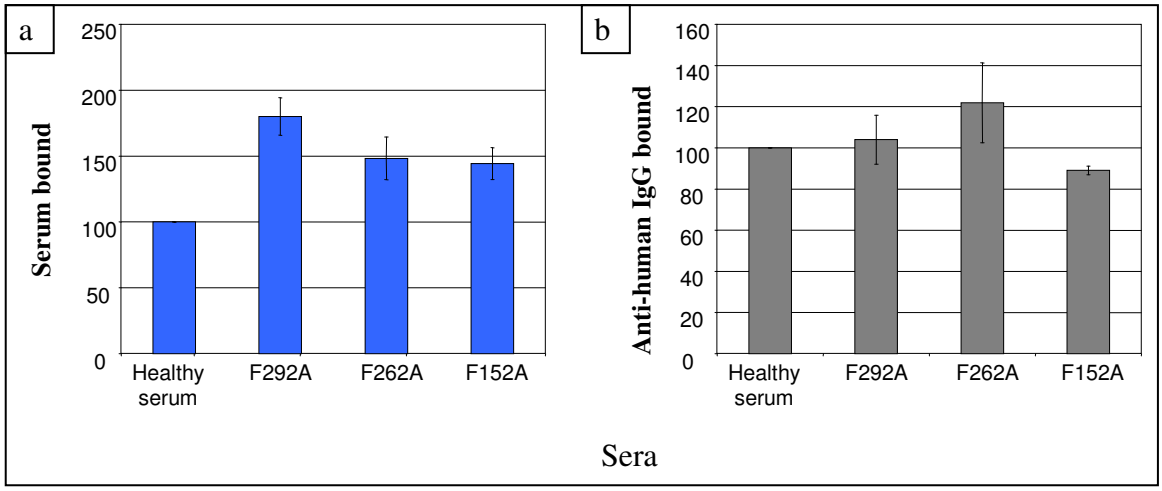


Figure 3-19: Comparing GBS patient sera to healthy serum binding as well as the subsequent goat anti-human IgG binding.a: The net amount of serum binding to the GM1 liposomes were normalized to bound control serum. b: The net amount of goat anti-human IgG (HRP conjugated, 160 µg/ml) binding after allowing patient serum binding to GM1 liposomes. Results were normalized according to the amount of anti-human IgG binding after control serum binding. Sera titres to GM1 determined by ELISA were as follows: F292A – 12800 (IgG); F262A – 25600 (IgG); F152A – 12800 (IgG), < or = 100 (IgM); Control serum < 100. Results are the average of duplicate experiments. The y-bars indicate represents the two values for the duplicate experiments.

The exploratory experiments on the IAsys biosensor suggest that GBS patient sera bind more than healthy control sera to 5% GM1-PC liposomes. This difference was not significant though. The differences in binding ranged from 40-80% of preferential GBS sera binding, which fall within the variability range obtained for the previous patient serum tested which could not be significantly distinguished from control serum binding (refer to figure 3-17). The differences between patient and healthy sera binding are therefore not large or significant. Furthermore, goat anti-human immunoglobulin binding could not discriminate patient sera from healthy serum, but does show that both patient and control sera bind antibodies. It was also observed that an increase in patient serum binding compared to healthy serum (even though not significant) did not necessarily result in an increase in anti-human IgG binding (compare patient F292A and F152A to healthy serum). This could imply that an additional factor in GBS patient serum, other than antibodies that is not present in healthy serum, binds to the GM1-PC liposomes.

The anti-human IgG bound to both healthy and patient sera and it is therefore not certain if the anti-human IgG is binding specifically. The specificity of anti-human IgG for human antibodies bound needs to be ascertained in order to reject the possibility of mere non-specific protein adsorption by anti-human IgG after serum has been bound. This was addressed by comparing the anti-human IgG binding with that of anti-rat after patient serum was bound. The results are summarized in figure 3-20.

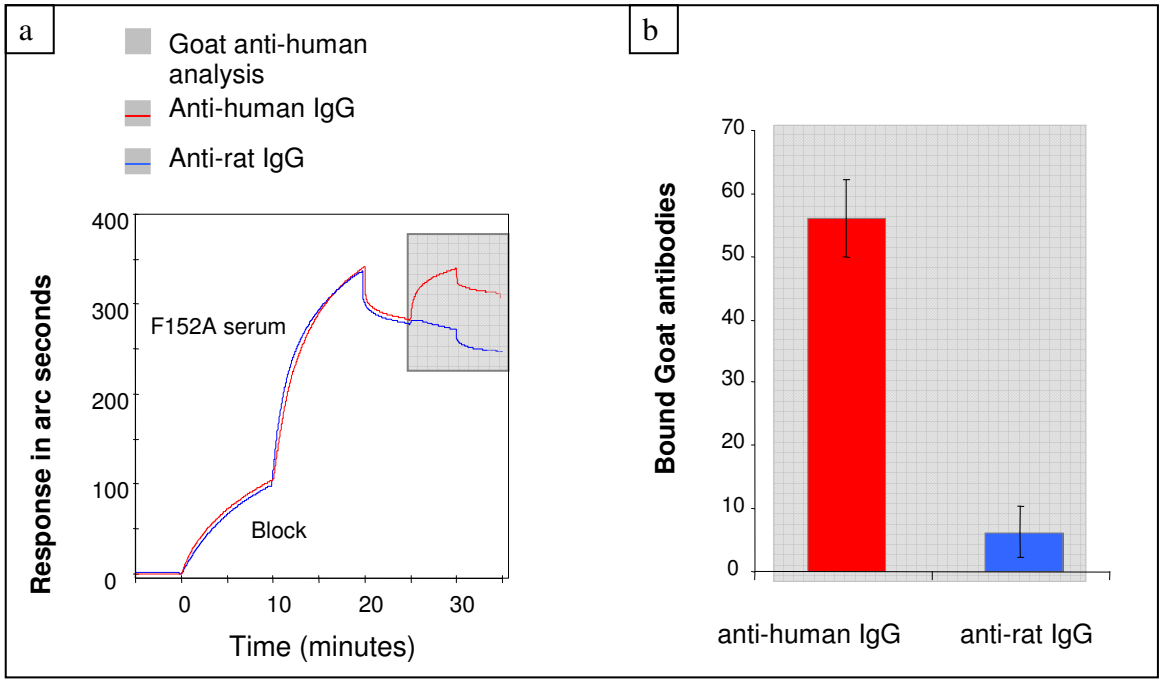


Figure 3-20: Comparing the specificity of anti-human IgG with anti-rat IgG after GBS patient serum binding. Graph a is a biosensorgram of blocking, serum binding, and goat anti-human/rat IgG (HRP conjugated, 160 µg/ml, after 5% GM1-PC liposomes were immobilized. b: A summary of the experiment repeated four times over two cells in a biosensor cuvette.

Anti-human IgG antibodies revealed a high mean binding value that was significantly higher than the amount of anti-rat bound after GBS patient serum binding. This is indicative of the specificity of the anti-human IgG for human antibodies bound after serum has been bound to GM1-PC liposomes.

The results therefore confirm that antibodies from both patient and healthy serum are binding to the gangliosides in the liposomes or to the liposomes themselves, but the specificities of these antibodies in whole sera remain undefined. Nevertheless, it is clear that anti-GM1 antibodies in at least some GBS patients can be detected with the evanescent field biosensor, either purified or in a whole patient serum (F102A). The current study has also shown that GM1 is functionally presented in coated liposomes for

antibody binding and this is therefore not accountable for the lack of antibody binding from some patient sera.

Diluted healthy serum is used as a blocking agent in the current assay for the detection of anti-GM1 antibodies from serum. This will need to be addressed since chapter 2 of this current study already showed that components in healthy sera are able to inhibit anti-GM1 antibodies from binding to GM1 and could therefore interfere with binding in the biosensor. This was investigated by comparing the difference between GBS patient and healthy serum binding after blocking with either diluted healthy serum, or BSA. The difference between patient and healthy serum was expressed as a ratio of patient over healthy serum bound. The ratio of sera binding obtained for the two blocking agents used were compared.

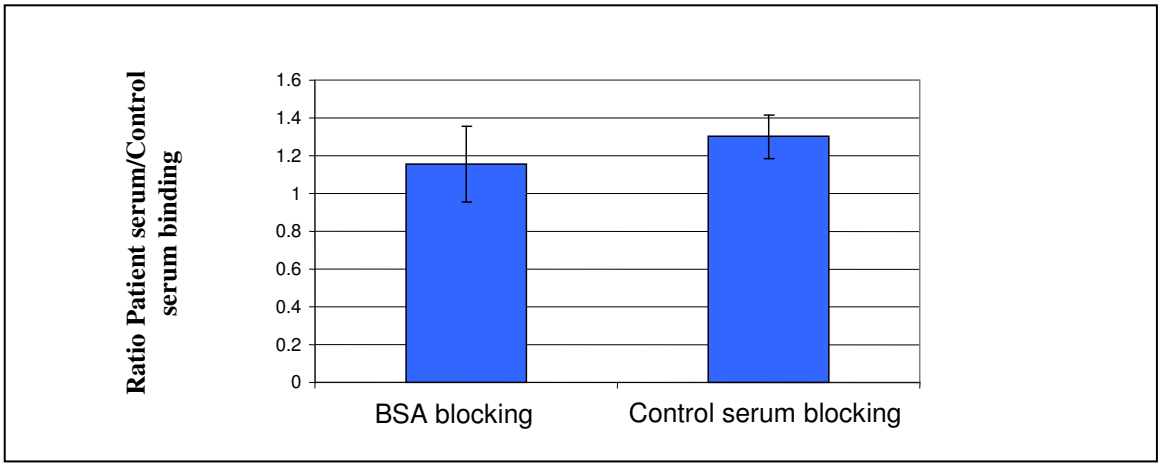


Figure 3-21: Comparing diluted healthy serum (2000X diluted) with 0.025% BSA as blocking agent for distinguishing the GBS patient from healthy serum binding (n = 5). The ratios of patient (F152A) over control serum binding was obtained using the average of two control serum binding analysis and comparing each patient analysis with the average control serum analysis.

The ratios of patient to control for the two treatments did not differ significantly thus implying that the differences between the patient and control serum binding using different blocking agents did not change (figure 3-21). It was therefore concluded that diluted control serum (2000X) as blocking agent does not inhibit patient serum from binding to GM1.

The binding of protein A purified immunoglobulin from GBS and control sera to ganglioside liposomes should be measured using the IAsys system. This will prove that

antibody binding to ganglioside liposomes can only be measured from purified antibodies and not directly from serum.

3-4. Discussion

In chapter 2 the binding of anti-GM1 antibodies was inhibited by various control sera. The long term aim after this finding is to investigate the mechanism of inhibition by control serum in the more sensitive IAsys system with the scope to investigate the role of idiotypic antibody interactions. A reliable method for the measurement of antibody-antigen interaction in GBS with the IAsys system needed to be formulated before inhibition studies could be pursued.

To improve on the ELISA approach, it was decided to use liposomes as a vehicle to immobilize GM1 and other antigenic gangliosides onto the solid support that will be used for interaction analysis on the IAsys system. The immobilization of GM1 liposomes has already been achieved earlier by another member in our group using diluted hydrochloric acid to prime the surface prior to liposome addition. The affinity of cholera toxin was thus determined using this GM-1 liposome-immobilization method and affinity results compared well with that in literature (Vrey 2003). In this study reproducibility for liposome immobilization appeared to improve when older cuvettes were used. Older cuvettes showed considerable amount of oxidation of the aluminium component of the cuvette, probably due to the high concentration of potassium hydroxide used as part of regenerating the solid support after analyses. Interestingly, various aluminium hydroxides are used industrially for coagulating, flocculating and removing natural organic matter as colloids from water (Zouboulis and Traskas 2005). Aluminium or rather its hydroxide species might therefore assist in coagulating and possibly immobilizing liposomes. From this observation it was decided to explore the use of potash alum (Potassium aluminium sulphate) in our system for immobilizing liposomes.

The first exploratory experiments combined with the electron micrographs showed that alum enhanced the coating efficiency of GM1 liposomes compared to using only buffer or hydrochloric acid to prime the surface. The hydrochloric acid used was unexpectedly found to be counter productive for liposomes' immobilization since it immobilized even less liposomes than when only buffer was used. Immobilizing liposomes using only buffer has been a common method used in various other studies (Glasmästar, Larsson *et al.,* 2002; Reimhult, Höök *et al.,* 2003; Seantier, Breffa *et al.,* 2005), and the effect of

low pH on immobilization has been found to be favourable (Cremer and Boxer 1999). The hydrochloric acid used does have a low pH and might therefore shield negative charges on both solid support and liposome surfaces. Conversely, the buffer (pH 7) used contains several counter ions, mainly cations, that might assist in stabilizing the interaction between the two surfaces.

When the properties of alum were analysed it was observed that a white precipitate was formed and that its formation was pH dependent, which supports literature findings (Zouboulis and Traskas 2005). The amount of precipitate forming and the speciation of alum at the specific pHs is not certain since the buffer solvent would have an influence. However, our results showed that pH values below 8.74 favoured liposome immobilization, implying that cationic aluminium hydroxide species, present at a lower pH, most probably play an important role in the immobilization of GM1 liposomes (Gregory and Duan 2001). The positively charged aluminium hydroxides might also play a role in flocculating liposomes out of solution through charge neutralization and subsequently indirectly accelerate immobilization of liposomes by acting as counter ions between the two negative surfaces, i.e. that of the solid support and of the liposome.

The composition of liposomes plays a role in the amount of liposomes being immobilized. More liposomes were immobilized when containing GQ1b compared to containing GM1, GD1a or no gangliosides. The difference between these gangliosides is the amount of sialic acids that they contain. Each sialic acid contains a negative charge. The ganglioside GQ1b contains 4 sialic acids while GD1a contains 2 and GM1 only 1. Therefore, GQ1b is 4 times more negatively charged than GM1. Negative charges on liposomes, due to incorporated gangliosides, are therefore probably aggregated by positive alum species to enhance their immobilization onto the HfO2 solid support. Alum at pH 7 also favoured immobilization of PC liposomes as seen with the electronmicrographs. Phosphatidylcholine is a zwitterionic lipid and the importance of counterions in immobilizing such lipids onto charged surfaces has been illustrated (Cha, Guo *et al.,* 2006). Hydrolyzed alum species that are positively charged could therefore also act as counter ions for immobilizing zwitterionic lipids in liposomes.

The experimental setup on the IAsys was further evaluated using monoclonal antibodies specific for gangliosides. Initial antibody binding analysis showed that anti-GM1

antibodies bound to the GM1 liposomes. The lipid concentration of the liposomes tested as well as the liposomes' sizes did not have such a great influence on the amount of liposomes immobilized, mass accumulated during blocking, and antibody binding. It has been reported that larger liposomes favour bilayer formation (Reimhult, Höök *et al.,* 2003), but for our system this did not affect antibody binding significantly.

Antibodies from two sources were tested for concentration dependent binding of GM1 to liposomes in the biosensor, namely mouse monoclonal and human polyclonal antibodies. The concentration dependent binding of human polyclonal antibody appeared to reach a plateau at a much lower concentration than mouse monoclonal antibody. This difference between the mouse and human antibody could be due to antibody isotype, affinity or even impurities in the sample. The isotypes of these antibodies have not been determined, but a higher concentration of IgM antibody in the human isolate would cause a greater refractive index change due to the size of the pentameric IgM compared to the monomeric IgG. It is difficult to decide on the affinity of the antibodies from the human isolate, since when assuming that only IgG antibodies are present, lower affinity antibodies in the human isolate might favour the binding of one antibody to one GM1 molecule. This occurs due to the steric hindrance of the bivalent antibody for binding two antigens. One could reason that high affinity bivalent antibodies might favour binding of antibody to two GM1 molecules resulting in less capacity for antibody binding, but higher initial rate of binding. Another possible influence on binding affinity could be the presence of anti-idiotypic antibodies in serum. Anti-idiotypic antibodies can bind into the paratope of the anti-ganglioside antibodies to reduce binding to antigen, but may also bind outside the antigen binding site to enhance antigen-antibody complex formation (Denisova, Zerwanitzer *et al.,* 2000). It is therefore not possible to provide a simple explanation for the much improved binding of purified human IgG from GBS patients in comparison to mouse monoclonal antibodies.

The IAsys setup for antibody analysis was further evaluated by determining its ability to determine the specificity of antibodies for different gangliosides in liposomes. The specificity was determined by comparing the amount of antibody binding to different gangliosides immobilized and specificities obtained correlated well with those from ELISA. As already mentioned, the amount of liposomes immobilized differed according

to the ganglioside incorporated, but this did not influence the ability of the IAsys setup for determining the specificity of monoclonal and polyclonal antibodies.

Initial serum analyses using one patient's serum showed that GBS patient's serum bound more than a healthy serum to immobilized GM1-PC liposomes. The difference in serum binding was significant at a 94% confidence level, but the differences in the mean values were small and did therefore not give such a big difference between patient and healthy serum as ELISA did. Three additional patient sera with high anti-GM1 titres were also compared with healthy serum binding to GM1-PC liposomes. The IAsys did not give much difference in binding as compared to ELISA and might be due to non-specific binding. Non-specific binding of proteins to supported phospholipid bilayers (EggPC) on silicon oxide was investigated before with SPR and a quartz crystal microbalance with dissipation (Glasmästar, Larsson *et al.,* 2002). The proteins tested included human fibrinogen, human serum albumin, bovine hemoglobin, horse heart cytochrome c, human immunoglobulin and 10% fetal bovin serum. The study concluded that the phospholipid bilayers were highly resistant to the non-specific binding of protein. Our study used HfO2 as a solid support for the formation of a phospholipid liposome layer. Complete coverage of the solid support was probably not possible and could be responsible for the degree of non-specific binding of serum components that was observed when comparing GM1-binding between GBS patient and control serum.

The addition of anti-human antibody binding after serum binding was performed in an attempt to differentiate specific antibody binding from non-specific binding. A similar method was used before by immobilizing pure gangliosides onto a carboxymethyl dextran surface and the interaction with antibodies measured on the BIAcore system (Alaedini and Latov 2000). Their results showed that anti-human immunoglobulin enhanced the positive anti-GM1 binding signal of antibodies directly from sera. Our results show that after binding GBS patient and healthy control sera, anti-human immunoglobulin could not differentiate reliably between all patient and control sera, despite the proven specificity of the anti-human immunoglobulin for human immunoglobulin. Therefore, antibodies from both healthy control serum and patient sera bound to the GM1-PC liposomes for some unknown reason that could be avoided by prior Protein A purification of the immunoglobulins from the sera.

In conclusion, alum assists in the immobilization of liposomes onto HfO2 and glass supports. Distinguishing between GBS patient and healthy control serum on the IAsys biosensor is possible for GBS patient sera containing high anti-GM1 titres, but the ELISA method gave clearer definition between these sera. The current protocol on the IAsys biosensor is sensitive for measuring antibody concentrations as well as antibody specificities when using purified antibodies. This method will allow the measurement of anti-idiotypic antibodies only if they are of particular isotypes. Purification of IgM and all other isotype antibodies by anti-human immunoglobulin H + L chains may be required to seriously investigate the presence of anti-idiotypic activities in human GBS patient sera from the peak of disease and recovered stages, as well as the presence of anti-idiotypic antibodies against the pathogenic auto-antibodies in healthy control sera. Another advantage of the biosensor approach is the incorporation of various ganglioside antigens in the liposomes that better represents the natural environment of these antigens in membranes of tissue cells. Future research along these lines may fulfil the requirements for testing the hypothesis that anti-idiotypic antibodies are crucial for the treatment, prevention and serodiagnosis of GBS.

Chapter 4
Concluding Discussion

The Guillain Barré Syndrome is the most common diagnosis for acute flaccid paralysis. It is fatal in 5% of cases and leaves 10% of patients with residual disability after recovery (Hughes, Swan *et al.,* 2006). Some host factors have been nominated for being responsible for the disease, but auto-antibody activity due to molecular mimicry between antigens from an antecedent infectious entity and the gangliosides from nerves has become a very attractive hypothesis for the aetiology of GBS. This is partly due to the demonstration that LPS from infectious agents induced antibodies that cross-reacted with the gangliosides, correlating with the development of GBS clinical symptoms in rabbits after long LPS immunization (Ang, Jacobs *et al.,* 2004; Yuki, Susuki *et al.,* 2004). The antibodies commonly associated with molecular mimicry in GBS are readily correlated with the axonal forms of GBS which is most commonly associated with anti-ganglioside antibodies. These anti-ganglioside antibodies are therefore considered to be very important for the pathophysiological effects in GBS and are therefore considered as a target for therapy.

Nowadays, attempts are made to remove the pathogenic antibodies in order to cure GBS. Plasma exchange therapy aims at removing auto-antibodies through filtration, while IVIg is believed to neutralize circulating pathogenic antibodies or even displacing those bound to auto-antigens due to idiotypic antibodies present in IVIg (Jacobs, O'Hanlon *et al.,* 2003; Zhang, Lopez *et al.,* 2004). These two approaches to treatment are considered to be equally effective. Treatment with IVIg was found to at least halve the severity of the disease (Hughes, Raphaël *et al.,* 2006; Willison, 2005). Unfortunately, not all patients respond the same to the treatment, which implies that IVIg treatment lacks universal immunological specificity by not adequately targeting the pathological origin similarly in all the patients. Treatment with IVIg is also very costly for the patient and therefore needs to be improved in order to target the origin of the pathology specifically.

Antibodies from 3000 to 10 000 donors are normally isolated and pooled to obtain IVIg, but pooled immunoglobulin from a smaller group (10 donors) has also exhibited the ability to adequately inhibit anti-ganglioside antibodies from binding. This was probably affected through idiotypic antibodies. It was more difficult to obtain direct evidence using

HPTLC to suggest that IVIg from smaller donor groups were able to displace auto-antibodies bound to self-antigen (Lopez, Irazoqui *et al.,* 2000). It was later suggested that the inability to displace antibodies could be due to the small number of donors (Zhang, Lopez *et al.,* 2004). In this current study it was hypothesized that the efficiency of treatment is not necessarily dependent on the size of the donor group, but on the immune status of individual donors, since donors differ in their ability to displace antibodies bound to antigen. The efficiency of displacement is dependent on both the character of the auto-antibody as well as the anti-idiotypic antibody responsible for displacement.

This study showed that different individual donors can displace the same GBS related auto-antibody differently from the ganglioside antigen, therefore emphasizing the importance of the differences of idiotypic antibodies to be displaced, or the anti-idiotypic antibodies that are required to affect this. The reason for the presence of different anti-idiotypic antibodies among healthy donors is likely brought about either by genetic inheritance, or the prevailing immune status of the donor. Paratope specificity between antibodies from different patients differ according to slight differences in antigens they have been exposed to as well as their individual genetic makeup which would subsequently cause different idiotopes to be produced. Furthermore, new idiotopes are also produced after hypermutation of genes, which adds to the diversification of idiotopes on auto-antibodies and subsequent anti-idiotypic antibodies (Reale, Manheimer *et al.,* 1986; Lemke and Lange 2002).

Sera from different donors may differ in their ability to displace auto-antibodies from different patients, since these auto-antibodies have different characteristics. This study could accordingly group sera from healthy donors into at least four groups according to their ability to displace anti-GM1 antibodies individually sourced from two GBS patients; namely group 1 showing poor displacement of bound antibodies from both patients, group 2 showing displacement of bound antibodies from both patients sufficiently and similarly, and group 3 and 4 showing displacement of antibodies preferentially from either the one or from the other of the two patients. The third and fourth group clearly show that the character of the auto-antibody differs between patients, which requires specific donors to displace antibodies. Group 2 donors probably contain anti-idiotpic antibodies recognizing cross-reactive idiotopes from different auto-antibodies, although

the presence of multiple anti-idiotypic antibodies each with the ability to displace a different patient idiotypic antibody is not excluded.

This finding has several implications for treatment. One could for instance test and correlate each patient's auto-antibody repertoire against various donor sera to identify a donor or IVIg batch that optimally displaces a specific pathogenic set of antibodies. This study has accordingly developed an ELISA displacement test that could be used to determine the potential of donors for displacing specific patient antibodies. Donors' sera that show similar displacement potential for a specific type of auto-antibody can be grouped and antibodies pooled for IVIg treatment. Once a GBS patient has been identified and found to contain high concentrations of auto-antibodies, the patient's serum can be screened with the different groups of IVIg for displacing the auto-antibodies. The IVIg batch showing the best displacement potential can subsequently be selected for treatment. Grouping healthy sera from donors into IVIg batches that displace specific auto-antibodies and using them as treatment will improve the immunological specificity of treatment, subsequently reducing the severity of the disease more efficiently and speeding up recovery. By selecting healthy sera to be pooled, fewer donors might be required per IVIg batch that might reduce the costs. Another possibility that has not been considered that might especially benefit developing countries is simple plasma transfusions with healthy sera that show displacement of antibodies. The main concern is whether the amount of anti-idiotypic antibody in one healthy donor plasma is sufficient for inhibiting and or displacing auto-antibodies to below pathogenic levels.

Before healthy sera can be classified into groups based on their ability to displace particular auto-antibodies, the latter antibodies need to be well characterized. Progress has been made in determining the specificity and affinity of auto-antibodies to gangliosides (Willison 2005), but only recently was the complexity of antibody specificity realised when it was observed that certain antibodies bound preferentially to gangliosides in membranes (Kaida, Morita *et al.,* 2004; Kaida, Kanzaki *et al.,* 2006). The current study aimed at developing a method to characterize the interaction between auto-antibodies and ganglioside antigens, where the latter was presented in liposomes, in order to appreciate the effect of membrane components on the antigenicity of the gangliosides. The IAsys biosensor was used to measure these interactions due to its sensitivity and ability to measure molecular interactions in real time without the labelling of compounds.

For this purpose a new method of liposome immobilisation using alum was developed. It was found that this method can measure the specificity of purified antibodies for gangliosides sensitively. The results corresponded well with those obtained by ELISA, indicating that the biosensor can be applied to characterize purified antibodies in respect of affinity and specificity, including the effects of membrane embedding of the ganglioside antigen.

In spite of the promising results obtained with purified antibody, analysis on whole serum was less successful. Only one patient serum of five tested could be distinguished from healthy control serum, and the difference was not as pronounced as obtained by ELISA. This could be due to non-specific binding of non-immunoglobulin components of the serum to the biosensor glass surface, as well as to the liposome layer. Non-specific binding to solid supports has been a barrier for sensor application in medical diagnostics when analysing serum. Plasma proteins are known to non-specifically adsorb immediately to the sensor surface upon contact (Brynda, Houska *et al.,* 2002). In addition to adhesion to the surface, the serum proteins undergo processes that lead to activation of coagulation and complement, which result in more proteins adsorbing to the surface. In order for the specific binding signal to exceed the non-specific binding signal in the biosensor, the specific analyte in the blood needs to be in high concentration and of high affinity and molecular mass. Anti-ganglioside antibodies' concentration varies a lot and may be very low in some cases. Furthermore, the affinity of anti-gangliosides are believed to be low and the mass of IgG that is often found in GBS patient sera may not be high enough to produce a specific signal that would exceed the non-specific binding signal. It is therefore clear that the non-specific binding will need to be reduced substantially in order to obtain a specific antibody binding signal that can be used to characterize anti-ganglioside antibodies. Previous attempts to reduce non-specific binding in optical biosensors include the use of hydrogel surfaces like carboxymethyl dextran (CMD) and polyethylene glycol (Brynda, Houska *et al.,* 2002). Liposomes were immobilized onto CMD surfaces and affinities of purified antibodies to gangliosides in these liposomes determined (Boffey, Odaka *et al.,* 2005). The effect of the CMD surface on the mobility of the immobilized liposomes is still unclear, but it is a good candidate for use in the IAsys system that can present gangliosides in liposomes to reveal antibodies that might otherwise remain undetected, such as in the AIDP subtype of GBS.

A second interesting possibility for the lack of detecting anti-ganglioside antibodies in whole serum with the current IAsys protocol could be the presence of idiotypic antibodies in the patients' sera. Serum from GBS patients used in this study was taken at the peak of disease and anti-idiotypic antibodies that neutralize anti-ganglioside antibodies might have already been produced. The IAsys biosensor measures antibody-antigen interactions at physiological conditions, which is ideal for antibody dimer formation, while the ELISA system measures these interactions at 4 ^{o}C with gangliosides immobilized directly onto the ELISA plate. In addition, the isolated antibodies from patient sera were purified by means of Protein A, that is known to miss IgM, A and E, as well as some of the less abundant IgG isotypes. Protein A purification could therefore have failed to isolate the anti-idiotypic antibody, if the latter is one of these isotypes.

This will not be the first time that the idiotypic network is recognised for its role in concealing the presence of auto-antibodies. In healthy individuals auto-antibodies to the ribosomal P proteins (anti-P antibodies) are masked by presumably Ab2γ antibodies (Pan, Anderson *et al.,* 1998). The presence of these idiotypic antibodies in healthy individuals suggests that all individuals contain anti-P antibodies, but these antibodies are regulated by the idiotypic network. In about 42% of patients with systemic lupus erythematous (SLE) overt anti-P antibodies are detected serodiagnostically using immunosorbents and cognate antigens (Stafford, Anderson *et al*., 1995; Pan, Anderson *et al.,* 1998). It is believed that regulation of these auto-antibodies is lost in SLE patients resulting in liberated anti-P antibodies causing disease. Interestingly, these idiotypic antibodies were shown to inhibit anti-P antibody binding activity. This inhibition was increased 20 fold when purified anti-idiotypic antibodies were used. These anti-idiotypic antibodies were purified using an affinity column onto which $F(ab)_2$ fragments of antigen affinity purified anti-P antibodies were immobilized. In that study some anti-Id activity is presumed to be lost after purification due to chaotropic and acidic conditions, but can be retained using milder conditions. This could therefore explain why the purification of anti-ganglioside antibodies from humans in our study resulted in the successful detection of anti-ganglioside antibodies in the IAsys biosensor while sera analysis remained less successful.

In SLE the idiotypic network regulating auto-antibodies is disregulated resulting in elevated auto-antibodies that can be neutralized by idiotypic antibodies from healthy sera

(Pan, Anderson *et al*., 1998). This disease therefore closely resembles GBS which could also be caused by idiotypic disregulation. Anti-ganglioside antibodies are found in healthy individuals, at much less activity and therefore not pathogenic, and our study supported other studies stating that healthy sera contain anti-idiotypic antibodies (Mitzutamari, Kremer *et al*., 1998; Lopez, Irazoqui et al., 2000). Therefore, this idiotypic network might subsequently also be disregulated by for instance specific infections or perhaps host factors, causing an increase in autoantibody production in GBS.

Like GBS, SLE with anti-P antibodies might benefit from using IVIg that is comprised of immunoglobulins pooled from healthy sera that could inhibit auto-antibodies specifically. One might therefore extrapolate the concept of pooling IVIg for treatment of idiotype network derived autoimmune diseases to the benefit of other human diseases where idiotypic interactions have been shown to be involved. Relevant examples of these are the anti-factor VIII disease characterized by antibodies to factor VIII, vasculitis associated with anti-neutrophil cytoplasmic autoantibodies (ANCA), thrombocytopenic purpura with antibodies to thyroglobulin, myasthenia gravis with antibodies to acetyl choline receptor and Lambert-Eaton myesthemic syndrome (LEMS) characterized by antibodies to VGCC causing decreased acetylcholin release (Rossi and Kazatchkine 1989; Bayary, Dasgupta *et al.,* 2006). Intravenous immunoglobulins pooled from selected healthy individuals could also benefit diseases where IVIg was shown to inhibit auto-antibodies from binding for example anti-phospholipid antibodies in APS (anti-phosphospholipid syndrome) (Rossi and Kazatchkine 1989; Bayary, Dasgupta *et al.,* 2006). Not all diseases that benefit from IVIg treatment have been found to contain specific auto-antibodies, but this might be due to the auto-antigen that is not yet known, or the masking of autoantibodies in detection due to the effect of anti-idiotypic antibodies.

Anti-idiotypic antibodies have been isolated from healthy sera in an earlier study by using antigen affinity columns to obtain Ab1 (anti-ribosomal P autoantibodies), and using a second affinity column with $F(ab)_2$ of Ab1 immobilized to obtain Ab2 antibodies specific for the variable region of Ab1 (Pan, Anderson *et al.,* 1998). This approach can also be used to isolate anti-idiotypic antibodies to anti-ganglioside antibodies from healthy serum showing the highest displacement of bound auto-antibodies. Anti-idiotypic antibodies can subsequently be assayed for anti-Ab1 activity by using the displacement

protocol in this study, or by immobilizing $F(ab)_2$ fragments of the purified patient antibodies (using antigen affinity column) on an ELISA surface, contacting it with healthy human immunoglobulin fractions and using anti-human Fc to reveal Ab2 binding. The amino acids making up these anti-idiotypic antibodies can subsequently be sequenced, gene sequence obtained and recombinant whole antibodies designed and produced in cell cultures. Purified anti-idiotypic antibodies could therefore be used to treat GBS. As already mentioned and illustrated in this study different anti-ganglioside antibodies are found and would require that different anti-idiotypic antibodies be isolated for each auto-antibody. The current drawback of this approach is firstly the lack of sufficient human monoclonal antibodies of different specificities to gangliosides, and secondly the lack of sufficient isolated antibodies from GBS patients.

A second attractive approach is the use of phage display to select for anti-idiotypic antibodies from gene libraries of human immunoglobulin variable regions representative of healthy individuals by showing displacement activity for each of the relevant auto-antibodies, i.e. recognizing idiotopes that are similar between auto-antibodies from different patients. Instead of isolating anti-idiotypic antibodies using affinity columns, genetic material (RNA) from isolated B-cells has previously been used to create random combinations of cloned heavy and light chain immunoglobulin genes. This approach succeeded before in an attempt to produce idiotypic antibodies against anti-ribosomal P antibodies in SLE (Zhang and Reichlin 2005). In GBS the so-called single chain variable fraction (scFv) antibodies produced by the phage system can be screened for anti-idiotypic activity against various patient sera using the ELISA displacement method used in this current study. Monoclonal anti-idiotypic antibodies can be produced for all the different types of anti-ganglioside antibodies, or may even be pooled as a general improved treatment above normal IVIg for GBS patients. This study went some way to demonstrate the feasibility of this vision for the future management and treatment of GBS and other auto-immune diseases that manifest as disturbances of the antibody idiotype network.

SUMMARY

Guillain Barré Syndrome in humans is characterised by ascending paralysis. It is often associated with preceding infections two to four weeks prior to nadir and is fatal in five percent of cases. Antibodies specific to several nerve components are frequently associated with clinical symptoms in GBS. These antibodies were found to be specific to various gangliosides and ganglioside complexes. It was also found that antibody reactivity to gangliosides is affected by membrane components. The most prevalent (20-30%) immunoglobulin in GBS is anti-GM1 (20-30%), which also binds to the LPS of the PEN O:19 *Campylobacter jejuni* serotype. This is the most common infectious agent associated with GBS and emphasizes the importance of infection and anti-ganglioside antibodies in disease development.

Intravenous infusion of pooled immunoglobulin from healthy donors, also called intravenous immunoglobulin (IVIg), halves the severity of disease manifestation. The action mechanism of IVIg in curing GBS is not clear, but intravenous immunoglobulin was shown to neutralize anti-ganglioside binding activity and its pathogenic effects. It was further found that anti-idiotypic antibodies in IVIg inhibit anti-ganglioside antibody activity. Treatment with IVIg is not equally effective in all GBS cases, which might be due to the inability of IVIg to neutralize anti-ganglioside antibodies in all patients adequately. Therefore, the treatment of GBS with IVIg needs to be better understood in order to improve its use as a cure for GBS.

This study confirmed previous findings that the interaction of patient serum anti-GM1 antibodies and ganglioside auto-antigens is greatly impaired by components in healthy serum. Bound anti-GM1 antibodies could be displaced by (presumably) anti-idiotypic antibodies from healthy donor serum. This study found that the displacement potential between donor sera differs. Anti-GM1 antibody displacement was found to be dependent on the character of both anti-GM1 and anti-idiotypic antibody. This demonstrated the feasibility of improving the efficiency of treatment by IVIg by sourcing it from only those sera that test best for displacing auto-antibodies from their ganglioside antigens in ELISA. IVIg selection may therefore greatly benefit from the use of recombinant phage display antibodies to distinguish between the various types of GBS for treatment.

To develop a method to characterize anti-ganglioside antibodies sensitively, an evanescent field biosensor was employed in which gangliosides were presented in a liposome environment. This provided a more physiological way of antibody antigen recognition. The optimized method determined the ganglioside binding specificity of purified IgG from a GBS patient, and mouse monoclonal anti-GM1 and anti-GD1a antibodies accurately. The results compared well with those from ELISA. The results obtained with purified IgG were far better than that obtained with whole serum analysis. This could be due to non-specific binding or the presence of inhibiting anti-idiotypic antibodies in patient sera. The biosensor method for antibody detection in GBS may allow the detection of anti-idiotypic antibodies in patients in future, because it requires no prior labelling of antibodies. Anti-idiotypic interaction may be detected by displacement of Ab1 from antigen, or by capturing Ab2 on Ab1 immobilized on the biosensor surface.

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