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High throughput measurement of antibody and complement mediated immunity to meningococcal disease

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High throughput measurement of

antibody and complement mediated

immunity to meningococcal disease

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ORIGINAL THESIS

High throughput measurement of antibody and complement mediated immunity in meningococcal disease

PhD Thesis Charlotte Brookes

The accepted correlate of protection for meningococcal disease is serum bactericidal activity. The importance of bactericidal activity was established by Goldschneider *et al.* (1969) who demonstrated an inverse relationship between disease incidence and bactericidal titres of ≥1:4. The importance of antibody and complement mediated immune mechanisms is also emphasised by the increased susceptibility of complement deficient individuals to meningococcal disease.

However, the absence of bactericidal activity does not necessarily indicate susceptibility and protection has been demonstrated in the absence of bactericidal activity. This has indicated the importance of other protection mechanisms such as opsonophagocytosis.

In this study a high throughput antibody-mediated complement deposition assay has been developed to measure the deposition of both C3b/iC3b and C5b-9 onto *Neisseria meningitidis*. Antibody mediated C5b-9 deposition measured in this assay has been shown to correlate highly with bactericidal activity for several strains and C3b/iC3b deposition has been shown to correlate with opsonophagocytosis. This assay has also been shown to be reproducible. Measurement of antibody mediated C5b-9 and C3b/iC3b deposition will not be a replacement for opsonic killing assays or the accepted correlate SBA, but could be used as a tool to evaluate large panels of sera against multiple strains due to the very low serum volumes required.

A respiratory burst assay has also been developed to measure both the uptake of fluorescently-stained bacteria and the induction of a respiratory burst response. This assay was optimised with respect to the reagents used to measure the respiratory burst response, bacterial stain and the assay buffers.

Complement is an important reagent used in immunoassays to evaluate antibody mediated immunity to *N. meningitidis*. Due to the high levels of nasopharyngeal carriage in adults it is difficult to obtain a complement source without cross-reactive bactericidal activity, often resulting in the requirement for a source from a different individual for each strain. An IgG-depleted complement source has been developed for use in immunoassays. The method developed has been shown to be reproducible and effective at removing the IgG whilst retaining functional complement and will be a valuable tool for assessing immunity to *N. meningitidis*.

For Mum, Dad and my husband Stuart

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Contents

Abbreviations

ACDP	Advisory committee for dangerous pathogens
BB	Blocking buffer
BCECF-AM	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester
BSA	Bovine serum albumin
CC	Clonal complex
CDA	Complement deposition assay
CSF	Cerebral spinal fluid
CoV	Coefficient of variance
dCDA	Duplexed complement deposition assay
dRBA	Duplexed respiratory burst assay
DHR	Dihydrorodamine
DHE	Dihydroethidium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
EDDHA	Ethylenediaminedi (O-hydroxyphenylacetic) acid
EDTA	Ethylenediamine tetraacetic acid
FcγR	Fc gamma receptor
FFI	Flexible film isolator
fH	Factor H
fHbp	Factor H binding protein
FM 1-43	N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide
FI	Fluorescence index
FI- C'	Fluorescence index minus the complement only control
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
GNA	Genome neisserial antigen
HBSS	Hanks buffered saline solution
HL60	Promylocytic suspension cell line
H_2O_2	Hydrogen peroxide
hSBA	Serum bactericidal activity using human complement
IgG	Immunoglobulin class G
IgA	Immunoglobulin class A

IFN-γ	Interferon gamma
IL-4	Interleukin 4
IP	Intraperitoneal
LCCD	Late complement component deficiency
LOS	Lipooligosaccharide
mAb	Monoclonal antibody
MAC	Membrane attack complex
MLST	Multilocus sequence type
NHBA	Neisserial heparin binding antigen
NIBSC	National Institute of Biological Standards and Controls
OMP	Outer membrane protein
OMV	Outer membrane vesicle
OP	Opsonophagocytosis
OPA	Opsonophagocytosis assay
OPKA	Opsonophagocytosis killing assay
pAb	Polyclonal antibody
PBS	Phosphate buffer solution
PBT	Positive bactericidal threshold
PCR	Polymerase chain reaction
PI	Propidium Iodide
PMNs	Polymorphonuclear leukocytes
PMSF	Phenylmethylsulphonyl fluoride
RBA	Respiratory burst assay
ROS	Reactive oxygen species
RP	Relative potency
rSBA	Serum bactericidal activity using rabbit complement
SC	Subcutaneously
SBA	Serum bactericidal activity
SLA	Surface labeling assay
SNARF-AM	SNARF -4F 5-(and-6)-carboxylic acid, acetoxymethyl ester
ST	Sequence type
TLR	Toll like receptor
TCC	Terminal complement component
WBA	Whole blood assay
WT	Wild type
7-AAD	7 aminoactinomycin D

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Chapter 1

Meningococcal disease

1.1.1 *Neisseria meningitidis*

Neisseria meningitidis is a Gram negative encapsulated diplococcus of between 0.6–1.9 μm in diameter. Of the *Neisseria* genus, only *N. meningitidis* and *N. gonorrhoeae* are able to cause disease, and other *Neisseria* strains including *N. lactamica*, *N. flavascens*, *N. cinerea* and *N. subflava* are regarded as commensals. The human nasopharynx is the only known reservoir for *N. meningitidis*, with colonisation rarely resulting in disease. Thirteen serogroups of *N. meningitidis* have been described based on differences in capsular polysaccharide, and of these only sergroups A, B, C, W135, Y and X are responsible for the majority of invasive disease. Non-serogroupable strains are often isolated from the nasopharynx. It is thought that a lack of capsule may be preferable for carriage (Claus *et al.* 2002). Classification of *N. meningitidis* is based on capsule type (serogroup) and then subcapsular antigens PorB (serotype) and PorA (serosubtype) and lipooligosaccharide (immunotype).

Strain variation has been measured using panels of monoclonal antibodies (Frasch *et al.* 1985; Abdillahi and Poolman 1988), as well as sequencing of the genes encoding these antigens (Maiden *et al.* 1991). Multilocus enzyme electrophoresis (MLEE) has been used to characterise the electrophoretic mobility of cellular enzymes and allows meningococci to be characterised by an electromorph type (ET) (Selander *et al.* 1986). ET-5 and ET-37 were associated with hyperinvasive *N. meningitidis*, with ET-5 causing a large proportion of serogroup B meningococcal disease in the 1980s (Caugant *et al.* 1986). MLEE has disadvantages, including difficulty of comparing results between laboratories.

MLEE has therefore been replaced by multilocus sequence typing (MLST), a measurement of variation in DNA sequence in eight housekeeping genes, *aroE*, *pgm*, *adk*, *abcZ*, *pdhC*, *porA*, *fumC* and *gdh* (Maiden *et al.* 1998; Holmes *et al.* 1999; Birtles *et al.* 2005). This method has been shown to be reproducible, cost effective and high throughput. Based on MLST, meningococci are allocated a sequence type (ST) and a group of STs which have 4 or more of the eight loci in common are known as a clonal complex (cc). The most prevalent cc types in the UK are 41/44, 213, 269 and 32 and across Europe these same cc represent the majority disease causing isolates (Lucidarme *et al.* 2009). In fact the majority of disease-causing strains globally are caused by a few hypervirulent lineages, whereas nasopharyngeal carriage strains are much more diverse in ST type. This observation indicates these strains may be genetically predisposed to cause disease.

1.1.2 Carriage

Understanding meningococcal carriage is important in understanding the epidemiology and transmission of disease. Carriage of meningococci is generally described at 10% in a population, but the carriage rate varies highly between age groups. Duration of carriage has been shown to be up to 6 months, and could extend past this period, although studies have not yet assessed this (Glitza *et al.* 2008). Nasopharyngeal colonisation of *N. meningitidis* induces bactericidal antibodies effective against homologous and heterologous strains, and thus carriage is important in the development of natural immunity, but also maintenance of antibody levels (Goldschneider *et al.* 1969; Reller *et al.* 1973). The relationship between carriage and disease incidence is unclear, where some studies have demonstrated an increase in disease with an increase in carriage others have found the opposite (Olsen *et al.* 1991; Fernandez *et al.* 1999). Some of the

differences observed in these studies could be attributable to differences in the sampling site used, swab handling and also the selection of the population to be swabbed (i.e. were they randomly selected?). A recent meta-analysis of 89 carriage studies from European countries with predominantly serogroup B and C disease, predicted the carriage rate in infants to be 4.5%, 7.7% in 10year olds, peaking at 23.7% in 19 year olds, and decreasing in the older adult age groups to 7.8 % in those over 50 years old (Christensen *et al.* 2010).

Carriage of *N. lactamica* has been shown to be high in young infants whilst meningococcal carriage is relatively low, and as levels of *N. lactamica* carriage decline increasing meningococcal carriage is observed (Gold *et al.* 1978; Cartwright *et al.* 1987). Nasopharyngeal carriage with *N. lactamica* has been shown to restrict carriage of *N. meningitidis* and induce opsonophagocytic antibodies cross reactive with meningococci (Evans *et al.* 2011). This study did not observe the induction of cross reactive bactericidal antibodies, while a previous study demonstrated children carrying *N. lactamica* have higher titres of bactericidal antibodies which are cross reactive with meningococci, in comparison with non carriers (Gold *et al.* 1978).

Carriage of meningococci can be elevated in certain populations, including military recruits, and university students, resulting from living in confined areas. In addition, smoking has also been shown to increase the carriage rate (Cartwright 1995; Bakir *et al.* 2001). An increase in meningococcal carriage has also been described in children who were also colonised with *Streptococcus pneumoniae*, and *Haemophilus influenzae* (Bakir *et al.* 2001).

1.1.3 Pathogenesis

The human nasopharynx is the only known reservoir for *N. meningitidis*, and colonisation of the nasopharynx occurs via respiratory droplets and secretions. Colonisation is most frequently asymptomatic, and only rarely progresses to invasion of the mucosal surfaces and bloodstream resulting in sepsis and/or meningitis.

Following colonisation, *N. meningitidis* adheres to the mucosal surface initially through the expression of type IV pili which bind with human CD46 (Kallstrom *et al.* 1998). Attachment is followed by down-regulation of capsule (Deghmane *et al.* 2002) allowing for closer adhesion of the bacteria via interactions such as Opc and Opa with CEACAMs/CD66. Following adhesion, meningococci are able to invade epithelial and endothelial cells. It has been observed that some lipooligosaccharide (LOS) mutants are unable to invade efficiently, and it has been suggested that the toxicity of LOS may be important in invasion (Dunn *et al.* 1995). The exact mechanism of how meningococci cross the epithelial and endothelial layer is unknown, but it has been suggested that meningococci cross these cell layers by transcytosis, or an alternative hypothesis is that they are transported within a vacuole inside the cells. It is possible that meningococci invade the epithelial cells in order to evade an immune response. Evasion of host immunity is critical, with capsule and LOS important in resistance to complement mediated immunity (Geoffroy *et al.* 2003). Following colonisation IgA1 protease is produced by the bacteria which neutralises IgA1 by cleavage on the hinge region. *N. meningitidis* also expresses lactoferrin binding protein (Lbp) enabling the acquisition of iron from human lactoferrin in the mucosa (Gray-Owen *et al.* 1997).

Following penetration of the epithelial and endothelial layers some bacteria occasionally cross into the circulation, where bacteria can multiply rapidly causing bacteriaemia. During bacteriaemia meningococci shed outer membrane vesicles (OMVs) in the circulation, which has the effect of amplifying inflammation and immune activity and overwhelming the host immune system. Meningococci express LOS and capsular polysaccharide which are important in resistance to complement-mediated killing and are key virulence factors, and without which the bacteria are unable to survive within the circulation (Geoffroy *et al.* 2003). In addition, meningococci express transferrin binding proteins (TbpA and TbpB) which enable the bacteria to acquire iron from the human iron transport protein transferrin. *N. meningtidis* has evolved mechanisms to vary surface proteins in order to evade host immunity. Phase variation, a process of on/off gene expression is utilised and occurs by slipped strand mispairing; a process where a repetitive DNA sequence upstream of a gene or within an open reading frame, gains or losses a nucleotide, which leads to a change in the protein expression levels. Alternatively, DNA can be taken up from the environment via horizontal transfer, which can result in, for example, capsule switching. Antigenic variation can occur via intragenomic recombination and results in variation of the expressed surface proteins (Hill *et al.* 2010).

After invasion of the circulation, bacteria are able to cross the blood brain barrier (BBB). The BBB consists of the choroid plexus, consisting of cuboidal epithelial cells and capillary endothelia both with tight junctions. It is thought that adhesion occurs once again via pili, although the exact method used to cross the BBB is unknown. There are several possible theories: attached meningococci are transcytosed and are able to enter the meninges, signalling to endothelial cells results in disruption of the tight junctions and bacteria are then able to cross the

BBB (Carbonnelle *et al.* 2009) or through direct damage of the endothelia by LOS (Dunn *et al.* 1995). Once meningococci have crossed the BBB, this leads to the inflammation of the meninges (meningitis).

1.1.4 Clinical aspects of disease

Clinical presentation of disease ranges from asymptomatic carriage of meningococci to fulminant septicaemia and meningitis. Fulminant meningococcal septicaemia includes patients with sepsis, or a combination of septicaemia and meningitis. Some patients present with meningitis only and there are also milder forms of systemic infection which do not develop into persistent septicaemia or meningitis. Case fatality rates vary between different clinical presentations of disease. Individuals with fulminant septicaemia have a case fatality rate of between 15-30% which can reach 50% in developing countries, patients with septicaemia and meningitis have a case fatality rate of 10-25%, and patients presenting with meningitis have a fatality rate of <5%. The overall case fatality rate is 5-10%. In addition, there are also a number of more unusual presentations including for example pneumonia, conjunctivitis, myocarditis and septic arthritis (Cartwright and Ala'Aldeen 1997).

Meningococcal disease can develop rapidly and normally develops over 2-48 hours. Patients present with general flu-like symptoms initially, which makes early diagnosis very difficult to distinguish from other milder viral infections. Patients demonstrate fever, loss of appetite, headache and seizures (meningitis), and upto 50% of patients also have neck pain. Several types of haemorrhagic rash are associated with fulminant septicaemia, these include macropapular rashes which fade when pressure is applied, and petechial rash which will not fade when pressure is applied. The formation of haemorrhagic rashes results from ruptured

blood vessels, which occur following the inflammatory response and the release of high concentrations of LOS and OMVs in the circulation.

Diagnosis of meningococcal disease is difficult due to the common symptoms during the initial stages of disease. Benzylpenicillin is given to patients prior to referral by general practitioners in suspected cases of meningococcal disease. Children presenting with petechial rash and symptoms of meningococcal disease are immediately given intravenous ceftriaxone on arrival at hospital (NICE 2010).

Chemoprophylactic treatment is used in most European countries to remove nasopharyngeal carriage of *N. meningitidis*. Treatment with rifampicin or ciprofloxacin is recommended prior to hospital discharge for patients recovering from meningococcal disease in the UK. The carriage rate is only 3% in patients who have received benzylpenicillin to treat meningococcal disease prior to chemoprophylactic treatment, but this method has been evaluated and shown to reduce disease in close household contact by 89% (Purcell *et al.* 2004). The protection afforded by chemoprophylactic treatment is short lived and disease in contacts has been reported following chemoprophylactic treatment, so in addition to this it is also thought that vaccination of patient family and friends, may further reduce disease in patient contacts (Hoek *et al.* 2008).

10-20% of patients who recover from meningococcal disease suffer long term sequelae. This can range from headaches, skin scarring to limb amputation deafness and in some cases learning disabilities.

1.2 Epidemiology

1.2.1 Global epidemiology of meningococcal disease

Meningococcal disease occurs as sporadic case outbreaks and epidemics, with epidemiology varying with geographic location (figure 1.2.1.). Surveillance of meningococcal disease is important to investigate epidemiology. Various surveillance mechanisms are used. Laboratory culture confirmation from either cerebrospinal fluid (CSF) or blood can be used to identify the bacterial strain from a patient with invasive meningococcal disease. Culture confirmation is not always successful, and therefore other mechanisms have been developed. Polymerase chain reaction (PCR) is also used for diagnosis of meningococcal disease, however the disadvantage of this method is that an isolate is not then available for further characterisation. However genotypic characterisation can be carried out, and serogroup specific PCR can be used to determine the serogroup. Multilocus sequence typing (MLST) is also utilised to characterise disease-causing meningococcal isolates.
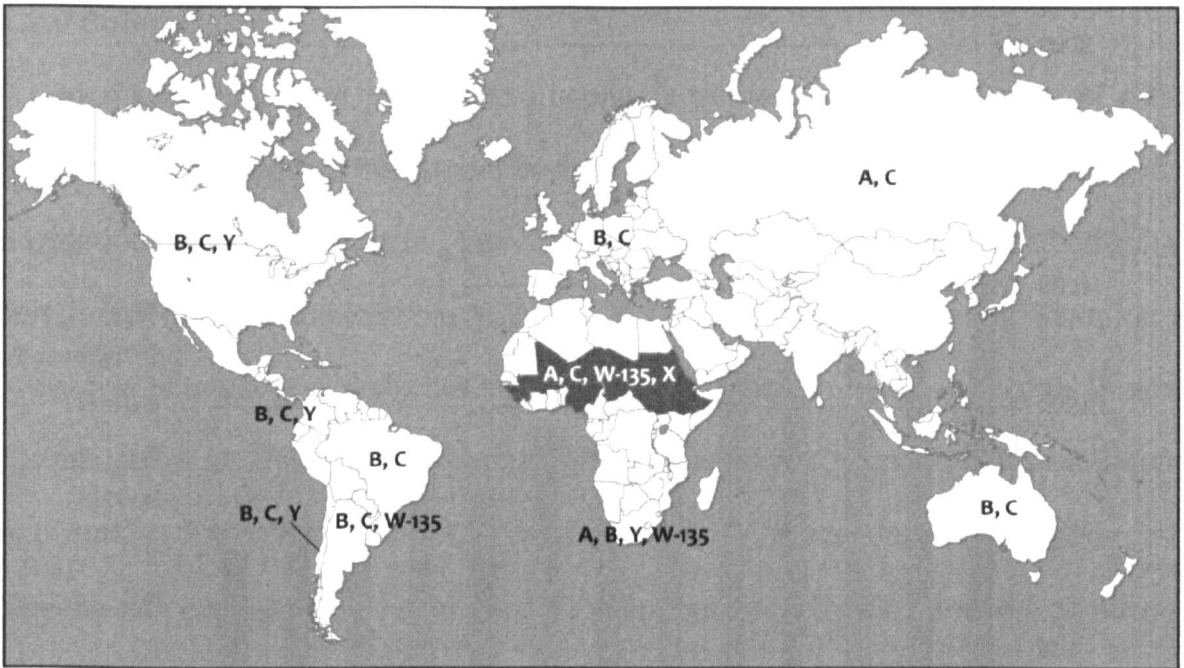
Figure 1.2.1 Global distribution of meningococcal disease by serogroup (Harrison *et al.* 2009).

1.2.2 England and Wales

In England and Wales in 2010 a total of 896 cases of meningococcal disease were reported, 88% of which were serogroup B (Gray *et al.* 2011). ST269 accounted for 31% of disease, and ST41/44, ST213 and ST32 also represented a large proportion of disease. Serogroup C disease in 1999 represented 35% of invasive meningococcal disease. This has reduced to 2% of disease in 2010 as a result of the successful introduction of the serogroup C conjugate vaccine in 1999 and the herd protection this has induced (www.HPA.org.uk 2011)(figure 1.2.2). Serogroup Y represents a small proportion of invasive meningococcal disease, but an increase in disease has recently been reported. 7% of disease was reported as serogroup Y in 2010, which is more than double that reported in 2004. This small but detectable trend has also been reported in many other European countries (Jacobsson *et al.* 2011; Parent du Chatelet and Taha 2011; Toropainen *et al.* 2011).
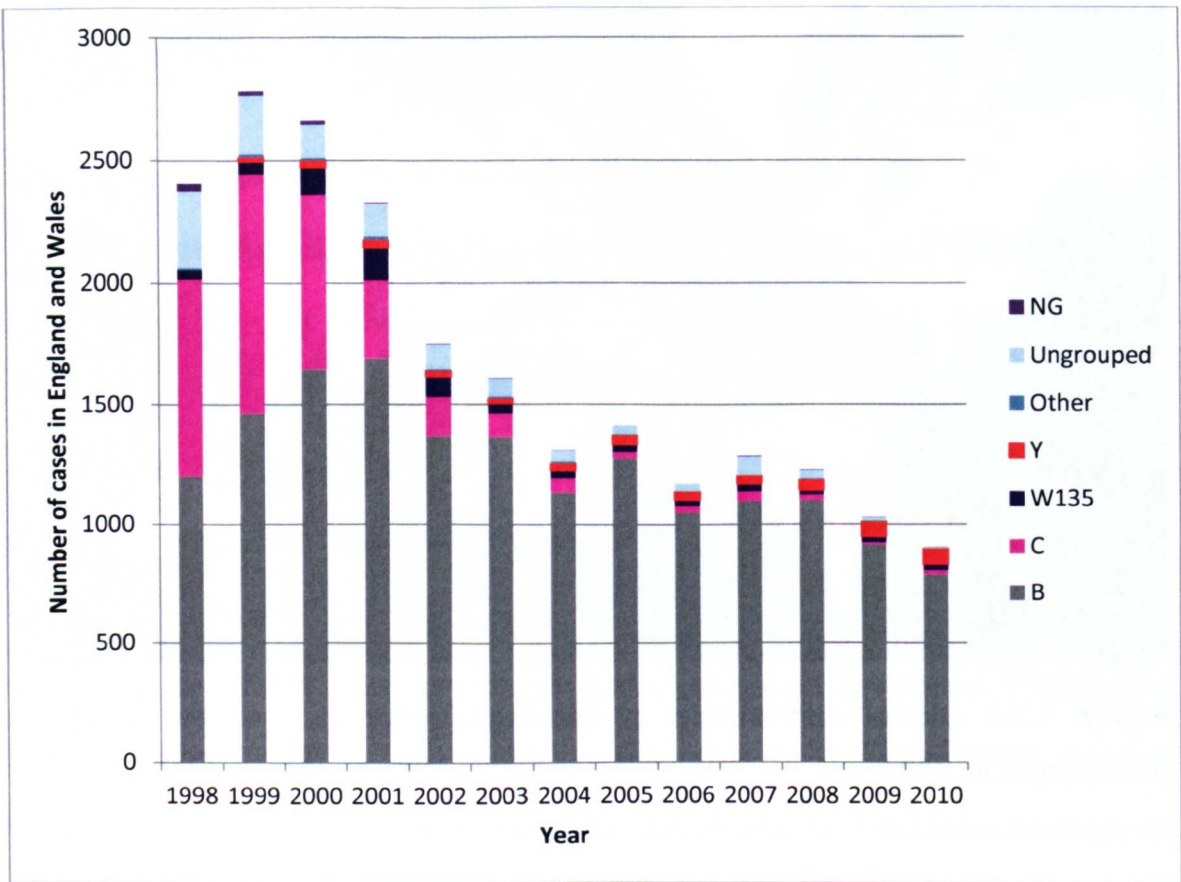
Figure 1.2.2 Meningococcal disease incidence in England and Wales 1998-2009 (www.HPA.org.uk 2011).

1.2.3 United States

Serogroups B, C and Y are responsible for the majority of meningococcal disease in the United States, with disease levels varying between 0.5 and 1.7 per 100000 population for the last 50 years. Serogroup Y represents 33% of meningococcal disease in the United States, and has increased gradually from very low levels reported in 1991. The tetravalent conjugate polysaccharide A,C,Y,W135 vaccine is recommended for adolescents, however the uptake of vaccine remains poor (Harrison *et al.* 2009).

1.2.4 Sub-Saharan Africa

A region stretching from Senegal to Ethiopia is termed the meningitis belt of sub-Saharan Africa, and has the largest burden of meningococcal disease in the world. Serogroup A meningococci has been the predominant epidemic-causing serogroup, however infections with W135, Y, C and X are also present. Serogroup X disease is unusual globally but has caused epidemics in Niger and North Ghana (Leimkugel et al. 2007) and serogroup W135 disease has caused disease in Niger and Burkina Faso (Fonkoua 2002; Taha et al. 2002). Serogroup A meningococcal disease can exceed 1000 cases per 100000 population. This is a much higher rate of disease than experienced elsewhere. Meningococcal epidemics tend to occur in cycles and in sub-Saharan Africa these coincide with the dry season (Harrison et al. 2009). It has been shown however that carriage rates, which range from 3 - 30%, are not variable by season (Trotter et al. 2007).

1.2.5 Age-related incidence

Invasive meningococcal disease is most prevalent in infants. Children under 6 months of age show some immunity as a result of maternal antibodies (Goldschneider et al. 1969), but as maternal antibodies wane incidence of disease increases peaking at 6 months of age at 75 incidences per 100,000 per year. Disease levels continue to fall until 8 years of age where low levels of disease are observed (<3 incidence per 100000). This low level is maintained until a further smaller peak of disease is observed in young adults 16-24 years old (www.HPA.org.uk 2011). This pattern of disease is altered during epidemics of disease, where increased numbers of older children present with invasive disease who have low levels of antibodies against the epidemic strain (Peltola et al. 1982).

1.2.6 Seasonal Occurrence

Meningococcal disease occurs in seasonal epidemics, in temperate climates the infection numbers are greatest in winter months. This is thought to result from close contact during the colder weather and also an increase in viral disease, which can cause nasopharyngeal mucosal damage, thus increasing the risk of contracting meningococcal disease (Cartwright 1995). In sub-Saharan Africa, epidemics mainly occur during the dry season between December and June. Dust storms, low humidity and upper respiratory tract infections may play a role in compromising the mucosal barrier (Moore 1992). In addition to this it is thought that overcrowded housing and pilgrimages also play a role in increasing the risk of nasopharyngeal carriage.

1.3 Immune defence against *N. meningitidis*

1.3.1 Complement

The complement system has three pathways: the classical pathway, the alternative pathway, and the lectin pathway (figure 1.3.1), and is composed of at least 35 proteins which are both fluid phase and membrane bound. Complement plays an important role in protective immunity to meningococcal disease. It is important in the inflammatory response, opsonisation, chemotaxis, serum bactericidal response and also plays an important role in the development of an antibody response and can augment the immunological memory (Walport 2001; Dunkelberger and Song 2010).

The classical pathway is initiated by the binding of C1q to the Fc portion of clusters of antibody (IgG or IgM) complexed with antigen. This activates C1r and C1s; the two other components of the C1 complex. This then enables C4 and C2 to be cleaved forming C4bC2a which is the classical pathway C3 convertase. This then cleaves C3, producing C3b and C3a. C3a initiates local inflammatory responses and C3b is able to coat the surface of the pathogen.

The lectin pathway has a starting point very similar in structure to the classical pathway. Mannose binding lectin (MBL) or ficolins bind mannose residues, other sugars and sialic acid, which activates MASP1 and MASP2; which are homologous to C1r and C1s. This then leads to the cleavage of C4 and C2 forming a C3 convertase as in the classical pathway.

The alternative pathway relies on low levels of spontaneous hydrolysis of C3, which directly binds to the surface of the bacteria. Factor B binds to C3b in the

presence of Factor D leading to the production of C3b and Bb. C3bBb is then stabilised by binding properdin, and then cleaves C3 to C3a and C3b by acting as the alternative pathway C3 convertase. There are also other factors involved in the alternative pathway which act as regulatory proteins, for example factor H and factor I. Factor H leads to the inactivation of the C3b component to iC3b, as a co-factor to factor I (Schneider *et al.* 2007). In addition to properdin's role in stabilising C3bBb, it has also been suggested that properdin can bind directly with the bacterial surface and provide a platform where the alternative pathway convertases can form and function (Spitzer *et al.* 2007).

The conversion of C3 to C3a and C3b is where the three complement pathways converge. As part of the classical or lectin pathway, C3b binds C4b2a (C4b2a3b) or as part of the alternative pathway C3b binds C3bBb (C3bBb3b), making a C5 convertase which cleaves C5 to C5a and C5b. This leads to the assembly of the membrane attack complex (MAC) with complement components C5b,C6,C7,C8 and C9. This forms a multiprotein pore-like structure which inserts into the lipid bilayers of the bacteria membrane resulting in membrane disruption and cell lysis. C3b can also act by binding directly to the surface of bacteria acting as an opsonin able to bind with complement receptors (CRs) present on the surface of phagocytes inducing phagocytosis, whereas the cleaved C3a acts as an anaphylatoxin by acting as a potent chemoattractant. Factor H acts by inactivating C3b into iC3b which can also opsonise bacteria. C3b and iC3b are able to bind with CR1, CR3, and CR4, which are present on the surface of phagocytes, and mediate uptake via phagocytosis (Ehlenberger and Nussenzweig 1977).

The complement system contains regulation factors to prevent inappropriate activation and subsequent host cell damage. Soluble receptors (C4BP, factor H and C1-inhibitor) are circulated in the bloodstream and at mucosal surfaces to prevent inappropriate complement activation by downregulating the production of C3 convertases. S protein (vitronectin) is an important fluid phase regulator of the MAC, preventing insertion into the membrane. SC5b-9 is usually associated with the fluid phase, but it is known that <0.4moles S protein per 1 mole of C5b-9 can be found in membrane bound C5b-9, although membrane bound SC5b-9 may not be lytic (Bhakdi *et al.* 1988). Host cell membranes also contain negative regulators to prevent damage, these include decay-accelerating factor, CR1, and CD59. CD59 inhibits the formation of C5b-9 on host cell surfaces.

Complement components also play an important role in the adaptive immune response. B cells are able to bind C3 and its degraded components, which results in a lowering of the activation threshold for bound antigen with B cells. Complement also plays a role in the selection and maintenance of B1 cells, and may also play a role in the differentiation of B cells into memory B cells and affinity maturation. The observation that CD4 and CD8 T cell priming is reduced in C3 deficient mouse model also indicates a role for complement in T cell immunity, although the mechanisms for this are still unknown (Kemper *et al.* 2008; Dunkelberger and Song 2010).
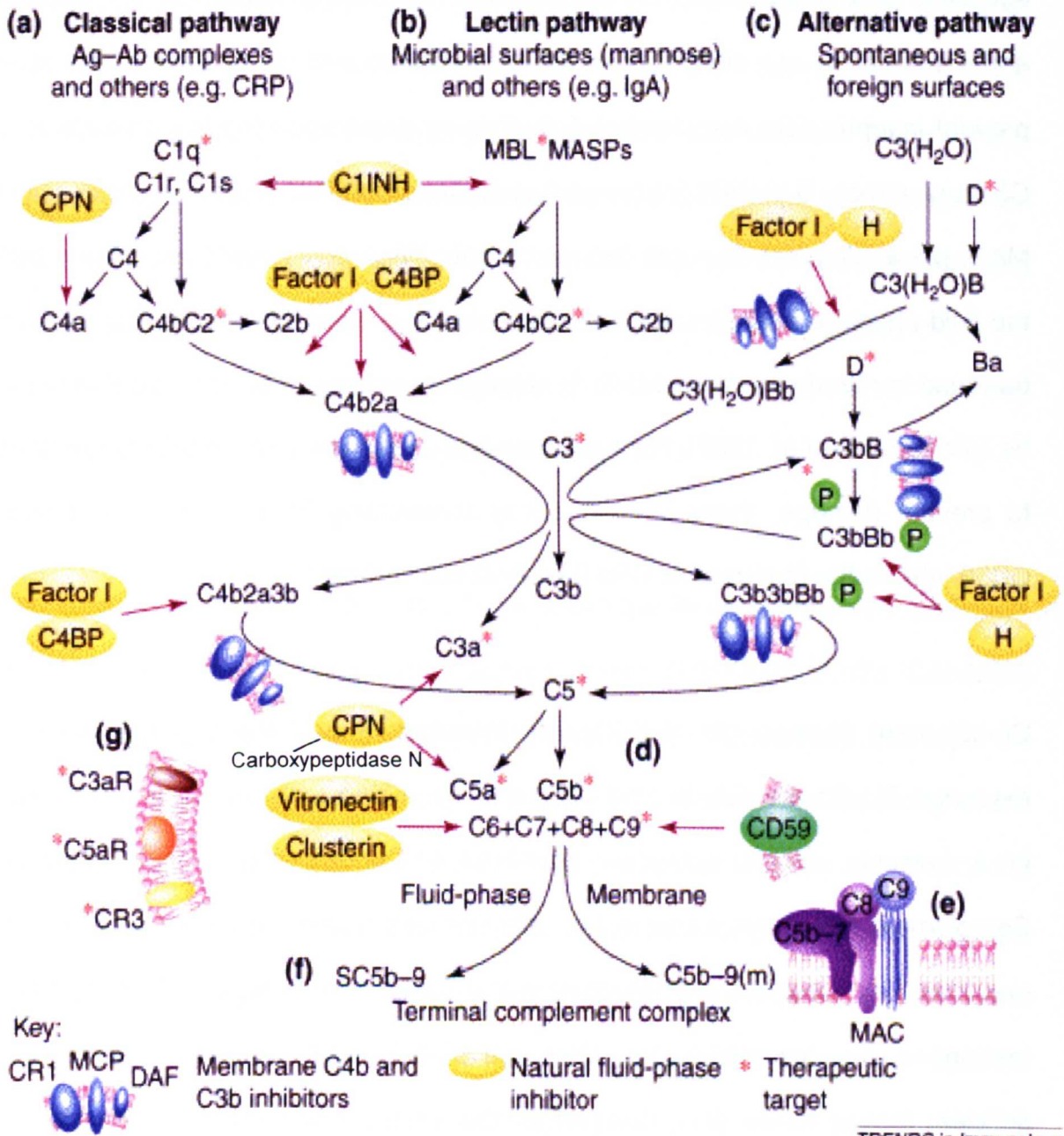
Figure 1.3.1 Complement cascade

Taken from Mollnes,T.E.*et al.* illustrating the classical, alternative and lectin complement pathways (Mollnes *et al.* 2002).

1.4 Complement in meningococcal disease

1.4.1 Serum bactericidal activity (SBA)

Goldschneider first described an inverse relationship between hSBA (SBA performed with human complement) titres of ≥1:4 and serogroup A, B and C disease by age. Whilst maternal antibodies persist disease incidence is low, but when these wane disease incidence increases, and then declines during childhood peaking again in late teenagers. In addition 14744 military recruits were sampled at the beginning of their training, and during the investigation period 54 cases of serogroup C meningococcal disease were recorded. Only 3 out of 54 recruits who developed disease had a detectable bactericidal titre, this was in contrast to 444 of 540 of healthy controls. This established an hSBA titre of ≥1:4 as an appropriate correlate of protection (Goldschneider *et al.* 1969).

This study led to SBA becoming a 'gold standard' for measuring vaccine-induced immunity and is now a requirement for licensure of meningococcal vaccines. This study (Goldschneider *et al.* 1969) was performed using human complement in the bactericidal assay. However, baby rabbit complement has frequently been used to evaluate the responses to meningococcal polysaccharide vaccines as it is easily standardisable. rSBA (SBA performed with baby rabbit complement) has been used to evaluate meningococcal serogroup C polysaccharide conjugate vaccines in immunogenicity and safety studies and vaccines were introduced in the UK in 1999, without a phase III efficacy trial (Miller *et al.* 2001).

There is strong evidence for a role of SBA in protective immunity to meningococci, but there is evidence that other mechanisms are also involved. Outer membrane vesicle (OMV) vaccines have shown a correlation with vaccine efficacy and % of

subjects with SBA titres ≥4, but the % of subjects with SBA titres ≥1:4 has also been observed to underestimate vaccine efficacy. This trend was observed following OMV vaccination in Chile, Brazil, Cuba and Norway (Granoff 2009).

Goldschneider first described an inverse relationship between hSBA titres of ≥ 4 and serogroup A, B and C disease by age. A more recent serogroup C seroprevalence study, performed with age-stratified sera collected from 1689 UK subjects prior to the introduction of the serogroup C conjugate vaccine, showed much lower levels of protection than observed in the Goldschneider study (Trotter et al. 2003). A further seroprevalence study, performed with 2415 age stratified sera, demonstrated a different relationship between serogroup B disease and bactericidal titre. Data showed that in the 2-12 year old age range there was a low disease rate whilst only low bactericidal antibody titres could be detected (Trotter et al. 2007). This may indicate the involvement of additional mechanisms in protection.

Whole blood from individuals with a SBA titre <4 has been shown to be able to kill meningococci (Ison et al. 1999; Welsch and Granoff 2007), and passive protection studies carried out in the infant rat model, have shown protection in the absence of bactericidal activity (Toropainen et al. 2001; Welsch et al. 2003; Welsch and Granoff 2004; Welsch and Granoff 2007), although caution must be used in interpreting data from the infant rat model due to the specificity of fHbp for human fH (Granoff 2009). Susceptibility of recurrent meningococcal infection is described for individuals with late complement component deficiencies (LCCD), who are unable to form MAC and induce SBA. However, it has been shown in this group of individuals that vaccination with a tetravalent meningococcal polysaccharide

vaccine is able to reduce the risk of developing meningococcal disease (Platonov *et al.* 2003).

1.4.2 Opsonophagocytosis (OPA)

As already described, there is evidence to suggest that there are additional immune mechanisms that provide protection against meningococcal disease. Opsonophagocytosis has been suggested as an alternate mechanism important in natural and vaccine-induced immunity and has been shown to be effective at killing meningococci (Ross *et al.* 1987; Schlesinger *et al.* 1994). Evidence supports a role for opsonophagocytosis (reviewed in (Granoff 2009)) and it has been shown that vaccination of LCCD patients with a tetravalent polysaccharide vaccine results in a reduction in recurrent infections; most probably due to the induction of opsonic antibodies (Andreoni *et al.* 1993; Platonov *et al.* 2003). In addition to this an increase in opsonic activity is also observed in convalescent patients (Halstensen *et al.* 1989) and also in response to OMV vaccination (Gorringe *et al.* 2009). Whole blood from subjects with no detectable bactericidal activity is able to kill meningococci (Welsch and Granoff 2007) and passive protection has been achieved in the infant rat model in the absence of SBA (Welsch and Granoff 2004; Toropainen *et al.* 2006).

An opsonic killing assay using polymorphonuclear leukocytes (PMNs) with a complement source which was either C6-depleted, and therefore unable to perform membrane attack complex (MAC) deposition, or native complement, has been used to investigate anti-PorA mAb and its ability to kill meningococci. No bactericidal activity was detected with the C6-depleted complement but with the addition of PMNs, efficient killing was observed. In addition, adult serum with no detectable bactericidal activity was able to induce killing in the presence of PMNs

(Plested *et al.* 2009), indicating an important role for opsonophagocytosis in immunity to meningococcal disease.

1.4.3 Evasion of the complement system

N. meningiditis has developed mechanisms for complement resistance. The most important of which is the presence of polysaccharide capsule and sialyated LOS. This was confirmed from a study aiming to isolate meningococcal genes involved in complement resistance. 4500 mutants were studied and their susceptibility to complement killing assessed and it was estimated that mutants were achieved in between 80-90% of *N. meningitidis* non-essential genes. Of these 4500 mutants 37 mutants were susceptible to complement killing. The investigation identified that all 37 mutants had disruption in 18 genes which had lost function for either capsule biosynthesis or expression of truncated LOS (Geoffroy *et al.* 2003).

In addition to capsule and sialyated LOS, meningococci also express surface exposed proteins factor H binding protein (fHbp) and PorA, which are able to bind with factor H and C4bp respectively (Schneider *et al.* 2007). In addition, NspA has also recently been shown to bind fH (Lewis *et al.* 2010). The functional relevance of C4bp binding has not yet been established. Binding of factor H has been suggested as a mechanism of complement resistance, as bound fH is able to interact with bound C3b, inactivating this to iC3b and thus limiting complement activation. It is also thought that binding of fH by meningococci during bacteriaemia may deplete the fH in the bloodstream, which may cause the host tissue to become more susceptible to complement attack, and thus contribute to the development of the haemorrhagic rash, which is common in fulminant meningococcal disease (Serruto *et al.* 2010).

1.4.4 Immunological activity of different antibody subclasses and isotypes

Antibody-driven immunity is very important for protection against meningococcal disease (Flexner 1913). This is confirmed by the observation that agammaglobulinaemic patients suffer recurrent life-threatening meningococcal infections (Hobbs *et al.* 1967). Carriage of *N. meningitidis* strains or commensal *Neisseria* species leads to the generation of natural immunity to meningococcal infection, which consists of IgG, IgM and IgA (Goldschneider *et al.* 1969).

IgA is present in both the mucosa and serum, as either IgA1 or IgA2. These two subclasses are found as either monomeric, dimeric, polymeric or a secretory form, which consists as dimeric IgA with a J chain. IgA has been shown to be capable of inducing phagocytosis albeit to a lesser degree than IgG, and to be unable to induce bactericidal activity (Vidarsson *et al.* 2001). In addition an FcαR has been identified and is expressed on macrophages and PMNs (Boross 2008). IgA is unable to bind C1q and does not therefore activate the classical complement cascade, but it has been shown that polymeric IgA is capable of activating the lectin complement pathway by binding MBL (Roos *et al.* 2001). IgM is efficient at complement activation due to its pentameric structure, but does not contribute to phagocytosis, as the FcμR is not located on the surface of PMNs and macrophages (Boross 2008).

IgG exists as four main subclasses: IgG1, IgG2, IgG3 and IgG4. Serum concentrations are IgG1 >IgG2 > IgG3 = IgG4, with IgG1 making up approximately 50% of IgG. Following vaccination with meningococcal capsular polysaccharide conjugated to CRM197, a strong IgG2 response has been observed with lower levels of IgG1 (Huo *et al.* 2005), whereas vaccination with OMVs induced a predominantly IgG1 and IgG3 response with low levels of IgG2 and IgG4. IgG

antibodies are able to activate the complement cascade, resulting in SBA and binding with FcγRs to induce OPA (Naess *et al.* 1999). A comparison of chimeric human IgG1,2,3,4 and IgA1, 2 with identical V genes against PorA showed that the ability of subclasses to bind complement was IgG1=IgG3>IgG2>IgG4 and IgG3>IgG1>IgG2>>IgG4 for phagocytic activity (Vidarsson *et al.* 2001).

1.5 Genetic polymorphisms and disease susceptibility

One third of the risk of contracting meningococcal disease is thought to be due to the host's genetic factors (Haralambous *et al.* 2003).

1.5.1 Disease susceptibility in complement deficient individuals

The importance of complement is emphasised by the susceptibility of complement deficient individuals to disease, resulting in approximately 10-20% of individuals who develop meningococcal disease (Ellison *et al.* 1983) and 31% of individuals who suffer recurrent meningococcal disease. Individuals with LCCD demonstrate recurrent invasive disease, which is usually milder than disease in complement sufficient patients possibly due to the reduction in release of bacterial products following MAC-induced lysis. Disease caused by serogroups W135 and Y and other rarer serogroups are more frequently seen in complement deficient individuals although serogroups B and C are still the most commonly isolated strains. Alternative pathway defects are also associated with recurrent meningococcal infection. Individuals with the alternative pathway deficiency demonstrate a higher mortality rate in comparison with complement sufficient patients. Properdin deficient individuals with meningococcal disease demonstrate a particularly poor prognosis possibly resulting from an inability to perform both opsonophagocytosis and serum bactericidal activity (Schneider *et al.* 2007). Individuals with polymorphisms which increase the amount of factor H, demonstrate a higher incidence of meningococcal infection. This is because binding of Factor H to Factor H binding protein (FHbp) enhances the survival of the bacteria (Haralambous *et al.* 2006; Kugelberg *et al.* 2008). MBL polymorphisms effect the amount of MBL in plasma and low levels of MBL have been associated with an increased susceptibility to meningococcal disease, polymorphisms in MBL gene are more common than LCCD and therefore

potentially responsible for a larger number of cases of meningococcal disease (Vermont and van den Dobbelsteen 2002).

1.5.2 Disease susceptibility and FcγR polymorphisms

Antibody is important in the initiation of complement-mediated killing of bacteria through either MAC deposition and lysis, or opsonophagocytosis. Polymorphisms in FcγRs have been associated with increased risk of developing meningococcal disease. IgG bind with three main receptors: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) present on the surface of the phagocyte, and polymorphisms in FcγRIIa and FcγRIIIb are thought to be important in meningococcal disease (Domingo *et al.* 2002).

FcγRIIa occurs in two allelic forms resulting from a single amino acid difference at position 131, FcγRIIa-R131 (arginine) and FcγRIIa-H131 (histidine). Homozygote's for FcγRIIa-R131 are poorer at binding IgG2, resulting in a reduced ability to induce phagocytosis. It has been found that 44% of children who survive meningococcal septicaemia are FcγRIIa-R131 homozygote, compared to the 23% occurrence of this FcγRIIa type in the general population (Bredius *et al.* 1994; Domingo *et al.* 2002). This suggests a role for FcγRIIa in the protective response in meningococcal disease.

FcγRIIIb also has two allelic forms NA1 and NA2. FcγRIIIb- NA1 binds IgG3 with higher affinity than NA2, and IgG1 and IgG3 bound to NA1 is more effective at inducing phagocytosis than FcγRIIIb - NA2 (Boross 2008).

1.6 Meningococcal vaccines

There is currently no effective vaccine against all meningococcal disease. However, vaccines have been developed to combat serogroups A, C, Y, W135 and strain-specific serogroup B OMV vaccines have been developed to combat clonal epidemic disease. The main focus of recent vaccine research is the development of a vaccine effective against all serogroup B disease.

1.6.1 Polysaccharide vaccines

Vaccines composed of high molecular weight capsular polysaccharide have been available since the 1970s where this type of vaccine was shown to be immunogenic in clinical trials conducted in military recruits, and was able to prevent disease in adults (Gotschlich *et al.* 1969). Polysaccharide vaccines have been widely utilised and have been shown to be very safe (Khatami and Pollard 2010). The immunogenicity and efficacy of these vaccines has been shown to be age-related, with poor immunity demonstrated in infants with older children producing more, higher avidity antibody. It has also been observed that immunity wanes quickly in all age groups but particularly in infants (Kayhty *et al.* 1980).

Polysaccharide vaccines have been shown to induce a T cell-independent immune response, and this is the reason why poor memory responses are observed with this type of vaccine. Capsular polysaccharide is able to directly stimulate a B cell response by cross linking B cell receptors, explaining the requirement for polysaccharide to be high molecular weight to be immunogenic (Kabat and Bezer 1958). Cross linking of B cell receptors results in the formation of B plasma antibody producing cells, which produce predominantly the IgM isotype. This is because there is no T cell help for inducing class switching, affinity maturation or production of B memory cells.

1.6.2 Conjugate vaccines

Conjugation of capsular polysaccharide to a carrier protein like CRM_{197} or tetanus toxoid enables the induction of a T cell-dependent response. The capsular polysaccharide and carrier protein are taken up by antigen presenting cells which present peptides on MHC II molecules and are recognised by CD4 T cells. T helper cells are then able to activate B cell proliferation and differentiation into memory B cells, and induce isotype switching and affinity maturation.

Conjugate vaccines are more immunogenic in infants than polysaccharide vaccines, and have been shown to induce herd protection by reducing nasopharyngeal carriage. In 1999 the UK became the first country to introduce a *N. meningitidis* serogroup C conjugate vaccine, with a three dose schedule at 2, 3 and 4 months, and was offered to everyone under 18 years in a phased catch up campaign. This resulted in a 67% reduction in carriage in adolescents 1 year after the introduction of the vaccine (Maiden and Stuart 2002), and this resulted in herd protection in the unvaccinated population (Ramsay *et al.* 2003; Maiden *et al.* 2008).

Tetravalent conjugate vaccines have been developed to target A, C, W135 and Y disease, and have been shown to be immunogenic in all age groups. There is no conjugate serogroup B polysaccharide vaccine due to similarities between serogroup B capsular polysaccharide and host sialylated neuronal cell adhesion molecules (Finne *et al.* 1987). Thus, there is a theoretical concern that vaccination with this polysaccharide would induce auto-antibodies. Attempts have been made to modify the polysaccharide to prevent the induction of autoantibodies. N-acetyl groups were been replaced with N-propionyl groups, which was able to induce

bactericidal responses in mice (Jennings *et al.* 1987) but was poorly immunogenic in humans (Bruge *et al.* 2004). Work has therefore focused on developing an effective serogroup B vaccine using noncapsular approaches including using outer membrane vesicles and/or recombinant proteins.

1.6.3 Outer membrane vesicle vaccines (OMV)

Detergent-extracted OMV vaccines are made by separating OMVs from bacteria and treating with detergents to remove LOS and thus reduce endotoxin activity. OMVs are released from the bacteria whilst they are multiplying, and contain immunogenic sub-capsular outer membrane proteins (OMPs), which when used as a vaccine have been shown to be safe and efficacious. OMV vaccines have been used against epidemic serogroup B strains in Cuba (Sierra *et al.* 1991), Norway (Bjune *et al.* 1991), New Zealand (Holst *et al.* 2005), and Brazil (de Moraes *et al.* 1992). OMV vaccines show poor cross-reactivity between strains. The main immune response has been shown to be directed against PorA, a highly immunodominant protein which demonstrates high levels of antigenic variability, and thus these vaccines are only efficacious for clonal epidemics caused by strains with a common PorA antigen. A broader immune response has been demonstrated in adults than in infants, who show a particularly strain specific response. A possible reason for this could be natural priming of the immune system following nasopharyngeal carriage resulting in a boost of these responses following vaccination with OMVs. This was demonstrated by an immunogenicity trial held in Chile where OMV vaccines manufactured to combat the epidemics in Cuba and Norway were used to vaccinate children (2-4 years) and infants (0-1 years) and adults (17-30 years). Neither vaccine was able to induce a significant increase in SBA (4 fold increase in titre) response against a heterologous strain (Chilean epidemic strain) in vaccinated infants, whereas 31-35% of children and

37-60% of adults demonstrated a four-fold rise in bactericidal titre against the heterologous Chilean epidemic strain. 90% of infants demonstrated a four-fold rise in bactericidal titre against the homologous strain (Tappero *et al.* 1999).

In order to broaden protection afforded by OMV vaccines, a vaccine consisting of OMVs from two recombinant strains which have each been modified to express 3 PorA serosubtypes, has been developed in the Netherlands. This hexavalent OMV vaccine (HexaMen), showed some PorA types to be less immunogenic than others and that specific priming with monovalent OMVs was required to improve this response (Luijkx *et al.* 2006). In order to broaden the protection afforded by the HexaMen vaccine further, a nonavalent OMV vaccine (NonaMen) has been produced. The Nonamen OMV vaccine has been used in a phase 1 clinical trial and has been shown to be safe, however there is currently no immunogenicity data available. The large diversity in PorA from serogroup B disease causing strains makes a vaccine which targets mainly PorA likely to confer only limited protection.

N. lactamica OMVs have also been used as a vaccine. *N. lactamica* is a commensal bacterium which is similar to *N. meningitidis* but lacks capsular polysaccharide, PorA (Perrin *et al.* 1999), and the ability to produce IgA protease (Mulks and Plaut 1978).

Carriage of *N. lactamica* in childhood has been associated with the development of immunity to meningococcal disease. Children carrying *N. lactamica* demonstrate an increase in cross-reactive bactericidal antibodies when compared with non-carriers (Gold *et al.* 1978). Carriage levels of *N. lactamica* have been shown to exceed *N. meningitidis* in the under 5 year old age group, whilst *N.*

meningitidis carriage becomes more frequent in later childhood (Cartwright *et al.* 1987). A more recent study investigated experimental nasopharyngeal colonisation following inoculation of adult volunteers with *N. lactamica* and the resulting protective immunity, and this demonstrated induction of cross-reactive OPA antibodies but not bactericidal antibodies to *N. meningitidis*. In addition, this study also found that *N. lactamica* carriage inhibited the acquisition of *N. meningitidis* carriage (Evans *et al.* 2011).

Vaccination with *N. lactamica* OMVs was protective against challenge with a panel of *N. meningitidis* strains in a mouse model of meningococcal septicaemia, supporting the conclusion that this could be a potential *N. meningitidis* serogroup B vaccine (Oliver *et al.* 2002). *N. lactamica* OMVs were used in a phase I clinical trial with male adult volunteers in the UK. Although cross-reactive protection was observed with OP, SBA levels were shown to be similar to those observed with a meningococcal OMV vaccine against heterologous strains (Gorringe *et al.* 2009). Therefore *N. lactamica* OMVs are unlikely to be used as a meningococcal serogroup B vaccine.

Detergent extraction of OMVs lowers the level of endotoxin content, but it has also been shown to damage or remove important immunogenic lipoproteins such as factor H binding protein (fHbp). Use of native OMVs (nOMVs) as a parenteral vaccine is impossible because of the very high levels of endotoxin, but an nOMV has been administered via the intranasal route (Drabick *et al.* 1999; Katial *et al.* 2002). Two studies have been carried out by the Walter Reed Army Institute (USA) into intranasal vaccination of an nOMV vaccine. The first study had 32 healthy adult volunteers intranasally vaccinated with an nOMV vaccine, this was shown to be safe and able to induce a fourfold rise in SBA titre in 75% of subjects.

The antibodies generated were shown to be specific for PorA and LOS (Drabick *et al.* 1999). The second study vaccinated 40 healthy adult volunteers with a range of dosages of the same nOMV vaccine. This study induced an increase in homologous meningococcal IgA and IgG ELISA titres, but only 43% of subjects showed a fourfold or greater rise in bactericidal titres.

The Walter Reed Army Institute has also developed a nOMV vaccine composed of three antigenically diverse serogroup B strains which have been genetic modified to disable *lpxL1* resulting in lower levels of endotoxin content, and *synX* gene preventing capsule formation and LOS sialyation. This vaccine strain has also had OpcA high expression stabilised, an additional PorA inserted and an additional fHbp from H44/76 to increase expression of fHbp. Initial studies have shown this vaccine to be capable of inducting cross reactive bactericidal antibody in mice (Zollinger *et al.* 2010) and also in humans in a phase I clinical trial (Keiser *et al.* 2011).

The use of recombinant proteins in vaccines has been heavily investigated with many proteins identified as potential vaccine antigens. These include Neisserial adhesion protein (NadA) (Comanducci *et al.* 2002), Neisserial surface protein A (NspA) (Halperin *et al.* 2007), Transferrin binding proteins (Tbp) (West *et al.* 2001), Opacity proteins (Opc) (Jolley *et al.* 2001), fHbp (Masignani *et al.* 2003; Fletcher *et al.* 2004), and FetA (Thompson *et al.* 2003). However, the potential of these vaccine antigens is limited by antigenic and phase variability and also varying expression levels. Therefore a vaccine made up of these recombinant antigens is likely to require the inclusion of more than 1 recombinant antigen.

Identification of potential vaccine antigens has been carried out using reverse vaccinology. This is a process whereby 600 potential surface-exposed or secreted proteins were identified by genome mining the MC58 genome. Out of these 600 potential surface exposed or secreted proteins 350 were expressed in *Escherichia coli*, purified and used to immunise mice. Serum was then assessed on its ability to induce a bactericidal response, and bind to the surface of the bacteria. 29 proteins were able to induce a bactericidal response in the SBA and were evaluated for sequence conservation across a panel of diverse strains. 5 vaccine antigens identified by this process have been included in the Bexsero vaccine along with NZ98/254 OMVs(Section 1.6.4)(Pizza *et al.* 2000; Bambini and Rappuoli 2009).

1.6.4 Factor H binding protein containing vaccines

Factor H binding protein (fHbp) is a surface-exposed lipoprotein able to bind complement alternative pathway factor H (fH), a protein able to down regulate the classical and alternative complement pathways by converting C3b into iC3b and thus enhance resistance to complement-mediated killing. fHbp has three variants termed variants 1,2, and 3 or subfamilies A (variants 2 and 3) and B (variant 1). This protein has been identified as an important vaccine antigen by reverse vaccinology (Bambini and Rappuoli 2009) and by assessment of protein fractions (Fletcher *et al.* 2004) and is included in the Novartis and Pfizer vaccines which are currently in clinical trials.

Bexsero is a multicomponent vaccine composed of: *N. meningitidis* NZ98/254 OMV, used in the mass vaccination in response to a meningococcal outbreak in New Zealand; NadA, important in attachment and invasion (Capecchi *et al.* 2005); NHBA (Neisserial heparin binding antigen) present as a fusion protein with

genome neisserial antigen (GNA) 1030. NHBA is possibly able to enhance resistance to complement mediated killing by binding heparin. The final recombinant vaccine antigen is also a fusion protein of fHbp with GNA 2091. This vaccine has been administered to adults (phase 1) (Toneatto *et al.* 2011) and infants and toddlers (phase 2 and 3) as both 3 and 4 dose schedules (Findlow *et al.* 2010; Snape *et al.* 2010). The vaccine has been shown to be safe and able to induce bactericidal antibodies against a panel of strains selected to be homologous with one of the vaccine antigens. Vaccine coverage has been assessed by using the meningococcal antigen typing system (MATS) assay (section 1.7.5), indicating 72.9% of 2007/2008 UK serogroup B disease would have been covered by this vaccine (Findlow 2011).

A bivalent fHbp vaccine composed of lipidated LP2086 from fHbp subgroup A and B, has also been developed. This vaccine has been administered to adults (phase 1) and adolescents 11-18 years (phase 2) and a phase 3 study is being planned in 2011. Results from phase 1 and 2 studies show that vaccination induced bactericidal antibodies and >80% of vacinees had an hSBA titre 1:4 or greater against a panel of diverse serogroup B strains. This vaccine is currently being targeted for administration to adolescents (Jansen *et al.* 2011). Potential coverage of this vaccine will be established using the MeASure assay (section 1.7.6) (McNeil *et al.* 2011).

1.7 Current assays for measurement of immunity to meningococcal disease

Measurement of immune responses responsible for protection and identification of correlates/surrogates of protection are a requirement for the development of effective vaccines. There are animal models which have been used to assess passive and active protection, and assays have been developed to assess both

functional and non-functional immune responses. For the leading vaccine candidates it has become important to assess the expression of the key antigens used to assess vaccine coverage and thus assays have been developed to measure antigen expression.

1.7.1 Serum bactericidal assay (SBA)

Bactericidal antibodies are important in host protection to meningococcal disease. Antibody bound to meningococci engage C1q via the Fc portion of antibody, activating the classical complement cascade, which is then enhanced by the positive amplification loop of the alternative pathway leading to deposition of C3b. C3b deposition leads to formation of C5 convertase and the cleavage of C5 and formation of the MAC complex. MAC inserts into the lipid bilayer forming a pore-like structure which leads to lysis. An SBA uses serial dilutions of sera, complement (human or rabbit) and bacteria, and a titre is assigned to the dilution of serum which results in ≥50% killing compared with a control.

The serum bactericidal assay is the accepted correlate of protection elicited by vaccination (Frasch *et al.* 2009), however this assay is labour intensive and uses significant volumes of sera. Additionally, work with live *N. meningitidis* requires containment due to the respiratory infection risk of this ACDP2 organism.

A source of complement is a key reagent used in the SBA. Obtaining a human complement source for use within the SBA is complicated by the levels of cross-reactive antibodies acquired following nasopharyngeal carriage of *N. meningitidis* and commensal *Neisseria* species. These antibodies can evoke meningococcal bacteriolysis in the absence of test antiserum and cause interference in the results of the assays. Large numbers of volunteers are screened to find serum or plasma

which contains little or no cross-reactive antibody to the strain of interest. Often this results in a different complement source from a different individual being used in assays against different strains, leading to poor inter-strain and inter-laboratory assay comparability. As a result, baby rabbit complement has been used frequently to evaluate meningococcal polysaccharide vaccine responses. rSBA (SBA performed with baby rabbit complement) has been used to evaluate meningococcal serogroup C polysaccharide conjugate vaccines in immunogenicity and safety studies and vaccines were introduced in the UK in 1999, without a phase III efficacy trial (Miller *et al.* 2001). Meningococci are more readily lysed by rabbit complement due to the specificity of meningococcal fHbp to human fH and therefore with rabbit complement this complement regulatory protein cannot convert C3b to iC3b and thus reduce lysis of the bacteria (Granoff *et al.* 2009).

1.7.2 Opsonophagocytosis assay (OPA)

Opsonophagocytosis (OP) is a correlate of protection of *S. pneumoniae* vaccines (Romero-Steiner *et al.* 2003) and evidence suggests that OP also plays an important role in protection against meningococcal disease (Ross *et al.* 1987; Balmer and Borrow 2004; Plested and Granoff 2008; Granoff 2009).

OP occurs following the binding of antibody to the surface of meningococci, and the Fc portion of this antibody is able to bind C1q and initiate the classical complement cascade which is enhanced by the positive amplification loop of the alternative pathway leading to deposition of C3b. C3b can then act as an opsonin, or C3b can be inactivated by factor H and factor I into iC3b which can also act as an opsonin. The Fc portion of antibody binds with Fc receptors and the deposited C3b/iC3b bind with complement receptors (CRs) 1 or 3 which are expressed on

the surface of the phagocyte. These receptors crosslink and induce rearrangement of actin, extension of pseudopodia and bacteria are then engulfed rapidly.

A variety of techniques have been developed to assess antibody-dependent opsonophagocytosis. Published OPAs measure the uptake of either a fluorescent bacterial target, OMV-coated fluorescent beads, the induction of a respiratory burst response or direct bacterial killing. A classic OPA measures the uptake and killing of bacteria via PMNs or HL60 cells, and the assay readout is a colony count of viable bacteria (Plested *et al.* 2009). There are several examples of OPA measuring the uptake of fluorescent OMV coated polystyrene beads (Lehmann *et al.* 1998; Bassoe *et al.* 2000) or labelled meningococci (Taylor 2010). Lehmann *et al.* 1999 measured the uptake of FITC-labelled ethanol-fixed bacteria. The FITC stain binds to protein and therefore could mask surface epitopes and thus alter the measurable opsonophagocytosis response. In addition, the bacteria were fixed using ethanol which has also been shown to expose otherwise hidden epitopes (Michaelsen *et al.* 2001). Other protocols have aimed to stain the bacteria internally with BCECF-AM, a lipophilic stain which is non-fluorescent and able to directly cross the bacterial membrane. Non-specific esterase activity is then able to cleave the AM group, converting the dye to a lipophobic fluorescent dye unable to leak out of the bacteria (Gorringe *et al.* 2009). In addition the bacteria were killed with sodium azide and PMSF a method used to preserve the outer membrane. OMV-coated fluorescent beads have also been used as a target in OPAs (Lehmann *et al.* 1998), with the advantage that they are easily standardisable, whereas variation occurs between cultures. However, OMV-coated beads have the disadvantage that they do not possess capsule and as a consequence may expose epitopes not usually available on the surface of meningococci.

Alternatively other assays have been developed to measure the respiratory burst response. These assays measure the conversion of dihydrorodamine 123 to fluorescent rodamine 123 (which measures hydrogen peroxide) or dihydroethidium to ethidium (which measures superoxide) following a respiratory burst response initiated by opsonophagocytosis (Aase *et al.* 1995; Lehmann *et al.* 1998). Lehmann *et al.* (1999) simultaneously measured both uptake of fluorescent OMV-coated beads and respiratory burst.

The majority of opsonic assays measure the uptake of fluorescently-labelled OMV-coated latex beads or meningococci, but do not measure the stimulation of a respiratory burst response; the bactericidal function which kills the phagocytosed bacteria. Similarly some assays measure respiratory burst response only and not opsonophagocytosed bacteria which may overestimate protection as it does not account for the respiratory burst response stimulated for example by N-formylmethionylleucylphenylalanine (FMLP) (Elbim *et al.* 1994), TNF-α (Menegazzi *et al.* 1994) and IFN-γ (Ellis and Beaman 2004). Phagocytosis is not always necessary for respiratory burst to occur (Phillips *et al.* 1990; Elbim *et al.* 1994).

Published OPA also vary not only in the incubation times, target and concentration of assay components, but also in the phagocytic effector cells used. PMNs are used in many assays, however this limits the throughput of the assay as this is reliant on human donation. They have however been used successfully in small scale investigations (Lehmann *et al.* 1999; Bassoe *et al.* 2000; Aase *et al.* 2003). Other assays use the HL60 cell line which can be differentiated into granulocytic-like phagocytic effector cell. This has the advantage that high thoughput screening can be performed, and HL60 cells have been used for the assessment of

meningococcal vaccines (Taylor 2010) and also for the assessment of pneumococcal vaccines (Martinez *et al.* 1999; Romero-Steiner *et al.* 2003).

OPAs also vary in the complement source used. Some assays have used baby rabbit complement (Taylor 2010), an easily standardisable source which has been used in the SBA to assess serogroup A,C,Y and W135 vaccine responses (Maslanka *et al.* 1997; Andrews *et al.* 2003; Keyserling *et al.* 2005; Kshirsagar *et al.* 2007). Rabbit complement can be purchased in bulk therefore reducing assay variation. Higher SBA responses are observed with rabbit complement in comparison with human complement, and therefore the use of rabbit complement can overestimate the protective response (Granoff 2009). It has been suggested that this is a result of fH binding with fHbp. fH is able to cleave C3 and turn C3b (a part of the C5 convertase) into iC3b a component able to contribute to opsonophagocytosis but not in the formation of C5b-9 and thus a lower bactericidal titres (1-2 log drop) are observed using human compared with rabbit complement.

1.7.3 Whole Blood Assay

The whole blood assay (WBA) has been used to assess the total bactericidal activity in the blood, which includes both SBA and OPA (Ison *et al.* 1995). WBA can also be used to investigate host:bacterial interactions as it is a model of meningococcal bacteraemia. The WBA has been used in the assessment of bactericidal activity in convalescent children where SBA and WBA were directly compared. WBA was shown to be sensitive and able to detect higher levels of bactericidal activity than the SBA, which is explained by the ability of this assay to measure the contribution of other protective mechanisms (i.e. OP) (Ison *et al.* 1999). The main limitations of this assay are its requirement for fresh whole blood,

which limits its uses for the assessment of clinical trial serum as it cannot be performed in a high throughput manner and can only be performed at the sampling site and repeat assays are not possible. Requirements for whole blood also could contribute to high assay variation. Published WBA methods use heparin or citrate as an anti-coagulant in the whole blood, both of which adversely affect complement function.

1.7.4 T cell assays

Cell-mediated immunity has been assessed using both T-cell proliferation assays and also measurement of cytokine production (Pollard *et al.* 1999). Immunity to *N. meningitidis* is humoral, however, T-cell help is important in developing immunological memory response. This includes inducing antibody class switching, affinity maturation, as well as inducing B cell proliferation. Proliferative responses have been carried out using incorporation of $[^3H]$ thymidine into cells. A scintillation counter can then be used to measure the radioactivity recovered from cells following stimulation and this enables an assessment of the amount proliferation that has occurred. Cytokine responses have been measured by a variety of methods, including ELISA, Luminex bead arrays and intracellular cytokine staining.

T-cell proliferation assays have been used to determine the reasons for poor responses seen in infants following OMV vaccination. T-cell proliferative responses and cytokine production has been compared between convalescing infants and older children. Higher proliferative responses were observed in children >10 years in comparison to younger children, and infants produced a T_H1 profile, whilst older children produced a T_H2 profile (Pollard *et al.* 1999). Following intranasal vaccination with an OMV vaccine, T cell proliferative responses have

also been shown to correlate highly with IgG and IgA titres measured in an OMV ELISA (Oftung *et al.* 1999).

1.7.5 Meningococcal Antigen Typing System (MATS) assay

The development of meningococcal vaccines composed of proteins, which vary in sequence and expression level in different meningococcal isolates, requires a mechanism of establishing vaccine coverage. The MATS assay is a non-functional sandwich ELISA developed to inform on the potential vaccine coverage if the Bexsero novel vaccine is introduced. Bacteria are grown and detergent-treated to extract capsule and expose antigens, a capture antibody specific for the antigen of interest is coated onto a 96 well ELISA plate and then the detergent-treated bacterial extract is added, this is followed by the addition of a biotinylated detection antibody and incubation with streptavidin-HRP. The level of antigen in each isolate is measured against a reference standard and assigned a relative potency (RP). The RP is related to the positive bactericidal threshold (PBT) established where 80% of strains show an hSBA titre with pooled infant vaccinee sera. The vaccine coverage is then derived from the proportion of strains that score a RP above the PBT for at least one of the vaccine antigens (Donnelly *et al.* 2010). It is unlikely to be able to predict the contribution of NadA to bactericidal killing as it has been observed that this antigen is down-regulated in culture conditions (Biolchi *et al.* 2011).

1.7.6 MeASure assay

An alternative assay has been developed to assess the expression levels of fHbp for Pfizer's bivalent factor H binding protein vaccine. Studies have demonstrated this vaccine is able to induce bactericidal antibodies against Men B strains, and variation in surface expression levels of fHbp influences the ability of anti-fHbp

antibodies to induce bactericidal activity. Thus, a meningococcal antigen surface expression (MeASure) assay has been developed to characterise surface expression of all fHbp variants on the surface of intact bacteria. Bacteria are incubated with a cross reactive mAb, followed by incubation with a fluorescent secondary detection antibody before being analysed by flow cytometry. This method was shown to be reproducible and robust. This assay will be utilised to measure the expression levels of fHbp on the surface of circulating strains, and to use this data to predict which serogroup B strains will be susceptible to vaccine-mediated bactericidal activity (McNeil *et al.* 2011).

1.7.7 ELISA

Enzyme-linked immunosorbant assay is a commonly used non-functional assay, which measures the antibody binding to either whole meningococci, OMVs or purified proteins of interest. In addition this assay can be used to look at the levels of different antibody subclasses and isotypes to deduce the contribution of these in the immune response to meningococcal vaccines. ELISA has shown good correlation with the functional SBA and OPA responses with homologous vaccine strains (Aase *et al.* 1995), but also shown to correlate poorly with SBA against a strain heterologous to the vaccine strain (Milagres *et al.* 1994). Evidence has shown ELISA to be a poor measure of protection in an efficacy study in 1-21year olds in Chile following vaccination with a meningococcal group B (15:P1.3) OMV vaccine (Boslego *et al.* 1995), and in addition a study investigating vaccine immunogenicity following vaccination of 15- 20 year olds in Iceland comparing two OMV vaccines showed good correlation between the SBA and ELISA results (Perkins *et al.* 1998). The inconsistency seen could be a result of the measurement of total IgG by ELISA, which does not take into account the difference in SBA and OPA activity of different IgG subclasses. Comparisons of

IgG subclasses measured by ELISA with functional assay responses showed a strong positive correlation between IgG1 and SBA and OPA (Naess *et al.* 1999).

Measurement of antibody avidity using ELISA could also be an important factor in the antibody-induced protection for meningococcal disease. Avidity ELISA uses sodium thiocyanate to discriminate weakly bound antibody from high affinity antibody. An avidity index can then be used to compare sera (Vermont *et al.* 2002). In a study comparing avidity ELISA, total IgG ELISA and IgG1 and IgG3 ELISA with SBA, good correlations were shown between SBA and all ELISA assays tested. Therefore no improvement in correlation was observed when using an avidity ELISA over measuring total IgG, IgG1 or IgG3 ELISA (Vermont *et al.* 2002).

1.7.8 Animal models

Animal models are the only way to measure the immune response to a pathogen in the context of all parts of the immune system and tissues. *N. meningitidis* specifically binds human transferrin and lactoferrin in order to acquire iron, and interactions of bacterial pili is specific for human CD46 as is the interaction of Opa and Opc with CEACAM1; both of which are required for bacterial attachment and invasion of the bacteria. In addition to this, *N. meningitidis* binds only human fH (Granoff 2009), and therefore developing an animal model for this disease is complex. In addition, the pathogenesis and disease progression from mucosal colonisation to septicaemia and/or meningitis also makes the development of an animal model complex.

There are several models described, including the mouse intraperitoneal challenge model. This model does not follow the natural pathogenesis of meningococci,

however septicaemia can be achieved following infection. High challenge doses are required and this needs to be administered with an exogenous iron source (Holbein 1981). This model does allow the investigation of protection by both active and passive immunisation and bacteria can be recovered from the blood (Gorringe et al. 2001).

Use of an exogenous iron source with the mouse infection model can cause hyperferraemia, which can result in the activation of the complement system, a reduction in opsonophagocytosis and also affects the T lymphocyte subsets. Specifically this effects the receptors expressed on the lymphocyte surface and the expansion of T cell subsets (Walker and Walker 2000). In order to avoid this, a transgenic mouse model has been developed to express human transferrin, and bacteraemia has been demonstrated in this model which was sustained for at least 48 hours, when compared with a wild-type mouse strain which cleared infection quickly (Zarantonelli et al. 2007).

Intranasal mouse models have been difficult to develop as this route of infection resulted in colonisation followed by pneumonia and then bacteraemia. This is in contrast to the course of human disease. A transgenic humanised mouse model has been developed to express CD46, which has resulted in an intranasal challenge model (following antibiotic treatment) without the requirement of an exogenous iron source. Bacteraemia resulted and bacteria were able to cross the blood brain barrier in this transgenic strain (Johansson et al. 2005).

Vaccines based on fHbp have been developed, and the absence of human fH from the mouse and rat models may indicate that these models overestimate protection because fHbp only binds human fH (Granoff 2009). A human fH

transgenic mouse model has been developed. Immunisation of this transgenic model with fHbp demonstrated lower bactericidal titres than that observed in the wild type mouse model, attributed to the binding of fH with fHbp. However, no differences were observed between transgenic and wildtype mice following immunisation with a serogroup C polysaccharide vaccination (Beernink *et al.* 2011).

An infant rat model of infection is also used to assess passive protection provided by mammalian serum to i.p infection with meningococci. This model does not require an exogenous iron source but cannot be used to assess active immunisation due to the short duration of meningococcal infection (Salit 1981). In addition, this model also requires a high challenge dose (Toropainen *et al.* 1999). This model has shown that sera with a bactericidal titre are able to protect from development of bacteraemia and in addition to this sera without a measurable bactericidal titre were also able to confer protection (Welsch and Granoff 2004).

1.8 Project aims

This project aimed to develop a high throughput, reproducible antibody-mediated C3b/iC3b and C5b-9 deposition assay. This assay would then need to be correlated with the functional SBA and OPA to determine whether it can be utilised to predict the functional response.

This project also aimed to develop a high throughput duplexed OPA, able to measure the uptake of fluorescent bacteria and the induction of an respiratory burst response. This would then be correlated with an opsonic killing assay to assess whether measuring both parameters would correlate with killing to a greater degree than measuring either parameter on its own.

Complement is an important component of most of the assays used to assess vaccine-induced immunity to meningococci. The final aim of this project was to develop an antibody-depleted human complement source that could be used with any strain of *N. meningitidis* to improve assay to assay comparability.

1.9 Hypothesis

Assays used to evaluate meningococcal vaccine sera, such as the SBA, are laborious and require large volumes of sera and therefore there is the requirement to develop assays which are high throughput and use low volumes of sera to allow evaluation of antibody-mediated responses against large strain panels. A high throughput, flow cytometric assay measuring antibody-mediated C3b/iC3b and C5b-9 will be developed and the hypothesis is that the values obtained from this assay will correlate with results obtained in SBA and OPA assays. If this hypothesis is correct the antibody-mediated complement binding assay will be useful in identifying sera able to induce either bactericidal or opsonic activity. A secondary hypothesis is that a threshold level of C5b-9 deposition will need to be achieved for bactericidal activity to occur.

OP has been suggested as an additional immune mechanism important in immunity to *N. meningitdis*. Current OPA assess either uptake of bacteria or induction of a respiratory burst response. The hypothesis to be addressed in this study is whether measurement of both uptake and induction of a respiratory burst response will correlate with OPKA to a greater level than just measuring bacterial uptake or respiratory burst alone.

Complement is an important reagent used in the assessment of meningococcal vaccine-induced immunity and identifying a suitable human complement source can be complicated by the high levels of pre-existing immunity. A third hypothesis to be tested in this study was whether antibody could be removed from a complement source whilst retaining complement function.

Chapter 2

Chapter 2: Materials and Methods

2.1 Laboratory reagents

2.1.1 List of Chemicals

Table 2.1.1.1 List of chemicals

Chemical	Company of purchase
Alternative pathway radial immunodiffusion assay kit	Binding site
Aluminum hydroxide	Brenntag Biosector
Ammonium Chloride	Sigma Aldrich
Bovine Serum Albumin (BSA)	Sigma Aldrich
C1q	Quidel
C7depleted complement	Quidel
Calcium Chloride	Sigma Aldrich
Cleanse fluid	Beckman Coulter
Crystal violet	Sigma Aldrich
Deoxycholic acid	Sigma Aldrich
Dimethylformamide (DMF)	Sigma Aldrich
Dimethyl sulfoxide (DMSO)	Sigma Aldrich
EDTA	Sigma Aldrich
Ethylenediamine Tetraacetic Acid (EDTA)	Sigma Aldrich
Ethylenediaminedi (O-Hydroxyphenylacetic) Acid (EDDHA)	Sigma Aldrich
Flow check Fluorospheres	Beckman Coulter
Fetal calf serum (FCS)	Biosera
Glucose	Sigma Aldrich
Glycine	Sigma Aldrich
Goat anti-human IgG - FITC	Jackson Immunoresearch
Goat anti-mouse IgG - FITC	Jackson Immunoresearch
Goat anti-rabbit IgG - FITC	Jackson Immunoresearch
Hanks buffered saline solution (HBSS)	Gibco
Heparin	Becton Dickinson
Horse blood agar	Biomerieux
Hydrochloric acid	Sigma Aldrich
Kanamycin	Sigma Aldrich
L-glutamic acid	Sigma Aldrich
L-glutamine	Sigma Aldrich
Magnesium sulphate	Sigma Aldrich
Marvel	Premier International Foods, UK
Mouse anti human C3b/iC3b- FITC	Abcam
Mouse anti human SC5b-9	Dako
Mouse anti human SC5b-9	Quidel
N,N-Dimethylformamide	Sigma Aldrich
Phenylmethylsulphonyl fluoride (PMSF)	Sigma Aldrich
Phosphate buffered saline (PBS)	Sigma Aldrich
Potassium chloride	Sigma Aldrich
Potassium dihydrogen phosphate	Sigma Aldrich

Protein G sepharose	Amersham Bioscience
Total haemolytic radial immunodiffusion assay kit	Binding site
Lepirudin (Refludan)	Movianto
RPMI 1640	Invitrogen
Sheath fluid	Beckman Coulter
Sodium azide	Sigma Aldrich
Sodium chloride	Sigma Aldrich
sodium hydrogen carbonate	Sigma Aldrich
Sodium hydrogen phosphate	Sigma Aldrich
Sodium hydroxide	Sigma Aldrich
Sodium phosphate	Sigma Aldrich
Sucrose	Sigma Aldrich
Tris HCL	Sigma Aldrich
Trypan blue	Sigma Aldrich
Yeast extract	Sigma Aldrich
Zenon Mouse IgG labelling kit	Invitrogen

Table 2.1.1.2 List of Suppliers Address

Company	Address	Web address
Beckman Coulter	Beckman Coulter Ltd, High Wycombe, UK	www.beckmancoulter.con
Becton Dickinson	BD Ltd., Oxford, UK England	www.bd.com
Biomerieux	bioMérieux Ltd, Basingstoke, UK	www.biomerieux.com
Biosera	Biosera Ltd, East Sussex, UK	www.biosera.com
Gibco	Invitrogen Ltd. Paisley, UK	www.invitrogen.com
Invitrogen	Invitrogen Ltd. Paisley, UK	www.invitrogen.com
Movianto	Movianto Ltd, Bedford, UK	www.movianto.com
Premier International Foods, UK	Premier International Foods Ltd, UK	www.premierfoods.co.uk
Sigma Aldrich	Sigma-Aldrich Company Ltd., Poole, UK	www.sigma-aldrich.com
Brenntag Biosector	Brenntag Biosector Ltd, Denmark	www.brenntag-biosector.
Quidel	Quidel Ltd, Dorking, UK	www.Quidel.co.uk
Amersham Biosciences	Amersham Biosciences UK Ltd., Bucks., UK	www.amershambioscien
Jackson Immunoresearch	Jackson Immunoresearch Laboratories Inc. West Grove, PA, USA	http://www.jacksonimmui
Abcam		
Dako	Dako Ltd, Ely, UK	www.dako.com
Hycult	Hycult Biotech, Uden	www.hycultbiotech.com

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2.1.2 Laboratory stock solutions

Blocking buffer (BB) used for SLA/CDA

2% (w/v) bovine serum albumin (BSA) dissolved in phosphate buffered saline (PBS). This was then filter-sterilised and stored at 4^0C until use.

Dulbeccos phosphate buffered saline (DPBS)

0.14 M sodium chloride, 9 mM sodium phosphate, 3 mM potassium chloride and 1.4 mM potassium dihydrogen phosphate into 1 litre of distilled water. This was then sterilised and stored at 4^0C until use.

DPBS-EDTA

17 mM EDTA added to 500 ml DPBS. This was then sterilised and stored at 4^0C until use.

DPBS-GA

1% (w/v) bovine serum albumin and 0.1% (w/v) glucose was added to 500 ml DPBS. This was then filter-sterilised and stored at 4^0C until use.

Ethylenediamine-Di (o-Hydroxy-Phenylacetic acid) (EDDHA) Solution

To make 1.5 mg/ml stock, 60 mg EDDHA was dissolved in 27.2 ml of 0.36 M NaCl. The pH was adjusted to 7 using 6 M hydrochloric acid and then made up to 40 ml with dH$_2$O. This was then filter-sterilised and used immediately.

Frantz medium

0.1 M sodium chloride (NaCl), 0.04 M sodium hydrogen phosphate ($Na_2HPO_4.7H_2O$), 0.02 M ammonium chloride (NH_4Cl), 1 mM potassium chloride (KCl), 10 mM magnesium sulphate ($MgSO_4.7H_2O$), 0.03 M glucose, 0.01 M L-glutamic acid, 0.2% (w/v) dialysed yeast extract and 0.13 mM L-cystein.HCL.H_2O were prepared in 1 litre H_2O. The medium was pH adjusted to 7.4 with 5 M sodium hydroxide (NaOH). This was then filter-sterilised and stored at 4^0C until use.

Lysis buffer

0.15 M NH_4Cl, 9.5 mM sodium hydrogen carbonate ($NaHCO_3$) and 3 mM EDTA then filter-sterilised and stored at 4^0C until use.

OMV preparation buffer 1

0.1 M Tris-HCL pH adjusted to 8.6, 10 mM EDTA, 0.5% (w/v) deoxycholic acid. This was then filter-sterilised and stored at 4^0C until use.

OMV preparation buffer 2

50 mM Tris-HCL pH adjusted to 8.6, 2 mM EDTA, 1.2% (w/v) deoxycholic acid, 20% (w/v) sucrose. This was then filter-sterilised and stored at 4^0C until use.

OMV buffer 3

0.2 M glycine buffer pH adjusted to 8, and 3% (w/v) sucrose. This was then filter-sterilised and stored at 4^0C until use.

RBA/ dRBA/ OPA assay buffer

1.2 mM $CaCl_2$ and 1 mM $MgSO_4$ were added to 500 ml Hanks' balanced salt solution (HBSS without calcium or magnesium), filter-sterilised and stored at room temperature. Immediately before use, 2% (w/v) Marvel skimmed milk powder was added.

RBA assay buffer with azide

1.2 mM $CaCl_2$ and 1 mM $MgSO_4$ added to 500 ml Hanks' balanced salt solution (HBSS without calcium or magnesium), filter-sterilised and stored at room temperature. Immediately before use add 4% (w/v) Marvel skimmed milk powder and 0.01% (w/v) sodium azide to the required volume of solution.

Phenylmethanesulfonylfluoride (PMSF)

9.8 mM PMSF was prepared in 10 ml of isopropyl alcohol. This was then filter-sterilised and stored at 4^0C until use.

Sodium azide

A 20% (w/v) solution of sodium azide was prepared and was filter-sterilised and stored at 4^0C until use.

Tissue culture medium

2 mM L-glutamine, 20% (v/v) heat inactivated foetal calf serum was added to 395 ml RPMI-1640 medium and stored until use at 4^0C.

ELISA wash buffer

50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0 was filter-sterilised and stored at 4^0C until used.

ELISA Coating buffer

0.05 M carbonate-bicarbonate, pH 9.6. This was made up immediately before use in the assay and filter-sterilised.

ELISA blocking buffer

50 mM Tris, 0.14 M NaCl, 1% (w/v) BSA, pH 8.0 was filter-sterilised and stored at 4^0C until used.

ELISA stop solution

0.18 M H_2SO_4.

2.2 Neisseria meningitidis

2.2.1 Bacterial strains used

All strains provided by HPA Meningococcal Reference Laboratory

Strain	Serogroup	Serotype	Serosubtype	Sequence Type	Clonal Cluster
H44/76-SL	B	15	7,16	32	32
M01-240101	B	NT	19-1,15-11	1049	269
M01-240013	B	NT	22,9	275	269
M01-240149 (3061)	B	4	7-2,4	41	41/44
M01-240185	B	2a	5-1,10-8	11	11
M01-240355 (3063)	B	1	22,14	213	213
NZ98/254	B	4	7-2,4	41	41/44
Nm26	B	4	16	Unknown	Unknown
NmP27	B	15	7, 16	Unknown	Unknown

Table 2.2.1 *N. meningitidis* strains used in immunoassays.

2.3 Preparation of bacteria for use in immunoassays

2.3.1 Growth of unstained *N. meningitidis*

All manipulations of *N. meningitidis* were performed within a flexible film isolator (FFI). An aliquot was removed from the -70^{0}C freezer and was placed within the FFI, and defrosted by placing within the 37^{0}C incubator. A disposable sterile inoculation loop was then used to create a streak plate on horse blood agar (Biomerieux) to check for contamination. 100 μl (2 x 10^8 CFU/ml) of *N. meningitidis* was spread onto another horse blood agar plate to create a lawn of growth, and both were incubated overnight at 37°C with 5% CO_2. Streak plates were checked for contamination and growth from half a plate was resuspended into 10 ml Frantz medium (containing 0.5 mg/ml EDDHA for iron restriction) using a disposable sterile inoculation loop. This was then incubated for 4 h at 37^{0}C with shaking.

The bacteria in these cultures were killed by the addition of 100 μl of fresh 20% (w/v) sodium azide solution and 100 μl of fresh 10 mM solution of PMSF, and incubated for 48 h at 37^{0}C. A 100 μl of this bacterial suspension was then spread on a horse blood agar plate and incubated overnight at 37^{0}C with 5% CO_2 for confirmation that all bacteria had been killed. The 10 ml culture was then centrifuged at 3060g for 5 min and resuspended in 1 ml of PBS.

2.3.2 Cryopreservation of *N. meningitidis*

Aliquots of *N. meningitidis* were prepared by inoculating 10 ml Frantz media with 30% (v/v) glycerol with half a plate of *N. meningitidis* growth. This was then aliquoted and frozen at -70^{0}C until required.

2.3.3 Gram staining

A loop full of bacterial suspension was spread onto the surface of a microscope slide. This was then placed onto a hotplate to heat fix the sample to the slide. This was then stained with crystal violet solution (0.981 g/ml) for 1 min. The slide was then washed several times in sterile water before being submerged in iodine solution (1.005 g/ml) for a further 1 min, this was again washed thoroughly with sterile water and then briefly with ethanol. Finally the slide was counter-stained with safarin (0.986 g/ml) for 30 s, and then washed and air dried before examination under a light microscope. This procedure was done to check *N. meningitidis* and HL60 cells for contamination.

2.4 Staining of *N. meningitidis*

2.4.1 BCECF-AM fluorescent staining of live *N. meningitidis*

10 ml cultures of *N. meningitidis* were grown as described in 2.3.1, and were centrifuged for 5 min at 3060g, and resuspended into 900 µl of HBSS. 100 µl of 1 mg/ml solution of BCECF-AM was added and the solution incubated for 1 h with shaking at 37^0C and then centrifuged for 5 min at 3060g, before being resuspended in 10 ml of PBS.

The bacteria were killed by adding 100 µl of fresh 20% (w/v) sodium azide solution and 100 µl of fresh 10 mM of PMSF and incubated for 48 h at 37^0C. 100 µl of this bacterial suspension was then spread on a horse blood agar plate and incubated overnight at 37^0C with 5% CO_2 to confirm that all bacteria had been killed. This was then centrifuged at 3060g for 5 min and resuspended in 1 ml of PBS.

2.4.2 Bacterial staining using various nucleic acid stains (PI, 7AAD and SYTO 59)

Azide/PMSF-killed unstained bacteria were centrifuged for 5 min at 3060g and then resuspended to an $OD_{600\ nm}$ of 2 using HBSS containing a range of concentrations of nucleic acid stains (0-60 µM), before being incubated for 30 min at room temperature. The bacteria were then centrifuged for 5 min and washed in HBSS. This step was repeated 4 times to remove any excess stain.

2.4.3 Staining of *N. meningitidis* using 5-(and-6)-carboxy SNARF®-1, acetoxymethyl ester (SNARF-AM) and dihydrocalcein, AM.

10 ml of *N. meningitidis* cultures were grown as described previously (2.2.1) and were centrifuged for 5 min at 3060g, and resuspended into 900 µl of HBSS. 100 µl of various SNARF-AM concentrations (0-150 µg/ml) were added, and the solution incubated for 1 h with shaking at 37^0C. The stain bacteria were then centrifuged for 5 min at 3060g, and then resuspended in 10 ml of PBS.

The bacteria were then killed as described in 2.4.1.

2.4.4 Staining of *N. meningitidis* using 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine-5,5'-disulfonic acid (DiIC$_{18}$(5)-DS) and PKH-26 lipid anchoring stains.

Azide-PMSF killed unstained bacteria were centrifuged for 5 min at 3060g and then resuspended in 10 ml of HBSS with a range of concentrations of PKH-26 (0-1 µM) or DiIC$_{18}$(5)-DS (0-160 µM) and incubated for 30 min at room temperature. The bacteria were then centrifuged for 5 min at 3060g and washed in HBSS, this step was repeated 4 times to remove excess stain which was assessed by the lack of dye visible in the HBSS wash buffer.

2.5 Cell culture

2.5.1 Preparation of cell culture medium

All cell culture was performed in a class II microbiological cabinet, 120 ml of sterile heat-inactivated foetal calf serum (FCS) was added to 500 ml of RPMI-1640 media. 5 ml was discarded from the media and 5 ml of a 200mM L- glutamine solution was added. Medium was checked for sterility by incubating a sample of media overnight at 37^0C before being evaluated by gram staining (2.3.3) and a visual check by microscopy. Media was then stored at 4^0C until use.

2.5.2 Cell line maintenance

The HL60 cell line was obtained from the American Tissue Culture Collection (ATCC). HL60 cells are a suspension culture and display a promyelocytic morphology, with a doubling time of approximately 24 h. They carry a variety of surface antigens characteristic of immature myeloid cells and can be differentiated into granulocytic-like cells with the addition of N,N-dimethylformamide (DMF) (Birnie 1988). This cell line was subcultured daily by adding 100 ml of growing cells to 100 ml of media in a T175 flask. When cells were subcultured for 48 h, 50 ml of growing cells were added to 150 ml of media in a T175 flask. This was done so that the cells were seeded to approximately 5×10^5 cells/ml. Cells were incubated at 37^0C with 5% CO_2.

2.5.3 Cell counting and viability

Prior to differentiation, cells were counted and their viability was checked. Cells were quantified using a haemocytometer. The haemocytometer was prepared by sliding a glass cover slip over the cell counting chamber, to create a tight seal. A 20 µl sample of cells was taken from the culture and was added to 20 µl of trypan

blue (0.4% w/v), and incubated for 5 min at room temperature. A 10 μl sample was taken up using a pipette, and placed at the edge of the haemocytometer and then slowly expelled so that the contents were drawn into the chamber by capillary action. This was visualised under a microscope. Cells were counted from the grid (figure 2.5.3), cells which appeared on a grid line were only counted if they were on the top and right lines of each square.

Counting area
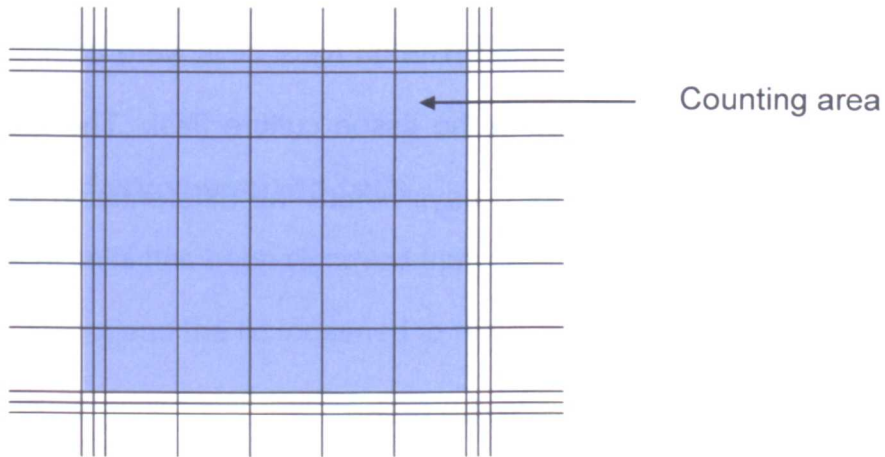
Figure 2.5.3 Haemocytometer grid used for cell counting.

The cell concentration was calculated by multiplying the live cell count by the dilution factor, which was then multiplied by 1×10^4 to give the total cells/ml. This number is derived from the volume underneath the cover slip being 0.0001 ml (length x width x height; i.e., 0.1 cm x 0.1 cm x 0.01 cm). Viability was determined by dividing the number of live cells which appeared clear, by the number of dead cells which absorbed trypan blue and therefore appeared blue, and multiplied by 100 to provide a percentage of live cells.

2.5.4 Differentiation of HL60 cells

Cells were initially counted and the number of viable cells calculated. The volume of cells required for a final concentration of 5×10^6 live cells/ml in 200 ml was calculated and placed into 50 ml centrifuge tubes and centrifuged for 5 min at 400g. The supernatant was discarded and the pellets resuspended into 50 ml of fresh culture media. In a separate 50 ml tube, 48.4 ml of media was mixed with 1.6 ml (0.8% (v/v)) of DMF. This was then added to a T175 tissue culture flask, where a further 100 ml of media was added. The 50 ml of cells were then added to this, to give a final volume of 200 ml in the tissue culture flask. The flask was then incubated at 37^0C at 5% CO_2 for 5 days before the differentiated cells could be used in an assay.

2.5.5 Leukocyte extraction from fresh blood

Blood was collected from a donor into vacutainer tubes (Becton Dickinson) containing heparin. The blood was diluted 1:10 in lysis buffer and incubated in a 37°C waterbath for 10 min, before being centrifuged at 400g for 5min. The supernatent was discarded and the pellet washed in 25 ml DPBS-GA. The cells were centrifuged for a further 5 min at 400g, before resuspension in 25 ml of lysis buffer. Once again this was incubated for 10 min in a 37°C waterbath to lyse any remaining red blood cells and the cells were centrifuged for 5 min at 400g and resuspended in 5 ml of DPBS-GA before being counted for use.

2.5.6 Cryopreservation and revival of HL60s

2.5.6.1 Crypopreservation

HL60s were made up to a concentration of 1×10^6 cells/ml in media with 10% (v/v) DMSO. This was then aliquoted into 1 ml cryovials and the placed into a 5100 Cryo 1°C freezing container which provides the 1°C/min cooling rate required for the successful cryopreservation of cells. This was then put into the -80^0C freezer overnight, and the aliquots were stored in liquid nitrogen until required.

2.5.6.2 Revival of cryopreserved cells

Once the vial of cells has been removed from liquid nitrogen, the vial was placed in a class II cabinet and the lid loosened to release any trapped liquid nitrogen, the lid was then re-tightened and then placed in a 37^0C waterbath until the cells were thawed. The vial contents were transferred into a centrifuge tube. This was then centrifuged for 5 min at 400g to remove any remaining DMSO. The pellet was then resuspended in 10 ml of fresh media and then transferred into a tissue culture flask and cultured at 37^0C with 5% CO_2.

2.6 OMV preparation

Frozen aliquots of *N. meningitidis,* stored at -70°C in Frantz media containing 30% (v/v) glycerol, were thawed and grown on horse blood agar overnight at 37°C with 5% CO_2, before being used to inoculate 20 ml of Frantz media with or without 0.5 mg/ml EDDHA for iron restriction, and incubated at 37°C with shaking (180 rpm) for 5 h. This was then used to inoculate 300 ml Frantz in a 2 L flask, and then incubated overnight at 37°C with shaking (180 rpm). Cultures were centrifuged at 4000g for 1 h and the supernatant discarded and pellets resuspended in OMV buffer 1 at a 5:1 buffer:biomass ratio. This was centrifuged for 30 min at 20,000g at 4°C, and the supernatant retained and centrifuged for a further 2 h at 125,000g. The supernatant was discarded and the pellet resuspended in 3 ml of OMV buffer 2 and centrifuged for a further 2 h at 125,000g. The final pellet was resuspended in 1 ml OMV buffer 3 and the OMVs were homogenised and stored at -20°C (Finney *et al.* 2008).

2.7 Generation of Immune sera

2.7.1 Animals

All mice used to generate immune sera were female NIH or BALB/c mice (Harlan) and were 6–8 weeks old at the beginning of the immunisation schedule. All rabbits used in the generation of sera were female NZ white rabbits (Harlan) which were 2-3 kgs at the beginning of the immunisation schedule.

2.7.2 Vaccine preparation

Mice were immunised with 10 µg OMVs (total protein concentration) prepared with 0.33% (v/v) alhydrogel adjuvant. Alhydrogel was made up in PBS to a concentration of 0.67% (v/v), this was mixed 1:1 with OMVs at the required concentration. Each mouse received two 100 µl doses subcutaneously at two separate locations, whilst rabbits received two doses of 500 µl subcutaneously. Vaccines were given in a three dose schedule on day 0, 21, 28 before being terminally bled on day 35 (Gorringe *et al.* 2001).

2.8 Labelling of monoclonal antibody

2.8.1 Zenon labelling of monoclonal antibody

Zenon (Invitrogen) mouse IgG labelling reagent (5 µl) was added to 100 µl of PBS containing 10 µg/ml antibody. The solution was incubated for 5 min at room temperature before 5 µl of Zenon blocking reagent was added and incubated for a further 5 min at room temperature before use.
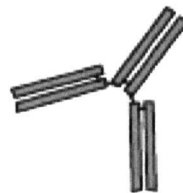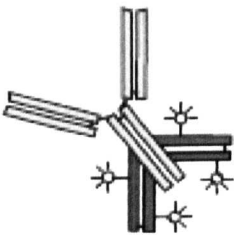
Figure 2.8.1 Zenon labelling method

2.8.2 Alexa fluor 647 monoclonal antibody labeling

Alexa flour 647 monoclonal antibody labeling kit was used as described in the manufacturer's protocol (Invitrogen, UK). Antibody was diluted to 1 mg/ml in 1 M sodium bicarbonate buffer and 100 µl of this antibody solution was added to a vial of Alexa fluor 647 reactive dye, and incubated for 1 h with rotation. The purification spin column was prepared during the hour incubation, 1 ml of purification resin was added to a spin column and allowed to settle before centrifuging at 1100g for 3 min to elute purification resin buffer. This was repeated until the resin bed was approximately 1.5 ml. After centrifugation, 100 µl of stained antibody was pipetted directly into the centre of the resin bed and then centrifuged for 5 min at 1100g and the eluted antibody collected and stored at 4°C until use.

2.9 Immunoassay methods

2.9.1 Surface binding assay (SLA)

Total antibody binding on the surface of meningococci was performed in U-bottom 96-well microtitre plates. 2 µl of each test serum (heat inactivated) was added to 198 µl of target bacteria at an O.D $_{600\,nm}$ 0.1 in blocking buffer and incubated for 30 min with shaking (900 rpm) at 25.0°C and then centrifuged at 3050g for 5 min and the supernatant removed and the pellet washed with 200 µl of blocking buffer. The wash step was repeated twice before the addition of 200 µl FITC-labelled goat anti-mouse/rabbit/human conjugate (Jackson Immunochemicals, USA) at 1:500 in blocking buffer. This was incubated for 20 min at 4°C, before being washed twice more with blocking buffer. The samples were then analysed by flow cytometry.

2.9.2 Complement deposition assay (CDA) original method

Antibody-mediated complement deposition on the surface of meningococci was measured using a flow cytometric assay performed in U-bottom 96-well microtitre plates. For the assay, 5 µl of each test serum (heat inactivated) was added to 90 µl of target bacteria at an O.D $_{600\,nm}$ 0.1 in a blocking buffer of 2% bovine serum albumin (BSA) in PBS followed by 5 µl of IgG-depleted human serum/plasma (2.10.4) and the mixture incubated for 30 min with shaking (900 rpm) at 25.0°C using a IEMs plate shaking incubator. This was then centrifuged at 3050g for 5 min and the supernatant removed and the pellet was then washed with 200 µl of blocking buffer. This was repeated twice before the addition of 200 µl FITC-labelled polyclonal sheep anti-human C3b/iC3b (Abcam) at 1:500 in blocking buffer. This was incubated for 20 min at 4°C, before being washed twice more with blocking buffer. The samples were then analysed by flow cytometry.

2.9.3 CDA using a secondary antibody.

Antibody-mediated complement deposition on the surface of meningococci was measured using a flow cytometric assay performed in U-bottom 96-well microtitre plates. For the assay, 5 µl of each test serum (heat inactivated) was added to 90 µl of target bacteria at an O.D $_{600\ nm}$ 0.1 in blocking buffer containing 2% BSA in PBS followed by 5 µl of IgG-depleted human serum/plasma and the mixture incubated for 30 min with shaking (900 rpm) at 25.0°C using a IEMs plate shaking incubator. This was then centrifuged at 3050g for 5 min and the supernatant removed and the pellet washed with 200 µl of blocking buffer. This was repeated twice before the addition of either 200 µl anti-human mAb C3b/iC3b (Abcam) at 1:500 in blocking buffer or anti human mAb C5b-9 (Quidel) at 1:2000 in blocking buffer. This was incubated for 20 min at 4°C, before being washed twice more with blocking buffer. FITC-labelled anti-human IgG (Jackson Immunoresearch) at 1:500 in blocking buffer was incubated for 20 min at 4°C, before being washed twice more with blocking buffer. The samples were then analysed by flow cytometry.

2.9.4 Duplexed complement deposition assay (dCDA), final method.

Antibody-mediated complement deposition on the surface of meningococci was measured using a flow cytometric assay performed in U-bottom 96-well microtitre plates. For the assay, 5 µl of each test serum (heat inactivated) was added to 90 µl of target bacteria at an O.D $_{600\ nm}$ 0.1 in blocking buffer of 2% BSA in PBS followed by 5 µl of IgG-depleted human serum/plasma and the mixture incubated for 45 min with shaking (900 rpm) at 37°C using a IEMs plate shaking incubator. This was then centrifuged at 3050g for 5 min and the supernatant removed and the pellet washed with 200 µl of blocking buffer. This was repeated twice before the addition of 200 µl anti-human C3b/iC3b (Abcam, UK) at 1:500 in blocking buffer and anti human C5b-9 (Quidel, USA) at 1:2000 in blocking buffer. This was

incubated for 20 min at 4°C, before being washed twice more with blocking buffer. FITC-labelled anti-mouse IgG (Jackson Immunoresearch, UK) at 1:500 in blocking buffer was incubated for 20 min at 4°C, before being washed twice more with blocking buffer. The samples can then be analysed by flow cytometry.

2.9.5 Total haemolytic complement activity radial immunodiffusion assay

Complement function was determined using a total haemolytic complement radial immunodiffusion assay kit (Binding Site, UK). Lyophilised calibrator and control samples were reconstituted using distilled water to the indicated volume on the vial, and all samples are kept pre-cooled on ice prior to the assay. 5 µl of neat test sample, calibrator dilutions (three doubling dilutions) or control samples were added to wells of a sheep erythrocyte gel sensitised with anti-sheep erythrocyte antibody and incubated overnight at 4°C before incubating at 37°C for 30 min. The diameter of the lysis rings was then measured and calibrator dilutions plotted and the complement activity calculated in CH100 units/ml from the standard curve.

2.9.6 Measurement of the alternative complement cascade using a radial immunodiffusion assay

Alternative complement pathway activity was determined using an alternative haemolytic complement radial immunodiffusion assay kit (Binding site, UK). Lyophilised calibrator and control samples were reconstituted using distilled water to the indicated volume on the vial, and all samples are kept pre-cooled on ice prior to the assay. 5 µl of neat test sample, calibrator dilutions (three doubling dilutions) or control samples were added to wells of a sheep erythrocyte gel. Incubate overnight at 4°C before incubating at 37°C for 30 minutes. The diameters of the lysis rings were then measured and calibrator dilutions plotted and the complement activity calculated from the standard curve.

2.9.7 OPA– original method

Opsonophagocytosis (OP) of meningococci by HL-60 cells which had been granulocytically differentiated with 0.8% (v/v) DMF for 5 days (2.5.4), was measured using a flow cytometric assay performed in U-bottom 96-well microtitre plates. 20 µl of each test serum (heat inactivated) was diluted 1:10 in OP buffer (Hanks balanced salts solution containing 2% (w/v) skimmed milk powder, 1.2 mM $CaCl_2$, and 1 mM $MgSO_4$) and added to 10 µl BCECF-AM labelled *N. meningtidis* (2.3.1) at 6.25 x 10^8 bacteria/ml in OP buffer, followed by 10 µl of baby rabbit complement and incubated for 7.5 min with shaking (900 rpm) at 37.0°C. Differentiated HL60 cells, (American Type Cultures Collection, Rockville MD, USA) at 2.5 x 10^7 cells/ml in OP buffer (50 µl) were added and incubation continued, with shaking, at 37.0°C for 7.5 min. OP was stopped by addition of 80 µl of ice-cold Dulbecco's PBS containing 0.02% (w/v) EDTA. The samples were then analysed by flow cytometry.

2.9.8 Duplexed respiratory burst assay (dRBA) final method.

DMF differentiated HL60 cells were incubated with 100 ng/ml IFNγ for 45 minutes at 37°C. The cells were then centrifuged at 500g for 5 min before being resuspended in HBSS with 50 µg/ml dihydrodamine 123 (DHR123) stain and incubated for 15 min with rotation to preload the cells with dye. 10 µl of $DilC_{18}(5)$-DS stained *N. meningtidis* at 6.25 x 10^8 bacteria/ml were incubated with 2 µl sera and 10 µl IgG-depleted human plasma as a complement source in 18 µl blocking buffer (HBSS containing 2.0% skimmed milk, 1.2 mM $CaCl_2$, 1 mM $MgSO_4$), at 37°C for 7.5 min. 50 µl DHR123 preloaded DMF-differentiated HL60 cells at 2.5 x 10^7 cells/ml were added and incubated at 37°C for a further 7.5 min. The assay was then stopped with addition of 80 µl of ice-cold Dulbecco phosphate-buffered

saline containing 0.02% (w/v) EDTA. The samples were then analysed by flow cytometry.

2.9.9 Opsonophagocytic killing assay (OPKA)

This assay was based on the method described by Plested and Granoff (2008). *N. meningitidis,* stored at -70°C in Frantz media containing 30% (v/v) glycerol, were thawed and grown on horse blood agar overnight at 37°C with 5% CO_2. This was used to inoculate 10 ml of Frantz media both with and without 0.5 mg/ml EDDHA for iron restriction, and incubated at 37°C with shaking (180 rpm) for 4 h. Bacteria were centrifuged and resuspended in BB before the OD $_{600\ nm}$ was measured and the bacteria were diluted in BB to a concentration of 5 x 10^4 CFU/ml.

50 µl of each dilution of heat inactivated serum was used in each well, with a starting dilution of 1:2 which was diluted across the plate to a final dilution of 1:1024 using doubling dilutions. To this, 20 µl of C7-depleted human complement (Quidel) was added, together with 10 µl of *N. meningitidis* at 5 x 10^4 CFU/ml to give 500 CFU/well, and 20 µl of DMF-differentiated HL60 cells. Each plate contained a HL60 cell-only control, bacteria-only control, cell, bacteria and complement-only controls in duplicate. Before the assay plate was incubated, 10 µl of the control wells were transferred onto horse blood agar using the tilt method, air dried and incubated overnight at 37°C with 5% CO_2. The assay was then incubated for 60 min at 37°C with shaking at 65 rpm. Each sample and control well was then plated out onto horse blood agar using the tilt method, air dried and incubated overnight at 37°C with 5% CO_2. The following day colonies were counted and a titre assigned to the reciprocal dilution which gave a >50% killing compared with the bacteria, cells and complement-only control.

2.9.10 SBA

The standard SBA protocol was used to assess the bactericidal activity of vaccine sera (Findlow *et al.* 2006). *N. meningitidis*, stored at -70°C in Frantz media containing 30%(v/v) glycerol, was thawed and grown on Columbia blood agar overnight at 37°C with 5% CO_2 and then used to inoculate 10 ml Frantz media, and incubated at 37°C with shaking (180 rpm) for 3 h. Bacteria were resuspended in bactericidal buffer (BB), (Hanks buffered saline solution (invitrogen) and 1% BSA (Sigma Aldrich)) before the OD $_{600\ nm}$ was measured and bacteria were diluted in BB to a concentration of 6 x 10^4 CFU/ml.

20 μl of heat inactivated serum was used in each well, and from a starting dilution of 1:2 was diluted across a microtitre plate to a final dilution of 1:1024 using doubling dilutions. 10 μl of 6 x 10^4 CFU/ml were added to every well followed by 10 μl of human complement. Each test contained three controls: bacteria and complement-only control, bacteria and heat inactivated complement-only control, and test serum, bacteria and heat inactivated complement control. The assay was then incubated at 37°C for 1 h with shaking at 65 rpm. Each sample and control well was then plated out onto horse blood agar using the tilt method, air dried and incubated overnight at 37°C with 5% CO_2. The following day colonies were counted and a titre assigned to the reciprocal dilution which gave a >50% killing compared with the bacteria, and complement-only control.

2.9.11 Antibody (IgG, IgM, or IgA) ELISA

Antibody ELISAs were performed using manufacturer's instructions (Bethyl Laboratories). 100 μl of coating antibody (in ELISA coating buffer (2.1.2)) was added to each well (96 well flat bottom plate) and incubated at room temperature

(20-25°C) for 1 h. The plate was then washed five times using ELISA wash solution. 200 μl of ELISA blocking buffer was added to each well and the plate incubated for 30 min at room temperature (20-25°C) before being washed a further five times with ELISA wash buffer. 100 μl of, either standard, or assay sample was added to each well and incubated at room temperature (20-25°C) for 1 h. The plate was then washed five times using ELISA wash solution. 100 μl horse raddish peroxidise conjugated detection antibody was added to each well incubated for 1 h at room temperature (20-25°C) and then 100 μl of 3,3',5,5'-Tetramethylbenzidine substrate solution was added to each well. The plate was left in the dark to develop for 15 min before the reaction was stopped using ELISA stop solution (2.1.2). Sample absorbance was measured at 450 nm (within 1 h of assay completion) and the OD of the standard curve used to extrapolate the sample concentration.

2.9.12 IgG subclass ELISA

IgG subclass ELISAs were performed using manufacturer's instructions (Invitrogen). 50 μl of coating antibody (in ELISA coating buffer) are added to each well (96 well flat bottom plate), 50 μl of test sample or assay standard, and then incubated at room temperature (20-25°C) for 30 min. The plate was then washed three times using ELISA wash solution. 100 μl HRP detection antibody was added to each well incubated for 30 min at room temperature (20-25°C). The plate was then washed three times using ELISA wash solution and then 100 μl of TMB substrate solution was added to each well. The plate was left in the dark to develop for 10 min before the reaction was stopped using ELISA stop solution. Sample absorbance was measured at 450 nm (within 1 hour of assay completion) and the OD of the standard curve used to extrapolate the sample concentration.

2.9.13 C1q ELISA

C1q ELISAs were performed using manufacturer's instructions (Hycult, UK). 100 µl of test sample or assay standard are added to precoated wells, and then incubated at room temperature (20-25°C) for 60 min. The plate was then washed four times using ELISA wash solution. 100 µl biotinylated detection antibody was added to each well incubated for 60 min at room temperature (20-25°C). The plate was then washed three times using ELISA wash solution and then 100 µl of streptavidin-peroxidase. Finally 100 µl of TMB substrate solution was added to each well. The plate was left in the dark to develop for 20 min before the reaction was stopped using ELISA stop solution. Sample absorbance was measured at 450 nm (within 1 hour of assay completion) and the OD of the standard curve used to extrapolate the sample concentration.

2.10 Complement

2.10.1 Collection of plasma or serum

Volunteer blood was taken using appropriate Vacutainer tubes (Becton Dickinson, UK) containing either: heparin, EDTA (anticoagulants), silica (clot activator) or no additive. Following collection, tubes without additive had 100 µg/ml of the anti-coagulant lepirudin (Movianto, UK) added. Tubes were then centrifuged at 1000g for 10 min to pellet the cells, the plasma, or serum was then removed and stored at -80°C.

2.10.2 Antibody depletion of human plasma initial method

IgG depletion of heparinised plasma was performed on ice and utilised a column containing 1 ml of ProteinG-Sepharose (Amersham Biosciences, UK). Using a 5 ml syringe, four column volumes of HBSS were pushed through the column to

remove the 20% (v/v) ethanol storage buffer. Very slowly, a 1 ml aliquot of complement source (human plasma or Pel-freez baby rabbit complement) was loaded onto the column to allow IgG binding and the column was incubated on ice for 5 min. 1 ml of ice-cold HBSS was pushed through the column to displace the plasma/complement and the eluent collected and stored on ice for immediate use. Four column volumes of HBSS was passed through to clean the column, followed by four column volumes elution buffer and finally two column volumes of 20% (v/v) ethanol storage solution and the column stored at 4^0C (Taylor 2010).

2.10.3 Antibody depletion of human plasma method during development

Antibody depletion of plasma or serum was performed on ice and utilised a column containing 1 ml of either Protein G, A or L Sepharose (Amersham Biosciences, UK). Using a 5 ml syringe, four column volumes HBSS was pushed through the column to remove the 20% (v/v) ethanol storage buffer. Very slowly, a 1 ml aliquot of complement source was loaded onto the column to allow IgG binding and the column incubated on ice for 5 min. 1 ml of ice-cold HBSS was pushed through the column to displace the plasma/complement and the eluent collected and stored on ice for immediate use. Four column volumes of HBSS were passed through to clean the column, followed by four column volumes elution buffer and finally two column volumes of 20% ethanol storage solution and the column stored at 4^0C.

2.10.4 Antibody depletion of human plasma, final method

Volunteers gave blood which was anti-coagulated with lepirudin. Donors consented to give blood and for it to be used for research, samples were taken and anonymised by a trained phlebotomist. The sample was centrifuged at 500g for 10 min and the supernatant was aliquoted and snap frozen until required for

use. Lepirudin anti-coagulated human plasma was used as a complement source and was antibody depleted using affinity chromatography with Protein G Sepharose column. The column was equilibrated with three column volumes of HBSS before one column volume of plasma was added (200 ml). This was incubated for 5 min at 4°C before one column volume of HBSS was used to displace the complement. The column was then washed with 3 column volumes of elution buffer (0.2 M glycine pH 2.7 (HCl)) and incubated for 5 min at room temperature before 3 column volumes of HBSS were used to displace the elution buffer. The column was then stored in 20% in deionised water.

2.11 Flow cytometry

All assays were analysed using a Beckman Coulter FC500 flow cytometer equipped with a 96-well microtitre plate reader.

2.11.1 Data Analysis using MXP software (Beckman Coulter): Gating
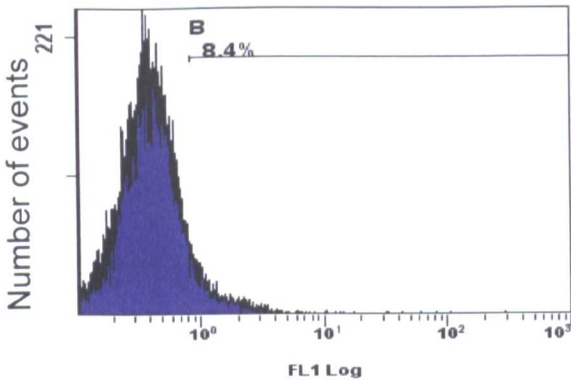
Regi...	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	24354	91.69	100.00	0.507	###
B	2034	7.66	8.35	1.84	###
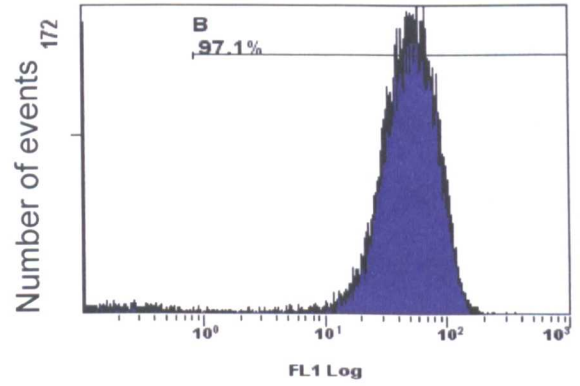
Regi...	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	22939	80.54	100.00	54	###
B	22274	78.20	97.10	55.6	###

A.   B.

Figure 2.11.1 Histogram to illustrate gating strategy used for flow cytometry data analysis.

Histograms illustrate a complement only background control (A) and a homologous positive control serum (B).

To analyse the data a horizontal gate (marked in fig. 2.11.1) was drawn to include at approximately 10% of the background control fluorescence. A Fluorescence Index (FI) was calculated for each sample, which involved the multiplication of the % of cells moving into the horizontal gate (%-gated), by the average fluorescence of that population (X-mean). From the example above and FI was calculated.

FI = 8.35 X 1.84 = 15.4 (figure 2.11.1).

A FI was generated for each test serum and the FI of the control was subtracted to result in an FI-C value.

E.g. 97.10 X 55.6 = 5398.8 – 15.4 = 5383.4 (figure 2.11.1).

2.11.2 Data Analysis using MXP software (Beckman Coulter): Compensation

Compensation was required in experiments which included two fluorochromes, and was done to eliminate any spectral overlap. Initially an unstained control was used to align the background auto-fluorescence to the first log decade in a dot plot of both fluorochromes (figure 2.11.2 A.). The x-mean and y-mean is taken on the background sample (0.479, 0.461). A positive control for the single fluorochrome sample was assessed (figure 2.11.2 B.) and shows spectral overlap in the second fluorochrome channel (in this case FL4). The data can then be compensated using the MXP software until the y-mean is within 0.1 of the background y-mean (figure 2.11.2 C).

A.

Region	Number	%Gated	X-Mean	Y-Mean
ALL	5202	100.00	0.515	0.462
C1	0	0.00	0	0
C2	1	0.02	34.2	2.54
C3	5197	99.90	0.479	0.461
C4	4	0.08	39.2	0.876

B

Region	Number	%Gated	X-Mean	Y-Mean
ALL	5437	100.00	69.9	5.92
C1	4	0.07	0.759	50.7
C2	3687	67.81	99.8	8.41
C3	865	15.91	0.571	0.365
C4	881	16.20	13.2	0.729

C.

Region	Number	%Gated	X-Mean	Y-Mean
ALL	4349	100.00	11.5	0.554
C1	0	0.00	0	0
C2	25	0.57	35.2	1.81
C3	583	13.41	0.854	0.466
C4	3741	86.02	13	0.56

Figure 2.11.2 Method of Compensation when using two fluorochromes within the same assay.

Histograms illustrate a background control (A), a homologous positive control for a single fluorochrome, and data once compensation has been completed (C).

2.11.3 Data analysis used in the surface labeling assay (SLA)

The bacteria were identified on the cytometer by their forward scatter (size) and side scatter (granularity) (figure 2.11.3.1). 7500 bacteria were analysed for fluorescence and a horizontal gate was drawn to include 10% of the population in the conjugate only control. A fluorescence index (FI) was calculated for all samples by multiplying the % gated by the x-mean (fluorescence intensity).

Duplicate samples were analysed and an average taken of the FI before subtracting the FI of the conjugate only control.
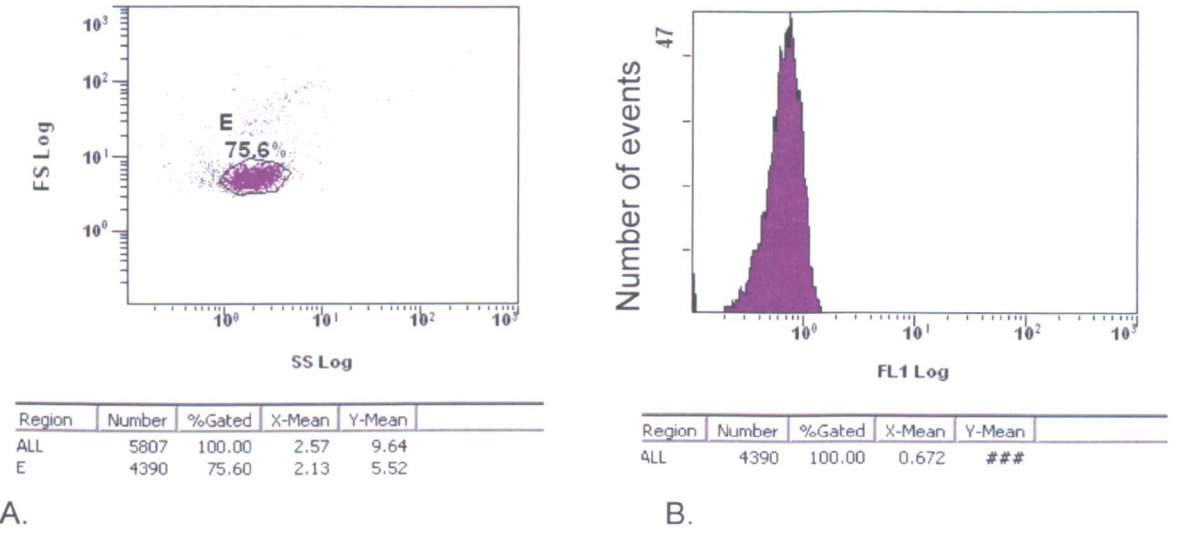
Figure 2.11.3.1 Gating strategy used in data analysis of a surface labeling assay.

Histograms illustrate forward scatter and side scatter gating for bacteria (A) and the background fluorescence observed in a bacteria and conjugate only control (B).
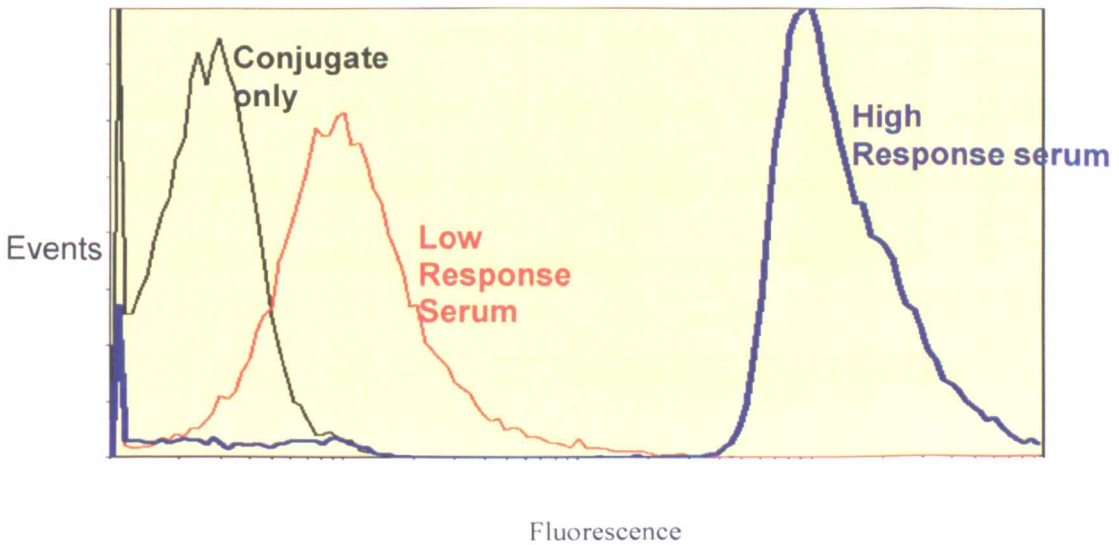
Figure 2.11.3.2 Overlay plot of SLA data.

This indicates the typical fluorescence observed with a conjugate only control, low responding serum and high responding serum.

2.11.4 Complement deposition assay (CDA)

N. meningitidis were identified on the cytometer by the forward scatter (size) and side scatter (granularity) (figure 2.11.4.1). 7500 bacteria were analysed for fluorescence and a horizontal gate was drawn to include 10% of the population in the conjugate and complement only control. A fluorescence index (FI) was calculated for all samples by multiplying the % gated by the x-mean (fluorescence intensity). Duplicate samples were analysed and an average taken of the FI before subtracting the FI of the complement only control.
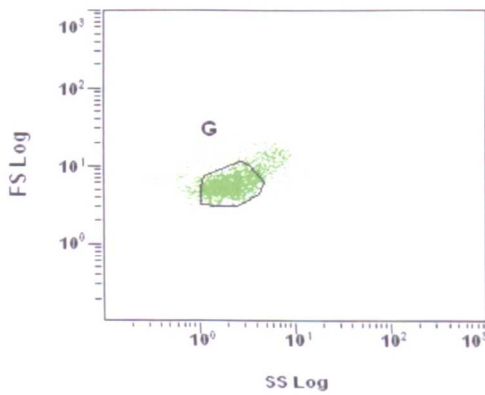
Region	Number	%Gated	X-Mean	Y-Mean
ALL	7242	100.00	2.61	6.77
G	5535	76.43	2.27	5.76

A.
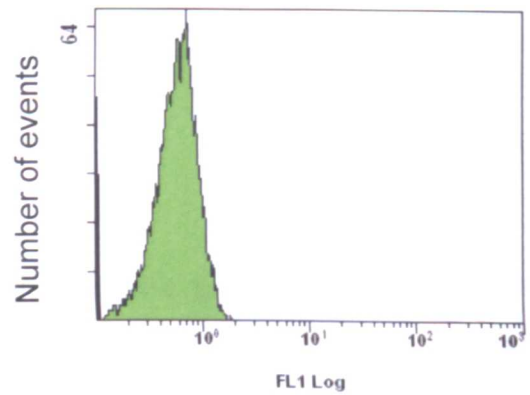
Region	Number	%Gated	X-Mean	Y-Mean
ALL	7242	100.00	0.625	###

B.
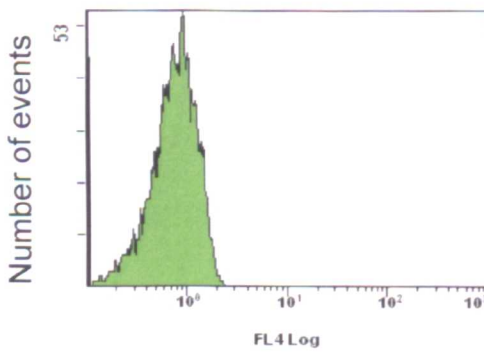
Region	Number	%Gated	X-Mean	Y-Mean
ALL	7242	100.00	0.791	###

C.

Figure 2.11.4.1 Gating strategy used in data analysis of a duplexed complement deposition assay.

The histograms show the forward scatter and side scatter gating for the bacteria (A) and the complement, bacteria and conjugate only control for both pAb C3b/iC3b (FITC) (B) and mAb C5b-9 (Alexafluor 647) (C).
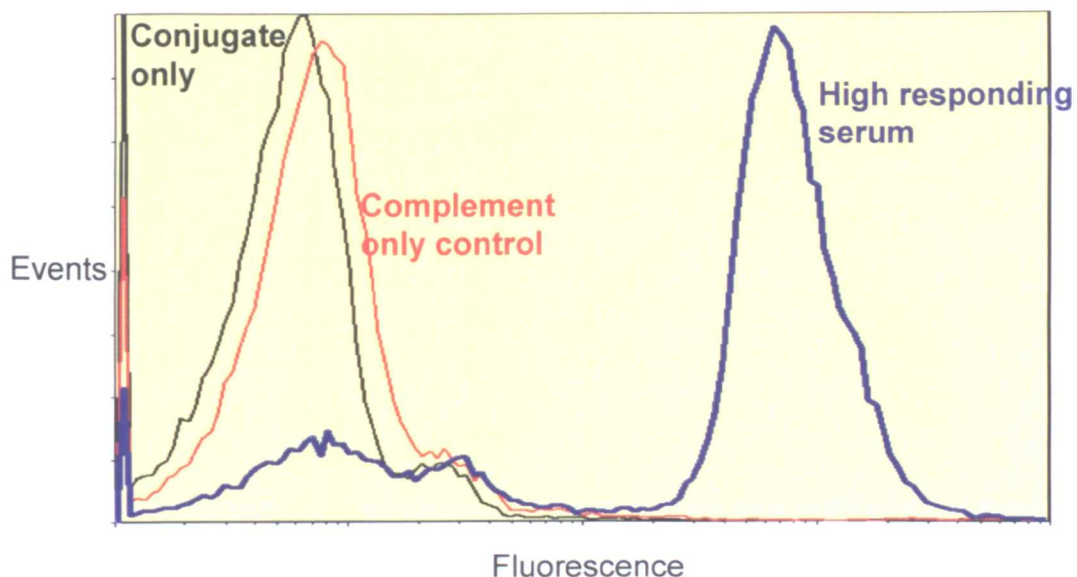
Figure 2.11.4.2 Overlay plot of CDA data.

This overlay indicates the typical fluorescence observed with a conjugate only control, complement only control and high responding serum.

2.11.5 Opsonophagocytosis Assay (OPA)

The HL60 cells were identified on the cytometer using forward scatter (size) and side scatter (granularity) (figure 2.11.5.1). 7500 HL60 cells were analysed for fluorescence and a horizontal gate was drawn to include 10% of the population in the complement only control. A fluorescence index (FI) was calculated for all samples by multiplying the % gated by the x-mean (fluorescence intensity). Duplicate samples were analysed and an average taken of the FI before subtracting the FI of the complement only control.

Panel A (FS Lin vs SS Lin):
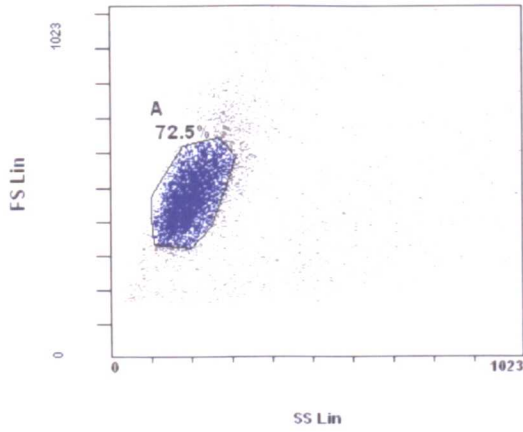
Region	Number	%T...	%Ga...	X-Mean	Y-Mean
LL	12147	100.00	100.00	242	486
,	8802	72.46	72.46	198	475

A.

Panel B (FL4 Log):
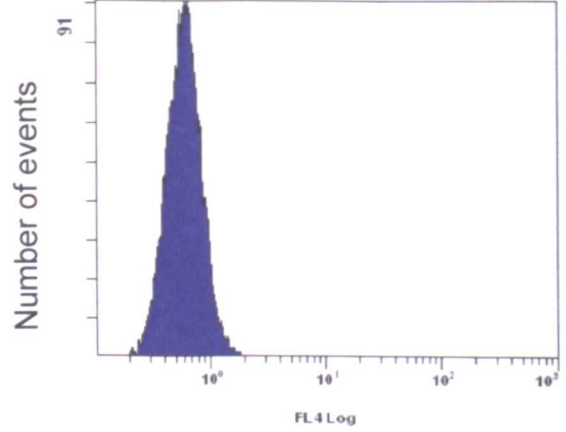
Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	8802	72.46	100.00	0.627	###

B.

Figure 2.11.5.1 Gating strategy used in data analysis of a duplexed complement deposition assay.

The histograms illustrate the forward scatter and side scatter gating used with the HL60 cells (A) and the fluorescence observed in a cells and $DilC_{18}(5)$-DS stained bacteria only control (B).
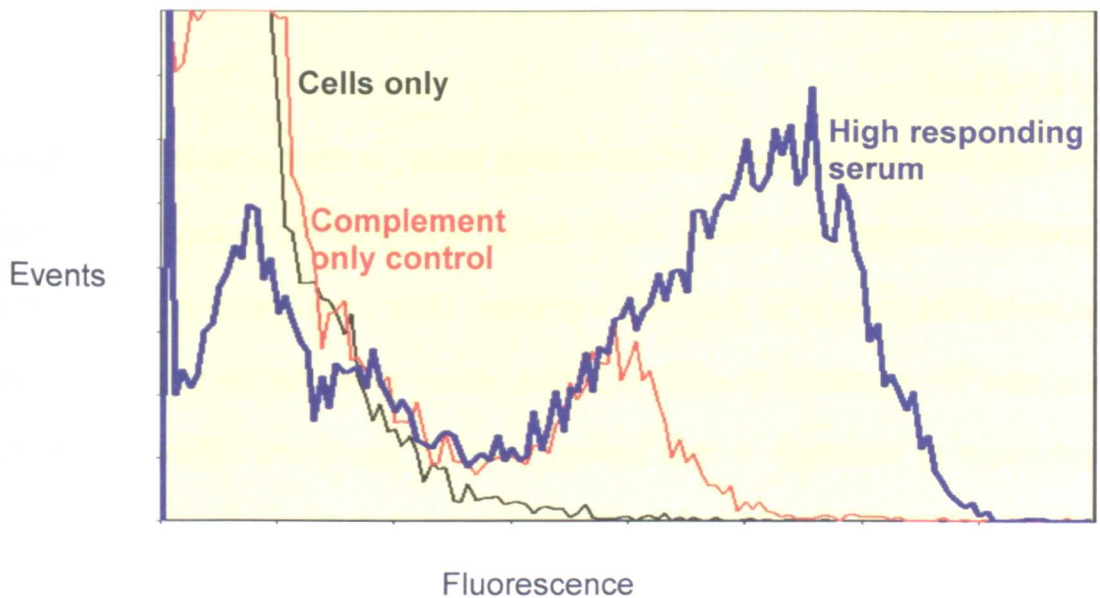
Figure 2.11.5.2 Overlay plot of OPA data.

This plot indicates a typical HL60 cells only control, complement only control and high responding serum.

2.11.6 Respiratory burst assay

Following compensation, horizontal gates in the $DiIC_{18}(5)$-DS channel (APC equivalent) of a flow cytometer (Beckman Coulter FC500) were set against a complement-only, no-antibody control to include approximately 10% of the population. Horizontal gates in the R123 channel (FITC equivalent) of a flow cytometer (Beckman Coulter FC500) were set against a complement-only, no-antibody control to include approximately 10% of the population For each sample, 7,500 live HL60 cells were measured, and the percentage of cells showing fluorescence in the appropriate gates (% gated) was multiplied by the mean fluorescence of the gated population (X-mean) to calculate a fluorescence index (FI); the Complement only control was subtracted from the FI to give a FI-C'.

2.12 Statistics

2.12.1 Z test

The data from the antibody surface binding assay, antibody-mediated complement deposition assay, respiratory burst assay and opsonophagocytosis assay are presented as means of duplicate samples. Data from these experiments were analysed for significance using a Z test, which uses the variance of the assay performed to calculate a test statistic, with a significant difference of $P<0.05$ represented by * and a significant difference of $P<0.01$ represented by ** unless otherwise stated. All error bars on figures represent standard deviations unless otherwise stated.

2.12.3 T-test

Statistical significance from ELISA and radial immunodiffusion assays was calculated using a two sample T-test. A significant difference of $P<0.05$ was represented by * and a significant difference of $P<0.01$ represented by **.

2.12.4 Pearson's correlation coefficient

Correlation between data was calculated using a Pearson's correlation providing a correlation coefficient r. Significance was assessed by a students t test of $P<0.05$ unless otherwise stated.

Chapter 3

Chapter 3 Development of a Duplexed C3b/iC3b and C5b-9 Antibody-mediated Complement Deposition Assay

3.1 Introduction

Complement plays an important role in protection against meningococcal disease. It contains more than 30 proteins, which play a role in inflammation, chemotaxis, and elimination of invading microorganisms by lysis or opsonisation, and plays a role in the development of an antibody response, and enhances the immunological memory response (Morgan et al. 2005).

A central role for SBA has been established in protection against meningococcal disease (described in 1.4.1). The importance of complement is also emphasised by the observed high susceptibility of complement deficient individuals to meningococcal disease (Walport 2001). SBA is accepted as a correlate of protection against meningococcal disease (Frasch et al. 2009) and is currently the primary method used in the assessment of vaccine elicited responses. SBA measures immune bactericidal lysis initiated by the classical pathway leading to the deposition of C3b, which combines with either components from the classical pathway or the alternative pathway to form a C5 convertase which cleaves C5 and leads to the formation of the membrane attack complex (MAC). This assay has some drawbacks: it requires large amounts of serum, the technique is laborious requiring containment facilities and strain selection can be restricted as the assay complement source kills some strains without addition of antibody.

Protection has been demonstrated in the absence of bactericidal antibody (Platonov et al. 2003; Toropainen et al. 2006; Welsch and Granoff 2007) and

opsonophagocytosis has been suggested as an additional mechanism of bacterial killing important in meningococcal disease.

Previous work has been conducted to develop an assay which measures the antibody-mediated complement deposition of C3b/iC3b using a sheep anti human C3b/iC3b polyclonal antibody (Biodesign) (Welsch *et al.* 2003; Taylor 2010).

This chapter describes the development of a duplexed complement deposition assay measuring both the deposition of C3b/iC3b and C5b-9 as potential indicators of opsonic and bactericidal antibody levels. In addition results from the duplexed assay are correlated with the functional SBA and OP assays.

3.2 Development of the assay

3.2.1 Measurement of C5b-9 antibody-mediated MAC deposition on meningococci

Assays measuring the antibody-mediated deposition of C3b/iC3b on the surface of meningococci have been previously described (Welsch *et al.* 2003; Findlow *et al.* 2006; Taylor 2010), and the aim of this study was to measure concurrently antibody-mediated deposition of C5b-9 membrane attack complex and C3b/iC3b then to correlate these two findings with SBA and OPA, respectively. Antibody-mediated deposition of C3b/iC3b assay was performed as previously described (2.9.2) and demonstrated antibody-mediated C3b/iC3b deposition with homologous and heterologous sera against strain M01240149 with a low deposition observed against an alhydrogel control serum (figure 3.2.1.1).

Two suitable monoclonal antibodies were identified which were able to detect C5b-9 deposition. mAb SC5b-9 (Quidel) was raised against purified C9 and has been shown to bind to a neoantigen only expressed on SC5b-9 (membrane attack complex which has bound to an S protein), MAC (C5b-9), Poly-C9 and on denatured C9 (Quidel product specification). This was compared with an alternative mAb C5b-9 (Dako), which was raised against poly C9 in the terminal complement complex (TCC). It has been shown to react with both membrane bound C5b-9 and the fluid phase SC5b-9 (Dako product specification sheet).

As neither of the monoclonals were conjugated to an fluorochrome, FITC-labelled secondary antibody was used, and the samples were analysed using flow cytometry. Several dilutions of each mAb were used to determine whether C5b-9 deposition could be detected. Both mAbs were able to detect deposition of C5b-9

on the surface of the bacteria (figure 3.2.1.2 and 3.2.1.3); the Dako mAb required a 1:50 dilution whereas the Quidel mAb was able to detect deposition at 1:500. 10-fold greater FI-C' values were obtained for binding at C3b/iC3b than with either C5b-9 (figure 3.2.1.1, 3.2.1.2 and 3.2.1.3). This might be expected as the C5b-9 is the terminal complement component and is made up of many of the final components of the complement cascade and C3b/iC3b are generated by several pathways (Walport 2001).
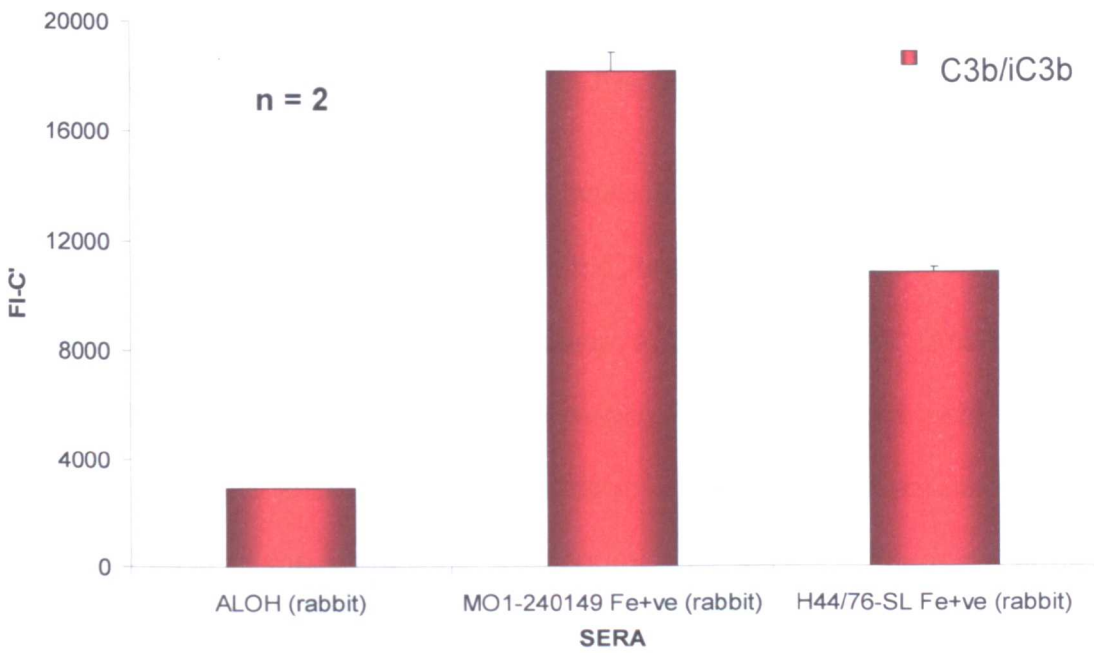
Figure 3.2.1.1 Antibody mediated C3b/iC3b deposition on *N. meningitidis*.
Antibody-mediated deposition of C3b/iC3b (1:500) on the surface of *N. meningitidis* M01-240149 as detected by pAb FITC labelled sheep anti-human C3b/iC3b. n=2 and error bars denote standard deviation.
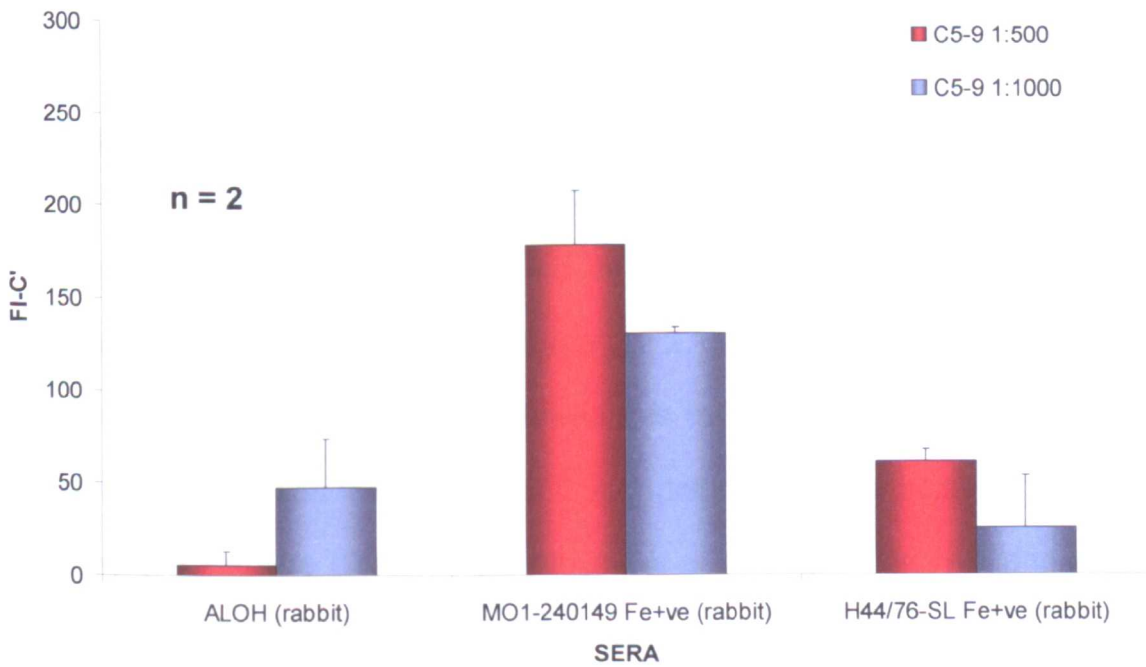
Figure 3.2.1.2 Antibody mediated C5b-9 deposition on *N. meningitidis* with Quidel mAb.
Antibody-mediated deposition of C5b-9 on the surface of *N. meningitidis* MO1240149 as detected by Quidel mAb with a secondary label of FITC goat anti-mouse IgG conjugate. n=2 and error bars denote standard deviation.
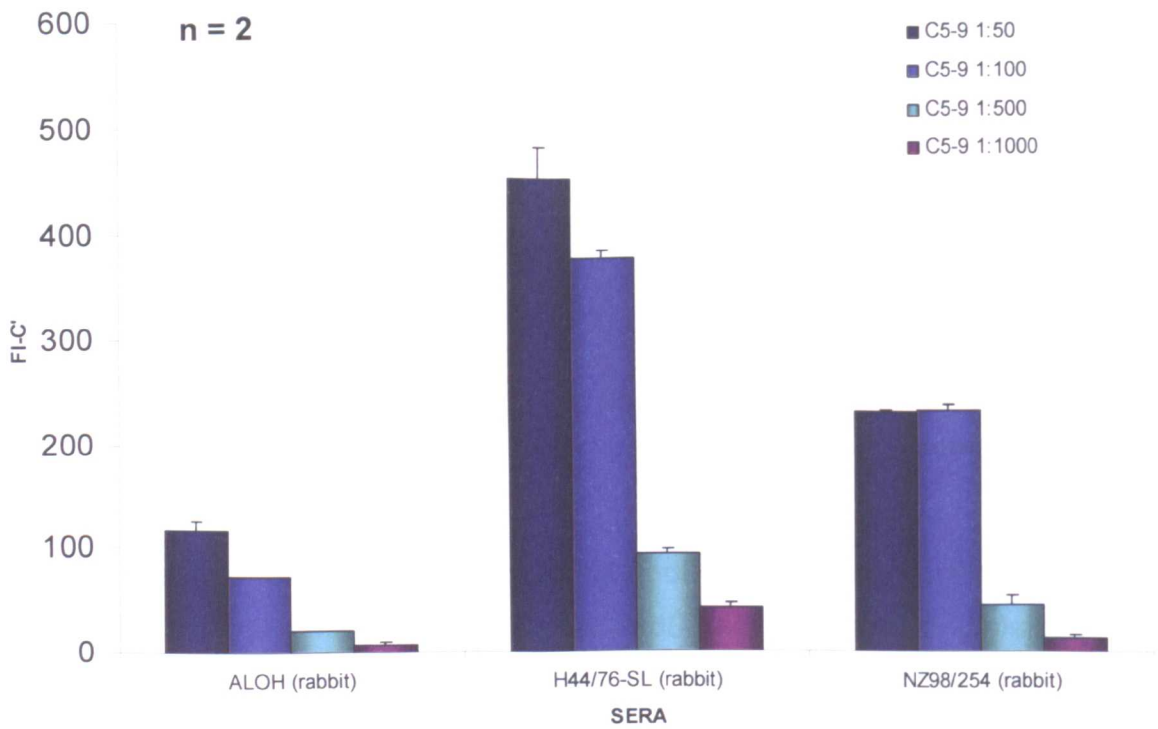
Figure 3.2.1.3 Antibody mediated C5b-9 deposition on *N. meningitidis* with Dako mAb.

Antibody-mediated deposition of C5b-9 on the surface of *N. meningitidis* MO1240149 as detected by the Dako mAb with a secondary label of FITC goat anti-mouse IgG conjugate. n=2 and error bars denote standard deviation.

3.2.2 Correlation of C5b-9 CDA with the SBA.

As both of the tested monoclonals were able to bind to both lytic and non-lytic forms of the MAC, a correlation with the functional bactericidal assay (2.9.10) was determined. A serum panel including a positive control rabbit serum and a selection of high, medium, and low responding human sera were used to assess each mAb. The antibody-dependent deposition of C5b-9 measured with either the Quidel mAb or the Dako mAb was compared with the bactericidal titres obtained against the same *N. meningitidis* strain M01240149. SBA titres and complement deposition Fl-C' values were Log10 transformed to align these ordinal and continuous data sets and a Pearson product moment correlation coefficient was calculated to establish the relationship between the bactericidal titre and the measured deposition of C5b-9 (2.12.4).

The antibody-mediated deposition of C5b-9 measured by the Dako mAb had a non- significant correlation (r=0.43), suggesting that this mAb had a poor ability to distinguish between sera able to induce a high bactericidal response and sera without a bactericidal response (figure 3.2.2.1). This result was consistent with Drogari-Apiranthitou *et al.* who showed this mAb was unable to demonstrate a relationship between the amount of C5b-9 detected on the bacterial surface and the bactericidal titre (Drogari-Apiranthitou *et al.* 2002). Drogari-Apiranthitou *et al.* (2007) used the Dako mAb in a study comparing C5b-9 deposition mediated by sera with or without an SBA titre and showed higher C5b-9 deposition in a high SBA titre serum compared to sera with low SBA activity. However, when using mAb PorA in a dose-dependent manner, they were able to induce dose-dependent bacterial lysis but not C5b-9 deposition.

The antibody-mediated complement deposition measured using the Quidel mAb was significantly correlated with bactericidal titre (r= 0.77, $P<0.05$) (figure 3.2.2.2) and was therefore used for all further assay development.
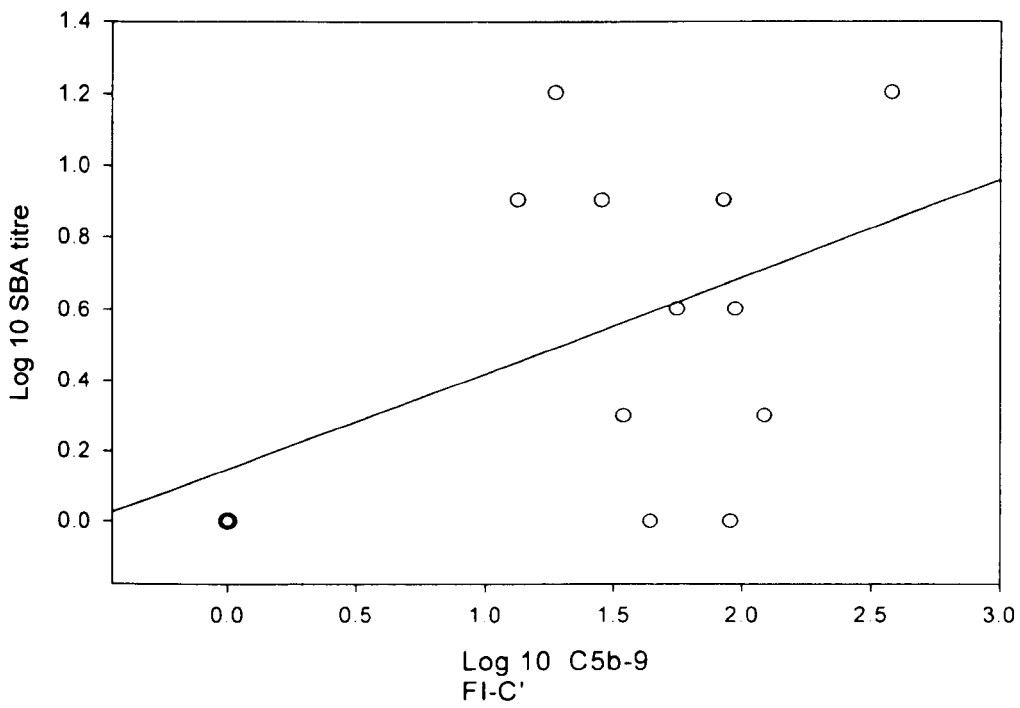
Figure 3.2.2.1 Correlation between log10 bactericidal titre and the log10 FI-C' antibody-mediated deposition of C5b-9 measured using the Dako mAb (r=0.43). Thick blue circle indicates where two points overlay.
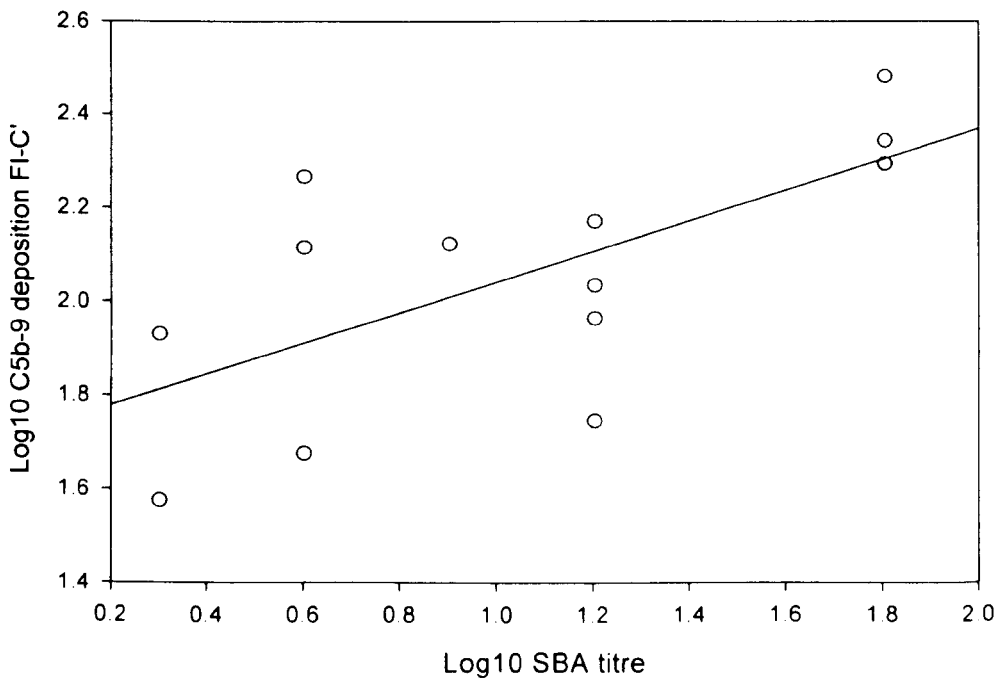
Figure 3.2.2.2 Correlation between log10 bactericidal titre and the log10 FI-C' antibody-mediated deposition of C5b-9 measured using the Quidel mAb (r=0.77, P<0.05).

Significance was determined by t-test.

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3.2.3 Conjugation of monoclonal antibody C5b-9.

The use of mAb C5b-9 (Quidel) and pAb C3b/iC3b antibodies in the same duplexed assay required each antibody to be conjugated to a different fluorochrome. This was advantageous to minimise the use of scarce reagents such as serum and complement. pAb sheep anti human C3b/iC3b antibody was purchased already conjugated to FITC, the Quidel mAb C5b-9 was only available to purchase unconjugated, and therefore the conjugation method and the use of an appropriate fluorochrome were investigated to be compatible with analysis using the 2 laser Beckman Coulter FC500 flow cytometer. Ideally the fluorochrome would be spectrally separate from FITC which is excited by the 488 nm laser. It would be advantageous to use a fluorochrome excited by the 633 nm laser as this would reduce or even negate the requirement for compensation, a procedure which removes all spectral overlap from the flow cytometry results. Firstly a Zenon Alexa fluor 647 nm labelling kit (Invitrogen) was used (2.8.1), which uses labelled Fab fragments directed against the Fc portion of the antibody being conjugated (in this case IgG2b). Any excess labelled Fab fragments remaining in solution are then removed by gel filtration. This system has an advantage over directly labelling the antibody as the process takes only 5 min. Labelling intensity can also be altered by adjusting the concentration of labelled Fab fragments added to the test antibody until a saturation point is reached.

The manufacturer's guidelines were followed for calculation of appropriate amounts of Zenon Alexa flour 647nm labelled Fab fragments. A large Stokes shift was observed between antibody-mediated C5b-9 deposition by the homologous OMV rabbit serum compared to the complement only background control (figure 3.2.3.1) when using Zenon Alexa flour 647nm conjugated C5b-9 mAb. The conjugation was repeated using the same method, however on several occasions

the staining procedure failed, leading to the conclusion that this would not be a consistent way of labelling the antibody.

In order to obtain a large volume of fluorescently labelled conjugate, which could be used for all assays in this study thus improving assay reproducibility, the Invitrogen custom conjugation service was used. Alexa fluor 647 fluorochrome was chosen as it was excited by the 633 nm laser and was spectrally different from the FITC-labelled C3b/iC3b conjugate, which was excited by the 488 nm laser (figure 3.2.3.3), thus reducing the amount of compensation required during analysis of CDA results. Use of the custom conjugated antibody in a CDA showed a detectable Stokes shift of antibody-mediated complement deposition of C5b-9 on to *N. meningitidis* 44/76-SL by the OMV positive rabbit serum compared with the complement only background control (figure 3.2.3.2).
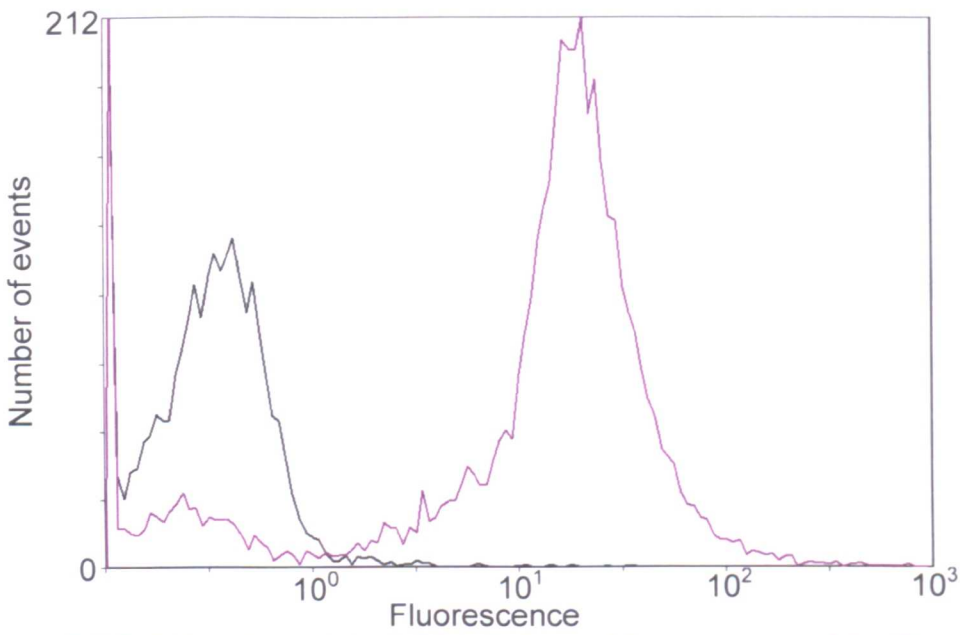
Figure 3.2.3.1 Overlay plot of C5b-9 deposition measured by Zenon labelled C5b-9 mAb.

Overlay plot of antibody-mediated deposition of C5b-9 on *N. meningitidis* 44/76-SL showing a complement only control (black) and a positive OMV rabbit control serum (purple) using Zenon 647 nm labelled mAb C5b-9 (Quidel).
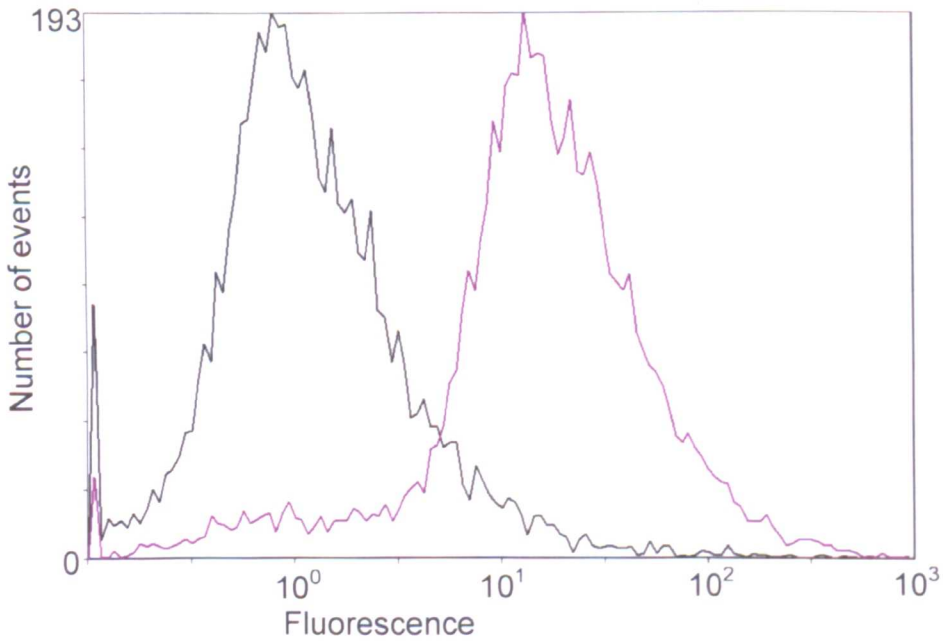
Figure 3.2.3.2 Overlay plot of C5b-9 deposition measured by Alexa fluor 647 labelled mAb.

Antibody-mediated deposition of C5b-9 deposition on *N. meningitidis* 44/76-SL of a complement only control (black) and a positive OMV rabbit control serum (purple) using custom conjugated Alexa fluor 647 mAb C5b-9 (Quidel).
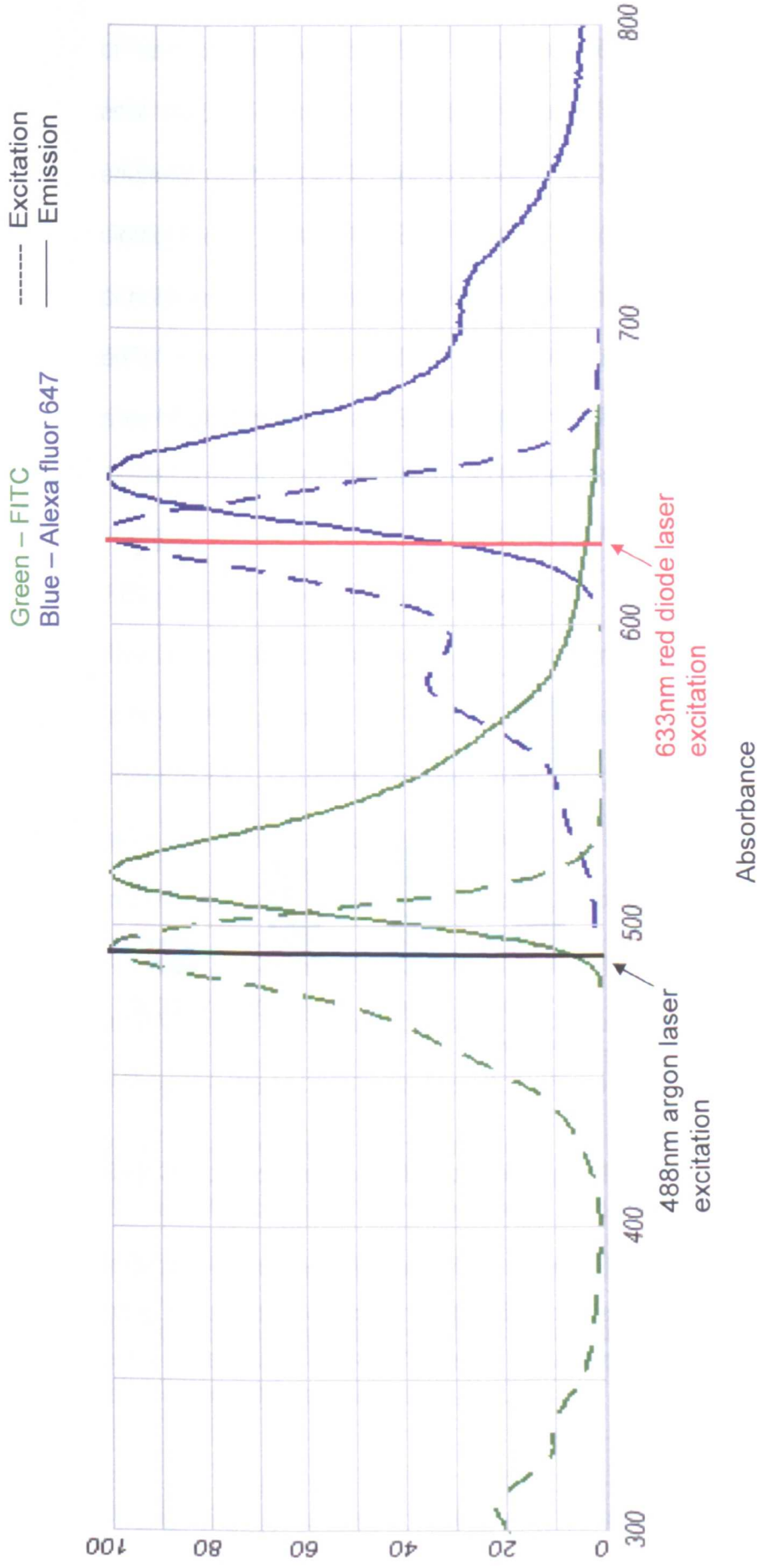
Figure 3.2.3.3 Excitation and emission spectra for FITC and Alexa fluor 647.

Alexa fluor 647 stain was choosen as a fluorochrome for both methods of conjugation.

3.2.4 Titration of monoclonal antibody C5b-9.

Following the successful conjugation of the mAb, a titration was performed to identify the optimal antibody concentration for use in the assay. A CDA was performed against *N. meningitidis* 44/76-SL using a range of mAb C5b-9 dilutions from 1:100 to 1:16000. Antibody-mediated C5b-9 deposition on *N. meningitidis* 44/76-SL with homologous strain OMV rabbit control serum showed the response began to decrease at the 1:4000 dilution with the optimal dilution found to be 1:2000 (figure 3.2.4.1). A significantly better response (Z-test) was observed at a dilution of 1:2000 than using the conjugate at 1:4000 ($P <0.01$).
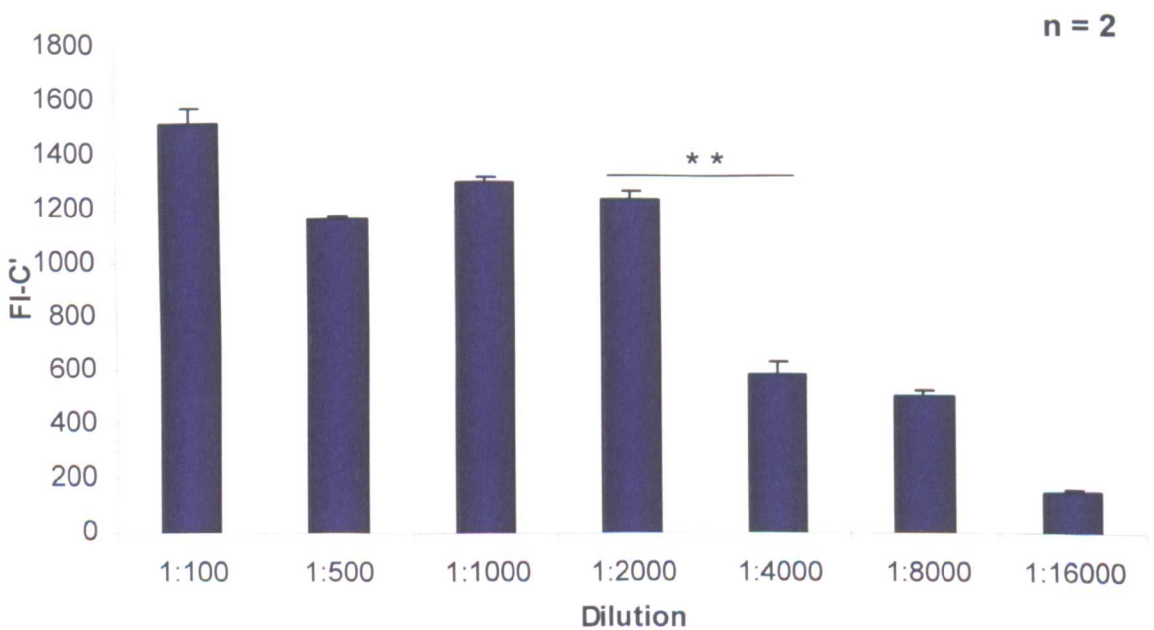
Figure 3.2.4.1 Optimisation of antibody dilution for detection of C5b-9 deposition.

Antibody-mediated deposition of C5b-9 using OMV homologous rabbit control serum on *N. meningitidis* 44/76-SL measured using different mAb C5b-9 conjugate concentrations. Significance was determined by z-test (**$P<0.01$), and error bars denote standard deviation.

3.2.5 Correlation of antibody surface binding on killed or live *N. meningitidis*.

The use of killed bacteria has advantages as it allows the ability to grow and kill a large stock for use over a large study, and it means that assays can be performed without containment, eliminating variation between cultures, which is a considerable problem when using *N. meningitidis* (Bai and Borrow 2010). It also enables the assay to be performed outside of containment in a high throughput manner, an important aspect of any assay required to assess large panels of sera against a panel of strains. However, there have been concerns that killing the bacteria could lead to normally hidden epitopes being exposed on the bacterial surface. Michaelsen *et al.* (2001) described the effects of a variety of different killing methods on the exposure of the PorB3 epitope, illustrating that ethanol fixation and heat killing methods were very damaging to the bacterial surface and altered the exposure of PorB3. Thus a comparison was made between the killing method (2.3.1) utilised for the *N. meningitidis* used in this assay (azide/PMSF), with live bacteria. Azide/PMSF bacterial killing method (2.3.1) was developed to minimise any disturbance of surface epitopes with the azide acting by irreversibly binding cytochrome c and therefore preventing respiration. PMSF was introduced to prevent any protease degradation of surface proteins. The comparison between killing methods was conducted using a total antibody surface binding assay, (2.9.1) with a panel of low, medium and high responding human sera. Live and azide killed bacteria were incubated with sera, washed and incubated with anti-human-FITC conjugate and then fixed with 2% formaldehyde for 1 h and analysed by flow cytometry. Total IgG binding for each test sera was compared between live and azide killed bacteria (figure 3.2.5.1).

A significant positive correlation was observed between the total antibody binding measured with live *N. meningitidis* against that measured with azide/PMSF-killed

bacteria (r= 0.93, *P* <0.01) (figure 3.2.5.1). Azide-killed bacteria showed a higher FI-C than the live bacteria. This could be possibly due to disruption of capsule by the killing process, but because of the high correlation any conclusions drawn about the response would be similar in either live or killed bacteria. However this reinforces the need to compare data obtained using killed bacteria with data obtained with live organisms, particularly where killing is the readout of the functional assay.
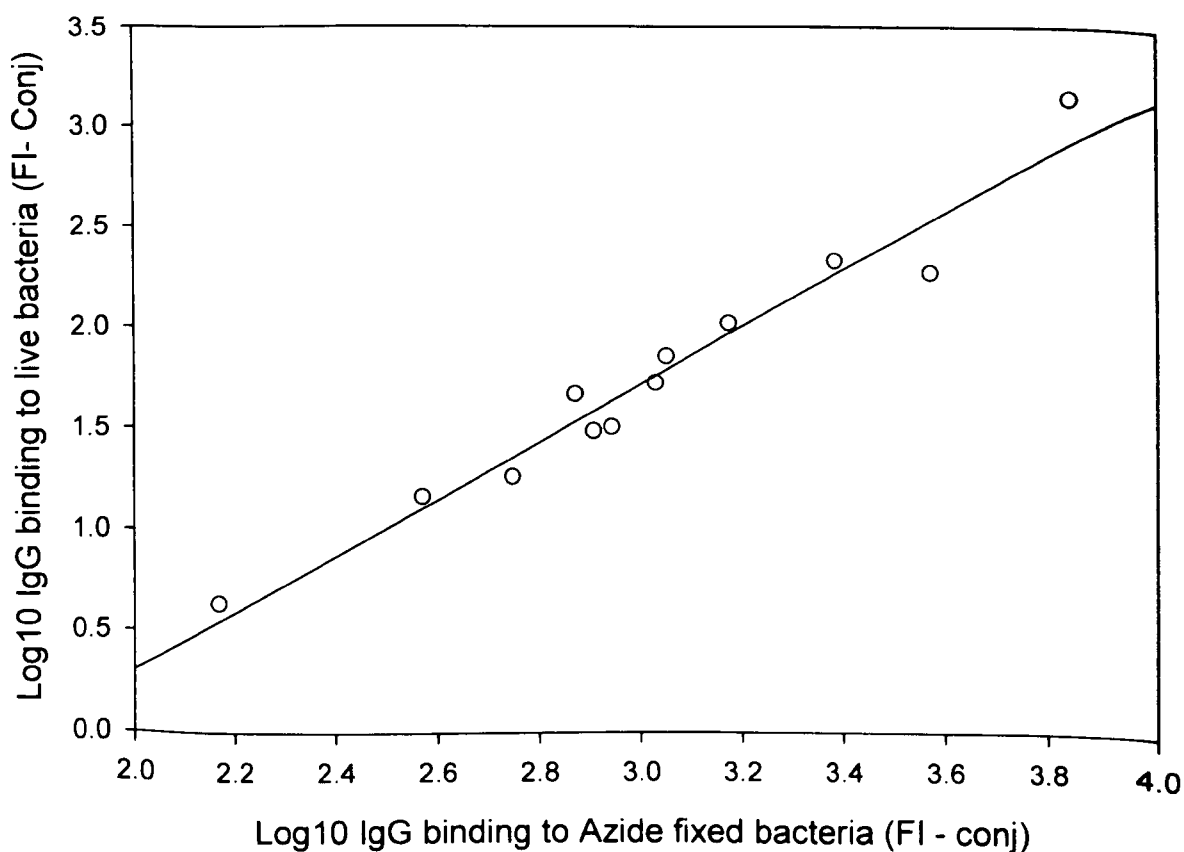
Figure 3.2.5.1 Correlation of total antibody binding between live and killed *N. meningitidis*.

Correlation of total antibody surface binding on live *N. meningitidis* strain H44/76 with azide/PMSF-killed *N. meningitidis* strain H44/76 (r=0.93, *P*<0.01). Significance was determined by t-test.

3.2.6 Duplexing the measurement of C3b/iC3b and C5b-9.

Duplexing the measurement of C3b/iC3b and C5b-9 required care to ensure that the complement deposition determined following duplexing was equivalent to C3b/iC3b or C5b-9 deposition determined in isolation. Duplexing of the flow cytometry analyses risks false readings resulting from either steric hindrance or from fluorescence resonance energy transfer (FRET). FRET occurs when two fluorochromes are 5 nm or closer and usually occurs between fluorochromes with similar emission spectra (Szollosi *et al.* 1998), this was not expected due to the chosen fluorochromes; but was investigated. A duplexed assay was performed (2.9.4) alongside separate assays measuring the C3b/iC3b (2.9.2) and C5b-9 deposition (2.9.3) and the results compared.

Antibody-mediated deposition of C3b/iC3b on to *N. meningitidis* M01240149 measured in the duplexed assay, correlated highly with the assay measuring this response individually (r=0.99, *P*<0.01) (figure 3.2.6.1a). The antibody-mediated deposition of C5b-9 measured in the duplexed assay also correlated strongly with the individual assay (r=0.95, *P*<0.01) (figure 3.2.6.1b). Differences between the Fl-C' values could be seen in the measurement of C5b-9 deposition, with the duplexed responses lower, possibly as a result of the small amount of compensation applied to the data during analysis to remove spectral overlap between the two fluorochromes used to label the antibodies. Therefore it was concluded that the duplexed assay could be utilised.
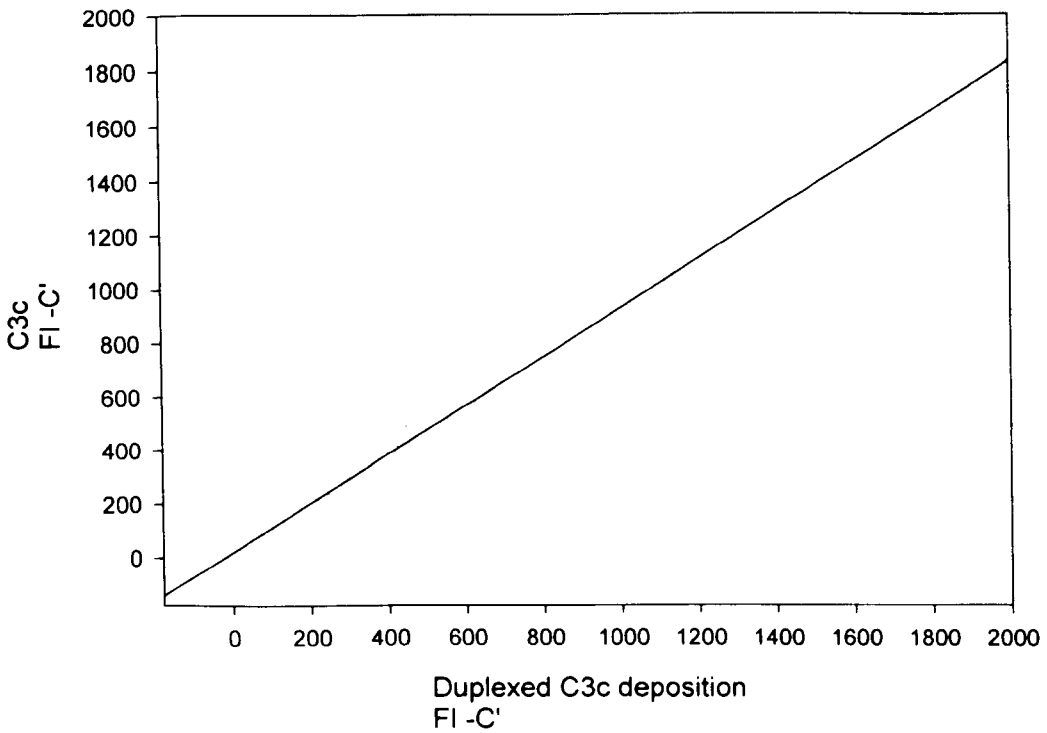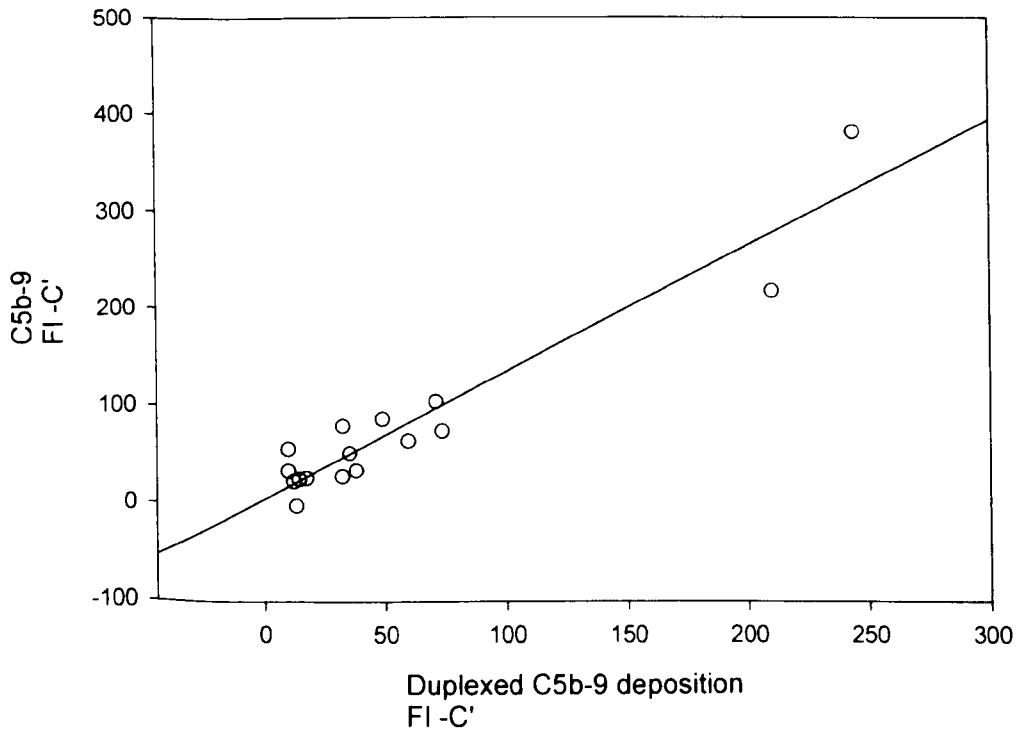
Figure 3.2.6.1 Correlation of duplexed antibody-mediated C3b/iC3b and C5b-9 deposition on *N. meningitidis* MO1240149 in comparison with C3b/iC3b measured separately (r=0.99, *P*<0.01) (a) and C5b-9 measured separately (r=0.95, *P*<0.01) (b).

Significance was determined by t-test.

3.3 Optimisation

3.3.1 Optimisation of duplexed complement deposition assay - effect of incubation time and temperature.

Previously published assays for measurement of antibody mediated C3b/iC3b deposition were performed at 25°C (Welsch *et al.* 2003; Taylor 2010). However, OPA and SBA both measure the functional effect of complement and are incubated at 37°C, the biologically relevant temperature. The effect of temperature was therefore investigated.

OPA uses 7.5 min incubation for opsonisation of antibody and initiation of complement deposition (C3b/iC3b), whereas the SBA uses an incubation of 60 min for the deposition of C5b-9 membrane attack complex to measure bacterial killing. Thus the optimal incubation time point for use in this duplexed assay was investigated. The CDA was performed at both 25°C and 37°C from 7.5 min up to 60 min.

There was a significant increase ($P<0.05$) in the deposition of C5b-9 and C3b/iC3b when the assay incubation temperature was changed from 25°C to 37°C (figure 3.3.1.2 and 3.3.1.1 A-E). Thus the results supported the use of 37°C for the first assay incubation temperature to provide the optimal response.

A smaller Stokes shift was observed when comparing C3b/iC3b deposition in the complement only control and the homologous rabbit positive control serum at 25°C, than at 37°C (figure 3.3.1.4 and 3.3.1.5). This pattern was also seen when comparing the deposition of C5b-9 (figure 3.3.1.6 and 3.3.1.7). Once again the

peak shift between the controls and the positive rabbit serum was greater at 37°C than at 25°C.

Different incubation time points were tested at 37°C showing significant ($P<0.05$) increases in antibody-mediated deposition of C5b-9 between 7.5 min and 15 min, 15 min and 30 min for homologous rabbit positive control serum tested. A significant ($P<0.05$) increase in response between 30 min and 45 min was observed (figure 3.3.1.3a). This pattern was also observed in the deposition of C3b/iC3b (figure 3.3.1.3b). There was no significant increase in response observed following 60 min incubation. Therefore 45 min incubation gave the optimum deposition of C5b-9. Deposition of C3b/iC3b was optimal at 30 min, however an incubation of 45 min did not cause any significant difference ($P<0.05$) in the deposition of C3b/iC3b.
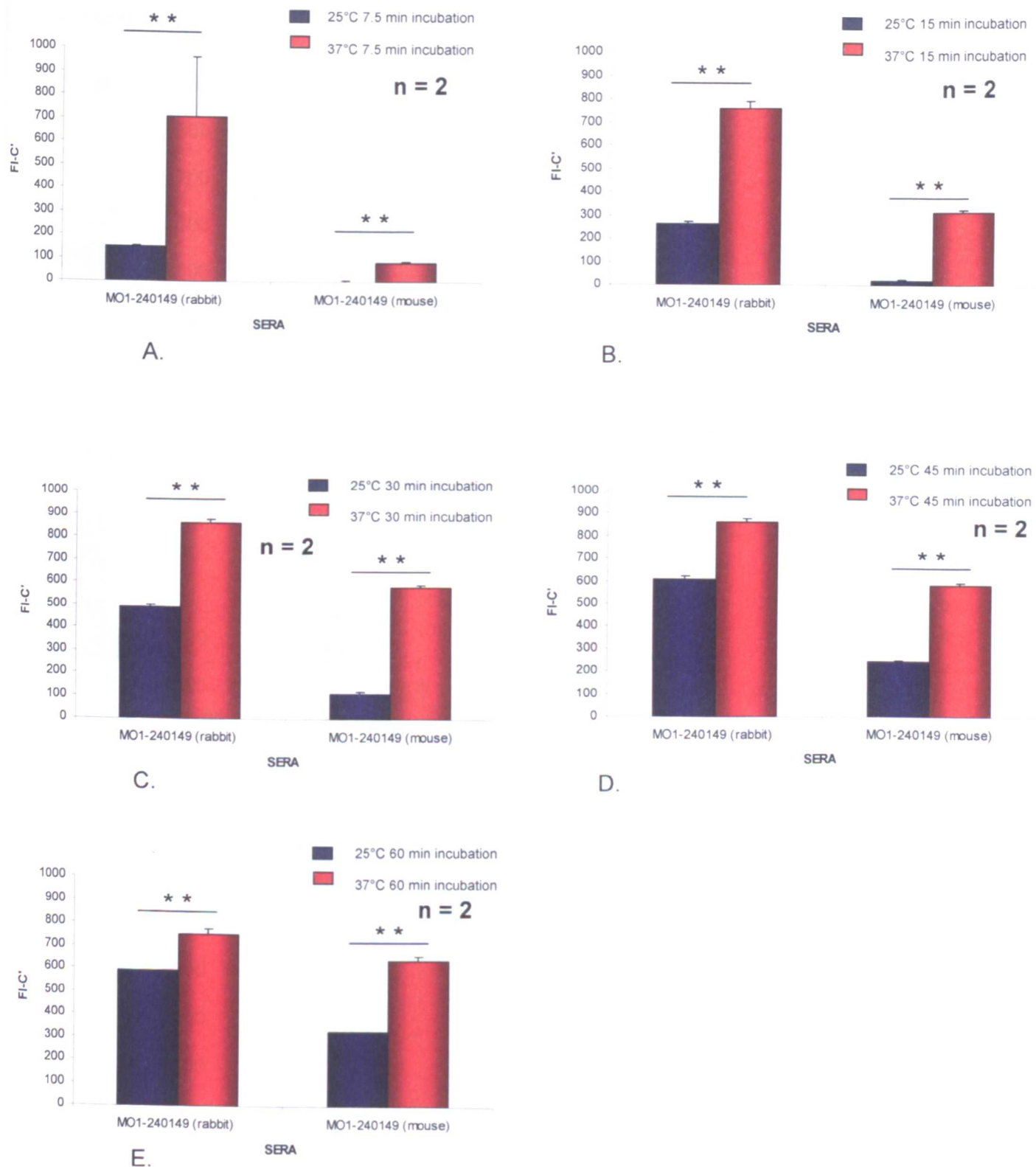
Figure 3.3.1.1 Optimisation of incubation temperature for C3b/iC3b deposition in the first assay incubation.

Effect of incubation time at 25°C or 37°C on antibody-mediated deposition of C3b/iC3b on *N. meningitidis* M01240149. Significance was determined by z-test (**P<0.01), and error bars denote standard deviation.
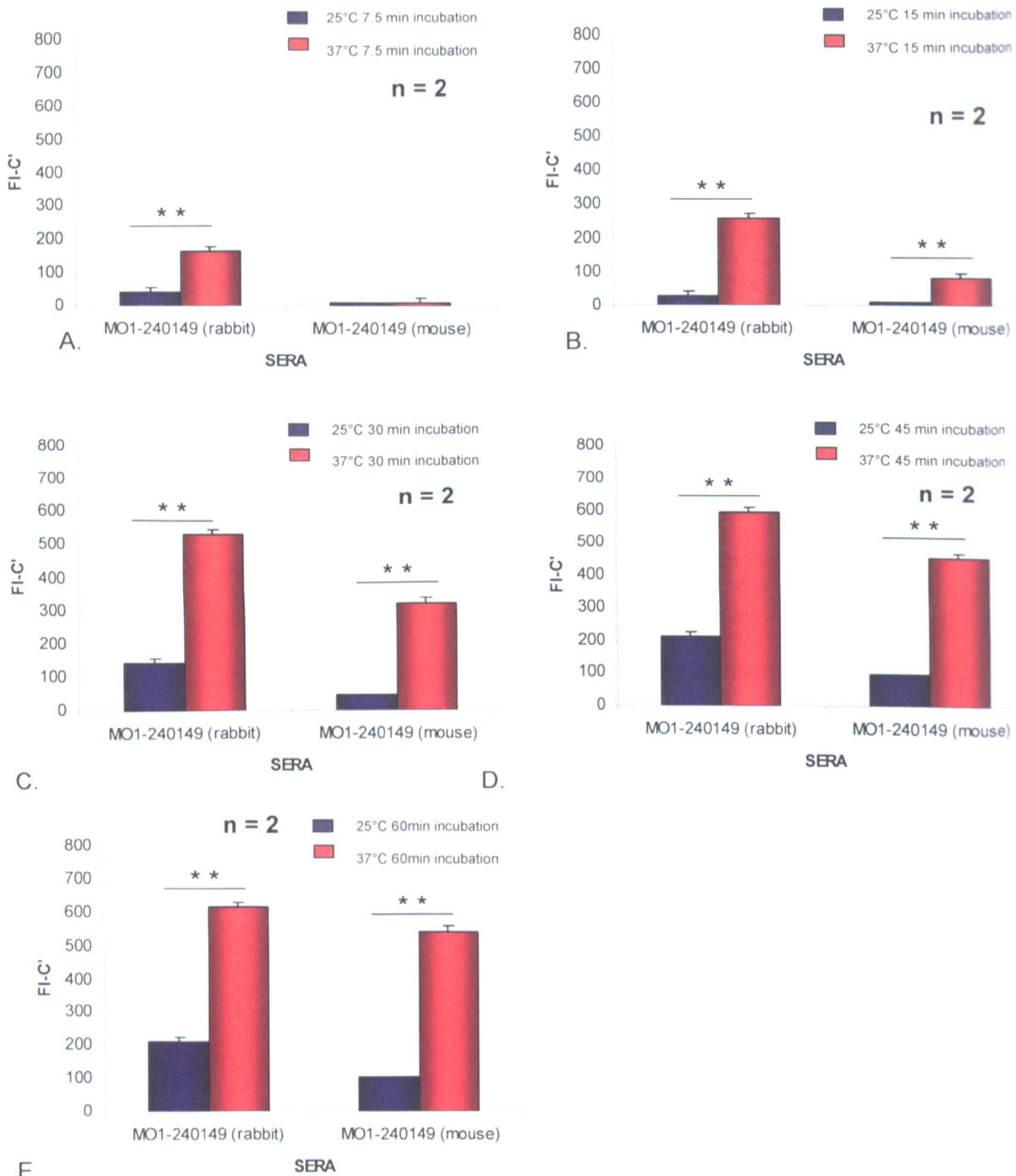
Figure 3.3.1.2 Optimisation of incubation temperature for C5b-9 deposition in the first assay incubation.

Effect of incubation time at 25°C or 37°C antibody-mediated deposition of C5b-9 on *N. meningitidis* M01240149. Significance was determined by z-test (**$P<0.01$), and error bars denote standard deviation.
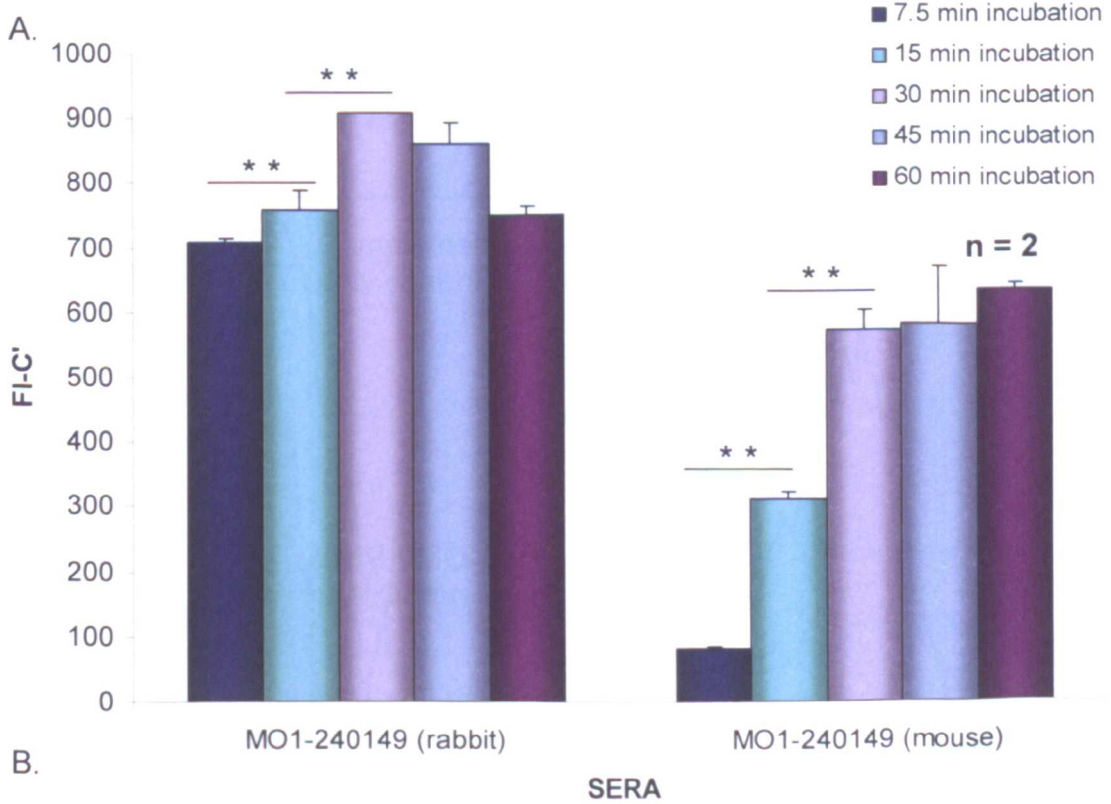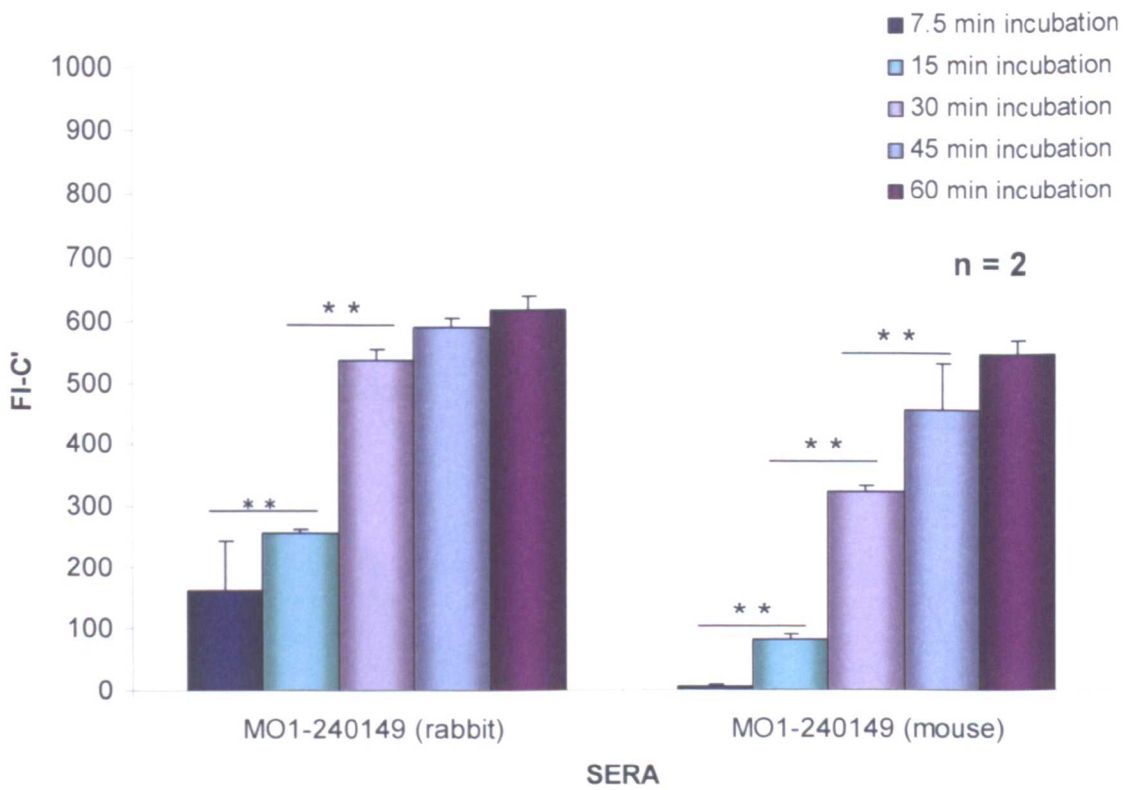
Figure 3.3.1.3 Optimisation of first assay incubation time on C3b/iC3b and C5b-9 deposition.

Effect of incubation time at 37°C on antibody-mediated deposition of A; C5b-9 and B; C3b/iC3b on *N. meningitidis* M01240149. Significance was determined by z-test (**$P<0.01$), and error bars denote standard deviation.
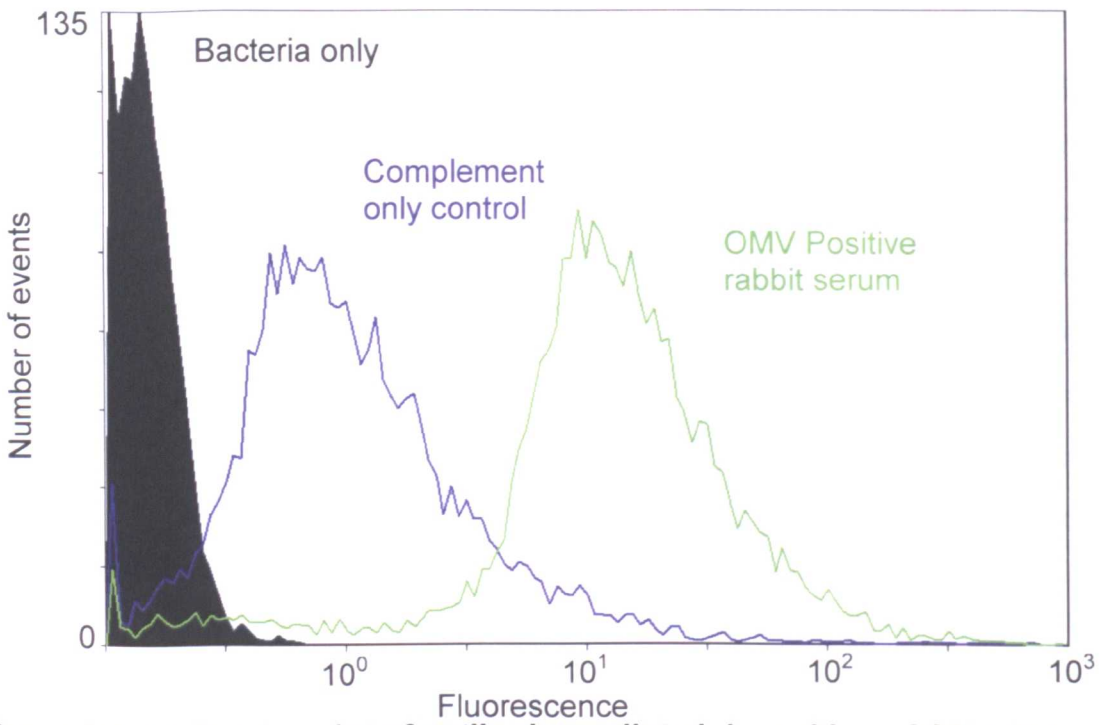
Figure 3.3.1.4 Overlay plot of antibody-mediated deposition of C3b/iC3b following incubation at 37°C.

Assay performed with *N. meningitidis* M01240149.
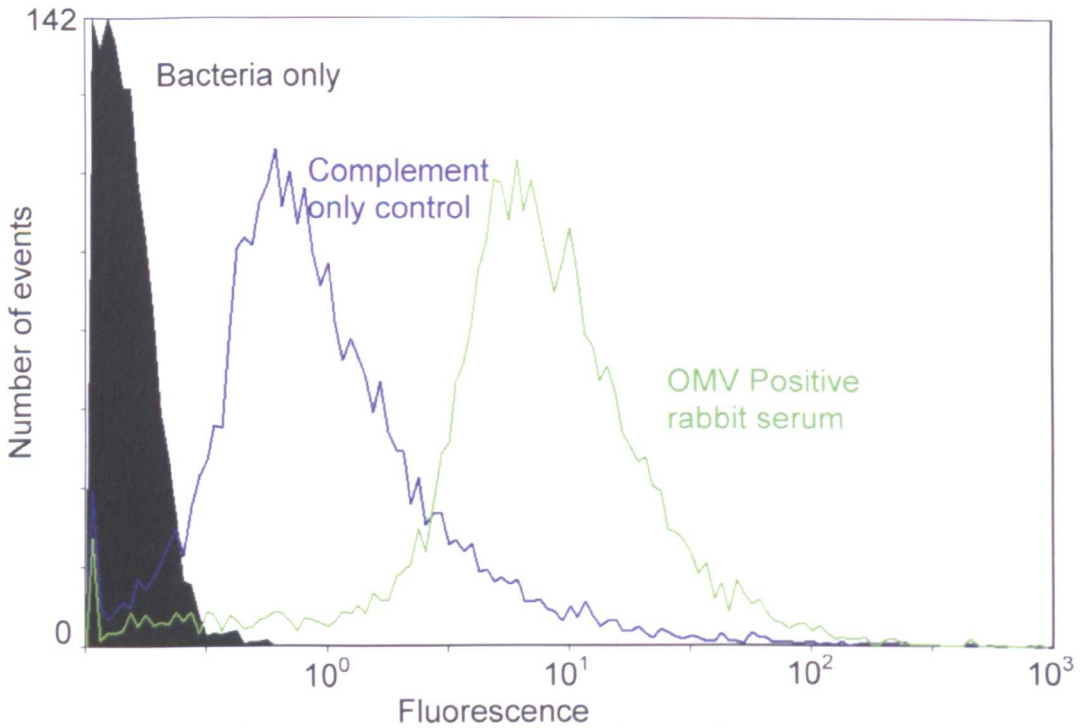
Figure 3.3.1.5 Overlay plot of antibody-mediated deposition of C3b/iC3b following incubation at 25°C.

Assay performed with *N. meningitidis* M01240149.
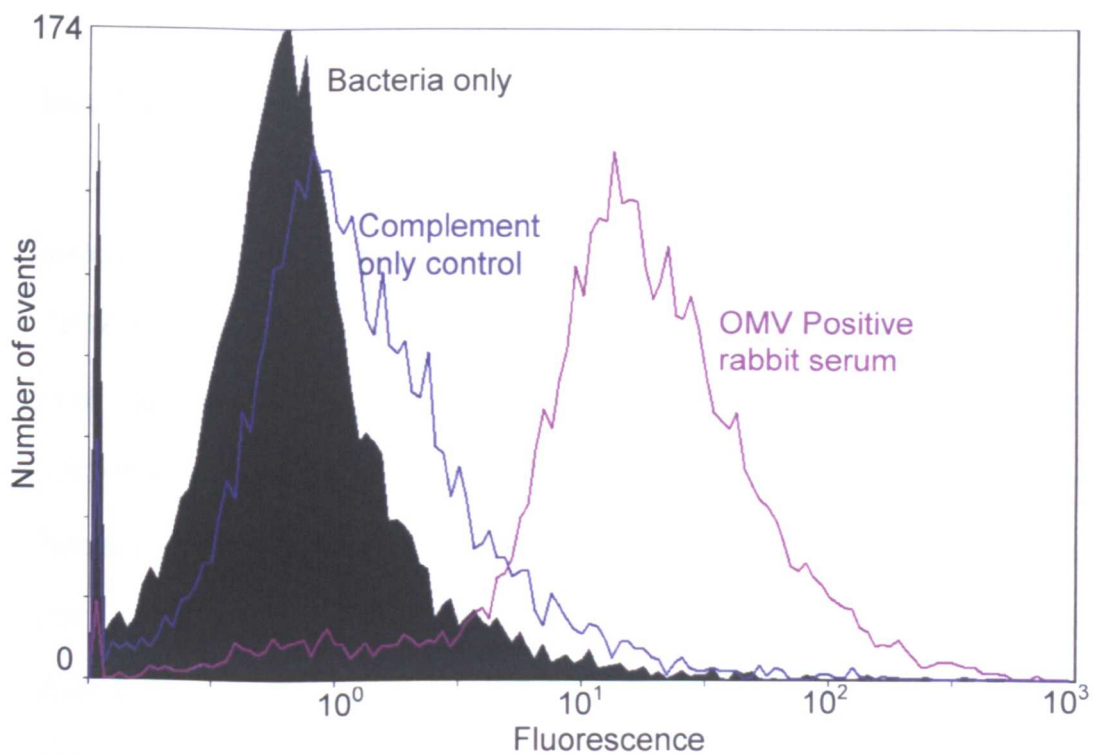
Figure 3.3.1.6 Overlay plot of antibody-mediated deposition of C5b-9 following incubation at 37°C.

Assay performed with *N. meningitidis* M01240149.
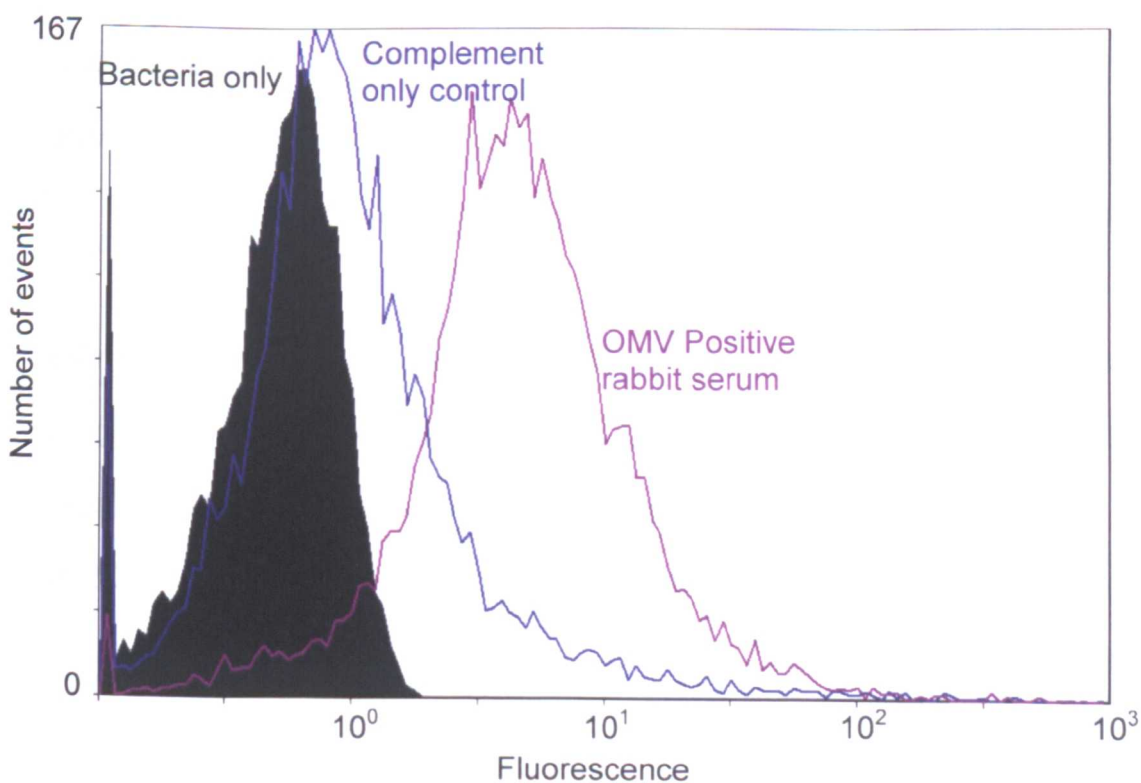
Figure 3.3.1.7 Overlay plot of antibody-mediated deposition of C5b-9 following incubation at 25°C.

Assay performed with *N. meningitidis* M01240149.

3.3.2 Comparison of antibody-mediated complement deposition at 25°C and 37°C and correlation with SBA.

The optimal temperature in the first assay incubation time point for the greatest response (antibody-mediated complement deposition) was observed at 37°C. Thus a study was performed to evaluate whether this change in temperature from 25°C affected the correlation of antibody-mediated complement deposition of C5b-9 with SBA and OPA with C3b/iC3b, and whether the temperature change affected assay reproducibility. A panel of 16 sera consisting of low, medium and high responding human sera and rabbit sera homologous (high SBA) and heterologous (low SBA) to the *N. meningitidis* test strain (M01240149) were assayed. Each serum was assayed in duplicate and the assay was repeated three times by a single operator and the results analysed.

Greater assay variation was observed in C3b/iC3b deposition at 37°C than at 25°C (table 3.3.2.1), with more duplicates showing a higher % CoV. This pattern was repeated in the antibody-mediated C5b-9 deposition results (table 3.3.2.2).

The correlation between antibody-mediated C5b-9 deposition and SBA titre was higher at 37°C than at 25°C (r=0.84, *P*<0.05 at 37°C and r=0.54 at 25°C) (figure 3.3.2.3 and 3.3.2.4). This was also seen when measuring antibody-mediated C3b/iC3b deposition and OPA (r=0.93, *P*<0.05 at 37°C and r=0.76, *P*<0.05 at 25°C) (figure 3.3.2.1 and 3.3.2.2).

Intra-assay variability at 25°C		<15%	<35%	>35%
16 sera in duplicate, performed three times by one operator on one day. n=42	16 Sera with defined CoV	13	1	2

Intra-assay variability at 37°C		<15%	<35%	>35%
16 sera in duplicate, performed three times by one operator on one day. n=42	16 Sera with defined CoV	2	8	6

Table 3.3.2.1 Summary of the CoV for the antibody-mediated C3b/iC3b deposition at 25°C and 37°C.
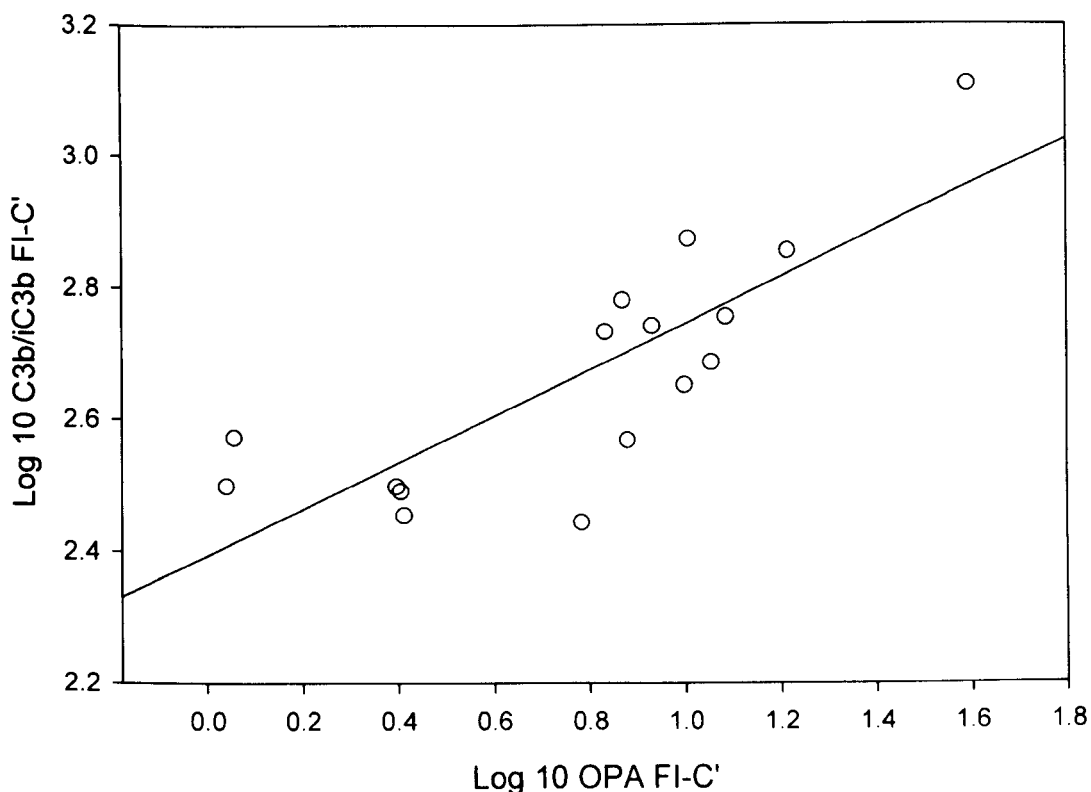
Figure 3.3.2.1 Correlation between OPA and C3b/iC3b deposition performed at 37°C.

Correlation of antibody-mediated C3b/iC3b deposition on *N. meningitidis* M01240149 performed at 37°C, with the OPA with 16 human sera ($r=0.93$, $P<0.05$). Significance was determined by t-test.

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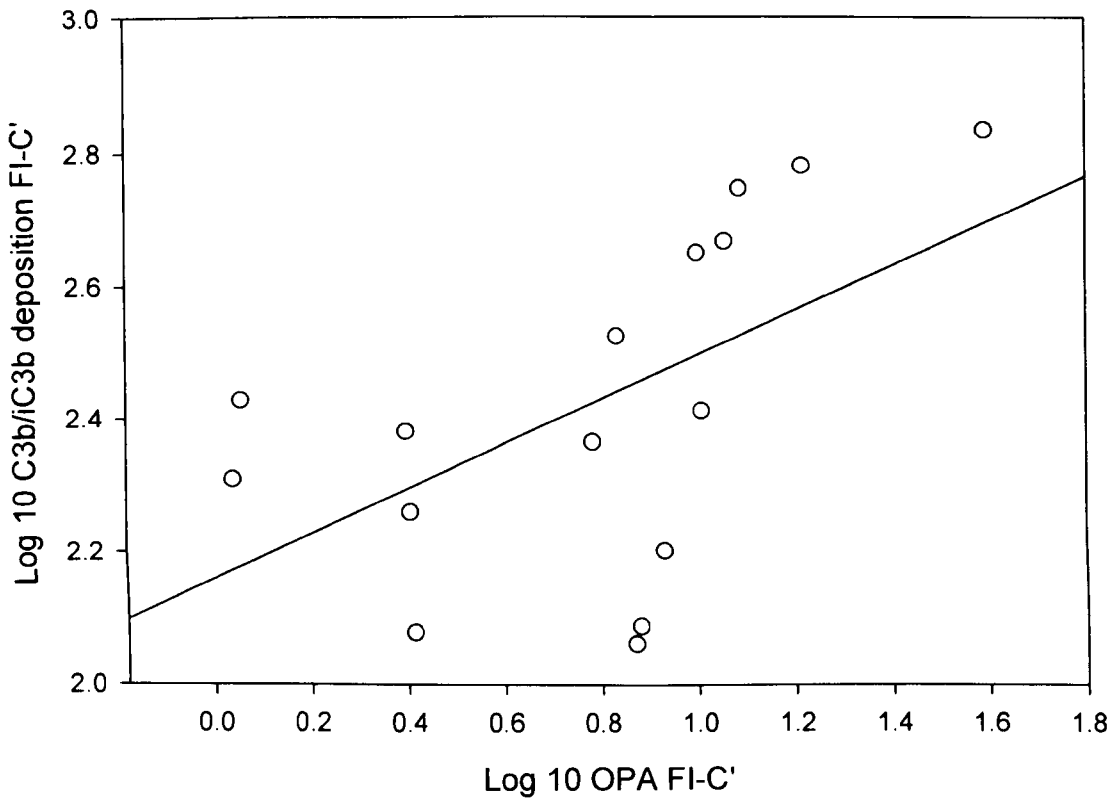
Figure 3.3.2.2 Correlation between OPA and C3b/iC3b deposition performed at 25°C.

Correlation of antibody-mediated C3b/iC3b deposition on *N. meningitidis* M01240149 performed at 25°C, with the OPA with 16 human sera ($r=0.76$, $P<0.05$). Significance was determined by t-test.

Intra-assay variability at 25°C		<15%	<35%	>35%
16 sera in duplicate, performed three times by one operator on one day. n=42	16 Sera with defined CoV	7	7	2

Intra-assay variability at 37°C		<15%	<35%	>35%
16 sera in duplicate, performed three times by one operator on one day. n=42	16 Sera with defined CoV	6	6	4

Table 3.3.2.2 Summary of the CoV for the antibody-mediated C5b-9 deposition for 25°C and 37°C.
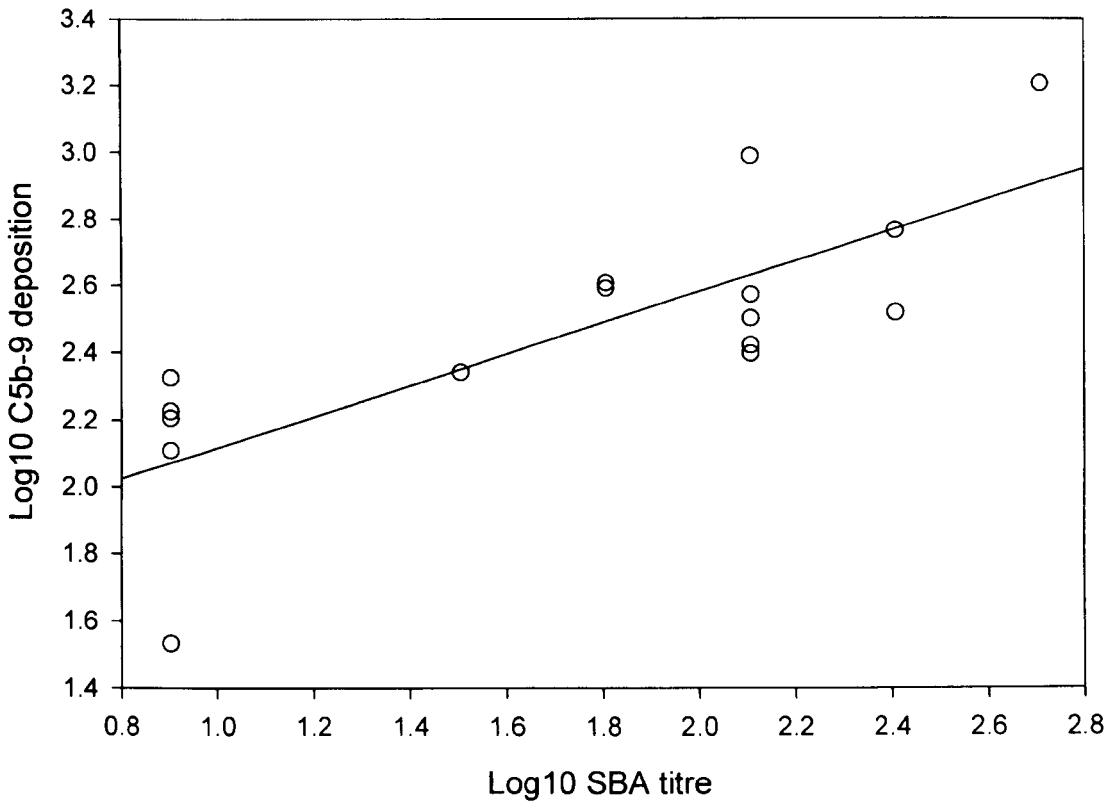
Figure 3.3.2.3 Correlation between SBA and C5b-9 deposition performed at 37°C.

Correlation of antibody-mediated C5b-9 deposition on *N. meningitidis* M01240149 and SBA titre at 37°C with 16 human sera (r=0.84, *P*<0.05). Significance was determined by t-test.
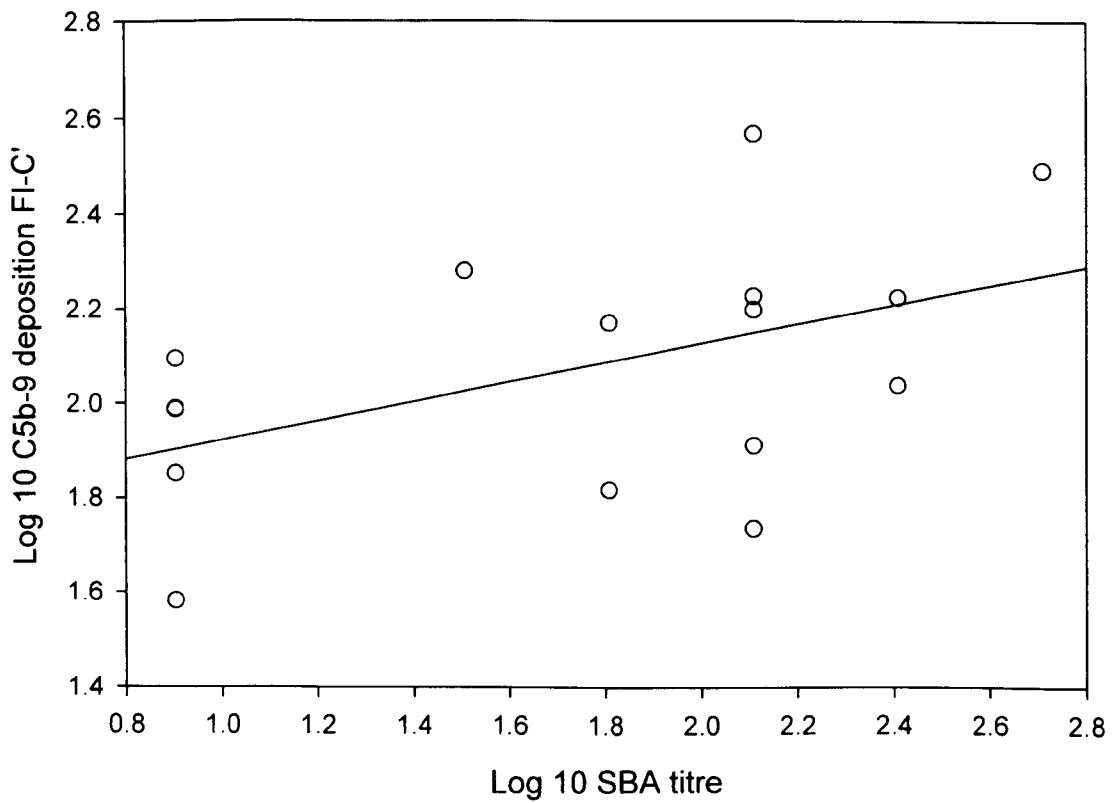
Figure 3.3.2.4 Correlation between SBA and C5b-9 deposition performed at 25°C.

Correlation of antibody-mediated C5b-9 deposition on *N. meningitidis* M01240149 and SBA titre at 25°C with 16 human sera ($r=0.54$, $P<0.05$). Significance was determined by t-test.

3.3.3 Optimisation of incubation time for anti C3b/iC3b and C5b-9 conjugates in the antibody-mediated complement deposition assay.

Following optimisation of the first incubation step combining serum, complement and bacteria, the effect of incubation time with conjugate was also determined at 4°C, and was performed either with shaking at 900 rpm or stationary. The second incubation temperature was kept at 4°C to prevent any further complement being deposited on the bacterial surface. Significantly higher antibody-mediated C5b-9 deposition responses were observed following 20 minutes incubation with anti-C5b-9, than at 10 min or 30 min ($P<0.05$) (figure 3.3.3.1a). There was no significant difference in the deposition of C3b/iC3b observed between any of the incubation times tested (figure 3.3.3.1b). Thus a second incubation of 20 min at 4°C would be adopted for the duplexed assay.

The effect of incubation with or without shaking was also determined. No significant difference in deposition of C5b-9 (figure 3.3.3.2a) and C3b/iC3b (figure 3.3.3.2b) was observed with and without shaking. Thus the second incubation step was maintained at 4°C without shaking.
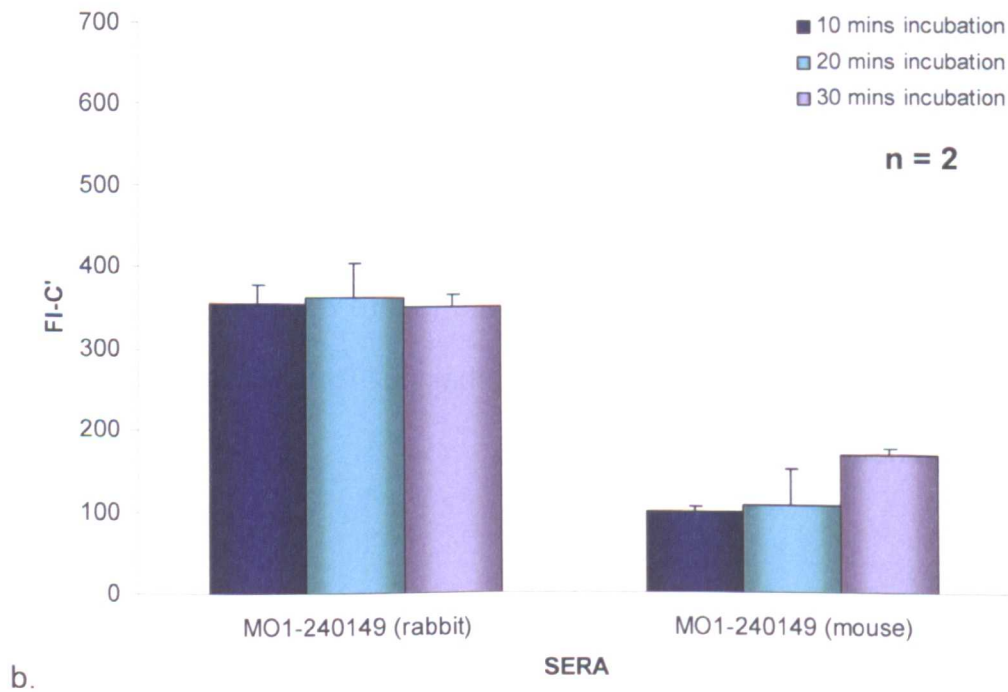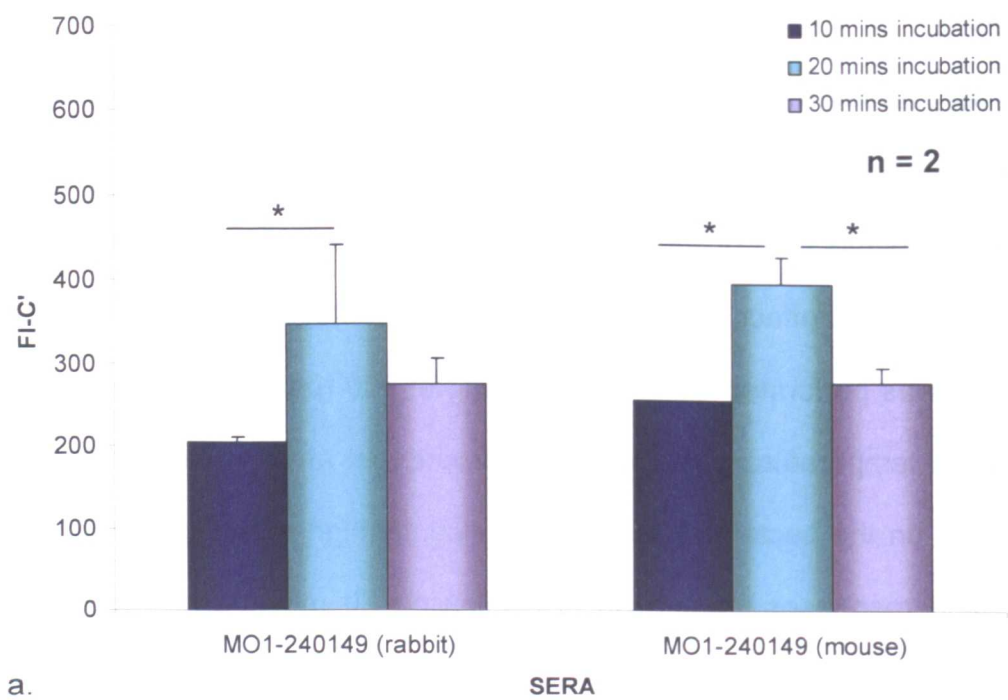
Figure 3.3.3.1 Effect of conjugate incubation time on antibody-mediated C5b-9 (a) and C3b/iC3b (b) deposition on *N. meningitidis* MO1240149.

Sera are rabbit or mouse sera raised against M01240149 OMVs. Significance was determined by z-test (*P<0.05), and error bars denote standard deviation.
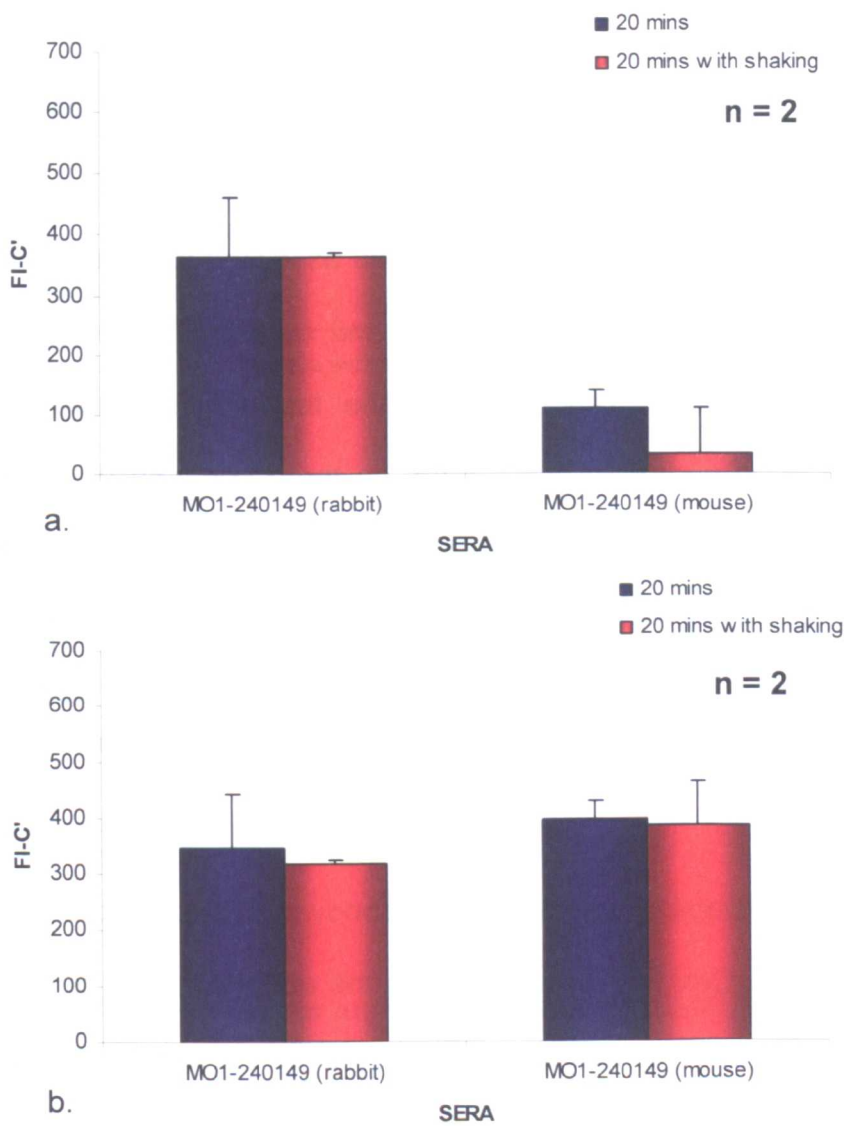
Figure 3.3.3.2 Effect of incubation with or without shaking on antibody-mediated C5b-9 (a) or C3b/iC3b (b) deposition on *N. meningitidis* MO1240149.

Sera are rabbit or mouse sera raised against M01240149 OMVs. Significance was determined by z-test and error bars denote standard deviation.

3.4 Precision of duplexed complement deposition assay measuring both deposition of C3b/iC3b and C5b-9.

The reproducibility of the duplexed assay was assessed using a panel of 20 low, medium and high responding human sera and sera from rabbits raised against homologous and heterologous OMVs to the test strain. These assays were completed by three operators in triplicate, on three separate days, and data for each serum was analysed to assess the precision of results. All assays were conducted using the same sera and complement source and then analysed using the same flow cytometer and protocol. The results were then analysed and the coefficient of variance (CoV) calculated.

The intra-assay variability was low for antibody-mediated C3b/iC3b deposition, with 14 sera with a CoV less than 15%, and the remaining sera all demonstrating CoV less than 35%. There was more variation observed when this was assessed with one operator completing three assays over three days, this was anticipated and still remained acceptable with 16 out of 20 of the sera demonstrating CoV less than 35% (table 3.4.1).

The intra-assay and inter-assay variability was low for antibody-mediated C5b-9 deposition, with 16 out of 20 sera showing CoV less than 35%. The inter-operator variability was again acceptable with the 19 out of 20 sera with CoVs less than 35%. (table 3.4.2). This data was correlated with the bactericidal titre once again and showed a highly significant correlation (r=0.82, *P*<0.01) (figure 3.4.1).

Intra-assay variability		<15%	<35%	>36%
20 sera in duplicate, performed three times by one operator on one day. n=60	**20 Sera with defined CV**	14	6	0

Inter-assay variability		<15%	<35%	>36%
20 sera in duplicate, performed three times by one operator on three days. n= 180	**20 Sera with defined CV**	3	13	4

Inter-operator variability		<15%	<35%	>36%
20 sera in duplicate, performed three times by three operators on one day. n=180	**20 Sera with defined CV**	14	5	1

Table 3.4.1 Assay precision for antibody mediated C3b/iC3b deposition.

Intra-assay variability

20 sera in duplicate,
performed three times by
one operator on one day.
n=60

	<15%	<35%	>36%
20 Sera with defined CV	15	5	0

Inter-assay variability

20 sera in duplicate,
performed three times
by one operator on
three days. n= 180

	<15%	<35%	>36%
20 Sera with defined CV	2	15	3

Inter-operator variability

20 sera in duplicate,
performed three times by
three operators on one day.
n=180

	<15%	<35%	>36%
20 Sera with defined CV	7	10	3

Table 3.4.2 Assay precision for antibody mediated C5b-9 deposition.
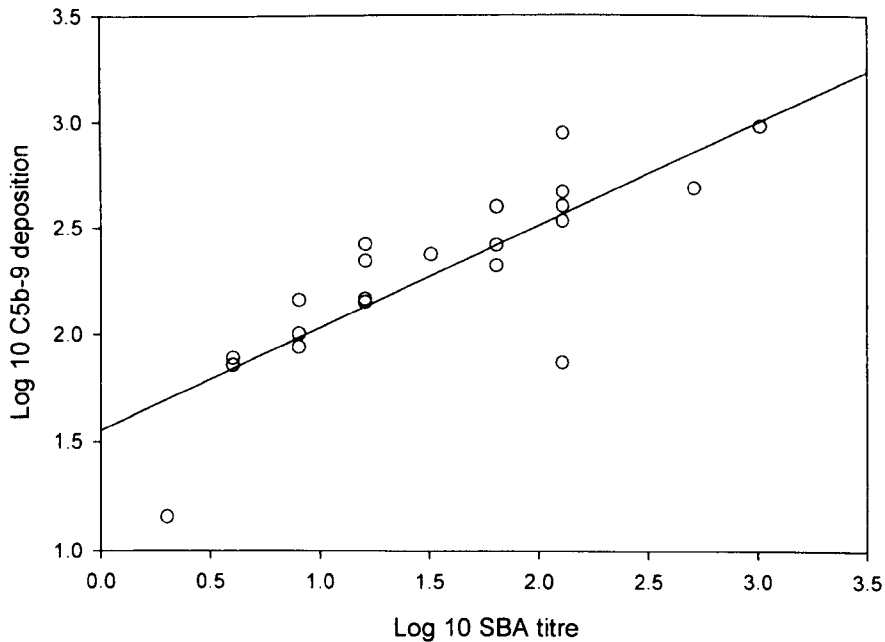
Figure 3.4.1 Correlation of SBA with C5b-9 deposition in optimised assay conditions.

Correlation of antibody-mediated C5b-9 deposition and bactericidal titre with a panel of 20 human sera and *N. meningitidis* M01240149 (r=0.82, *P*<0.05). Significance was determined by t-test.

3.5 Correlation of C5b-9 deposition with *N. meningtidis* H44/76 and M01240185.

The correlation of C5b-9 deposition and bactericidal titres is important to evaluate the fitness of the assay for more than one meningococcal strain. A significant correlation has already been demonstrated with C5b-9 deposition and bactericidal titres with *N. meningitidis* M01240149 (3.4.3). Using a panel of 40 high, medium and low responding sera, bactericidal titres were correlated with C5b-9 deposition with *N. meningitidis* H44/76 and M01240185.

Significant positive correlations were found with both H44/76 and M01240185 (figure 3.5.1). The correlation of C5b-9 deposition and bactericidal titre was lower with M01240185 (r=0.53, *P*<0.05), than H44/76 (r=0.78, *P*<0.05). Due to these varying levels of correlation any strain that is going to be used in the dCDA would need to be assessed for correlation with SBA.

Another important aspect of using the dCDA is relating the deposition of C5b-9 to the protective bactericidal level which is accepted as ≥1:4 (Goldschneider *et al.* 1969). A C5b-9 deposition level was assessed to establish a level where approximately 75% of sera samples tested could be correctly defined as from protected or unprotected individuals, a level which is consistent with the MATS assay. This level of C5b-9 deposition was established at 2.3 log10 C5b-9 deposition for H44/76 and 2.25 log10 C5b-9 deposition for M01240185 (figure 3.5.1).
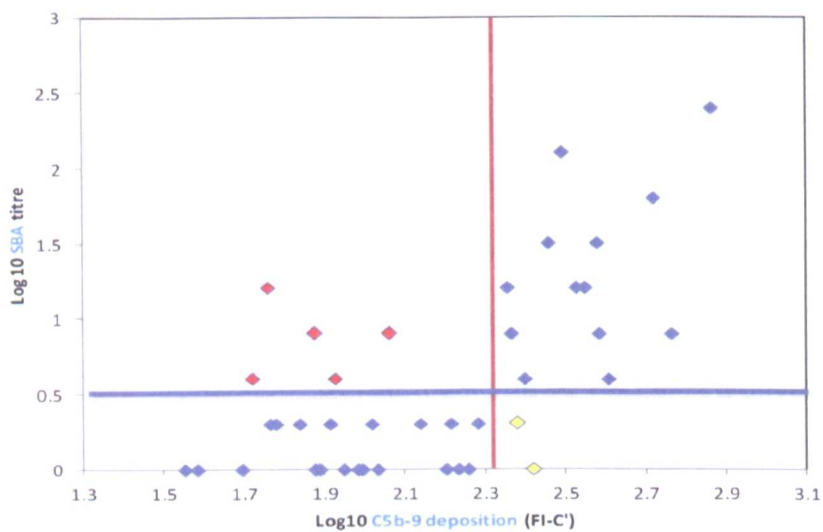
a.

- Indicates a false positive
- Indicates a false negative
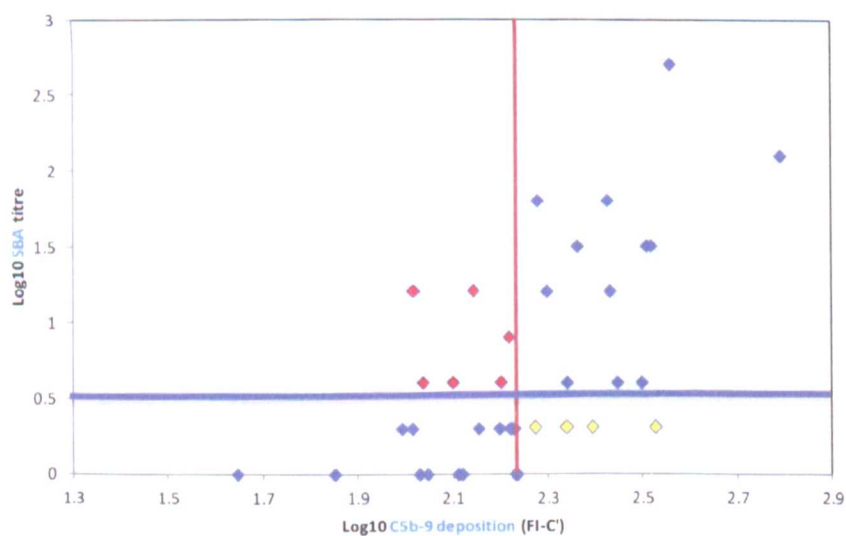
b.

Figure 3.5.1 Correlation of antibody-mediated C5b-9 deposition and bactericidal titre with H44/76 and M01240185.

Correlation of antibody-mediated C5b-9 deposition and bactericidal titre with H44/76 (r=0.78, $P<0.05$) (a) and M01240185 (r=0.53, $P<0.05$) (b).

3.6 Application of duplexed antibody-mediated complement deposition assay to evaluate a candidate meningococcal vaccine enriched in heat shock proteins.

The key objective for development of novel meningococcal vaccines is to provide cross-strain protection. The use of heat shock proteins (Hsps) has been suggested as a potential meningococcal vaccine as they are molecular chaperones that bind peptides, and are able to present these to the immune system. Hsps carrying peptides are able to induce innate immune responses, activate dendritic cells, upregulate surface expression of MHC, and are also capable of delivering peptides to dendritic cells leading to MHC antigen presentation, and development of adaptive immunity.

A vaccine has been designed with an enriched content of heat-shock proteins purified from *N. meningitidis* or *N. lactamica* in collaboration with Immunobiology Ltd. Mouse serum raised against these vaccines have been assessed in the duplexed antibody-mediated complement deposition assay against a panel of diverse UK meningococcal case isolates.

Antibody-mediated C5b-9 deposition showed that the Hsp vaccine developed from *N. lactamica* to have the greatest C5b-9 deposition against all strains tested. M01240355 and M01240185 demonstrated the greatest antibody-mediated C5b-9 deposition in the *N. lactamica* Hsp vaccine preparation in comparison to the *N. meningitidis* Hsp vaccines and positive OMV rabbit control, with NLV3 and NLV1 preparations giving a significantly ($P<0.01$) greater result than the buffer only control (figure 3.6.1). When antibody-mediated C5b-9 deposition for *N. meningitidis* H44/76 was compared with the bactericidal results obtained with the

same strain, both assays showed both of the *N. lactamica* Hsp vaccines to provide the greatest activity (figure 3.6.2).

Antibody-mediated C3b/iC3b deposition also showed the Hsp-enriched vaccine developed from *N. lactamica* to have the greatest C3b/iC3b deposition against all strains tested. Again M01240355 and M01240185 demonstrated greater antibody-mediated C3b/iC3b deposition in the *N. lactamica* Hsp-enriched vaccine preparation in comparison to the *N. meningitidis* Hsp-enriched vaccines and positive rabbit control, with NLV3 and NLV1 preparation giving a significantly ($P<0.01$) greater result than the buffer only control (figure 3.6.3).
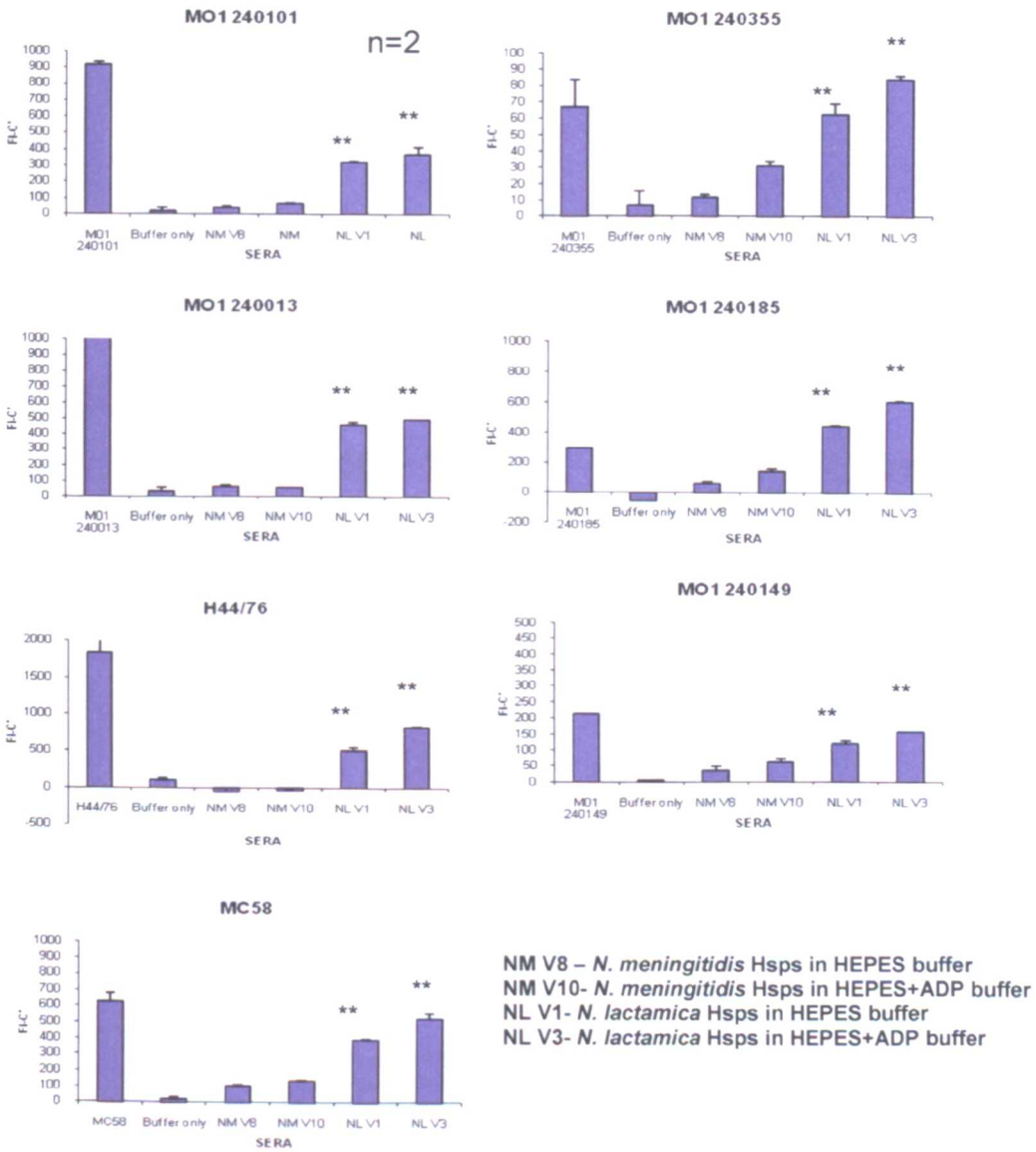
Figure 3.6.1 Antibody-mediated C5b-9 deposition mediated by mouse sera raised against different Hsp vaccine preparations, against a panel of circulating *N. meningitidis* strains.

** denotes significance (*P*<0.01) of vaccine in comparison to the buffer only control. Significance was determined by z-test and error bars denote standard deviation. All samples were performed in duplicate (n=2).

Antibody-mediated C5b-9 deposition

Serum bactericidal titre
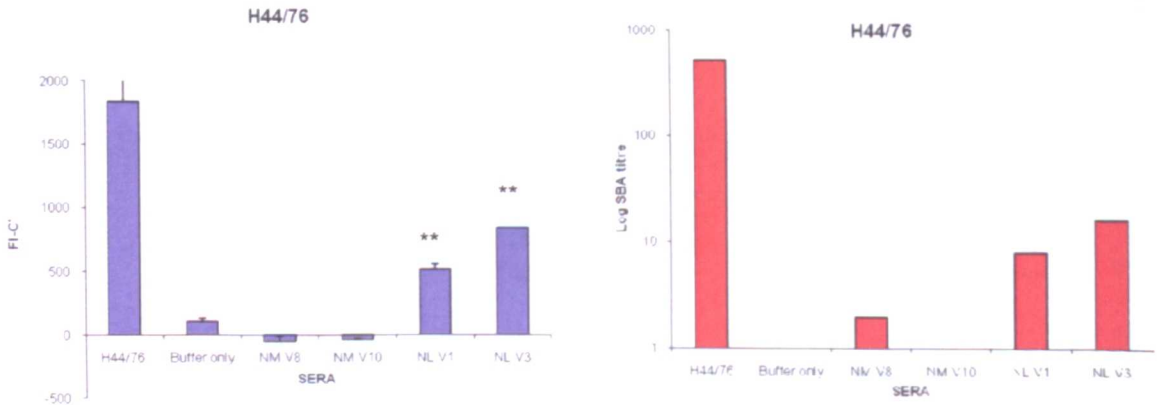
NM V8 – *N.meningitidis* Hsps in HEPES buffer
NM V10- *N.meningitidis* Hsps in HEPES+ADP buffer
NL V1- N.lactamica Hsps in HEPES buffer
NL V3- N.lactamica Hsps in HEPES+ADP buffer

Figure 3.6.2 Comparison of antibody-mediated C5b-9 deposition and bactericidal titre with mouse sera raised against Hsp enriched vaccines. Both the SBA and antibody mediated C5b-9 deposition assays were against *N. meningitidis* H44/76. ** denotes significance ($P<0.01$) of vaccine in comparison to the buffer only control. Significance was determined by z-test and error bars denote standard deviation. All samples were performed in duplicate (n=2).
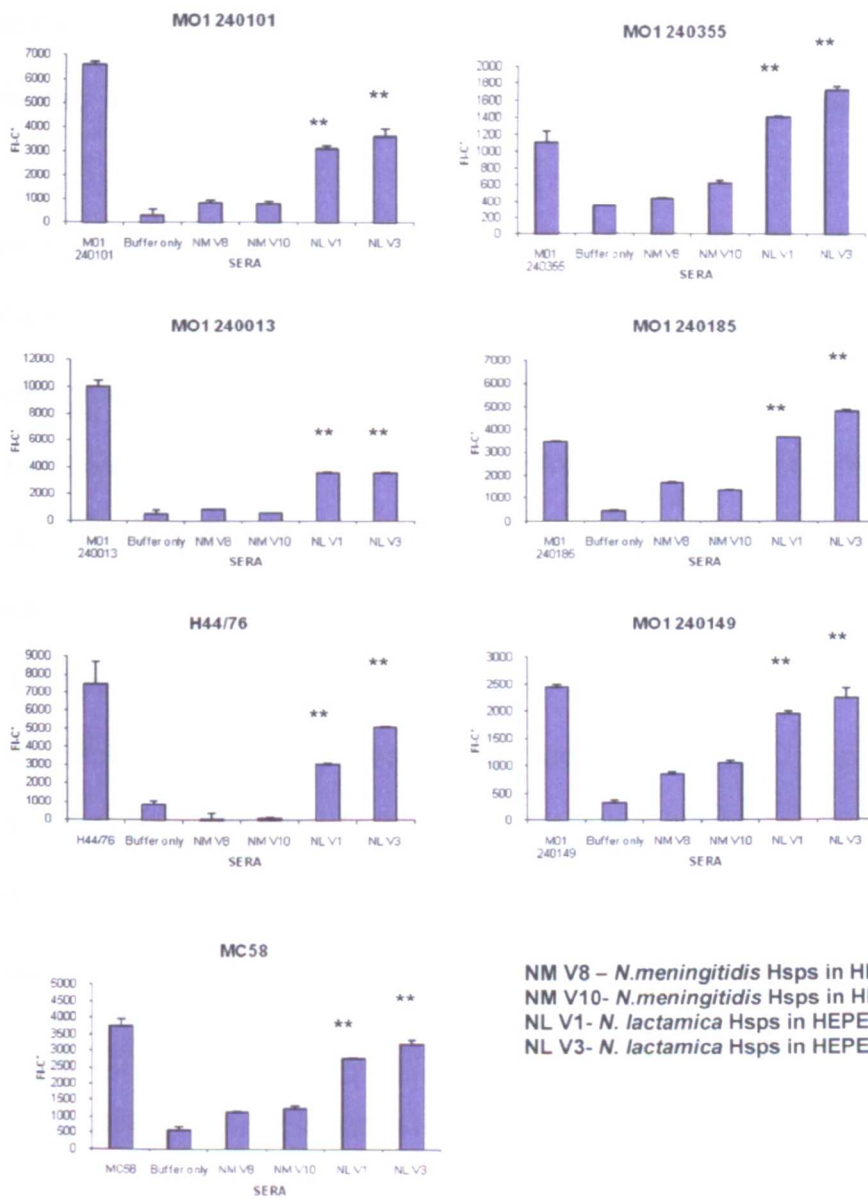
Figure 3.6.3 Antibody-mediated C3b/iC3b deposition of sera raised against different Hsp vaccine preparations, against a panel of circulating *N. meningitidis* strains.

** denotes significance (*P*<0.01) of vaccine in comparison to the buffer only control. Significance was determined by z-test and error bars denote standard deviation. All samples were performed in duplicate (n=2).

3.7 Application of duplexed antibody-mediated complement deposition assay to evaluate immunity induced by purified outer membrane protein complexes.

Outer membrane protein complexes were purified by Marzoa *et al.* so tha the complexes retained their native structure. This was done using *N. meningitidis* H44/76 and knockout mutants of this strain producing five different complexes: CxABR (PorA/PorB/RmpM), CxAB (PorA/PorB), CxBR (PorB/RmpM), CxB (PorB) and CxChap (Chaperonin MSP63). The dCDA along with an OPA and an SLA were used to investigate the immune responses induced by these protein complexes. Mouse sera raised against CxABR was able to induce antibody-mediated C3b/iC3b and C5b-9 deposition, opsonophagocytosis and bactericidal activity against the homologous strain, showing the greatest activity of the complexes (figure 3.7.1). Mouse sera raised against CxChap showed lower levels of antibody mediated C3b/iC3b and C5b-9 deposition in comparison with anti-CxABR but was found to be cross-reactive with heterologous strains inducing high levels of C3b/iC3b and C5b-9 deposition across a panel of six strains (figure 3.7.1b) (Marzoa *et al.* 2011).
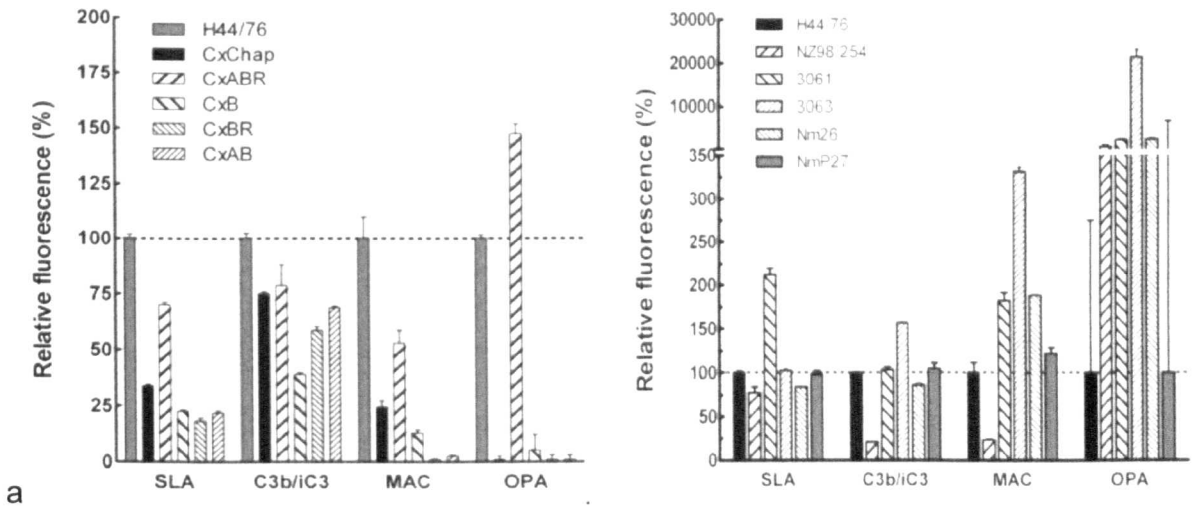
Figure 3.7.1 Evaluation of immunity induced by purified outer membrane protein complexes by SLA, dCDA and OPA.

Results of total antibody binding (SLA), C3b/iC3b deposition, MAC deposition and OPA with (a) *N. meningitidis* H44/76 with mouse sera raised against complexes. Results are presented as a percentage of anti-H44/76 OMV mouse sera. (b) A panel of *N. meningitidis* strains with anti-CxChap mouse sera. Results are presented as a percentage of anti-CxChap mouse sera against H44/76.

3.8 Application of duplexed antibody-mediated complement deposition assay to evaluate NHBA-GNA1030 stability.

The dCDA has also been used in a study investigating the structural characteristics and stability of NHBA-GNA1030, a component of 4CMenB vaccine. dCDA was used to assess the thermal stability of the fusion protein. Antibody-mediated C3b/iC3b and C5b-9 deposition, with mouse sera raised against NHBA-GNA1030 before and after heat stress for 10 days at 37°C, showed a reduction in the ability to induce antibody mediated complement deposition (figure 3.8.1), this same trend was seen with the avidity ELISA measuring the total IgG and also with SBA (Martino *et al.* 2011).
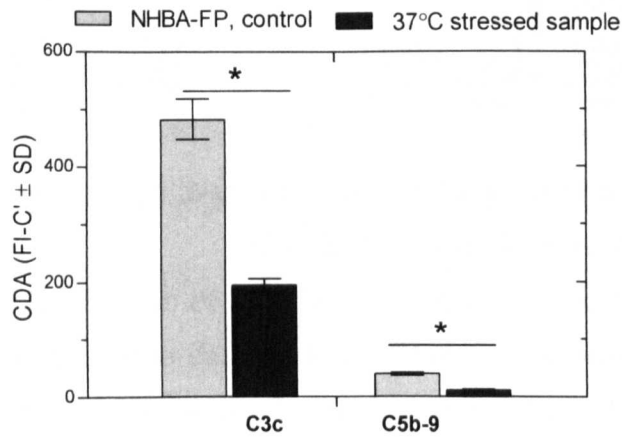
Figure 3.8.1 Antibody mediated C3b/iC3b and C5b-9 deposition assay with mouse sera raised against anti-NHBA fusion protein before and after heat stress against *N. meningitidis* M01-240101.

Significance was determined by z-test (*$P<0.05$), and error bars denote standard deviation.

3.9 Discussion and further work

A central role for SBA has been established as a read out of protection against meningococcal disease (Goldschneider *et al.* 1969; Fijen *et al.* 1989; Frasch *et al.* 2009; Granoff 2009). However, a number of observations that an absence of SBA does not necessarily indicate an absence of protection, suggests a role for other protective mechanisms. Opsonophagocytosis has been suggested as an alternate mechanism which may be important in serogroup B disease (Ross *et al.* 1987; Toropainen *et al.* 1999; Platonov *et al.* 2003; Plested *et al.* 2009). The importance of complement has also been emphasised by the increased susceptibility to meningococcal disease seen in individuals who are deficient in parts of the complement cascade which contribute to opsonophagocytosis and SBA (Figueroa and Densen 1991; Fijen *et al.* 1998; Hellerud *et al.* 2010). This is particularly highlighted amongst individuals who lack components of MAC, demonstrating an increased risk of meningococcal infection of 1000-10000 fold, in comparison to the general population (Lehner *et al.* 1992). Disease in these LCCD individuals has a lower fatality rate, thought to be attributable to less bacterial lysis and thus lower levels of bacterial LOS released into the blood (Schneider *et al.* 2007). Individuals who lack earlier components of the complement cascade also show an increased susceptibility to disease and in the case of properdin deficiency show a much poorer prognosis than LCCD individuals (Schneider *et al.* 2007).

This chapter describes the development of a duplexed antibody-mediated complement deposition assay able to measure both C3b/iC3b and C5b-9, these elements of the complement cascade are potential indicators of both opsonic and bactericidal responses. There are several assays developed to measure the deposition of complement on bacterial surfaces for a variety of organisms.

The relative importance of the classical or alternative complement cascade has been evaluated using C1q or factor B-depleted serum and investigation of the difference in C3b/iC3b deposition compared with control sera. C3b/iC3b deposition in innate immunity to *Streptococcus pyrogenes* was found to be dependent on the alternative complement cascade, and this was shown using a C3b/iC3b deposition assay (Yuste *et al.* 2006). This assay involved the incubation of bacteria with serum for 30 minutes at 37°C, followed by incubation with a fluorescent conjugate. Measurement of C3b/iC3b deposition has also been used to identify the relative importance of the classical complement pathway in innate immunity to *Streptococcus pneumoniae*, this was done using sera from mice deficient in complement components (Brown et al. 2002). This deposition assay required a 30 minute incubation of bacteria with serum at 37°C before incubating with a fluorescent conjugate. Deposition of C3b/iC3b was also used to investigate the role of *S. pneumoniae* capsule in the inhibition of complement deposition, demonstrating capsule was able to inhibit complement deposition via IgG dependent and independent mechanisms (Hyams *et al.* 2010), once again using the same assay parameters as in Brown *et al.* (2002). Involvement of both classical and alternative complement pathways in immunity to *Francisella tularensis* has been demonstrated using a complement deposition assay measuring the deposition of C3b/iC3b, and this study also investigated the deposition of C5b-9. It was shown that C5b-9 was not able to assemble on the surface of the bacteria. Deposition of both components was assessed in separate assays which combined fixed bacteria with serum and incubated for 60 min at 37°C (Ben Nasr and Klimpel 2008). In addition to this, complement deposition assays have been used to assess the effect of factor H binding to CspA in *Borrelia burgdorferi* on deposition of C3, C6 and C5b-9, once again all measured separately by incubating spirochetes with serum for 30 min at 37°C, and then with

a fluorescent conjugate (Kenedy *et al.* 2009). Assays developed to measure deposition of complement components have also been published for *N. meningitidis*, and have been used to investigate the function of NhhA. This protein involved in bacterial adhesion in the nasopharynx which also plays a role in complement resistance (Sjolinder *et al.* 2008) as the deposition of C5b-9 was increased in the Nhha knockout mutant. This assay incubated paraformaldehyde fixed bacteria with 25% serum for 30 min at 37°C. Welsch *et al.* (2003) investigated the ability of anti-GNA2132 to elicit C3b/iC3b deposition, using an assay which incubates bacteria for 30 min at room temperature.

Most of these assays use similar protocols, incubating bacteria with a complement source for between 20-60 min at 37°C, then incubating with mAb to detect the specific complement source component, followed by a secondary incubation with a secondary detection antibody (Brown *et al.* 2002; Drogari-Apiranthitou *et al.* 2002; Yuste *et al.* 2006; Sjolinder *et al.* 2008; Kenedy *et al.* 2009). In contrast, there are other assays which detect complement deposition using an incubation temperature of 25°C (Welsch *et al.* 2003; Taylor 2010).

Previous assays developed for use with *N. meningitidis* have used either azide/PMSF killed bacteria (Taylor 2010), or live bacteria (Welsch *et al.* 2003) and this study investigated the correlation of total antibody surface binding on killed or live *N. meningitidis* to establish whether using killed bacteria would be representative of the response seen with the live bacteria (3.2.5). A comparison of total IgG binding on killed bacteria or live bacteria showed a significant correlation ($r=0.93$, $P<0.01$) (3.2.5). An additional observation was made that although the correlation was high, the FI-C' values observed with the live bacteria were consistently lower than with the killed bacteria, and could have been a result of

capsule disruption during the killing process. However the high correlation gives confidence in results obtained with killed bacteria. Using azide/PMSF killed bacteria has several advantages over the use of live bacteria as it can reduce assay variation, thus removing differences between cultures of the same bacterial strain, which has been shown to be an important aspect of assay variation (Borrow et al. 2005). Using killed bacteria also allows the assay to remain high throughput as it can be performed outside microbiological containment (a requirement for any assay using live N. meningitidis in accordance with UK safety standards), and allows the assessment of large panels of sera against multiple strains, providing a way to standardise assays between laboratories. The effect of the killing method on the exposure of surface epitopes has also raised concerns about the use of killed bacteria, with an observed increase in the exposure of PorB3 occurring following killing with a variety of methods including azide, ethanol, antibiotic, paraformaldehyde fixation or heat killing (Michaelsen et al. 2001). However the strong correlation between total IgG binding between live bacteria and Azide/PMSF killed bacteria supports the conclusion that this is a suitable killing method to use.

Measurement of C5b-9 deposition required identification of a suitable anti-C5b-9 monoclonal antibody. Two different antibodies were identified, both of which were reportedly able to bind to both lytic and non-lytic complement components including fluid phase SC5b-9, TCC, MAC, poly C9 and also denatured C9 (product details Quidel and Dako). As this assay requires several wash steps following incubation with bacteria complement and serum, it would be unlikely to have any fluid phase SC5b-9 detectable in this assay. It has been shown that a small fraction <0.4 moles S protein per 1 mole of C5b-9 of membrane bound C5b-9 can contain the S protein and this may be non-lytic (Bhakdi et al. 1988). In addition to

this, poly C9 and denatured C9 are also non-lytic and thus it was important to establish whether the measured antibody-mediated deposition of C5b-9 correlated with SBA titre. The correlation of C5b-9 binding and SBA was poor (r=0.43) with the Dako mAb C5b-9. This suggested that this antibody was unable to distinguish between bactericidal and not bactericidal sera. However, the Quidel mAb C5b-9 correlated significantly (r=0.77, $P<0.05$) with SBA and was chosen for all further assay development.

Drogari-Apiranthitou *et al.* (2007) used the Dako mAb in a study comparing C5b-9 deposition mediated by sera with or without an SBA titre and showed higher C5b-9 deposition in a high SBA titre serum compared to sera with low SBA activity. However, when using mAb PorA in a dose-dependent manner, they were able to induce dose-dependent bacterial lysis but not C5b-9 deposition. These authors initially used both the Dako C5b-9 mAb and the Quidel C5b-9 mAb but in a whole cell ELISA the Quidel C5b-9 mAb showed high assay backgrounds, reacting with non opsonised meningococci, and thus the Dako C5b-9 was chosen for their studies, and no comparison was made between the antibodies with regard to the detection of bactericidal activity (Drogari-Apiranthitou *et al.* 2002). The lack of correlation between bactericidal response and C5b-9 deposition detected with the Dako C5b-9 mAb was consistent with a poor correlation observed with this mAb in this study (3.2.2.1). Other work has been conducted to investigate the role of antibody in the protection against non-typhoidal *Salmonella* bacteremia, which examined antibody-mediated C5b-9 deposition using the Dako C5b-9 mAb. This work showed a good correlation between antibody-mediated C5b-9 deposition detected with C5b-9 mAb (Dako) and bactericidal *Salmonella* killing (MacLennan *et al.* 2008).

Initial studies used unlabelled mAb and a FITC-labelled secondary antibody (2.8.1 and 2.8.2). For the use of C5b-9 mAb in a duplexed CDA this antibody required conjugation to a spectrally separate fluorochrome from C3b/iC3b FITC. It was important to choose the fluorochrome so that little compensation would be required during analysis and to reduce the possibility of FRET. Alexa Fluor 647 nm was chosen as it was excited by the 633 nm laser as opposed to the 488 nm laser used to excite FITC (conjugate for pAb C3b/iC3b). Zenon labelling with labelled Fab fragments directed against the Fc portion of antibody proved an inconsistent conjugation method. Thus covalently labelled conjugate was obtained using Invitrogen Custom Conjugation Service, which allowed a large volume of antibody to be conjugated in a single procedure. The use of a single conjugate batch was advantageous to reduce assay variation. The duplexed assay was performed in parallel with separate assays to determine whether there were adverse results observed when the fluorochromes were combined in a single assay. C3b/iC3b and C5b-9 deposition correlated significantly with the duplexed assay measurements ($r = 0.99$, $P<0.05$ and $r = 0.95$, $P<0.05$ respectively) showing that this assay could be duplexed for the measurement of both antibody-mediated C3b/iC3b and C5b-9 deposition in a single assay.

It was important to optimise the assay temperature and time points as they were considerably different to that used in the standard SBA protocol measuring the functional lytic response of C5b-9 deposition and opsonic protocols (Lehmann *et al.* 1998; Bassoe *et al.* 2000; Taylor 2010; Findlow 2011). In the SBA, bacteria, complement and serum are incubated for 60 min at 37°C, whilst the initial CDA method incubated bacteria, complement and serum for 30 min at 25°C. Results demonstrated a significant ($P<0.05$) increase in antibody-mediated C3b/iC3b and C5b-9 deposition was observed at 37°C in comparison to 25°C at all time points

tested with the optimum incubation time found to be 45 min. Correlation between bactericidal titre and complement deposition was also higher when performed at 37°C. In addition, the assay variation showed a reduction in reproducibility at 37°C. The incubation time point for addition of conjugates was also investigated and it was found that the original time 20 min at 4°C without shaking was optimum.

In order to assess assay precision a selection of 20 high medium and low responding sera were assayed by three operators three times on three separate days. The assay precision showed that for both C3b/iC3b and C5b-9 deposition, the coefficient of variance (CoV) was below 35% when the intra assay variability was measured. Inter assay CoV was also below 35% as was the inter operator variation, however 4 sera showed a CoV higher than 35% when comparing precision between operators. Assay precision was good, and was equivalent to other assays which measure the deposition of complement (Taylor 2010). CoV are higher than those seen with ELISA which are typically set with an acceptance criteria of a CoV ≤10% (Granoff *et al.* 1998) and is slightly higher than the reported CoV ≤15% for intra assay variation reported for the validated SBA used in the MeNZB vaccine trials (Martin *et al.* 2005). A meningococcal OPA CoV demonstrated a comparable level of variance of ≤30% for intra and inter-assay variation (Taylor 2010) which was lower than that observed with a pneumococcal OPA, where much higher CoVs were observed (Romero-Steiner *et al.* 1997).

The dCDA has been utilised to measure antibody-mediated complement deposition levels with sera raised against purified outer membrane protein complexes. Mouse sera raised against CxABR (complex of PorA/ PorB/ RmpM) showed the greatest activity of the complexes assessed, but mouse sera raised against CxChap (complex of chaperonin MSP63) showed lower levels of antibody

mediated C3b/iC3b and C5b-9 deposition in comparison with mouse sera raised against CxABR. However, CxChap was the only complex able to induce cross-reactive antibodies with heterologous strains inducing high levels of C3b/iC3b and C5b-9 deposition across a panel of six strains (figure 3.7.1)(Marzoa *et al.* 2011). The dCDA has also been utilised to show the reduction in immunogenicity of NHBA-fusion protein following heat stress (figure 3.8.1)(Martino *et al.* 2011) and also to evaluate heat shock protein enriched vaccines (figure 3.6.1, 3.6.2, and 3.6.3).

Further investigation could include the assessment of assay linearity, assay robustness and establishing the upper and lower limits of detection. Further work should also investigate correlation of C5b-9 deposition and bactericidal titre against further strains. It would be important to establish if there is a level of C5b-9 deposition which can predict a protective bactericidal titre of ≤1:4, and whether this varies between strains or whether a consistent level of C5b-9 deposition can be established.

This CDA is not a replacement for the functional assays measuring complement-mediated killing or phagocytosis of *N. meningitidis*. The SBA is an established assay, and is accepted as a correlate of protection. However, the duplexed CDA provides a mechanism for assessment of large panels of bacterial strains against large numbers of serum for clinical serological testing, this is because of the requirement for small volumes of serum for each assay. In addition, the assay requires no microbiological containment (all assays using live bacteria require containment) facilitating high throughput assays, and the use of killed bacteria reduces the growth to growth variation.

Chapter 4

Chapter 4: Development of a respiratory burst opsonophagocytosis assay

4.1 Introduction

Serum bactericidal activity is the accepted correlate of protection for serogroup A,B,C,Y and W135 disease vaccines (Frasch *et al.* 2009), but other mechanisms of protection, including opsonophagocytosis are likely to be important (Granoff 2009).

Opsonophagocytosis is the accepted correlate of protection for *S. pneumoniae* vaccines (Romero-Steiner *et al.* 2003) and evidence suggests that the interaction of complement binding antibodies and polymorphonuclear cells also plays an important role in protection against meningococcal disease (Ross *et al.* 1987; Balmer and Borrow 2004; Plested and Granoff 2008; Granoff 2009)(1.4.2).

Polymorphonuclear cells are important effector cells, phagocytosing opsonised meningococci and then exposing them to either reactive oxygen species (ROS) generated by the activation of NADPH oxidase, or hydrolytic granule proteins causing killing of engulfed bacteria.

Resting polymorphonuclear cells consume little oxygen as they generate ATP mainly by glycolysis, but following phagocytosis of opsonised bacteria they begin to take up large amounts of oxygen. The uptake of oxygen is initiated by the activation of an enzyme complex (NADPH oxidase) on the surface of the plasma membrane which oxidises NADPH.

$$NADPH + 2O_2 \longrightarrow NADP^+ + H^+ + 2O_2^{\bullet-}$$
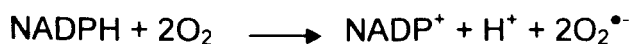
Oxidation of NADPH releases two electrons which are used to reduce oxygen into superoxide. Following phagocytosis of bacteria the intra-vacuolar pH increases from 7.4 to 7.8 and then falls to 6.0. This fall in pH occurs because superoxide radical is dismutated into hydrogen peroxide within the vacuole (Hampton *et al.* 1998).

$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$
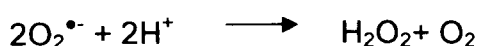
Whilst it has been have shown that superoxide has a some bactericidal effect on *N. meningitidis*, hydrogen peroxide (H_2O_2) has been found to be highly toxic to *N. meningitidis* (Dyet and Moir 2006). H_2O_2 and derived hydroxyl radicals are able cross the membrane and directly damage bacteria by lipid peroxidation, a process where electrons are removed from the lipids in the cell membrane leading to membrane damage (Hampton *et al.* 1998). In addition to this, H_2O_2 reacts with iron or copper salts to form hydroxyl radicals which cause DNA modification and strand breakages. Alternatively myleoperoxidase converts hydrogen peroxide into hypochlorous acid, which is highly potent, causing damage to membrane transport systems (Albrich *et al.* 1986), adenosine triphosphate generating systems (Barrette *et al.* 1989) and the origin of replication for DNA synthesis (Rosen *et al.* 1990).

Respiratory burst can be measured by a variety of different probes (Gomes *et al.* 2005) each detecting a different reactive oxygen species. A variety of techniques have been developed to assess antibody-dependent opsonophagocytosis. There are several examples of opsonophagocytosis assays measuring the uptake of fluorescent OMV-coated polystyrene beads (Lehmann *et al.* 1998; Bassoe *et al.* 2000) or labelled meningococci (Taylor 2010). In addition, killing assays have

been developed to directly measure a bactericidal opsonic response (Plested and Granoff 2008). However, these assays are laborious and require a large serum volume which makes them unfeasible for use in the assessment of clinical trial sera. Alternatively other assays have been developed to measure the respiratory burst response. These assays measure the conversion of dihydrorodamine 123 to fluorescent rodamine 123 (which measures hydrogen peroxide) or dihydroethidium to ethidium (which measures superoxide) following a respiratory burst response initiated by opsonophagocytosis (Lehmann *et al.* 1998). Lehmann *et al.* (1998) measured both uptake of fluorescent OMV-coated beads and respiratory burst. OMV beads have been used as targets in assays (Lehmann *et al.* 1998) as they are easily standardised in comparison to whole bacteria, although these have the disadvantage that they do not possess capsule and as a consequence may expose epitopes not usually available on the surface of meningococci. Assays have also been developed to measure both uptake of fluorescently stained *Staphylococcus aureus* and induction of respiratory burst response (Perticarari *et al.* 1991).

Previous studies measuring respiratory burst responses have used a variety of different reagents (Lehmann *et al.* 1998; Gomes *et al.* 2005), with many of these reagents measuring the production of either superoxide or hydrogen peroxide.

The majority of flow cytometric opsonic assays measure the uptake of fluorescently-labelled OMV coated latex beads or meningococci, but do not measure the stimulation of a respiratory burst response, the bactericidal function which kills the phagocytosed bacteria. Similarly some assays measure respiratory burst response only and not opsonophagocytosed bacteria which may overestimate protection as it does not account for the respiratory burst response

stimulated by N-formylmethionylleucylphenylalanine (FMLP) (Elbim *et al.* 1994), IFN-γ (Ellis and Beaman 2004) and TNF-α (Menegazzi *et al.* 1994). In addition, phagocytosis is not always necessary for a respiratory burst to occur (Phillips *et al.* 1990; Elbim *et al.* 1994). Lehmann et al. (1998) showed that the level of phagocytosis did not correspond with the level of respiratory burst response measured.

The work described in this chapter aimed to develop a high throughput assay to quantify both opsonophagocytosed bacteria and respiratory burst response within HL60 cells. The starting method used was an OPA developed to measure the uptake of bacteria stained with BCECF-AM using DMF-differentiated HL60 cells and baby rabbit complement (Taylor 2010) (2.9.7). This work aimed to develop this assay to measure the simultaneous measurement of respiratory burst and uptake of opsonophagocytosed bacteria. The measurement of both parameters would allow correlation with opsonic killing assays to assess whether the duplexed response correlates to a greater level than either opsonophagocytosis or respiratory burst response measured individually.

4.2 Results

4.2.1 Investigation of different reagents to detect respiratory burst response in differentiated HL60 cells.

Initial investigations compared different reagents able to measure hydrogen peroxide production and investigated detection of superoxide production (table 4.2.1.1). This aimed to identify the most appropriate reagent for measurement of reactive oxygen species in HL60 cells. All reagents tested were used at the recommended maximum concentration (Invitrogen technical information sheet).

No response was observed using H_2DCFDA and carboxy-H_2DCFDA, but CM-H_2DCFDA demonstrated an FI-C' of greater than 1600 (figure 4.2.1.2). However CM-H_2DCFDA demonstrated a high response for both positive control serum and negative non immune serum (figure 4.2.1.2). This result suggested that the reagent may have been maximally converted to H_2DCF. H_2DCFDA and its derivatives are photosensitive and can undergo some photoreduction converting them to fluorescent H_2DCF prior to exposure to hydrogen peroxide, this could have caused the non-specific response observed (Gomes *et al.* 2005). Due to the photosensitivity of these fluorochromes none would be suitable for use in a respiratory burst assay (RBA). Both dihydroethidium and DHR 6G also showed no detectable response (figure 4.2.1.3 and 4.2.1.4), whereas dihydrorodamine 123 was the only reagent which demonstrated a low response to negative control serum and a high response to the positive control serum (figure 4.2.1.2) and has been previously described (Lehmann *et al.* 1998; Bassoe *et al.* 2000; Aase *et al.* 2003; Walrand *et al.* 2003; Aase *et al.* 2007). DHR123 was chosen for all further work.

Non-fluorescent respiratory burst reagent	Fluorescent oxidised product	Additional information	ROS detected
2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA)	H$_2$DCF	n/a	Hydrogen peroxide
5- (and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA)	H$_2$DCF	Retained within the cell following covalent binding to intracellular components (intracellular glutathione).	Hydrogen peroxide
5-(and-6)-carboxy 2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H$_2$DCFDA)	H$_2$DCF	Negative charges impedes its leakage out of the cell.	Hydrogen peroxide
Dihydrorodamine 123 (DHR 123)	Rodamine 123	Reacts with H$_2$O$_2$ producing H$_2$O and fluorescent rodamine 123.	Hydrogen peroxide
Dihydrorodamine 6G (DHR 6G)	Rodamine 6G	Reacts with H$_2$O$_2$ producing H$_2$O and fluorescent rodamine 6G.	Hydrogen peroxide
Dihydroethidium (DHE)	Ethidium	Reacts with 2O$_2^{\bullet-}$ producing H$_2$O and ethidium.	Superoxide

Table 4.2.1.1 Summary of the respiratory burst detection reagents (Gomes *et al.* 2005).
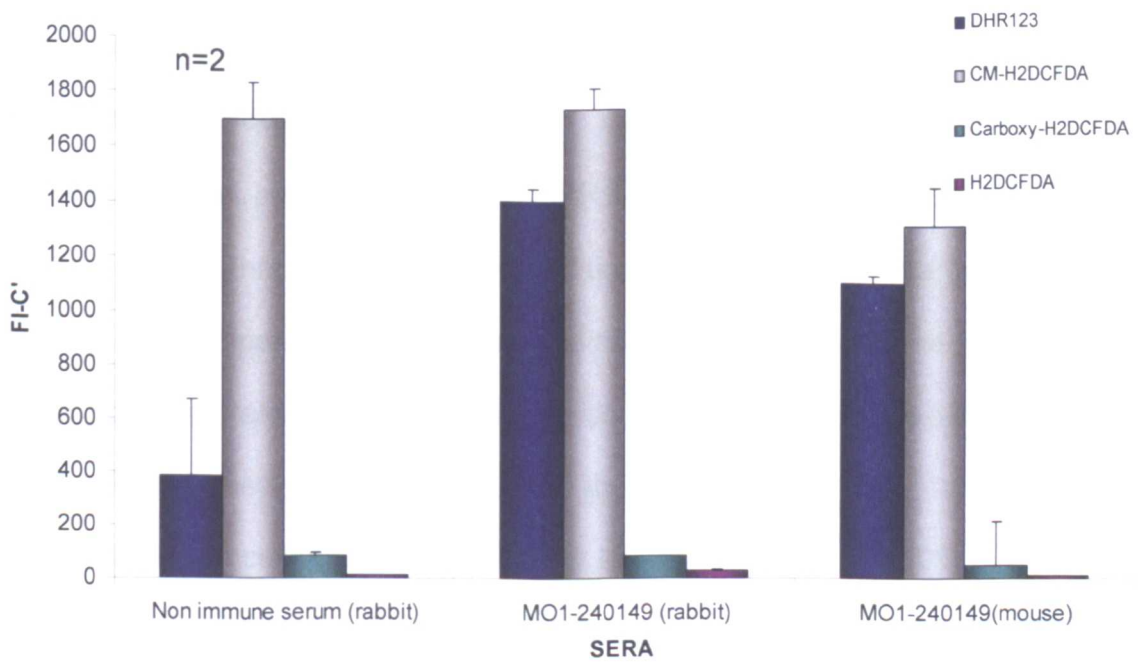
Figure 4.2.1.2 Comparison of four respiratory burst detection reagents in HL60 cells.

Comparison of four respiratory burst detection reagents in HL60 cells following opsonophagocytosis of *N. meningitidis* M01240149 with rabbit or mouse anti-OMV serum.
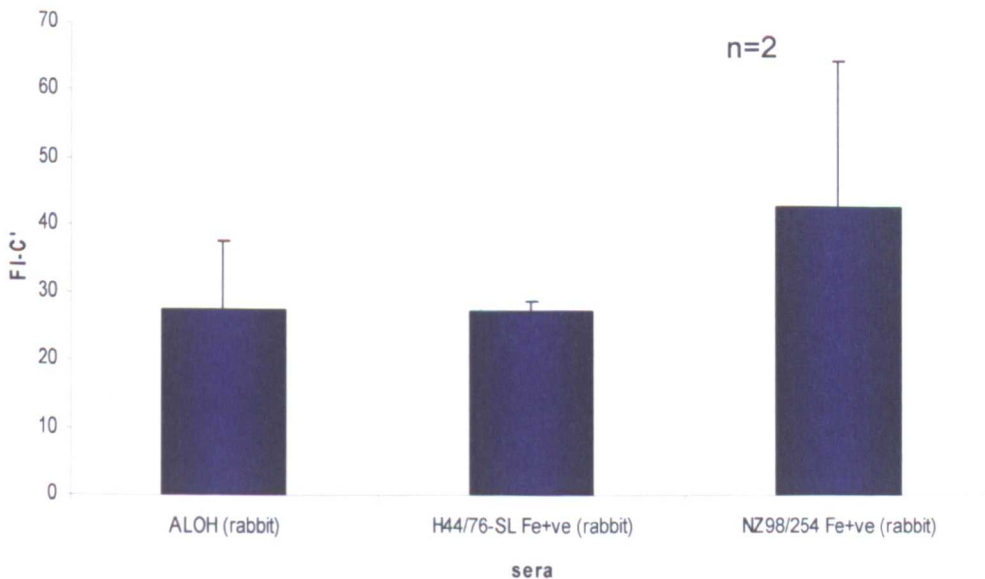
Figure 4.2.1.3 Investigation into the use of dihydrorodamine 6G (DHR6G) as a respiratory burst detection reagent in HL60 cells.

The use of respiratory burst detection reagent DHR6G in HL60 cells following opsonophagocytosis of *N. meningitidis* H44/76 with rabbit or mouse anti-OMV serum.
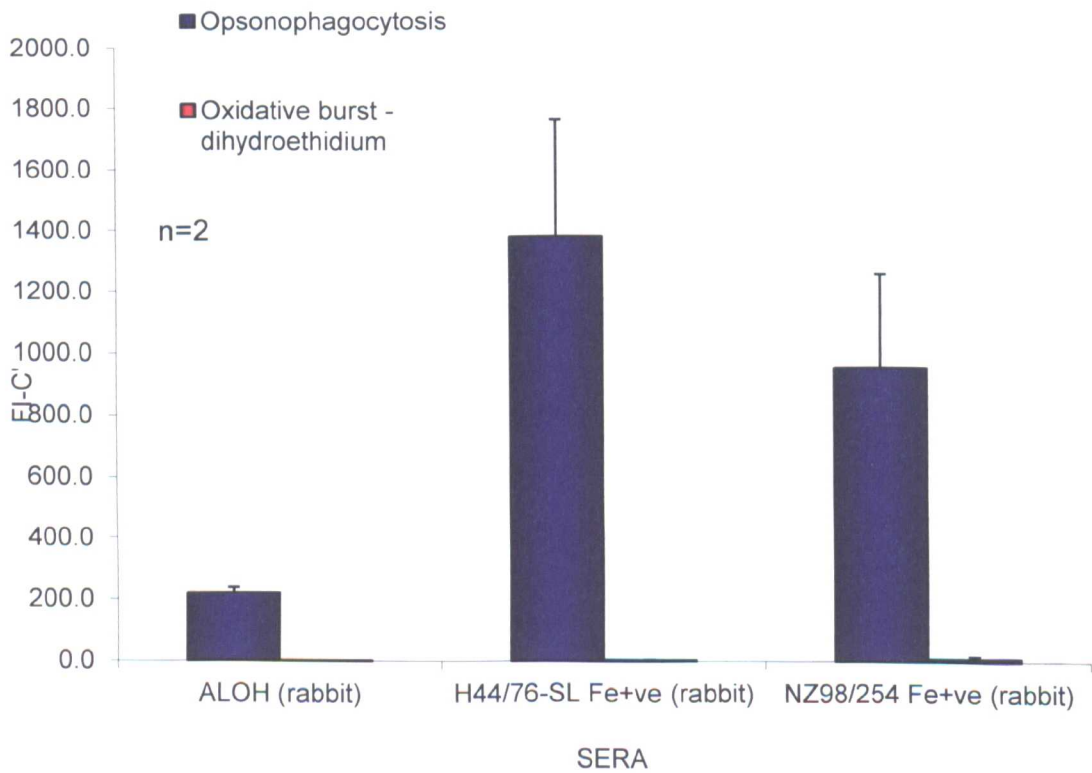
Figure 4.2.1.4 Investigation into the use of dihydroethidium (DHE) as a respiratory burst detection reagent in HL60 cells.

Use of respiratory burst reagent dihydroethidium in HL60 cells following opsonophagocytosis of *N. meningitidis* H44/76 with rabbit or mouse anti-OMV serum.

4.2.2 Optimisation of DHR123 application during the assay- Comparison of three different methods.

To establish the best protocol for use of DHR123 within the assay, a comparison was made between three methods. The addition of DHR123 with the bacteria (1st incubation) was compared with both the addition of DHR123 with the HL60s (2nd incubation) or with preloaded HL60 cells, which had been incubated with DHR123 before use in the assay. All methods used the same concentration of DHR123. Preloading the HL60 cells gave a significantly higher response ($P<0.01$) compared with the addition of DHR123 with either the cells or bacteria (figure 4.2.2.1). It was therefore decided to optimise this HL60 cell preloading concentration and incubation time with DHR123.
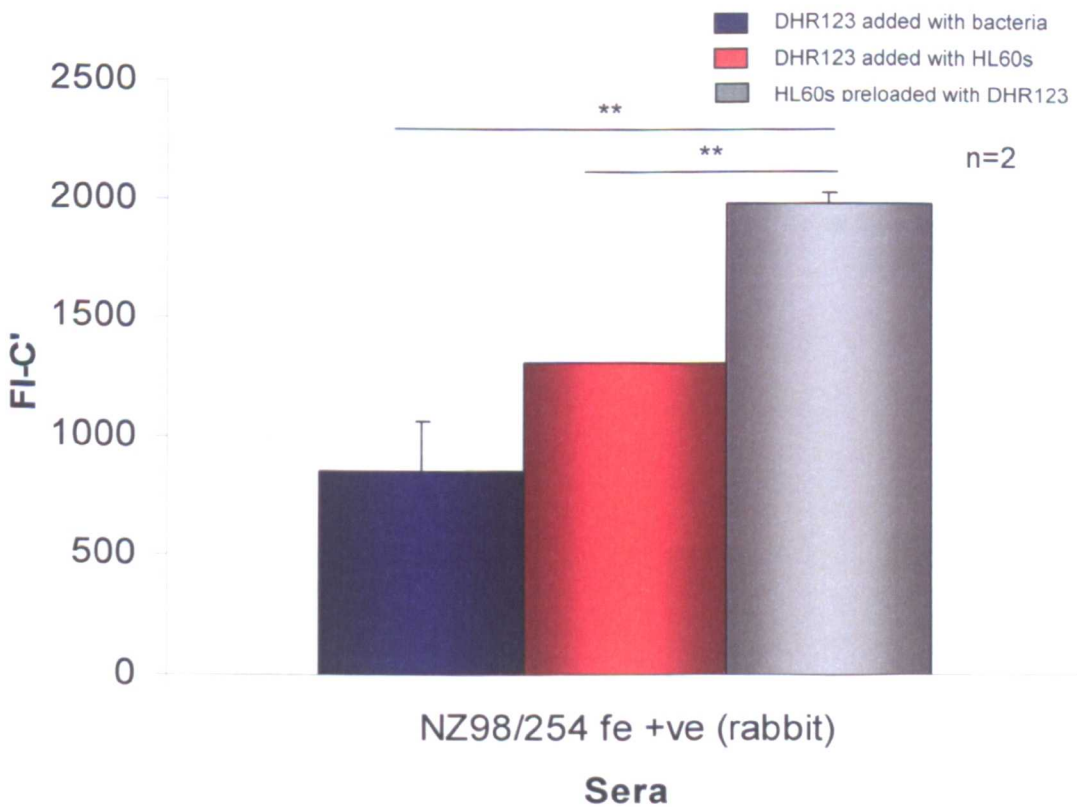
Figure 4.2.2.1 Comparison of different methods of DHR123 addition into the RBA using *N. meningtidis* NZ98/254.
Significance was determined by z-test (**$P<0.01$), and error bars denote standard deviation.

4.2.3 Optimisation of DHR 123 concentration and preloading incubation with the HL60 cells.

The optimum concentration of DHR123 for use in the assay was investigated by preloading the differentiated HL60 cells with a range of concentrations of DHR123. The optimum concentration was shown to be at 50 µg/ml, this gave a significantly ($P<0.01$) higher response than at any of the lower concentrations, with no significant increase in response observed with the higher concentrations tested (figure 4.2.3.1). The incubation time for preloading DHR123 into HL60 cells was also investigated. A significantly higher response ($P<0.01$) was observed following pre incubation of DHR123 with the HL60 cells for 15 min, and this response was significantly reduced ($P<0.01$) at the longer incubation times (figure 4.2.3.2).
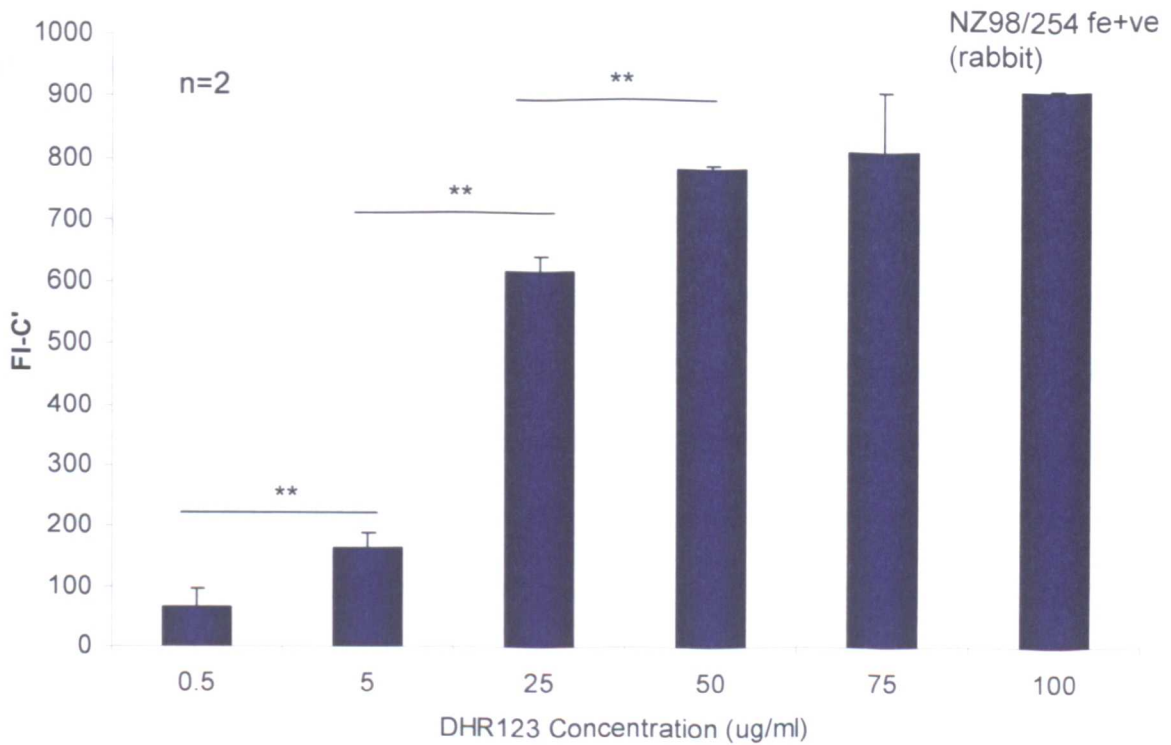
Figure 4.2.3.1 Optimisation of DHR123 concentration in RBA.

Effect of increasing DHR123 reagent concentration on respiratory burst following opsonisation of *N. meningitidis* NZ98/254 with rabbit anti-OMV serum. Significance was determined by z-test (**$P<0.01$), and error bars denote standard deviation.
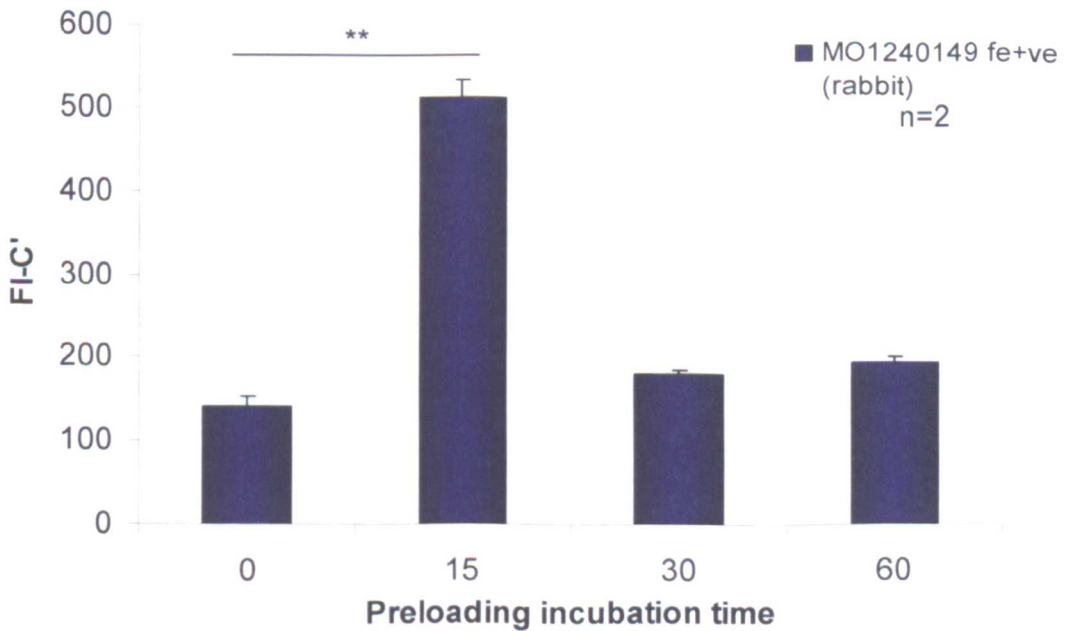
Figure 4.2.3.2 Optimisation of DHR123 preloading incubation time.

Effect of increasing preloading incubation time with HL60 cells before use in a respiratory burst assay on *N. meningitidis* MO1240149. Significance was determined by z-test (**$P<0.01$), and error bars denote standard deviation.

4.2.4 Comparison of HL60 cells and fresh human polymorphonuclear cells

The HL60 cells are at the promyelocyte stage of maturation, isolated from a patient diagnosed with promyelocytic leukaemia. Differentiation of the HL60 cells can be induced into granulocyte maturation by chemicals including DMSO and DMF. The differentiated HL60 cells provide a continuous supply of granulocytic like cells (Gallagher *et al.* 1979; Koeffler and Golde 1980) facilitating use in large assays enabling the high throughput nature of the assay. The use of fresh human PMNs has disadvantages: it is heavily reliant on volunteer donation, and the PMNs need to be isolated from the same volunteer on each occasion to allow reproducibility, as a alternative volunteer may express different polymorphisms of the FcγR. However, differences have been reported between HL60s and fresh human polymorphonuclear cells. HL60s contain no lactoferrin or alkaline phosphatase, and do not differentiate down the accepted maturation pathway for a promyelocyte (Birnie 1988). The HL60 cells have been shown to lack specific and secretory granules (Koeffler and Golde 1980; Nordenfelt *et al.* 2009), but can phagocytose, express complement and Fcγ receptors (Taylor 2010) and produce superoxide, and are often used in assays measuring opsonophagocytosis (Henckaerts *et al.* 2006; Guttormsen *et al.* 2008; Taylor 2010). It has also been shown that HL60 cells have NADPH oxidase and myeloperoxidase and are capable of producing a respiratory burst response (Teufelhofer *et al.* 2003). It was important to compare the respiratory burst response of the HL60 cells with that of human PMNs to ensure the response was representative, this was performed using a panel of high, medium and low responding human sera. A significant positive correlation was observed (r= 0.87, *P*<0.01) (figure 4.2.4.1). Thus, the HL60s are a suitable alternative to PMNs.
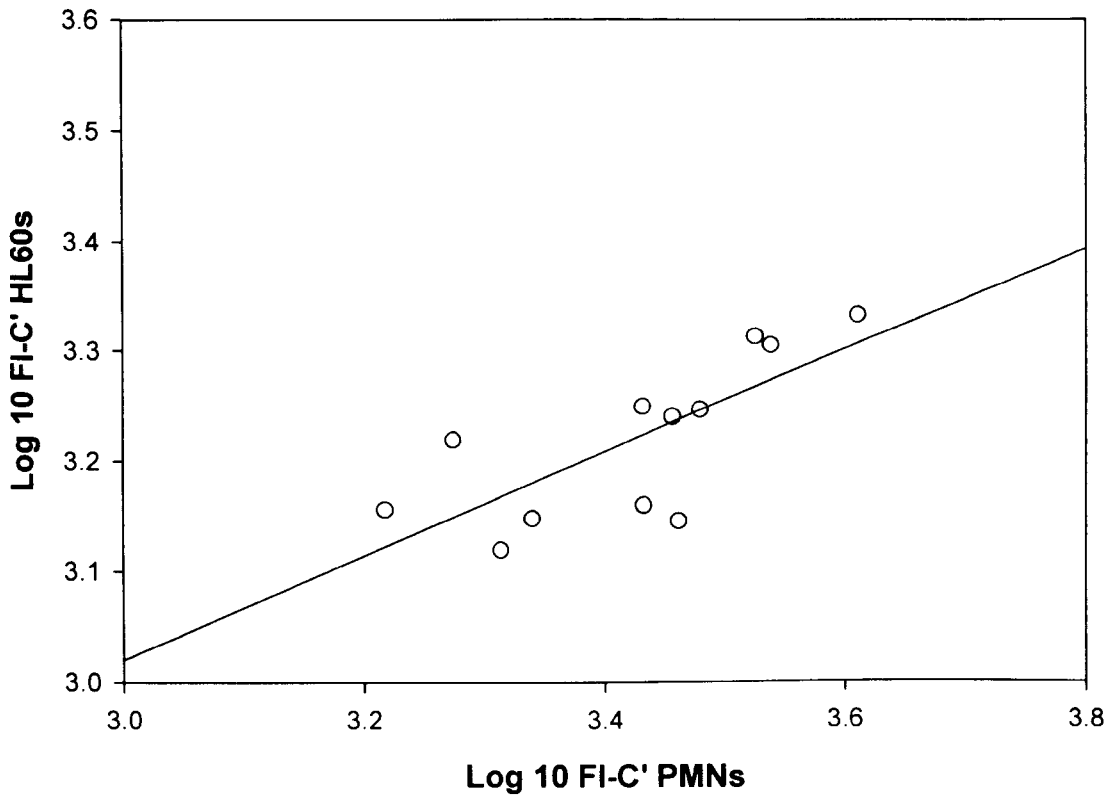
Figure 4.2.4.1 Correlation of respiratory burst observed with either HL60 cells or PMN cells.

Correlation of respiratory burst observed with either HL60 cells or PMN cells using *N. meningitidis* M01240149 (r=0.87, *P*<0.01). Significance was shown by t-test.

4.3 Optimisation of respiratory burst opsonophagocytosis assay (RBA)

4.3.1 Use of a priming agent

PMNs are stimulated *in vivo* by cytokines which are produced during immune and inflammatory responses to pathogens (Ferrante *et al.* 1988; Khwaja *et al.* 1992; Brechard *et al.* 2005). These cytokines can act in either priming sensitising neutrophils to be more responsive to the pathogens encountered, or they can directly stimulate the respiratory burst response. Previous studies have shown TNF-α, IL-8, GMCSF, and IFN-γ (Roilides *et al.* 1993; Elbim *et al.* 1994) to be effective as priming agents for the respiratory burst response. In addition, TNF-α and IFN-γ have also been shown to directly stimulate the respiratory burst response (Menegazzi *et al.* 1994; Ellis and Beaman 2004).

TNF-α, IL-8, GMCSF and IFN-γ were initially used at 100 ng/ml and were incubated with 2.5×10^7 HL60 cells/ml for 45 min prior to use in the assay. These conditions were as described by Elbim *et al.* (1994). IFN-γ gave a significantly ($P<0.05$) higher respiratory burst response in comparison with unprimed samples (figure 4.3.1.1), a moderate increase in background respiratory burst response was also observed indicating there was a low level of direct stimulation. IL-8, TNF-α and GMCSF did not induce a significant increase in respiratory burst response (figure 4.3.1.1). Responses were correlated which confirmed that priming the cells only altered the magnitude of response (figure 4.3.1.3).

The optimal IFN-γ priming concentration was found to be 100 ng/ml, which gave a significantly ($P<0.05$) greater response than the unprimed cells. No significant difference was observed between 100 ng/ml and either of the higher priming concentrations tested (figure 4.3.1.2).

The optimal incubation time was then established at 3 h (figure 4.3.1.4). At this incubation time point, primed cells induced a response that was six times greater than that observed with unprimed cells. It was also observed that the complement only control (assay background) increased following priming with IFN-γ, but was not significantly affected by the preloading incubation time (figure 4.3.1.5).
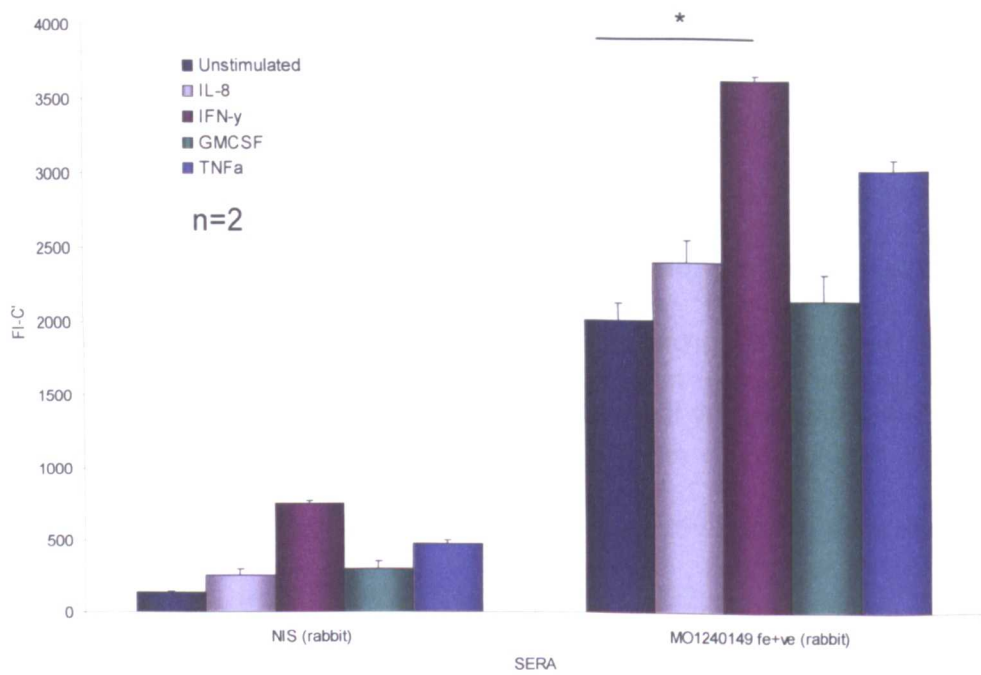
Figure 4.3.1.1 Comparison of cell primers in the RBA.

RBA to compare 100 ng/ml of either IL-8, IFN-γ, GMCSF and TNFα as cell primers using *N. meningitidis* M01240149 with rabbit serum. Significance was shown by z-test (*$P<0.05$), and error bars denote standard deviation.
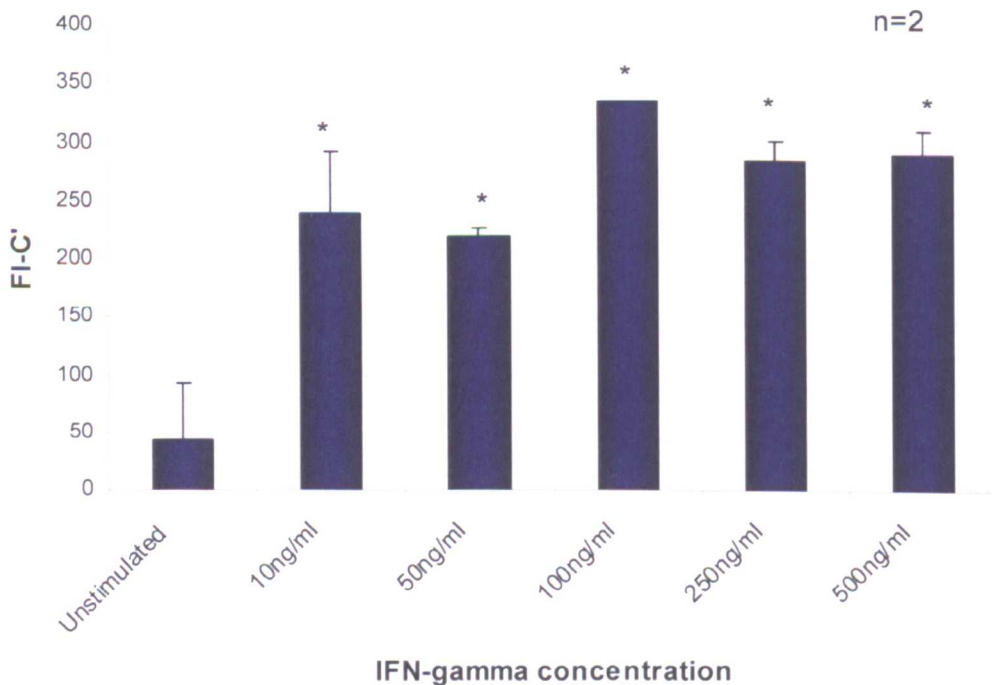
Figure 4.3.1.2 Optimisation of IFN- γ concentration for priming the cells in an RBA.

Determination of the appropriate IFN-γ concentration for priming the cells in an RBA using *N. meningtidis* M01240149 with rabbit serum. Significance was shown by z-test (*$P<0.05$), and error bars denote standard deviation.
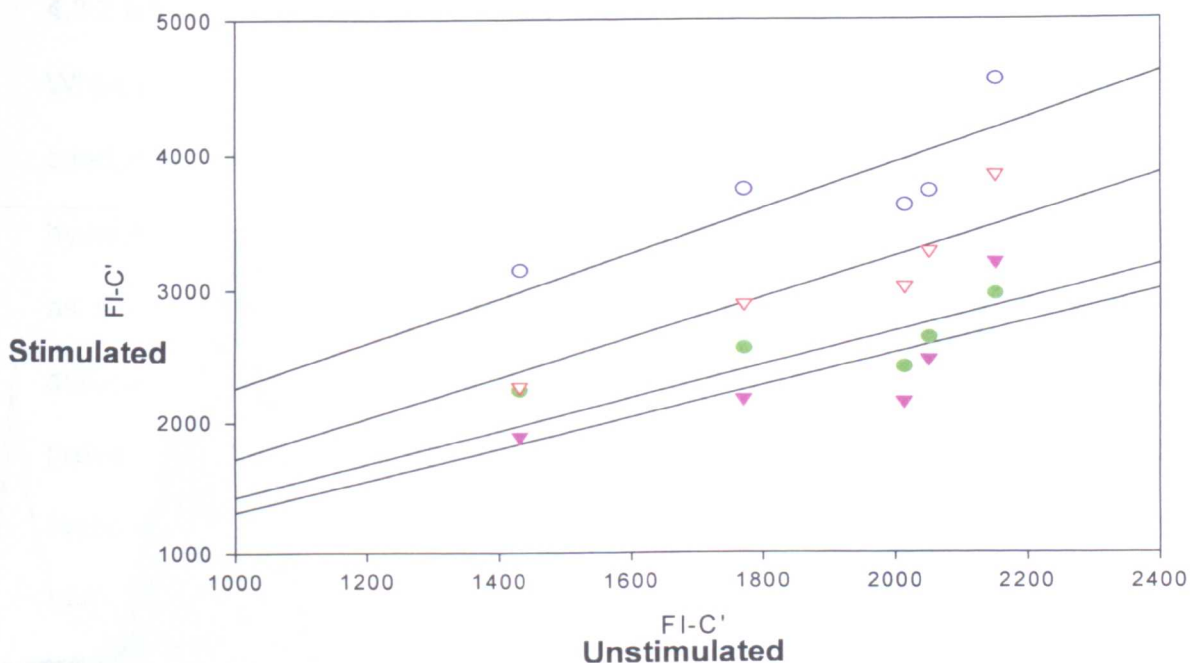
Blue – IFN-γ stimulated, r =0.97 (p<0.01)
Red –TNF-α stimulated, r =0.98 (p<0.01)
Green –IL-8 stimulated, r =0.98 (p<0.01)
Pink- GMCSF stimulated, r =0.94 (p<0.01)

Figure 4.3.1.3 Correlation between unstimulated and stimulated (100 ng/ml) samples in an RBA against *N. meningitidis* M01240149.

Significance was shown by t-test.
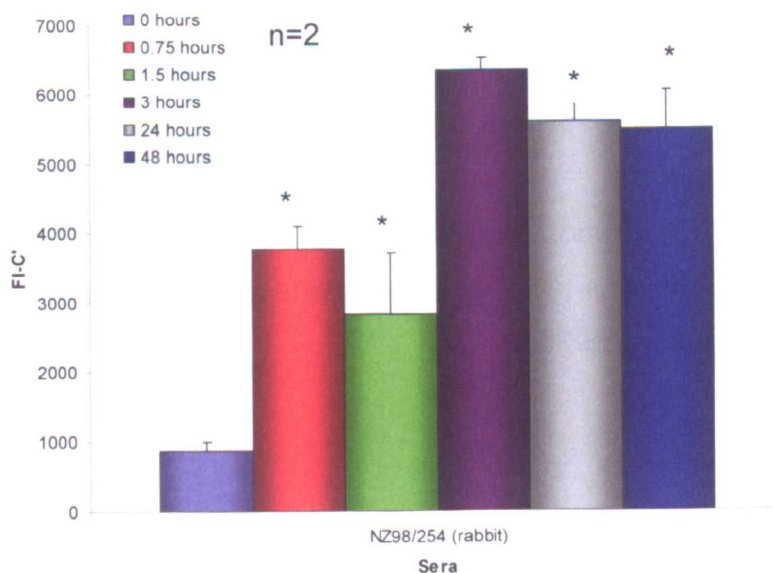
Figure 4.3.1.4 Effect of increasing incubation time with IFN-γ (100 ng/ml) stimulation in an RBA against *N. meningitidis* NZ98/254.

Significance was shown by z-test (*$P<0.05$), and error bars denote standard deviation.

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A. Complement only control

B. Rabbit anti-OMV positive control sera
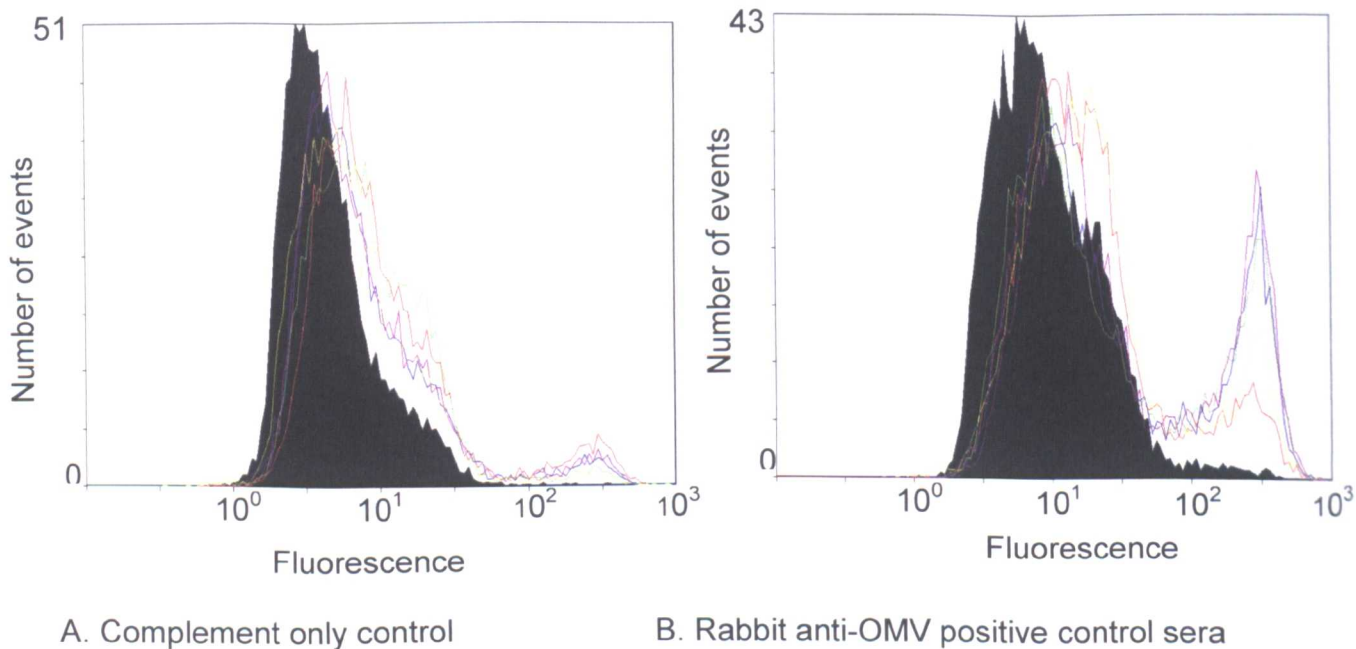
Black – Unstimulated
Yellow -0.75 hour IFN-γ stimulation
Red –1.5 hours IFN-γ stimulation
Green -3 hours IFN-γ stimulation
Purple -24 hours IFN-γ stimulation
Blue -48 hours IFN-γ stimulation

Figure 4.3.1.5 Overlay plots illustrating the effect of increasing incubation time with IFN-γ on the response observed in the complement only control and positive control sera.

Effect of increasing incubation time with IFN-γ on the complement only assay control (A.) and homologous rabbit sera positive control (B.) performed in an RBA against *N. meningitidis* NZ98/254.

4.3.2 Effect of sodium azide in the assay buffer

When the respiratory burst response is stimulated, H_2O_2 is produced, which is then catabolised into other ROS. Low levels of azide prevent this catabolism of H_2O_2 by inhibiting myeloperoxidase activity (Hampton *et al.* 1996). This was investigated as a component of the assay buffer, to further optimise the fluorescent signal achieved. The use of azide would not increase the amount of H_2O_2 produced but prevent catabolism into other ROS (Walrand *et al.* 2003). A significantly higher response ($P<0.05$) was observed with the addition of 0.01% sodium azide with the homologous strain OMV positive control serum in comparison with the control. In addition to this, no significant increase was observed in the non-immune control serum test and thus non-specific background had not increased (figure 4.3.2.1). This effect was seen with all sodium azide concentrations tested (figure 4.3.2.1).

Addition of sodium azide to the assay buffer, required the assessment of any effect on HL60 cell viability. This was investigated by comparing the cells incubated with or without the sodium azide buffer, for 2 h (the approximate length of assay plus analysis by flow cytometry) and viability was determined using trypan blue staining and enumeration with a haemocytometer (2.5.3). No significant difference was observed for cells incubated with or without 0.01% sodium azide addition (figure 4.3.2.2). Therefore 0.01% sodium azide was introduced into the assay buffer.
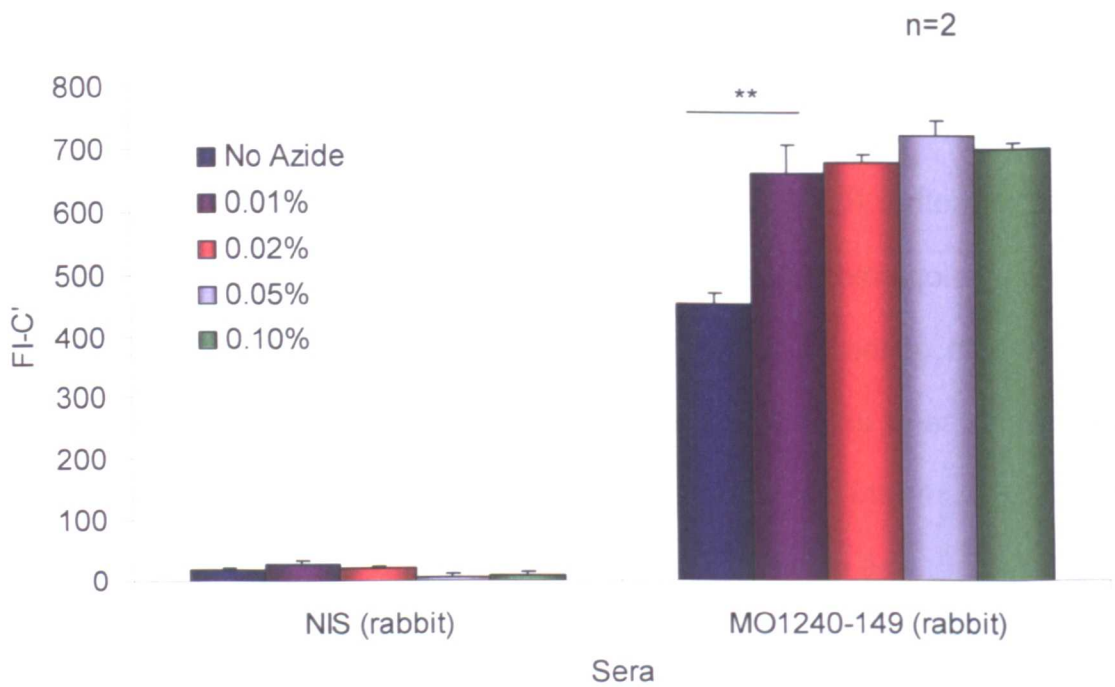
Figure 4.3.2.1 Investigation into the effect of sodium azide in RBA assay buffer.

Investigation into the effect of sodium azide on the respiratory burst response in HL60 cells using *N. meningitidis* M01 240149 with rabbit anti-OMV sera. Significance was shown by z-test (*$P<0.05$), and error bars denote standard deviation.
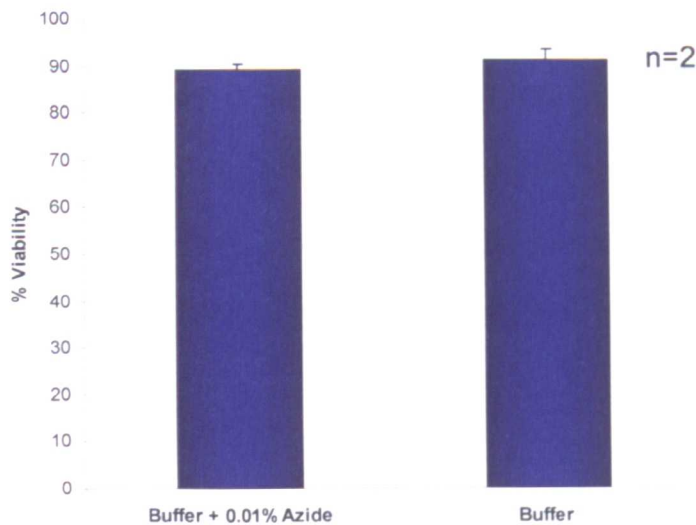

Figure 4.3.2.2 Investigation into the effect of sodium azide on HL60 cell viability following 2 hours incubation.

Error bars denote standard deviation.

4.3.3 Effect of glucose addition to the assay buffer.

It has been well established that neutrophils require glucose to generate both ATP through glycolysis and NADPH through the pentose phosphate pathway. The presence of NADPH is essential for the respiratory burst reaction as it is subsequently consumed by NADPH oxidase and leads to the production of superoxide, hydrogen peroxide and other ROS (Freitas *et al.* 2009). It was therefore important to evaluate the effect of glucose concentration in the assay buffer on the respiratory burst response. Increased concentrations of glucose lead to an increase in the respiratory burst response and the optimal concentration was found to be 1 mM. Significantly greater ($P<0.01$) responses were observed at this concentration compared with no glucose, 0.5 mM or 2 mM (figure 4.3.3.1).
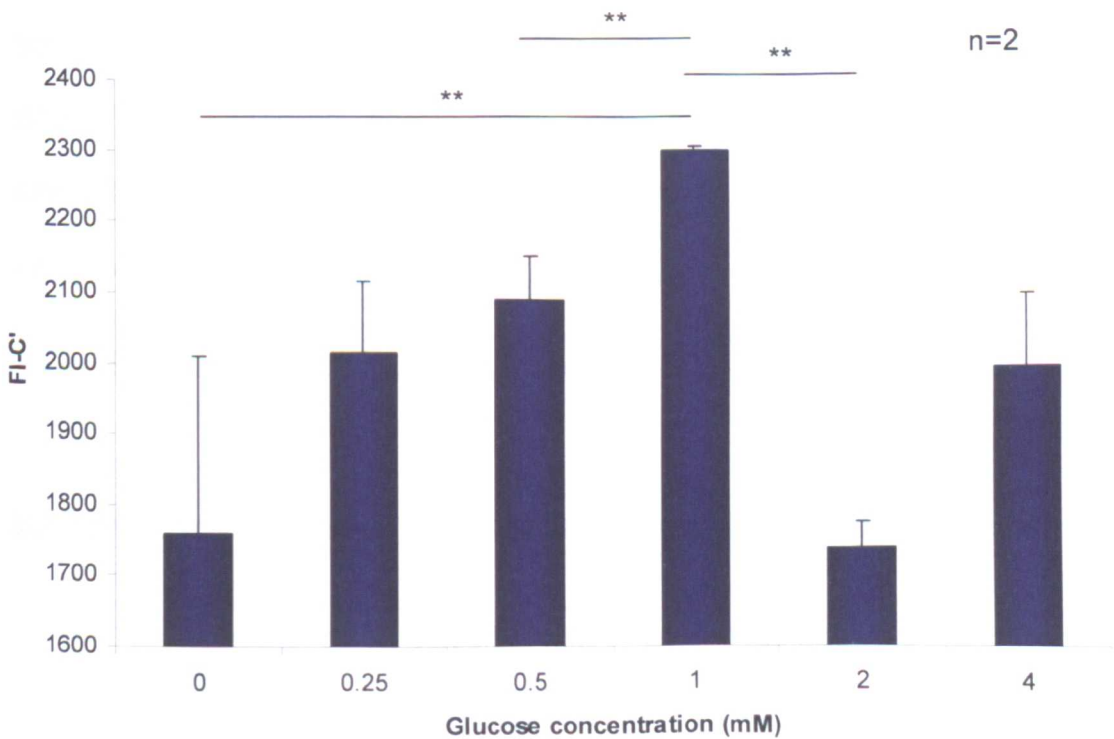
Figure 4.3.3.1 Effect of glucose addition to the RBA assay buffer.
Effect of increasing glucose concentration on RBA using *N. meningitidis* M01240149 and a high responding human serum. Significance was shown by z-test (**$P<0.01$), and error bars denote standard deviation.

4.4 Bacterial stains.

4.4.1 Investigation of alternative bacterial stains.

Dihydrorodamine 123 is excited by a 488 nm laser and emits fluorescence at 525 nm, this meant that we could not introduce the respiratory burst reagent directly into the existing opsonophagocytosis assay (2.9.7) as the bacterial stain BCECF-AM used in this assay would overlap spectrally, as it is also excited by a 488 nm laser and emits at 525 nm (figure 4.4.1.1). The use of alternative stains was thus investigated to complement the respiratory burst reagent.
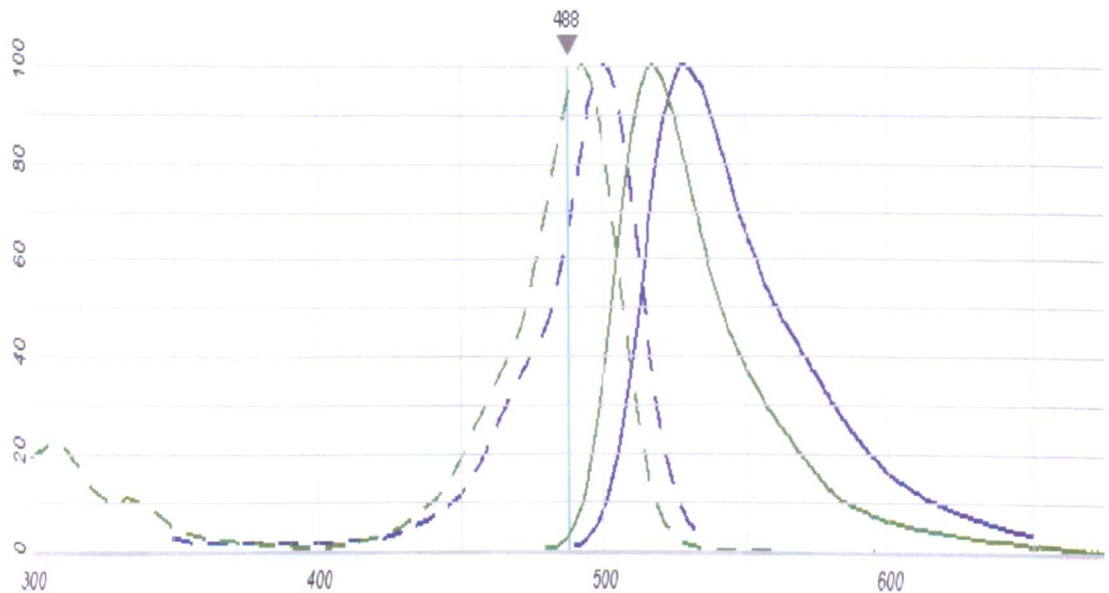
Figure 4.4.1.1 Fluorescent spectra for BCECF-AM (green line) and DHR 123 (blue line).

Excitation indicated by dashed line, emission indicated by solid line.

4.4.2 The use of Intracellular stains

BCECF-AM was a successful internal stain for the bacteria which was evident from the strong uniform fluorescent peak detected by flow cytometry (figure 4.4.2.1), because of this other similar stains were investigated, this included SNARF-AM (5-(and-6)-carboxy SNARF®-1, acetoxymethyl ester) which stains internally by crossing the bacterial membrane before the acetomethyl ester is cleaved by esterases leading to the stain being permanently retained. SNARF–AM demonstrated uniform staining (figure 4.4.2.2), however the bacteria were weakly fluorescent even at a stain concentration double that used for BCECF-AM and for double the incubation period. The optimal staining concentration was achieved at 150 μg/ml for 2 h (figure 4.4.2.2). SNARF-AM-stained bacteria were used in an OP assay to assess whether the staining was sufficient to measure a detectable bacterial uptake response (figure 4.4.2.3). No response was observed with SNARF-AM-stained bacteria in comparison with the control assay using BCECF-stained bacteria, indicating that SNARF-AM was not suitable for the detection of opsonophagocytosis.
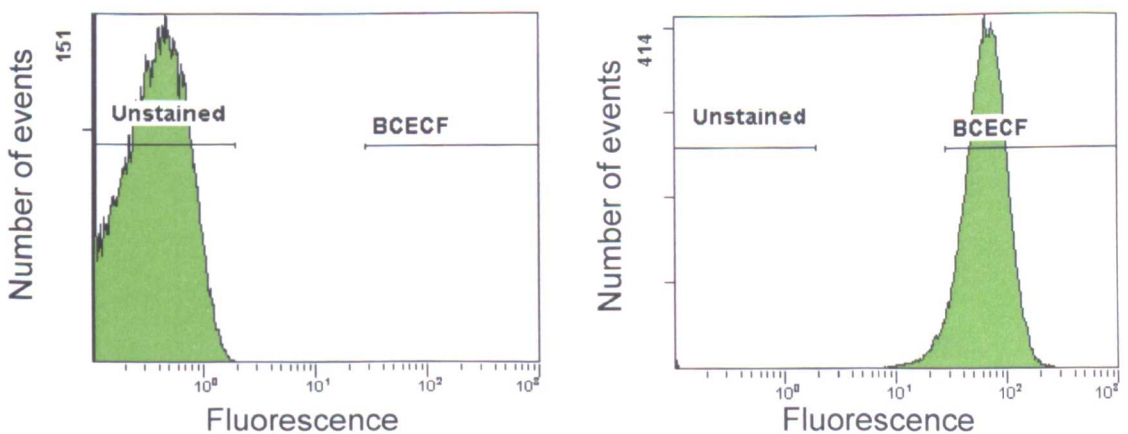
Figure 4.4.2.1 Flow cytometric analysis of *N. meningitidis* H44/76 staining using internal bacterial stain BCECF-AM.

Unstained bacteria

SNARF AM 100 µg/ml for 2 h

SNARF AM 150 µg/ml for 2 h
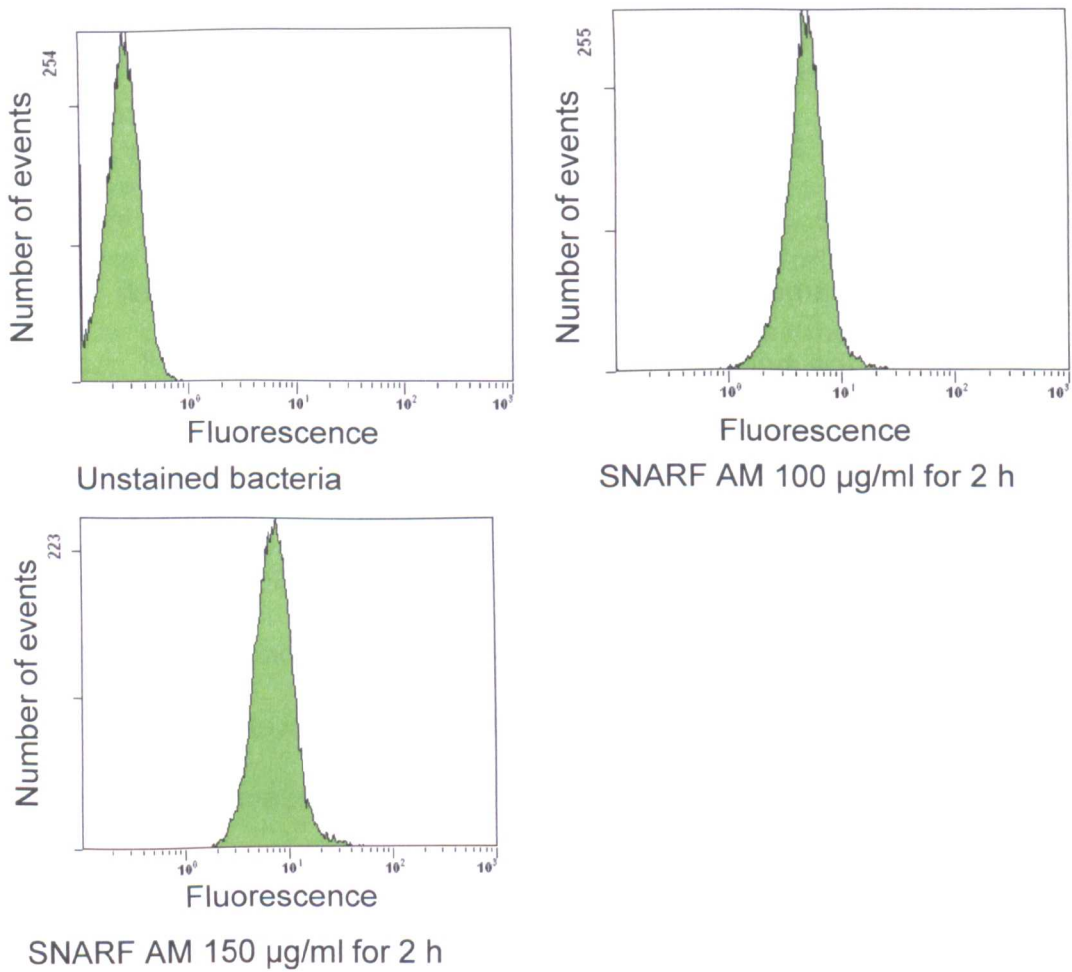
Figure 4.4.2.2 Flow cytometric analysis of *N. meningitidis* H44/76 staining using internal bacterial stain SNARF-AM.
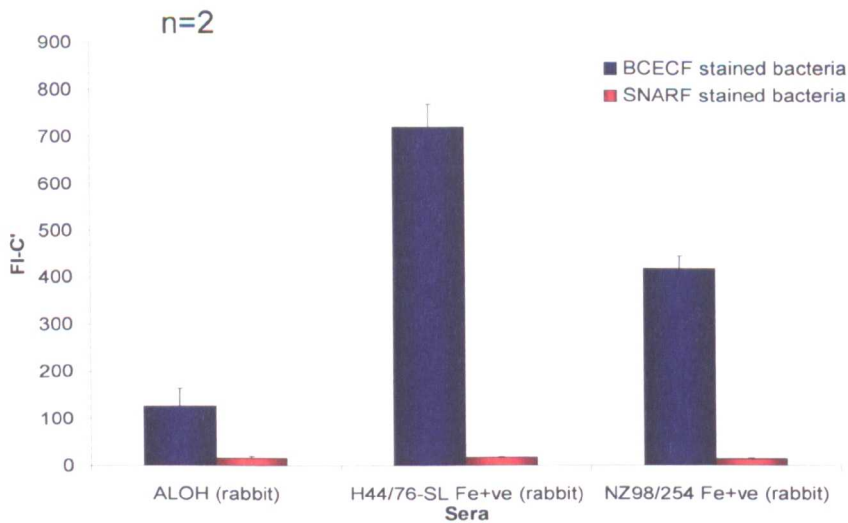
Figure 4.4.2.3 Opsonophagocytosis assay using SNARF-stained bacteria against *N. meningitidis* H44/76.

Error bars denote standard deviation.

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4.4.3 DNA bacterial stains

Various DNA stains were assessed for their ability to stain *N. meningitidis*. These were investigated as they would stain bacteria internally and not mask external epitopes. Propidium iodide (PI) and 7AAD-stained bacteria did not demonstrate sufficient fluorescence to be considered as replacement bacterial stains, although SYTO DNA stains were shown to be effective (figure 4.4.3.1). The SYTO dyes are cell permeant stains which are non-fluorescent until they bind with nucleic acids, and were chosen based on their emission spectra as they are excited by the 633 nm laser and emit at 670 nm, and if used this would result in a reduction of spectral overlap with the respiratory burst reagent (DHR123). This stain provided very bright and uniform staining (figure 4.4.3.1), and was used in an OP assay. During analysis of the assay it was found that all HL60 cells demonstrated bright staining, including undifferentiated cell populations and apoptosing and dead cell debris (figure 4.4.3.2). A possible explanation is that the SYTO dye had leached out of the stained bacteria and stained the HL60 cell nucleic acid. It was impossible to differentiate between cells which had phagocytosed fluorescent bacteria and cells just stained with free SYTO dye, therefore it was concluded that this type of stain would not be suitable in a respiratory burst assay.
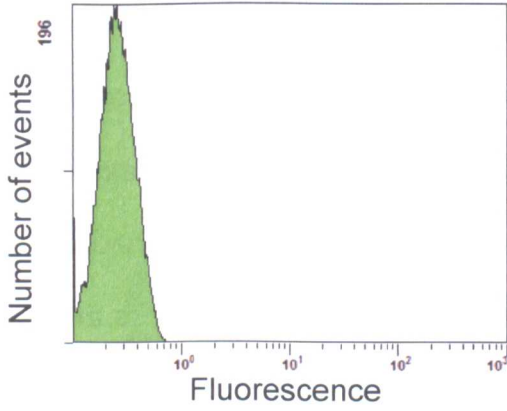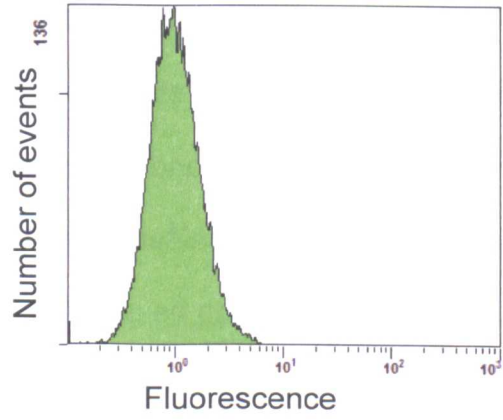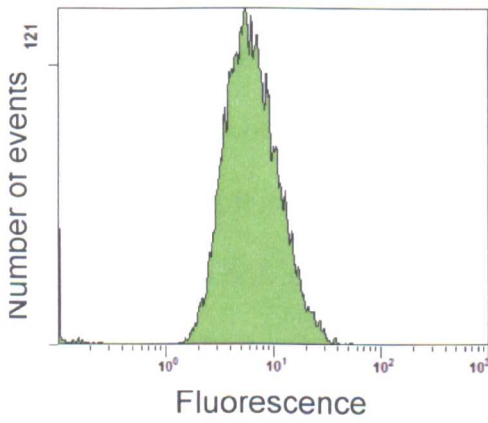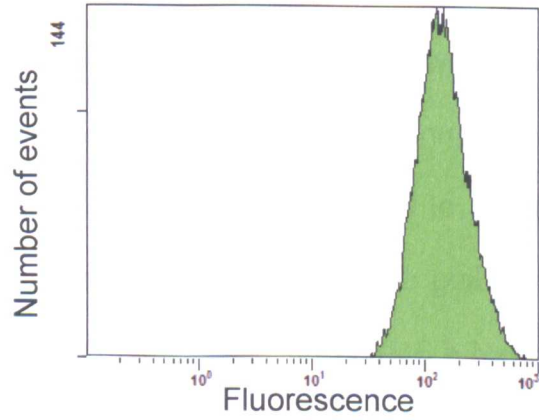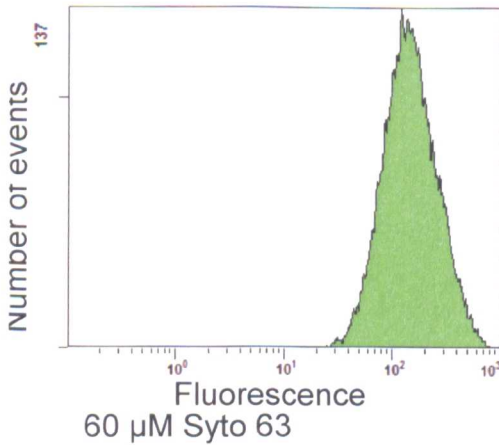
Figure 4.4.3.1 *N. meningitidis* H44/76 staining using Syto 63 nucleic acid stain.
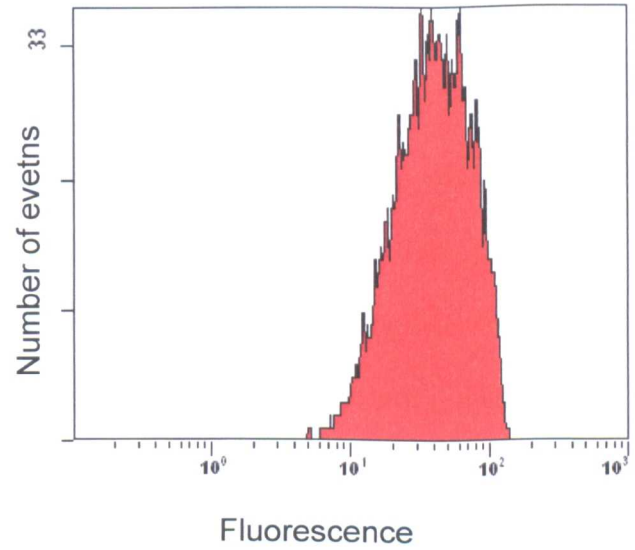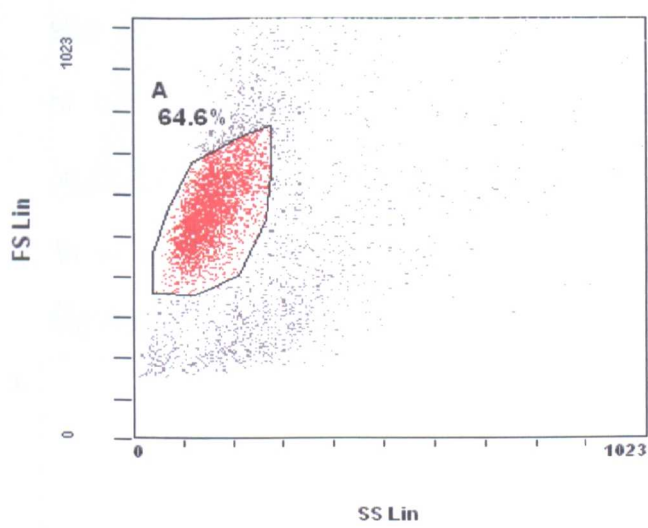
a.
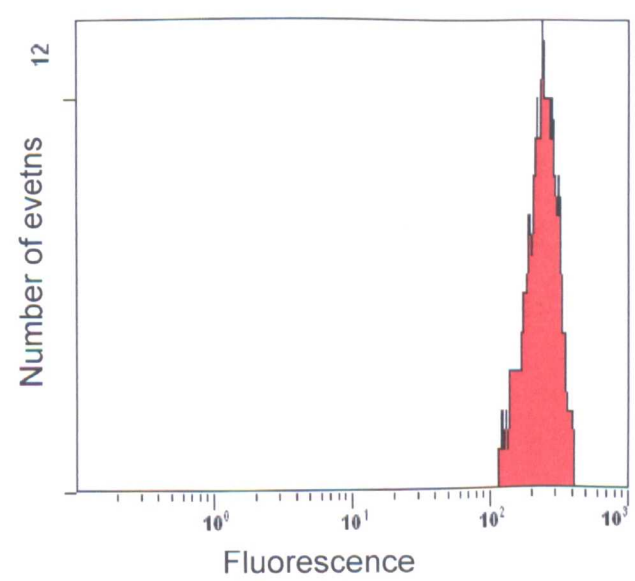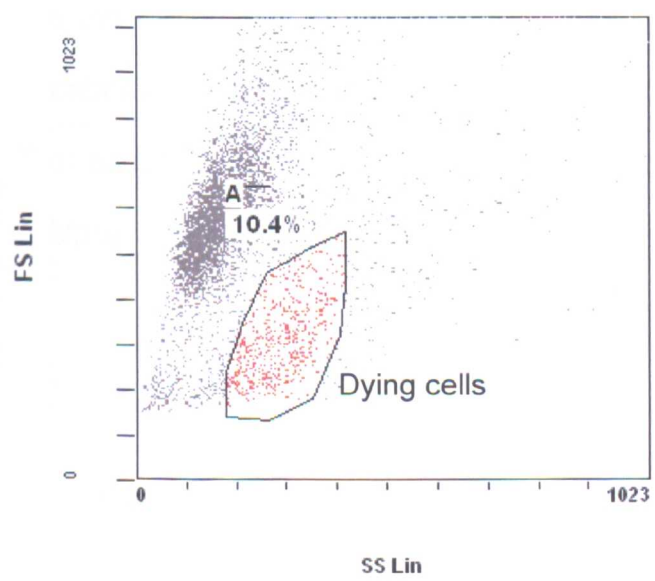
b.

Figure 4.4.3.2 Opsonophagocytosis assay performed using Syto 63 stained
N. meningitidis **H44/76.**

Forward scatter and side scatter plots with corresponding histogram showing the opsonic response observed with homologous positive control sera (a), and the Syto 63 stained bacteria, complement and differentiated HL60 cells only control looking at the dead cell and debris population (b).

4.4.4 Lipid stains

The lipophilic stain PKH-26 was assessed. This dye attaches to lipophilic tails before diffusing into the cell membrane and has been previously used in lymphocyte migration and proliferation studies (Givan *et al.* 1999; Batard *et al.* 2000) and also to stain *Bordetella pertussis* for use in an OPA (Hellwig *et al.* 1999). Bright and uniform staining was achieved with a concentration of 200 µM showing the optimal fluorescence (figure 4.4.4.1).

These stained bacteria were used in an OPA and compared with the OP response in a duplexed RBA (figure 4.4.4.2). The OPA response using PKH-26-stained bacteria was compared to BCECF-stained bacteria and very similar results were obtained (figure 4.4.4.2 a and b). In addition, the PKH-26 OP response from a duplexed RBA (PKH-26 stained bacteria and DHR123 respiratory burst reagent) showed a similar trend (figure 4.4.4.2 c). These responses provided evidence to suggest PKH-26 would be a suitable replacement stain for BCECF-AM, but would require further investigation with a larger panel of sera.

Unstained

1 μM PKH-26

10 μM PKH-26

50 μM PKH-26

100 μM PKH-26
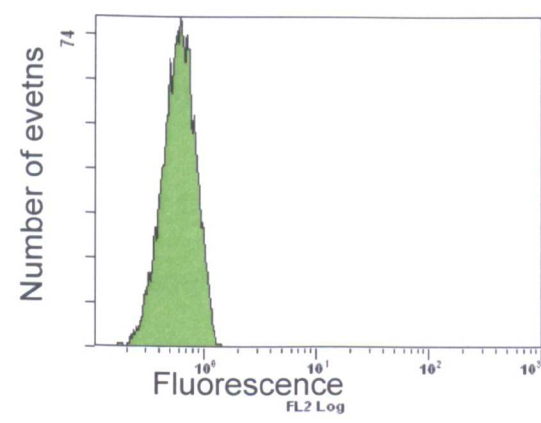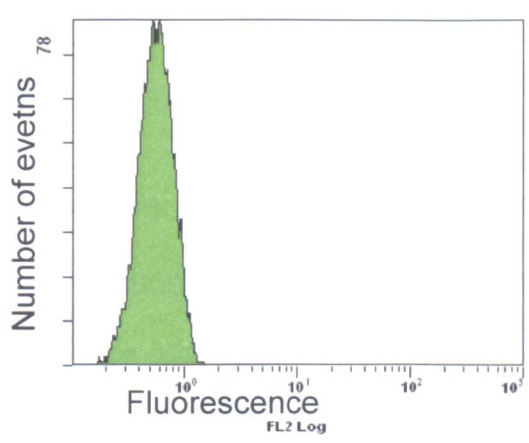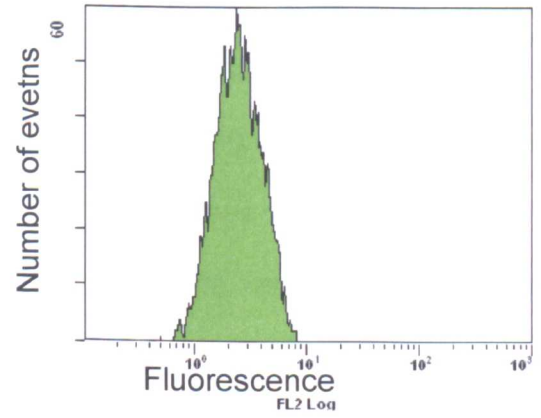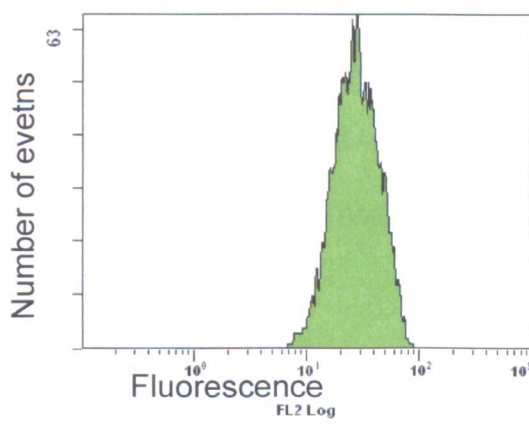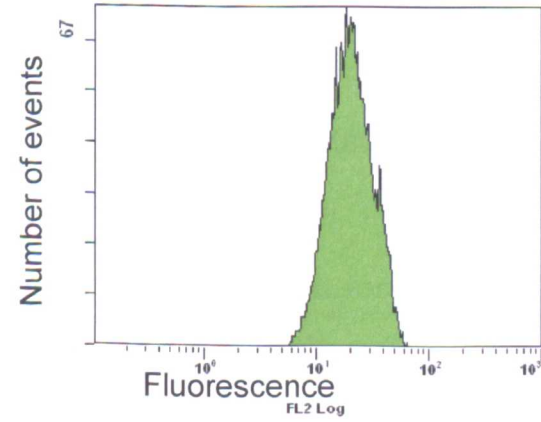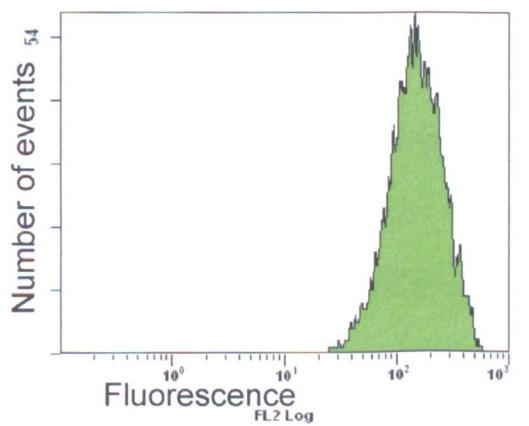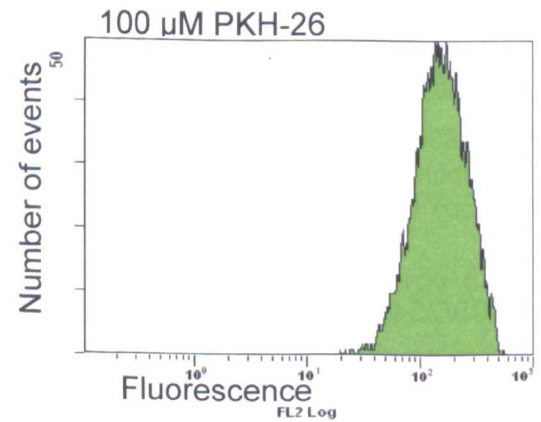
200 μM PKH-26

Figure 4.4.4.1 Staining of *N. meningitis* 44/76 using PKH-26 lipophillic stain.

400 μM PKH-26
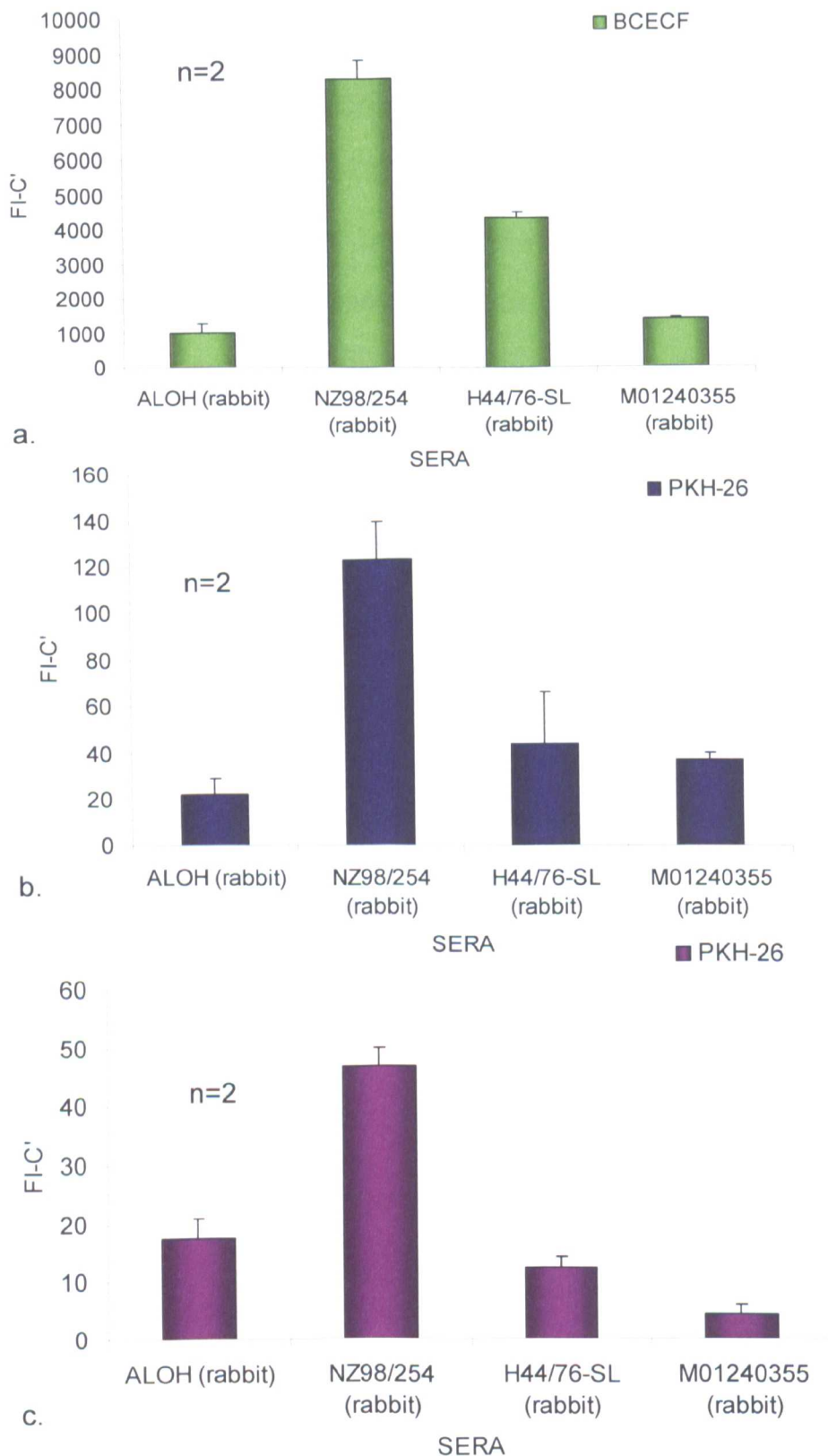
Figure 4.4.4.2 Use of PKH-26 in an OPA.

(a) An OPA using BCECF-stained *N. meningitidis* NZ98/254 and rabbit anti-OMV sera, (b) OPA using PKH-26 stained *N. meningitidis* NZ98/254 and rabbit anti-OMV sera and (c) duplexed RBA with the OP data using PKH-26 stained *N. meningitidis* NZ98/254 and rabbit anti-OMV sera. Error bars denote standard deviation.

4.5 Duplexing the assay

4.5.1 Correlation of duplexed assay responses with single assay responses.

A panel of low, medium and high responding human sera were used to investigate the correlation of the duplexed RBA responses with the OPA and RBA individual responses. All four assays were performed simultaneously: an RBA duplexed assay measuring both respiratory burst and opsonic responses, an OPA with PKH-26 stained bacteria, an OPA with BCECF-AM stained bacteria and an RBA measuring respiratory burst response only, using DHR123 and unstained bacteria. Responses were correlated to ascertain whether a duplexed assay was providing the same response as the single-plex assay.

The opsonic response measured using PKH-26 stained bacteria in a duplex assay correlated with the opsonic response using PKH-26 stained bacteria only, r=0.95 ($P<0.01$) (figure 4.5.1.1). Opsonic activity measured using PKH-26 labelled bacteria correlated with the opsonic response measured using BCECF-AM labelled bacteria, r=0.83 ($P<0.01$) (figure 4.5.1.2). The correlation between the respiratory burst response R123 and the duplexed assay measurement of R123 was non-significant (figure 4.5.1.3), r=0.62. All assays were repeated three times each giving similar correlations. The poorer correlation could have been attributable to the spectral crossover between R123 and PKH-26. A possible explanation for the poor correlation could be that when two spectrally similar dyes are in close proximity FRET (fluorescence resonance electron transfer) can occur, whereby excited energy from a donor molecule can be passed to an acceptor molecule resulting in an increase in emission in the acceptor molecule. Therefore this dye combination was not suitable for use in a duplexed assay with DHR123 as the respiratory burst reagent.

Figure 4.5.1.1 Correlation of opsonic response for PKH-26 stained bacteria in a duplexed assay vs the opsonic response of PKH-26 stained bacteria only (r=0.95, P<0.01).

Significance was determined by t-test.
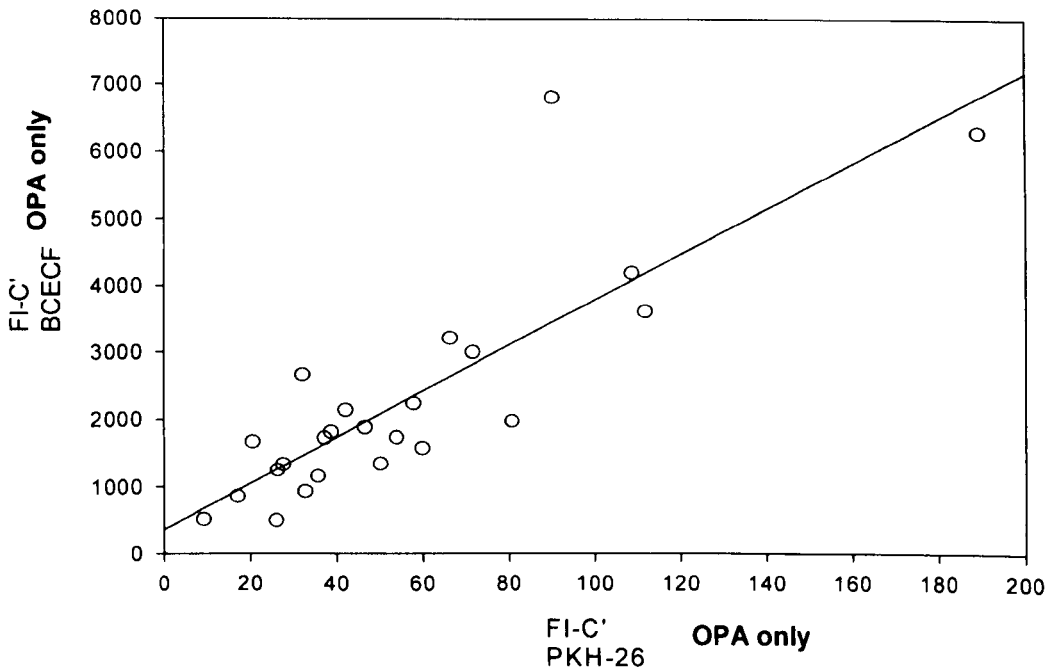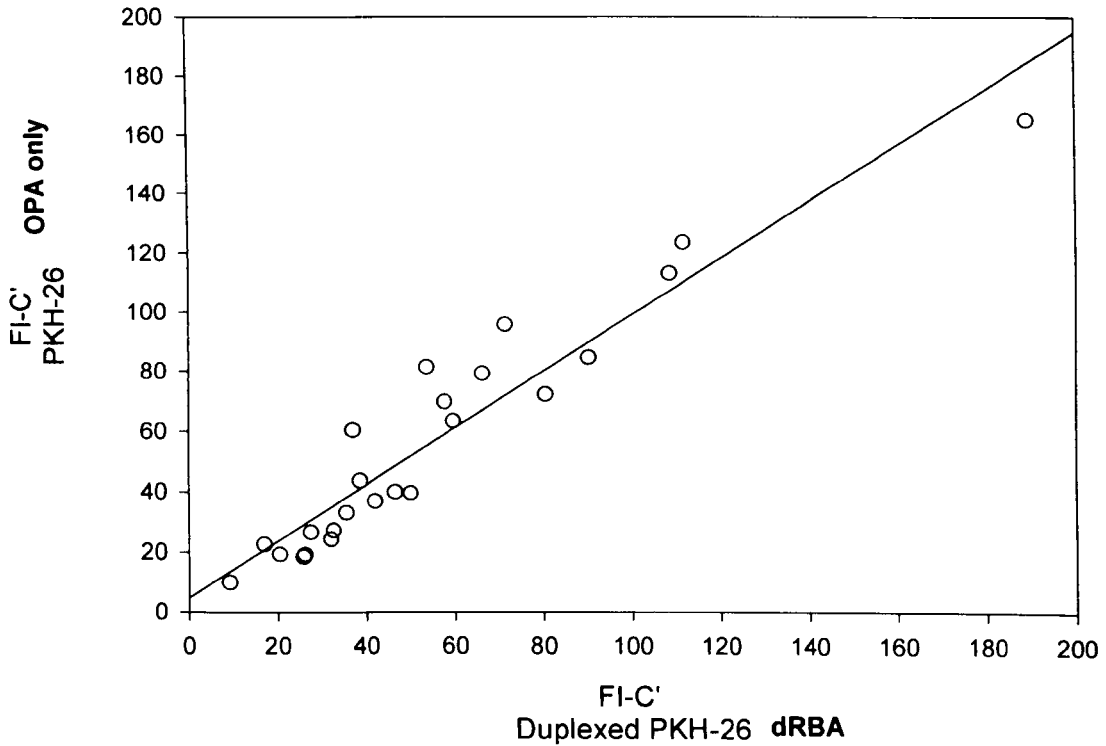
Figure 4.5.1.2 Correlation of opsonic response for PKH-26 stained bacteria in a vs the opsonic response of BCECF-AM stained bacteria only (r=0.83, P<0.01).

Significance was determined by t-test.
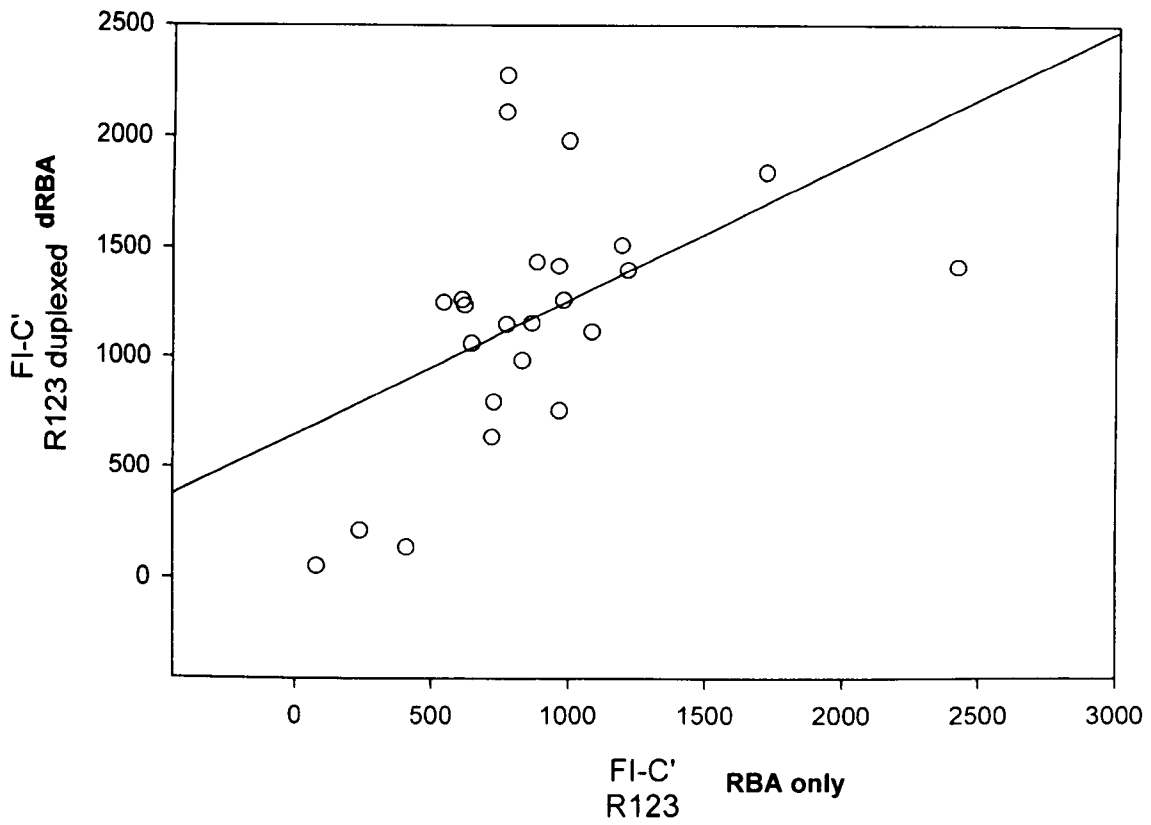
Figure 4.5.1.3 Correlation of respiratory burst response measured with DHR123 in a duplexed assay vs the respiratory burst response measured with DHR123 only (r=0.62).

Significance was determined by t-test.

4.5.2 Staining of killed bacteria with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine-5,5'-disulfonic acid (DilC$_{18}$(5)-DS).

Following the conclusion that PKH-26 would not be suitable for use in conjunction with DHR123, DilC$_{18}$(5)-DS was assessed as a possible alternative. This stain is also a lipophilic tracer which has been used for long term tracing applications and is excited by the 633 nm laser and emits at 675 nm. Therefore this stain should require much less compensation with R123 as it is spectrally different.

Azide/PMSF killed *N. meningitidis* were stained with different concentrations of DilC$_{18}$(5)-DS to optimise the staining. Greatest fluorescence was observed with 20 µM with equivalent staining observed at 40 and 80 µM (figure 4.5.2.1). At higher concentrations (160, 200 and 400 µM) a reduction in fluorescence was observed, with the lowest fluorescence occurring with the highest DilC$_{18}$(5)-DS concentration. At high concentrations of DilC$_{18}$(5)-DS precipitation of the stain can occur and thus may account for the poorer lipid staining on the bacteria. It was therefore concluded that 20 µM of DilC$_{18}$(5)-DS would be optimal staining concentration for bacteria at an OD$_{600}$ of 2.0.
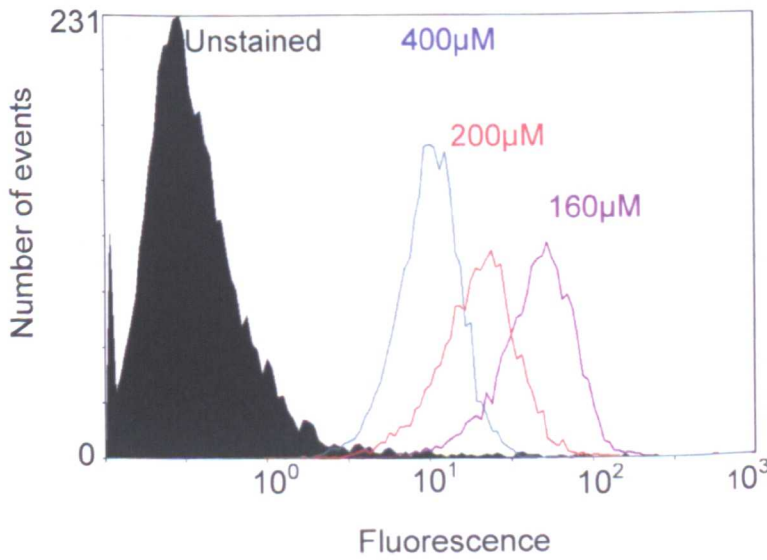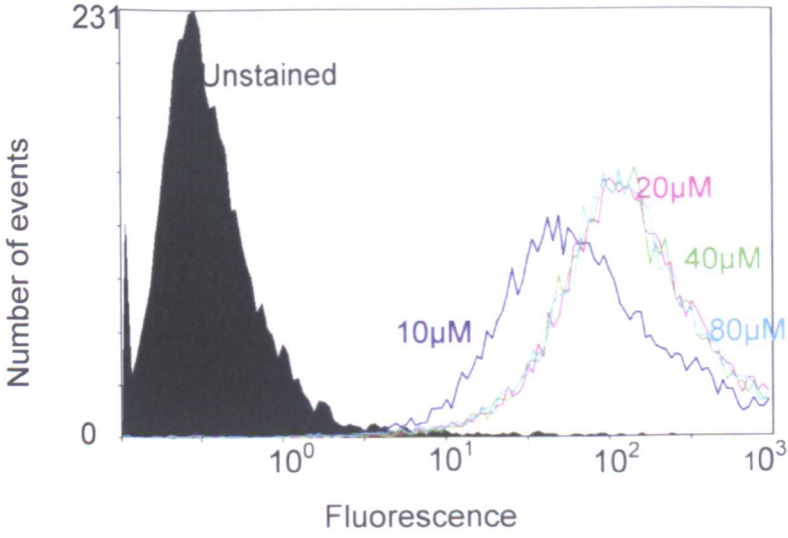
Figure 4.5.2.1 Overlay plot of increasing concentrations DilC$_{18}$(5)-DS staining of _N. meningitidis_ H44/76.

4.5.3 Comparison of total antibody binding to DiIC$_{18}$(5)-DS stained or unstained bacteria.

As DiIC$_{18}$(5)-DS is a lipid stain and there was a concern that this could mask surface epitopes and thus effect the amount of antibody able to bind to the bacteria and thus the opsonic response. To assess this a total antibody surface binding assay was performed on unstained bacteria, and bacteria stained with DiIC$_{18}$(5)-DS-stained bacteria or BCECF stained bacteria. This was conducted with a panel of 17 high, medium and low responding rabbit and human sera.

Antibody binding to DiIC$_{18}$(5)-DS-stained bacteria showed a strong positive correlation with antibody binding to unstained bacteria (r = 0.92, P<0.01)(figure 4.5.3.1). Antibody binding to BCECF-stained bacteria also showed a positive correlation with that to unstained bacteria, (r = 0.79, P<0.01) (figure 4.5.3.2). These results confirm that staining with DiIC$_{18}$(5)-DS did not adversely affect the total IgG binding to the bacteria and that when this was compared with BCECF, DiIC$_{18}$(5)-DS had the strongest correlation.
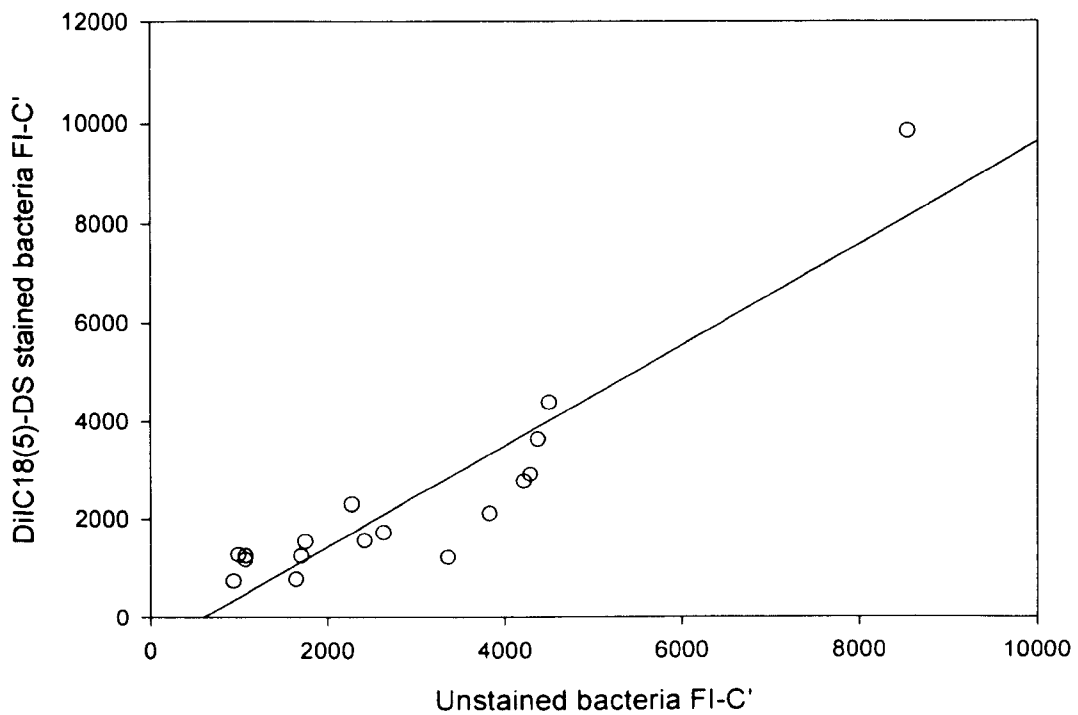
Figure 4.5.3.1 Correlation of DilC$_{18}$(5)-DS stained bacteria with unstained bacteria in a total antibody surface labelling assay against MO1240149, (r = 0.92, P<0.01). Significance was determined by t-test.
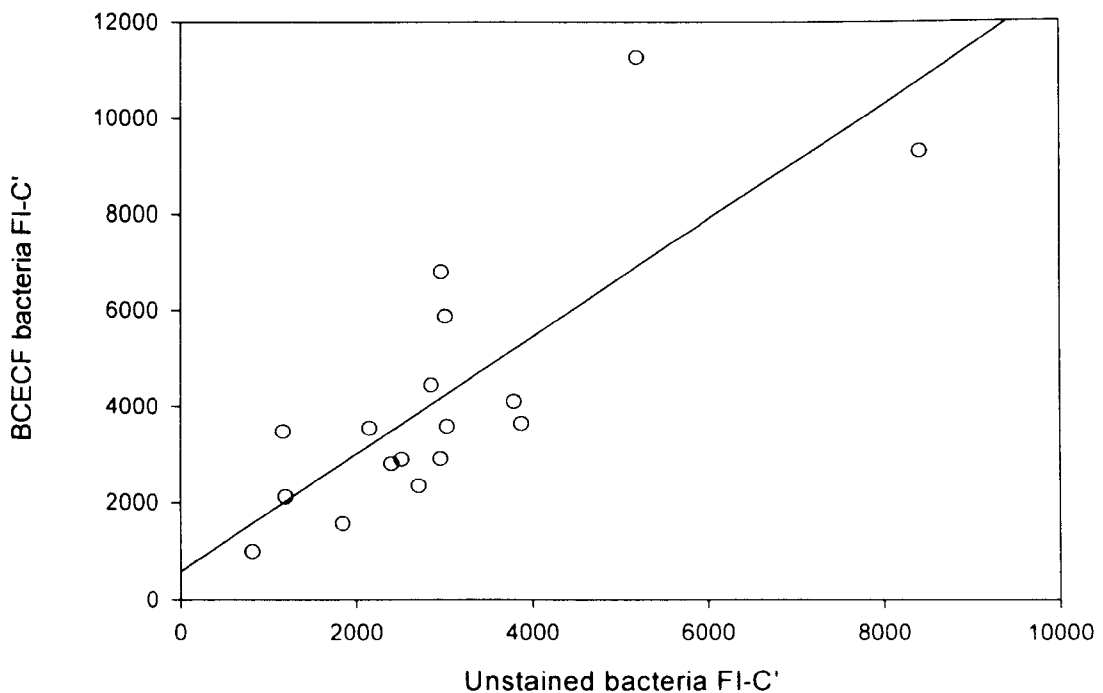
Figure 4.5.3.2 Correlation of BCECF stained bacteria with unstained bacteria in a total antibody surface labelling assay against M01240149, (r = 0.79, P<0.01).

Significance was determined by t-test.

4.5.4 Correlation of duplexed assay with single plex assay

It was important to compare an RBA using DHR123 and unstained bacteria, with a duplexed RBA using DHR123 and $DilC_{18}(5)$-DS stained bacteria, an OPA with $DilC_{18}(5)$-DS stained bacteria and an OPA with BCECF stained bacteria. The assays were performed with a panel of human and rabbit, low, medium, and high responding sera.

The opsonic response measured using the $DilC_{18}(5)$-DS stained bacteria in a duplexed assay showed a positive correlation when compared with the opsonic response measured using $DilC_{18}(5)$-DS stained bacteria only ($r=0.98$, $P<0.05$) (figure 4.5.4.1). The opsonic response measured using $DilC_{18}(5)$-DS labelled or BCECF-AM labelled bacteria also showed a positive correlation, ($r=0.89$, $P<0.05$) (figure 4.5.4.2). The respiratory burst response measured with DHR123 only or in a duplexed assay using DHR123 also demonstrated a positive correlation, $r=0.88$ ($P<0.05$) (figure 4.5.4.3).

$DilC_{18}(5)$-DS stained bacteria and DHR123 was used as a dye combination in all subsequent work.
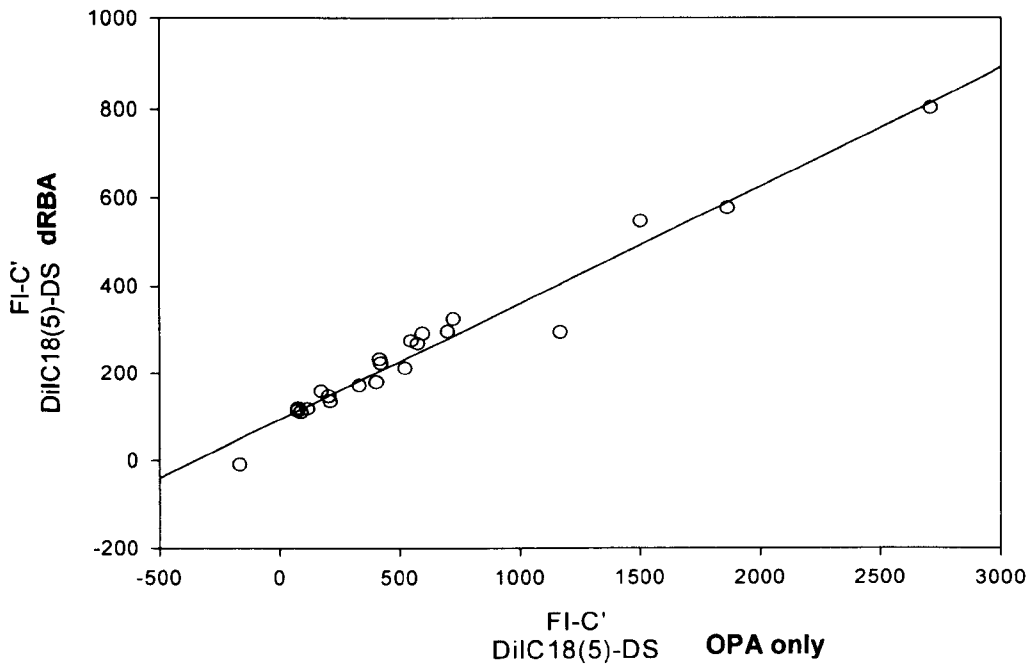
Figure 4.5.4.1 Correlation of opsonic response DilC$_{18}$(5)-DS labelled bacteria in a duplexed RBA with the opsonic response for internalised DilC$_{18}$(5)-DS labelled bacteria only, (r=0.98, P<0.01).

Significance was determined by t-test.
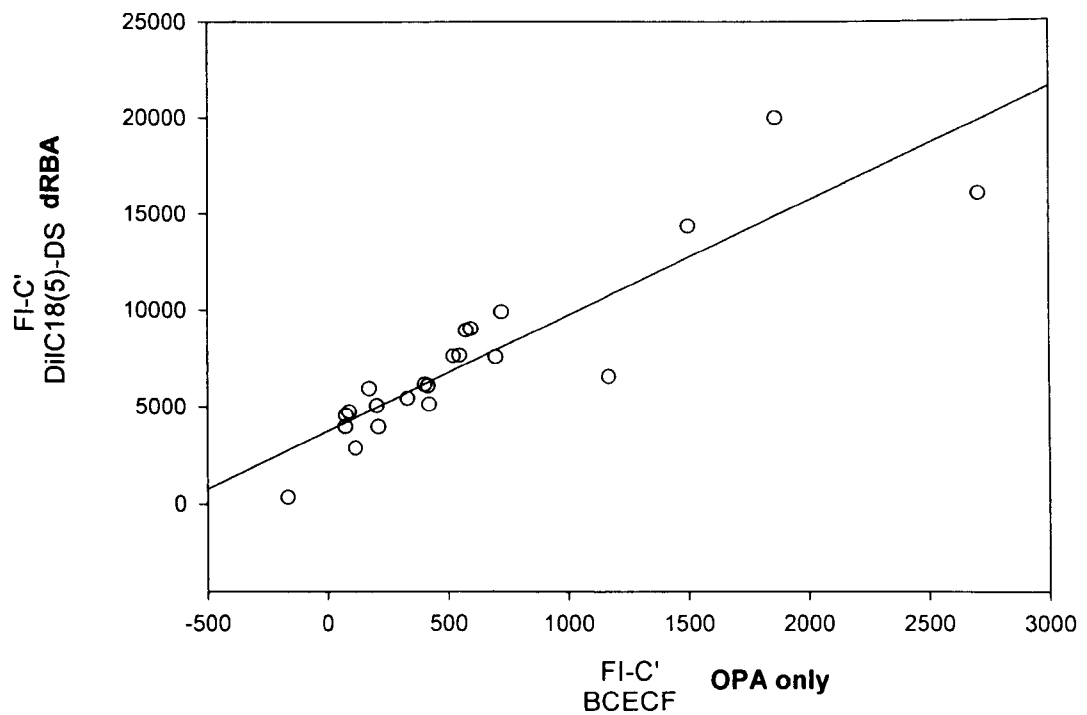
Figure 4.5.4.2 Correlation of opsonic response for DilC$_{18}$(5)-DS labelled bacteria in a duplexed assay with the opsonic response of BCECF-AM labelled bacteria only, (r=0.89, P<0.01).

Significance was determined by t-test.
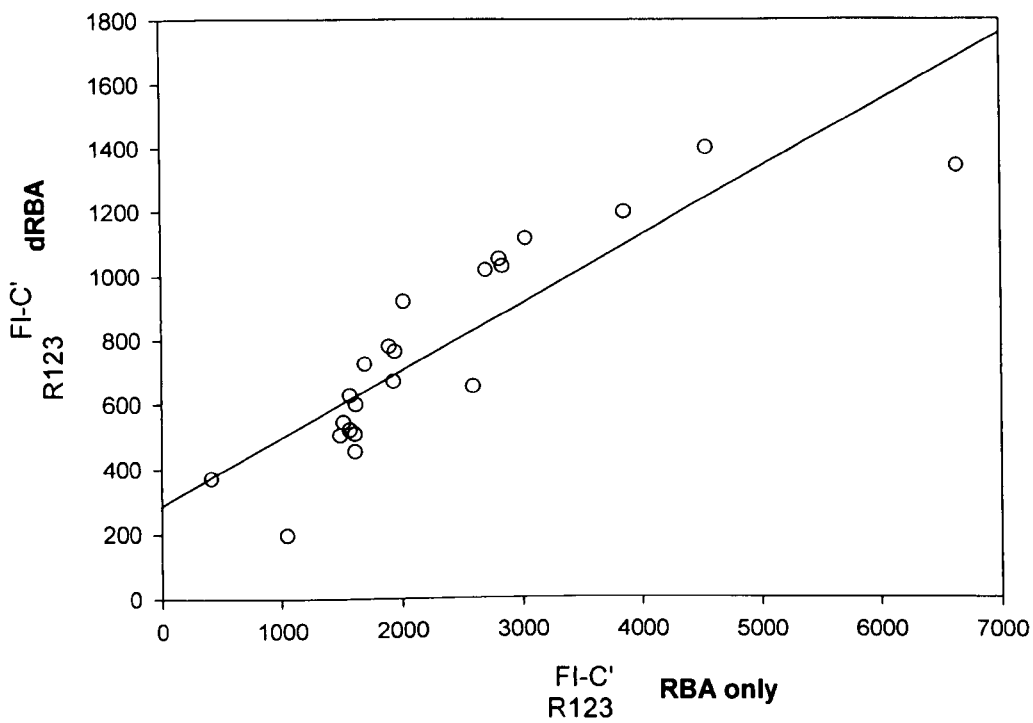
Figure 4.5.4.3 Correlation of respiratory burst response measured with DHR123 in a duplexed assay compared with the respiratory burst response measured with DHR123 only, (r=0.88, P<0.01).

Significance was determined by t-test.

4.6 Assay precision

4.6.1 Precision of duplexed respiratory burst assay measuring both opsonic uptake of *N. meningitidis* and the respiratory burst response.

The reproducibility of the duplexed assay for the measurement of OP activity and respiratory burst assay was assessed using a panel of 21 sera consisting of low, medium and high activity human sera and rabbit OMV sera homologous and heterologous to the test strain *N. meningitidis* M01240149. These assays were completed by three operators in triplicate on three separate days and data for each serum was analysed to assess the precision of results. The results were analysed by flow cytometry and the coefficient of variance (CoV) calculated from the mean values derived from the duplicate of each serum.

The intra-assay variability was high for 12 out of 21 sera tested with a CoV of more than 36%, and the remaining 9 sera demonstrating CoV less than 35%. There was a higher level of variation observed when precision was assessed with three operators completing three assays in one day, with 16 of the 21 sera tested with a CoV higher than 35% (table 4.6.1.1). Assay variation between two assays showed the percentage difference in response to vary between +100% and -100% when two assays were compared (figure 4.6.1.1). Percentage CoV was higher than that expected and higher than other comparable cell-based assays (Romero-Steiner *et al.* 2003; Martin *et al.* 2005).

When the data was reanalysed as the two separate components of the assay, respiratory burst response showed greater variation, with 15 of the 21 sera tested showing CoV higher than 35% (table 4.6.1.3). The opsonic response appeared to be much more reproducible with 16 out of 21 sera showing CoV less than 35%

(table 4.6.1.2). This indicated that the respiratory burst was the cause of the high levels of assay variation.

Thus further investigation was required to determine the cause(s) of assay variation, and whether this could be resolved by introducing a fixation step at the end of the assay.

Coefficient of Variance (%)

Intra-assay variability

		<15%	≤35%	>36%
20 sera in duplicate, performed three times by one operator on one day. n=60	**20 Sera with defined CV**	0	9	12

Inter-operator variability

		<15%	≤35%	>36%
20 sera in duplicate, performed three times by three operators on one day. n=180	**20 Sera with defined CV**	0	5	16

Table 4.6.1.1 Summary of duplexed RBA %CoV for 21 low, medium and high responding human sera.
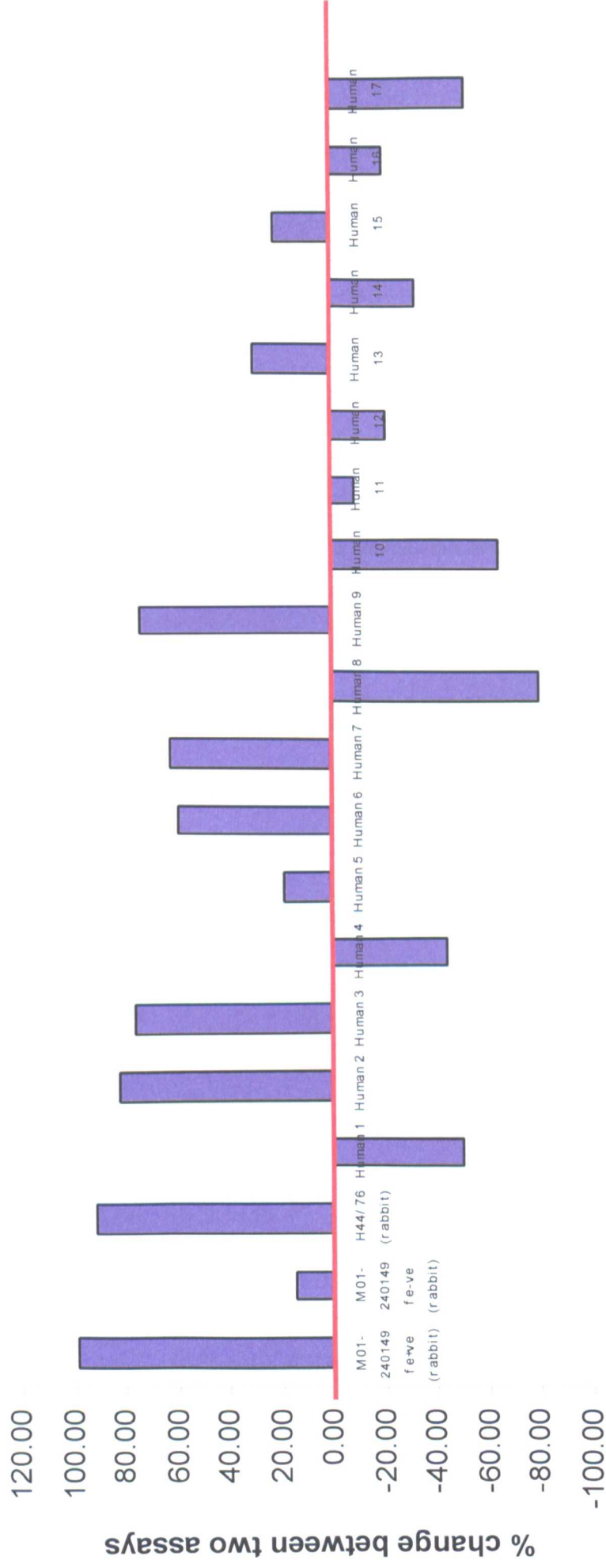
Figure 4.6.1.1 Percentage change between duplicate assays run as part of the precision testing.

Intra-assay variability		<15%	≤35%	>36%
20 sera in duplicate, performed three times by one operator on one day. n=60	20 Sera with defined CV	0	16	5

Table 4.6.1.2 Summary of opsonic response only, %CoV for 21 low, medium and high responding human sera.

Coefficient of Variance (%)

Intra-assay variability		<15%	≤35%	>36%
20 sera in duplicate, performed three times by one operator on one day. n=60	20 Sera with defined CV	0	6	15

Table 4.6.1.3 Summary of respiratory burst response only, %CoV for 21 low, medium and high responding human sera.

4.6.2 Effect of formaldehyde fixation of RBA

To establish whether fixation would reduce assay variation, a duplexed RBA was performed twice. One assay was completed without fixation and one assay was fixed with 1% formaldehyde for 15 min prior to being washed and then both assays were analysed by flow cytometry at 0, 60 and 120 min following completion of assay.

No variation was observed in the opsonic uptake response detected over 2 h without formaldehyde fixation. This was consistent with results shown previously in the precision testing of this assay (figure 4.6.2.1). When these results were compared with the opsonic response in a fixed assay, fluorescence index was reduced immediately and this further reduced over 2 h (figure 4.6.2.1).

High levels of variation were observed in the respiratory burst response over the 2 hours without formaldehyde fixation and confirmed the results observed during assay precision testing (figure 4.6.2.2). Following assay fixation the FI showed a 50% reduction at 0 h and this continued to reduce over 2 h (figure 4.6.2.3).

This demonstrated that following fixation, the fluorescence was adversely affected and this was not a suitable method to reduce assay variation. Therefore the RBA could not be used for high throughput analysis and only to assess small numbers of sera. It also indicated that the respiratory burst reagent degraded rapidly in the absence of fixation. This again indicated the assay was only suitable for analysis of small numbers of sera.
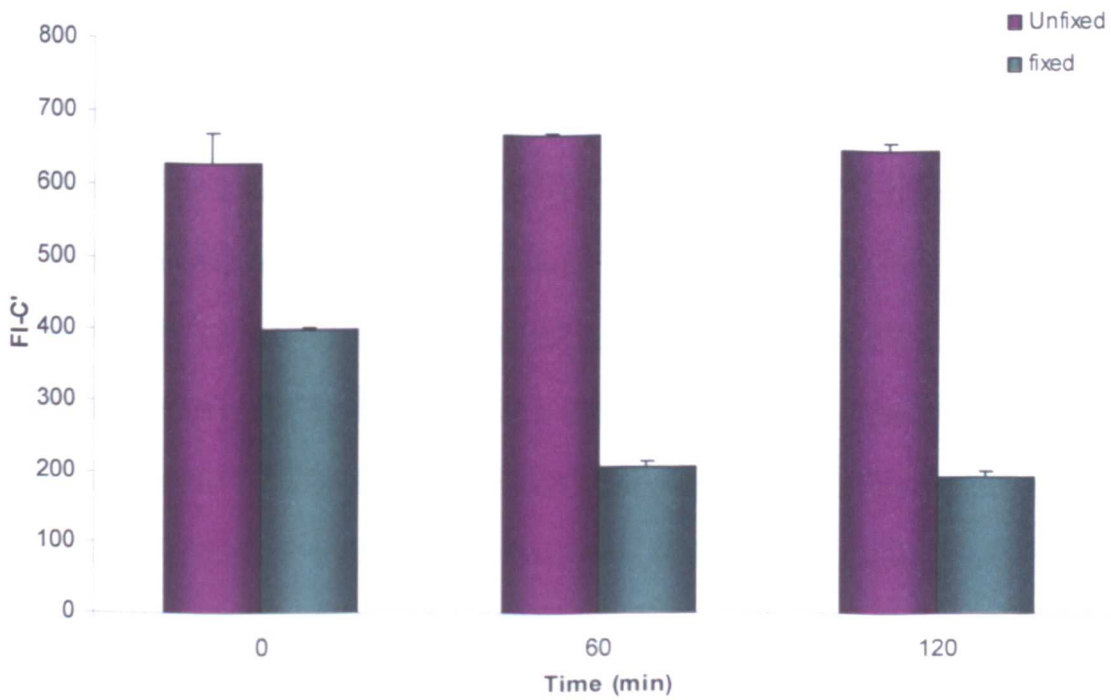
Figure 4.6.2.1 Opsonophagocytosis response measured over 120 min following completion of assay, with or without formaldehyde fixation.
Assay was performed with *N. meningitidis* M01240149 with mouse anti-OMV serum. Error bars denote standard deviation.
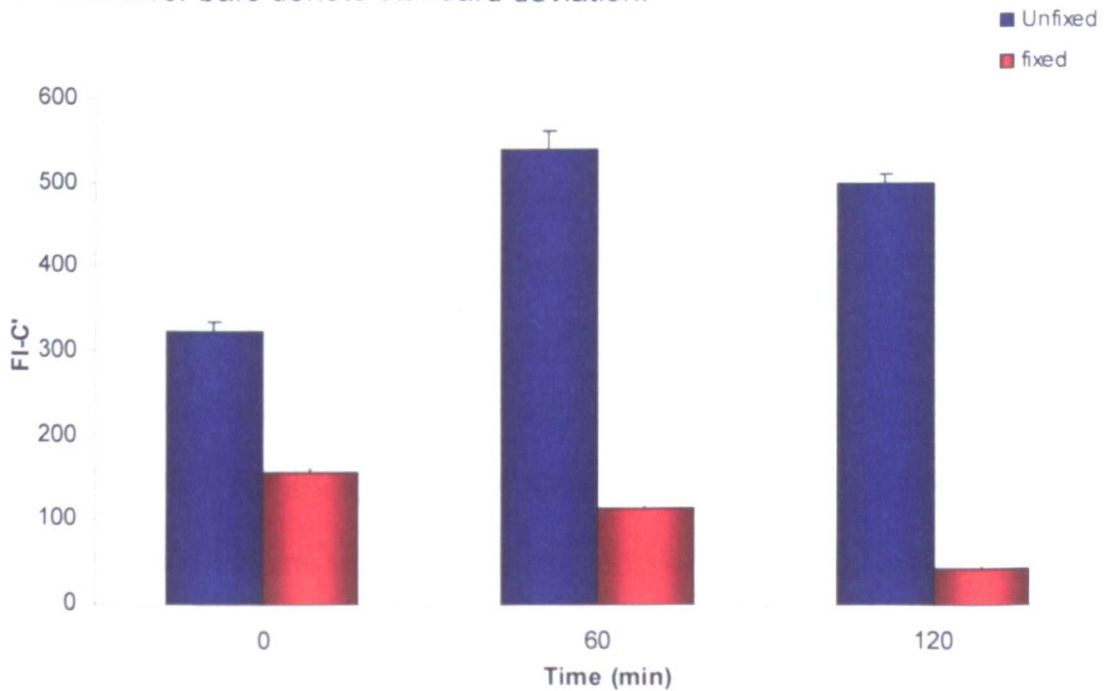
Figure 4.6.2.2 Respiratory burst response measured over 120 min following completion of the assay with or without formaldehyde fixation.
RBA was performed against *N. meningitidis* M01240149 with mouse anti-OMV serum. Error bars denote standard deviation.

4.7 Discussion

With the observation that absence of SBA does not necessarily constitute an absence of protection (Platonov *et al.* 2003; Welsch and Granoff 2007; Plested and Granoff 2008; Plested *et al.* 2009). Opsonophagocytosis activity has been suggested as an additional mechanism of protection (Granoff 2009), there have been several published opsonophagocytosis assays that have been developed to characterise protection from meningococcal disease. These assays have varied in detection method: measurement of fluorescent OMV coated polystyrene beads, uptake of labelled meningococci, measurement of the induction of the respiratory burst response, as well as opsonic killing assays (Aase and Michaelsen 1994; Lehmann *et al.* 1998; Plested and Granoff 2008).

This chapter describes the development of a high-throughput flow cytometric duplexed respiratory burst assay, measuring both the uptake of fluorescent bacteria and the induction of a respiratory burst response. Measurement of both parameters may correlate better with killing than either response measured individually. Measurement of respiratory burst individually does not account for the response stimulated by N-formylmethionylleucylphenylalanine (FMLP) (Elbim *et al.* 1994), IFN-γ (Ellis and Beaman 2004) and TNF-α (Menegazzi *et al.* 1994). Lehmann et al. (1998) showed that the level of phagocytosis did not correspond with the level of respiratory burst response measured. Low and medium response sera induced the same level of respiratory burst, whilst the medium response serum was able to induce much higher levels of phagocytosis.

Initial investigations attempted to measure a respiratory burst response in HL60 cells. The initial method used was an OPA developed to measure the uptake of

bacteria stained with BCECF-AM using DMF-differentiated HL60 cells and baby rabbit complement (Taylor 2010) (2.9.7). A number of different reagents for measurement of the respiratory burst response were then compared. H_2DCFDA, $CM-H_2DCFDA$, carboxy-H_2DCFDA, DHR 123, DHR 6G, and DHE were investigated, all of which measure the production of hydrogen peroxide except DHE which measures the production of superoxide. In order to detect a respiratory burst response, the detection of hydrogen peroxide was thought to be more appropriate than superoxide as hydrogen peroxide has been shown to be more bactericidal against *N. meningitidis* (Roos *et al.* 2001; Dyet and Moir 2006).

When the different reagents were compared in a respiratory burst assay, no responses were observed with H_2DCFDA, Carboxy-H_2DCFDA, DHR 6G, and DHE. Non-specific fluorescence was observed with $CM-H_2DCFDA$, with similar fluorescence seen with the negative control serum and the homologous OMV rabbit control serum. This was possibly a result of photoreduction of $CM-H_2DCFDA$ to H_2DCF during the course of the assay, as these compounds can be highly photosensitive (Gomes *et al.* 2005). DHR123 was the only reagent which demonstrated low fluorescence with the negative control and high fluorescence with the homologous OMV rabbit control sera, and was chosen for all further assay development. DHR123 is used for detection of respiratory burst in other assays (Aase *et al.* 1995; Lehmann *et al.* 1998; Bassoe *et al.* 2000; Walrand *et al.* 2003; Freitas *et al.* 2009). HE and H_2DCFDA have also been successfully used for detection of ROS. However, the assay protocol used to test these reagents was not necessarily optimal for all the reagents tested, but the assay had been optimised for the measurement for opsonophagocytosis of *N. meningitidis*, and therefore a reagent which could be used within the existing protocol was required.

Thus no further investigation was conducted into the other reagents as DHR123 was able to detect the ROS of interest and within the assay parameters.

Other investigators have introduced DHR123 into the assay protocol in different ways, some introducing DHR123 in the assay buffer with the cells (Lehmann *et al.* 1998) and others pre-loading the cells with the dye (Aase *et al.* 1995; McCloskey and Salo 2000; Walrand *et al.* 2003; Brechard *et al.* 2005; Aase *et al.* 2007). Three different methods were compared: preloading the HL60 cells, introduction of DHR123 during the first assay incubation (opsonisation of bacteria, sera and complement) or during the second assay incubation (addition of differentiated HL60 cells). A significantly (*P*<0.05) higher respiratory burst response was detected with DHR123 preloaded HL60 cells.

The preloading incubation time and the DHR123 concentration were then optimised showing a significantly higher response with 15 minutes preloading of HL60 cells with DHR123 at a concentration of 50 µg/ml.

DMF differentiation of HL60 cells produced granulocytic-like cells, and these are commonly used in opsonophagocytosis assays (Romero-Steiner *et al.* 2003). The use of PMNs is reliant on volunteer donation and this restricts the numbers of samples which can be assessed in an assay, using HL60s allows larger numbers of sera to be evaluated in a single assay. Use of PMNs from different volunteers also introduces an additional variable in the assay, which may result in poor assay reproducibility. HL60s are a promyelocytic leukaemia cell line, which do not differentiate down the accepted maturation pathway for a promylocyte (Birnie 1988). HL60s have been reported to lack both specific and secretory granules,

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important in delivery of the membrane bound component of NADPH oxidase and thus a lower respiratory burst response would be expected in comparison to neutrophils, however a respiratory burst has been demonstrated in HL60s (Nordenfelt et al. 2009) and they have been shown to be useful for investigations measuring ROS production (Teufelhofer et al. 2003). It was therefore important to compare HL60 cells with PMNs to assess whether the response of HL60s was representative. A positive correlation was found when PMNs and HL60s were compared in a respiratory burst with a panel of high, medium and low responding human sera, leading to the conclusion that HL60s are a suitable cell line for the detection of a respiratory burst response.

The introduction of a priming agent was investigated to see whether this could improve the respiratory burst signal. Reagents were selected on the basis to prime the cells, rather than directly stimulate the cells. FMLP, IFNγ and TNFα have all been shown to directly stimulate the respiratory burst response, but IFNγ and TNFα have also been shown to prime PMNs without stimulation of the respiratory burst response, perhaps due to differences in the concentration, incubation time and detection methods used when investigating the cytokine effects (Elbim et al. 1994; Menegazzi et al. 1994; Ellis and Beaman 2004). TNF-α, IL-8, GMCSF and IFN-γ have been shown to be able to prime neutrophils to be more responsive to pathogens, and produce greater quantities of oxygen radicals when stimulated (Phillips et al. 1990; Roilides et al. 1993; Elbim et al. 1994). TNF-α, IL-8, GMCSF and IFN-γ were compared, and IFN-γ showed a significant ($P<0.05$) increase in assay response. The concentration and incubation time of IFN-γ with HL60 cells were optimised to 100 ng/ml for 3 hours prior to use within the assay. It was observed that priming of HL60 cells with all of the cytokines tested moderately increased the assay complement-only background, but this increase was not

affected by the incubation time used. HL60 cells primed with cytokines were compared with unprimed HL60 cells with a panel of mammalian sera, and showed that cells primed with the different cytokines correlated with unprimed HL60 cells, thus increasing the magnitude of the response but not alter the pattern of response. Something not investigated during this work was the effect of combinations of priming cytokines. Further work should investigate whether this could further improve the respiratory burst within the HL60 cells. This may be more representative of *in vivo* conditions where multiple priming cytokines would be present during inflammation.

During the respiratory burst response hydrogen peroxide is produced, which during this assay reacts with the DHR123 to form fluorescent rodamine 123 and water (Invitrogen technical information sheet). The production of hydrogen peroxide during the respiratory burst leads to a number of bactericidal chemicals being produced following catabolism of hydrogen peroxide, a reaction which occurs very quickly (Hampton *et al.* 1996). Sodium azide was used to inhibit the myeloperoxidase activity and provide opportunity for the reaction with DHR123 to occur. Very low levels of sodium azide were introduced into the assay buffer and a significant (*P*<0.05) increase in assay respiratory burst response was observed. As sodium azide itself is toxic, and therefore a further investigation was performed to check for any impact on cell viability with sodium azide in the assay buffer. No difference was found with or without sodium azide.

One further assay parameter investigated was the optimisation of glucose levels in the assay buffer. Neutrophils require glucose to generate ATP and NADPH through the pentose phosphate pathway, and NADPH is essential for the respiratory burst response to occur, as it is consumed by the NADPH oxidase and

leads to the production of superoxide, hydrogen peroxide and other ROS. As the assay buffer contains 2% skimmed milk as a blocking agent the buffer already contained glucose but this was at an unknown level. A range of concentrations of glucose were introduced into the assay buffer and a significant ($P<0.01$) increase in assay response was observed at 1 mM additional glucose, at higher levels 2 and 4 mM glucose a significant reduction in response was observed. 1 mM glucose was therefore introduced into the assay buffer. Other published work investigating the optimum experimental conditions required for measurement of neutrophil respiratory burst, has found a glucose concentration of 0.5 mM to be optimum, not showing any significant difference at higher concentrations (Freitas *et al.* 2009).

An alternative bacterial fluorescent stain was required to measure uptake of bacteria because DHR123 and BCECF-AM had overlapping excitation and emission spectrums (figure 4.4.1.1). BCECF-AM was used as a bacterial stain because it stains internally, BCECF-AM is membrane permanent and once inside the bacteria the AM ester group is cleaved by non-specific internal esterases, resulting in a charged BCECF which leaks out of the bacteria at a low rate. This method of internal bacterial staining does not mask surface epitopes, an important consideration when utilising stained bacteria in assays assessing antibody induced immune responses. Fluorescent labelling of meningococci for use in flow cytometric assays has involved the use of the FITC, Rhodamine Green-X or Texas Red labelling (Lehmann *et al.* 1998). These stains bind to amine groups, which may mask surface epitiopes. Alternatively, OMV-coated fluorescent polystyrene beads have also been used as phagocytic targets (Lehmann *et al.* 1999; Bassoe *et al.* 2000; Martinez *et al.* 2002), however this method raises the issue of relevance to the intact bacterial targets.

Initial studies focused on an alternative fluorescent stain which would be able to stain meningococci internally. SNARF-AM was shown to stain *N. meningitidis* weakly even at very high stain concentrations and incubation times. When the stained meningococci were assessed in an OPA, no detectable OP response was observed. As this was the only suitable alternative AM dye, alternative stains were considered. Syto dyes are nucleic acid stains which passively diffuse through cell membranes to stain nucleic acid, and these stained *N. meningitidis* strongly and uniformly (figure 4.4.3.1). However, when Syto stained bacteria were used in an OPA, the stain was observed to leak out of the bacteria and diffuse into all HL60s, as shown by the bright staining of all cells including dead HL60s (figure 4.4.3.2). This made the stain unsuitable for use in the RBA.

PKH-26 dye attaches to lipohilic tails before diffusing into the cell membrane and has been shown in previous studies to be an effective stain for lymphocyte migration and proliferation studies (Givan *et al.* 1999; Batard *et al.* 2000). Bright uniform staining was achieved with *N. meningitidis*, and following an OPA a response was achieved at a lower level when compared with BCECF stained bacteria, but was able to distinguish between the ALOH control serum and the homologous OMV rabbit serum. In order to establish whether PKH-26 stained bacteria could be used in conjunction with DHR123 to measure both uptake and induction of a respiratory burst response, assays measuring either OP with PKH-26 stained bacteria or RB with DHR123 preloaded HL60 cells were compared with the duplexed assay measuring both parameters. A significant strong positive correlation was observed with PKH-26 in the duplexed and singleplex assays ($r=0.95$, $P<0.05$). No significant correlation was observed for DHR123 used in the singleplex and duplexed assay ($r=0.62$). As PKH-26 and DHR123 were excited by

the 488 nm laser, the observed poor stain compatibility could be attributed to the occurrence of FRET (fluorescence resonance electron transfer). FRET has been found to occur in situations where stains are within close proximity (5 nm), the energy from excitation of a donor molecule is able to pass to an acceptor molecule resulting in an increase in emission in the acceptor molecule (Ciruela 2008).

As PKH-26 was successful as a bacterial stain, a similar lipophilic dye to PKH-26 was assessed. $DilC_{18}(5)$-DS stains using the same mechanism as PKH-26, however it is excited by the 633 nm laser. $DilC_{18}(5)$-DS has been used previously to label and track embryonic cells, chosen for its ability to label strongly and permanently (Fraser 1996). $DilC_{18}(5)$-DS stained uniformly and maximally at 20 µM, with increasing concentrations staining less well, caused by stain precipitation (Invitrogen information sheet). When $DilC_{18}(5)$-DS stained N. meningitidis were tested for compatibility with DHR123, again assays measuring either OP with $DilC_{18}(5)$-DS stained bacteria or RB with DHR123 preloaded HL60 cells were compared with the duplexed assay measuring both parameters. Significant positive correlations were observed with both $DilC_{18}(5)$-DS stained N. meningitidis used in an OP and in the duplexed assay. Significant positive correlations were also observed between DHR123 measured in the duplexed assay and DHR123 measured in an RB.

Assay precision was investigated for the duplexed assay using a selection of high medium and low responding sera, assayed by three operators three times on a single day. High variance was observed between operators and by one operator with 16 of 21 sera having a CoV higher than 36%, which are higher than those seen with ELISA where a typical acceptance criteria is set a ≤10% (Granoff et al. 1998). Other functional assays have reported CoV ≤15% for intra-assay variation

reported for the validated SBA used in the MeNZB vaccine trials (Martin *et al.* 2005). Whereas a meningococcal OPA demonstrated a comparable level of variance of ≤30% for intra and inter-assay variation (Taylor 2010) which was lower than that observed with a pneumococcal OPA where much higher CoV have been observed (Romero-Steiner *et al.* 2003). Measurement of respiratory burst response by DHR123 has been reported to have good inter-assay variance (CoV <10%) when using high responding serum, this variance increased with lower responding serum (Lehmann *et al.* 1998). When the opsonic response and respiratory burst response were analysed separately, the respiratory burst response provided the greatest variation with CoV comparable to the measured duplex response, whereas CoVs for intra-assay opsonic response showed 16 of 21 sera with a CoV <35%. This indicated that the variation resulted from the R-123 signal (respiratory burst response). This variation has also been observed by Lehmann et al. who noted constant drift in R-123 signal with time after incubation (Lehmann *et al.* 1998). However, DHR123 has been used successfully by other investigators (Aase *et al.* 1995; Lehmann *et al.* 1998; Bassoe *et al.* 2000), and is perhaps not a problem if small numbers of samples are being tested, however this assay was being developed as a high-throughput assay and therefore sample stability over several hours is required whilst analysis by flow cytometry occurs.

To investigate whether this assay would be fixable, two duplexed assays were performed either fixed with 1% formaldehyde for 15 min or unfixed, both assays were analysed by flow cytometry at 0, 60 and 120 min after completion of the assay. The unfixed opsonophagocytic response was consistent over the time period tested, but fixation reduced the detectable opsonic response. Unfixed respiratory burst response was variable over time, but following fixation showed a progressively lower response over 120 minutes. Formaldehyde was therefore not

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compatible with either DHR123 or DilC$_{18}$(5)-DS. Loss of R-123 signal could have been because R-123 is sequestered by the mitochondria, and following fixation the mitochondrion's membrane potential is lost resulting in the R-123 signal being lost out of the cell (Poot *et al.* 1996). In addition to this, R-123 is photosensitive and this feature may also have contributed to the observed loss of signal and the variation in signal observed between assay duplicates. The results also showed that DilC$_{18}$(5)-DS was sensitive to formaldehyde fixation.

DHR123 was found to be variable in the developed assay, yet because the assay was developed for high-throughput use, the variation seen with the R-123 signal was unacceptable subsequently the assay is only suitable for assessment of small panels of sera. The R-123 signal was also found to be unfixable and therefore future work would need to assess whether any other ROS detection agent would be useable within the assay, focusing on a fixable reagent to improve assay precision. These results found do show that caution should be applied assay using DHR123 as a respiratory burst agent and that assay results need to be assessed for precision, before data can be interpreted.

In addition to reagent selection, it would also be important for the development of this assay to correlate the response of the duplexed assay (opsonophagocytosed bacteria and respiratory burst) with the OP killing assay, with respiratory burst alone and OPA (opsonophagcytosed bacteria) only. This would inform if a duplexed assay would show a stronger correlation with the OP killing assay than either phagocytosed bacteria or respiratory burst only.

The development of an assay which could measure both bacterial uptake and respiratory burst induction would be a valuable tool for the assessment of a

functional antibody response. The high-throughput nature would be a requirement for use to assess large numbers of clinical sera, something which is difficult, laborious and requires large volumes of serum with existing opsonic killing assays. However, this work has shown this not to be possible with currently available reagents. This work could be completed with the development of further respiratory burst reagents.

Chapter 5

Chapter 5: Development of a human complement source for use in immunoassays

5.1 Introduction

The pivotal role of complement in protection against meningococcal disease is evident from the marked increase in disease susceptibility seen in complement deficient individuals (Figueroa and Densen 1991; Hellerud *et al.* 2010). A central role for SBA has been established in the protection against meningococcal disease (1.4.1). Thus the serum bactericidal assay is currently the primary method used in the assessment of vaccine-elicited responses. However, other studies have shown protection in the absence of bactericidal antibody and that OP may play an important role in protection from disease (Granoff 2009). Antibody-mediated C3b/iC3b deposition is able to induce OP through deposition onto the bacterial surface followed by binding to CR1/3 receptors on the surface of the granulocyte (Ross *et al.* 1987).

The use of a human complement source in these functional assays has been problematic for many years (Santos *et al.* 2001). This results from the large amounts of cross-reactive antibodies in the human population acquired by nasopharyngeal carriage of different *N. meningitidis* and commensal *Neisseria* strains, which can evoke meningococcal bacteriolysis in the absence of test antiserum and cause interference in the results of the assays. A human complement source can be obtained from a hypogammaglobulinaemic patient (rare in the population and these individuals are treated with immunoglobulin preparations). Alternatively, large numbers of volunteers are screened to find serum or plasma which contains little or no cross-reactive antibody to the strain of interest. Often this results in a different complement source from a different

individual being used in assays against different strains leading to poor inter-strain and inter-laboratory assay comparability. Baby rabbit serum has been used as a complement source in both SBA and OPA (Findlow *et al.* 2007) due to the absence of intrinsic bactericidal antibody and this is used as the complement source for Men C and Men A SBAs (Maslanka *et al.* 1997; Andrews *et al.* 2003; Keyserling *et al.* 2005; Kshirsagar *et al.* 2007). However, investigators have shown that the use of rabbit complement results in increased bactericidal titres (Santos *et al.* 2001). Initial studies suggested this was caused by the presence of low avidity anti-MenB capsular antibody; absorption of these antibodies reduced bactericidal titres but they were still not comparable with titres achieved using human complement (Findlow *et al.* 2007). More recent work has now shown the increased bactericidal titres results from the specificity of meningococcal factor H binding protein for human factor H which leads to an overestimation of functional antibody activity using baby rabbit complement (Granoff 2009). The availability of a standard source of human plasma/serum for use as a complement source in both the SBA and OPA is thus a key issue for the assessment of meningococcal vaccines (Martin *et al.* 2005).

This study set out to develop a universal human complement source that could be utilised in any immunoassay against any strain of *N. meningitidis* by optimising the method for antibody depletion and assessing the effect on complement function. This study also investigated depletion of large batches of plasma and the reproducibility of the method.

5.2 Results

5.2.1 Optimisation of complement source used- Plasma or Serum?

Initially, the use of either plasma or serum and the effect of anti-coagulant were investigated in order to optimise the complement source used in meningococcal immunoassays. The complement source used in previous investigations (Taylor 2010) was derived from plasma bags purchased from the National Blood Service. It was impossible to know the length of time following extraction from the patient before the sample was frozen and therefore this complement source may have been variable as complement activates at room temperature and maintenance of an intact complement cascade, requires storage at -80°C (Bjorkholm *et al.* 1986). The products from the National Blood Service are routinely frozen at -20°C within 24 h, with the potential for loss of complement activity (Bjorkholm *et al.* 1986; Belmusto-Worn *et al.* 2005; Livorsi *et al.* 2010). Blood products collected at the National Blood Service are most commonly directly mixed with either Heparin (14-17 U/ml) or EDTA (1.8 mg/ml) (ethylenediaminetetraacetic acid) as an anti-coagulant. EDTA and heparin have been shown to have inhibitory effects on the complement cascade (Mollnes *et al.* 2002), and therefore the effect on the complement system would need to be investigated.

An alternative to Heparin or EDTA is to use lepirudin (also known as Refludan) as an anti-coagulant. Lepirudin is an anti-thrombic drug which has been developed for individuals presenting with an allergy to heparin. Lepirudin is a recombinant hirudin, a naturally occurring peptide in the salivary glands of leeches. This has been widely used in complement research as it reportedly does not interfere with the complement cascade (Mollnes *et al.* 2002). Alternatively serum could also be

used as a complement source. However, the activation of the coagulation pathway has also been shown to disrupt the complement cascade (Amara *et al.* 2008).

To investigate alternative complement sources, blood was taken from volunteers and anti-coagulated with EDTA (1.8 mg/ml), heparin (14-17 U/ml), lepirudin (100 µg/ml) or isolated as serum in a serum separator tube which contains clot activators (silica particles) and a gel layer used to separate the serum from the coagulated pellet of cells. Blood was taken from a volunteer and immediately processed (2.10.1) to minimise complement activation. All samples were then IgG-depleted using a 5 ml protein G Sepharose column (2.10.2) before being analysed in a CDA measuring antibody-mediated deposition of C5b-9 and C3b/iC3b.

No deposition of C5b-9 or C3b/iC3b was seen with rabbit serum raised against OMVs prepared from the same strain with EDTA anti-coagulated plasma as a complement source (figure 5.2.1.1 a and b). This was because EDTA is a very potent complement inhibitor as it binds Ca^{2+} and Mg^{2+} which are required for a functional complement pathway (James 1982).

No significant ($P<0.01$) difference in C3b/iC3b deposition was observed with homologous strain rabbit anti-OMV serum when comparing heparin or lepirudin anti-coagulated plasma. However, both of these complement sources resulted in significantly higher C3b/iC3b deposition than observed with EDTA anti-coagulated plasma and serum (figure 5.2.1.1a). Lepirudin anti-coagulated plasma had significantly ($P<0.01$) higher C5b-9 deposition with homologous strain OMV rabbit serum in comparison to heparin, EDTA and serum (figure 5.2.1.1b).

When serum was used as the complement source in the CDA a further resuspension step was required because the samples began to form a gel during the assay.

To further evaluate the complement sources, lepirudin, heparin and EDTA plasma and serum were compared in the OPA (2.9.7). Lepirudin, EDTA and heparin anti-coagulated plasma were compared with serum in the opsonophagocytosis assay. A significantly ($P<0.01$) higher opsonic activity was observed with lepirudin anti-coagulated plasma than any other complement source, with no opsonic activity observed at all with EDTA anti-coagulated plasma (figure 5.2.1.2).

This assay was also performed using serum as a complement source, the samples could not be analysed using the flow cytometer as the samples had become gel-like (as in the CDA). However, a further resuspension step failed, rendering this complement source unusable for the OPA.

Baby rabbit complement has been used as a complement source in published OP assays (Maslanka et al. 1997; Taylor 2010), as it can be bought in large batches and lacks cross-reactive antibodies, improving inter-strain and inter-assay comparability. A comparison was made between baby rabbit complement and lepirudin as a complement source in the OPA. Significantly ($P<0.01$) higher responses were observed with baby rabbit complement (figure 5.2.1.3). This is likely to have occurred as fHbp is specific for human factor H and the recruitment of this negative regulator only occurs in assays using human complement and thus the results using baby rabbit complement are higher than those with the human lepirudin-treated complement.

The results from this series of experiments supported the choice of lepirudin anti-coagulated plasma as a complement source.
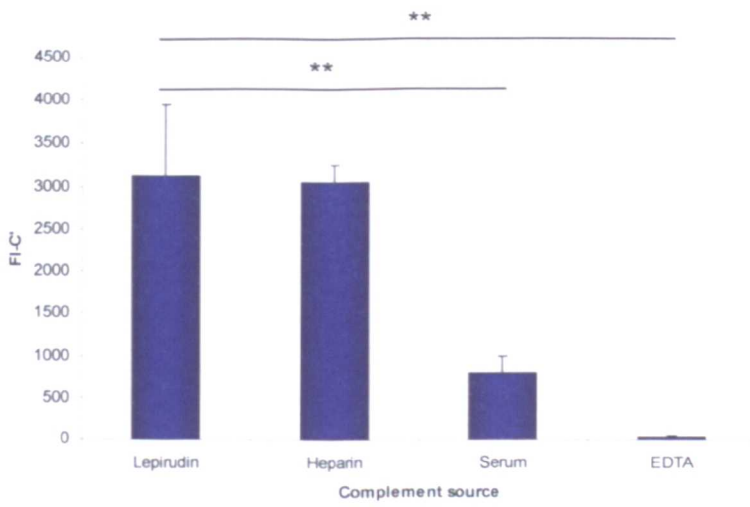
a.
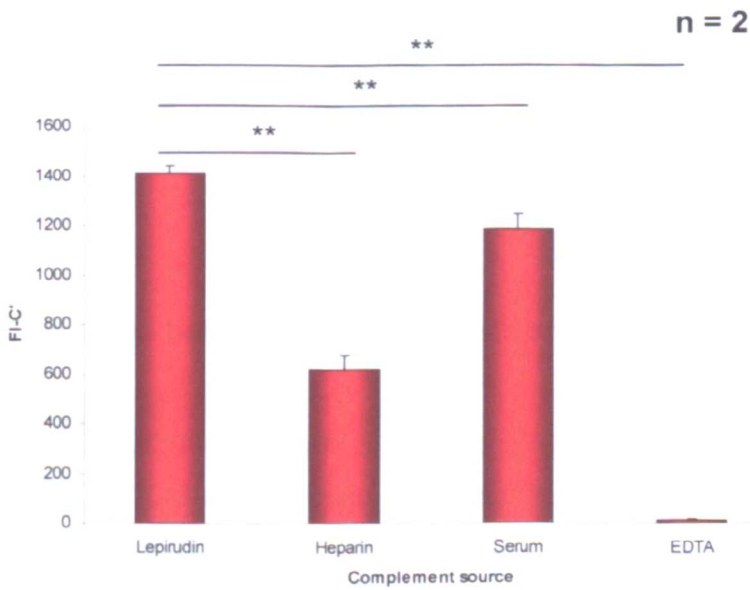
b.

Figure 5.2.1.1 Comparison of different anti-coagulated complement sources in the dCDA.

Antibody-mediated C3b/iC3b (a.) or C5b-9 (b.) deposition on the surface of *N. meningitidis* M01240149 using either lepirudin, heparin, EDTA anti-coagulated blood or serum as a complement source, mediated by homologous strain OMV rabbit serum. Significance was determined by z-test (**$P<0.01$), and error bars denote standard deviation.
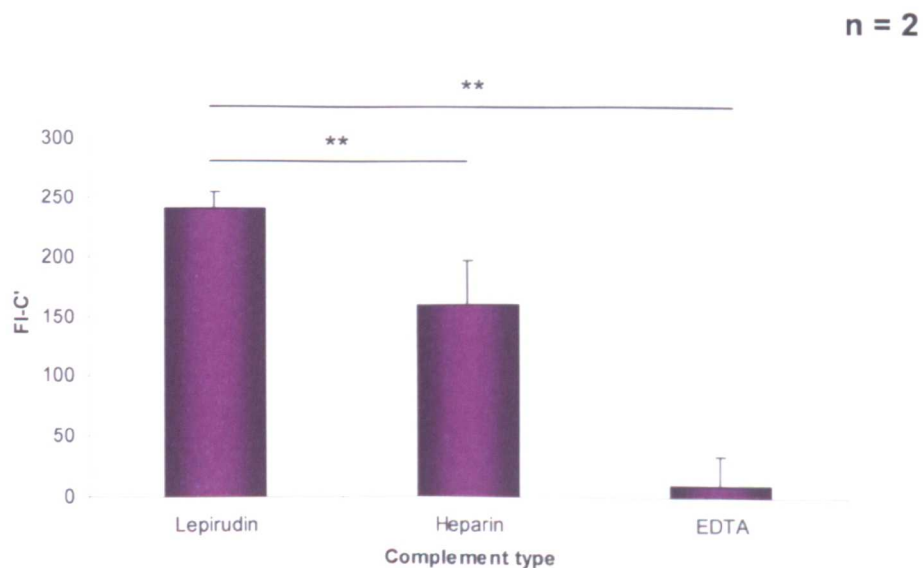
Figure 5.2.1.2 Comparison of different anti-coagulated complement sources in the OPA.

Comparison of OP activity using lepirudin, heparin and EDTA anti-coagulated complement sources on the surface of *N. meningitidis* M01240149 mediated by homologous rabbit anti-OMV serum. Significance was determined by z-test (**$P<0.01$), and error bars denote standard deviation.
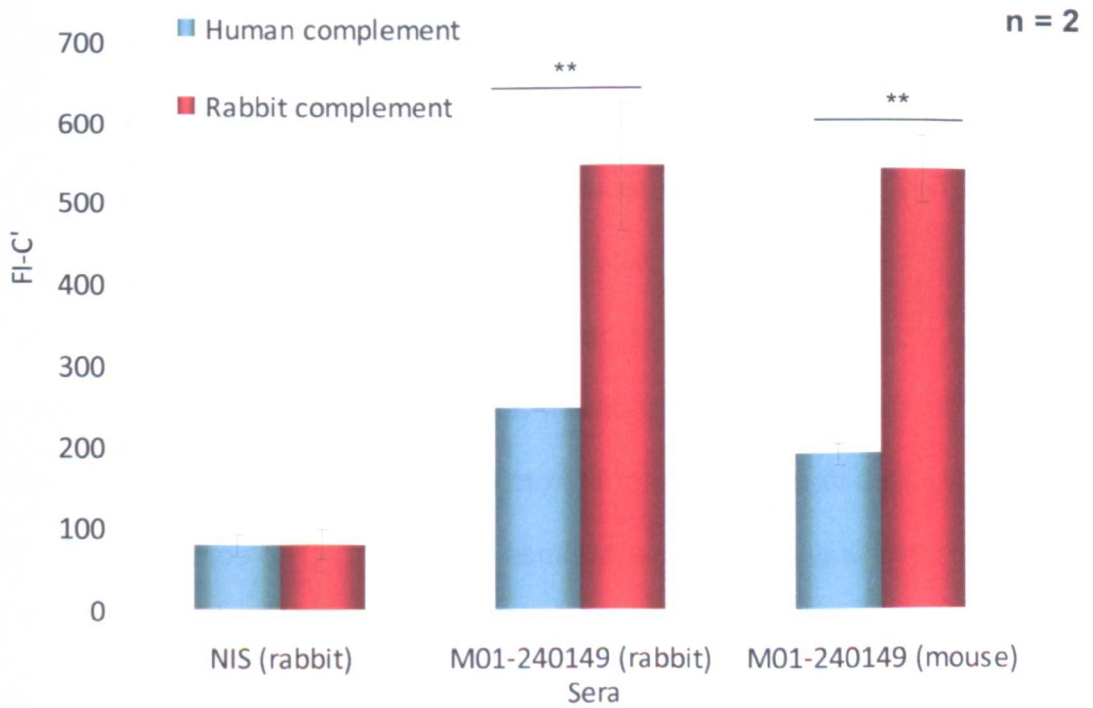
Figure 5.2.1.3 Comparison of baby rabbit and human complement in an OPA.
OPA with *N. meningitidis* M01240149 comparing the activity of lepirudin anti-
coagulated complement source and baby rabbit complement. Significance was
determined by z-test (**P<0.01), and error bars denote standard deviation.

5.2.2 Comparison of total haemolytic complement activity comparison of complement sources.

Plasma samples anti-coagulated with either heparin or lepirudin were compared with serum in a total haemolytic complement assay kit (The Binding Site, UK). This was performed to determine whether the classical pathway of the complement cascade was intact. The total haemolytic complement assay measures the classical and alternative complement cascade, by measuring the lysis circle formed following complement activation. Lysis circle radii from the standard curve and the control wells were used to derive the activity in the different complement types tested (figure 5.2.2.1). The heparinised blood demonstrated a double ring of complement lysis. This was a result of low haemolytic complement activity, possibly as a result of complement pathway dysfunction. Heparin has previously been shown to interfere with complement activity and has a factor H binding site thus potentially able to remove factor H from the complement, which might explain the haemolytic pathway dysfunction (Mollnes et al. 2002). The complement activity seen for lepirudin anti-coagulated blood was high at 1130 CH100 units/ml. This high level of activity was consistent with the antibody-mediated C5b-9 deposition results reported in 5.3.1 (figure 5.2.2.1i) and lepirudin's reported non-interference with the complement cascade (Mollnes et al. 2002). Lepirudin anti-coagulated complement activity was shown to be higher than that seen using serum as a complement source (figure 5.2.2.1). Lepirudin therefore showed the greatest classical pathway function (figure 5.2.2.1), opsonic activity (figure 5.2.1.2) and antibody-mediated C5b-9 deposition (figure 5.2.1.1). Lepirudin anti-coagulated plasma was thus used in all further studies.
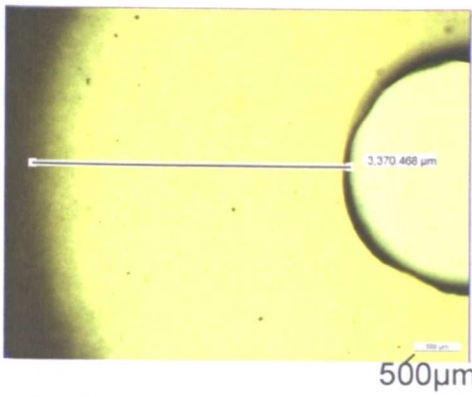
a.100% complement activity
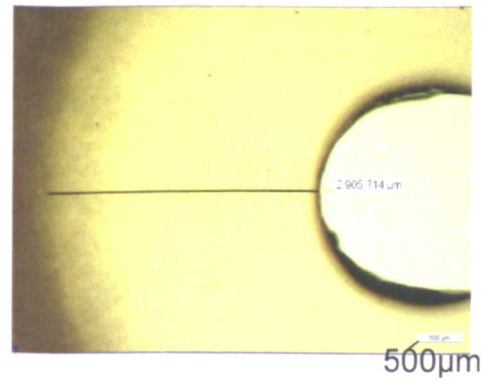
b. 50% complement activity
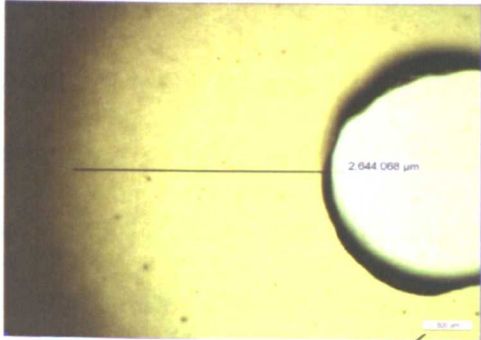
c. 25% activity sample   500µm
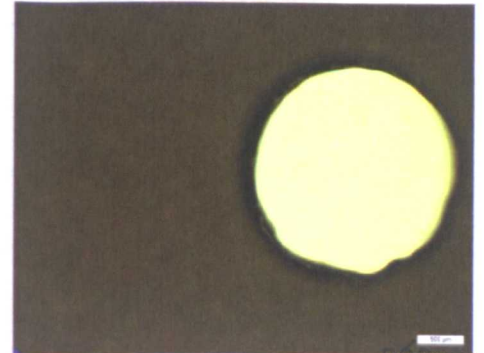
d.0% complement activity   500µm
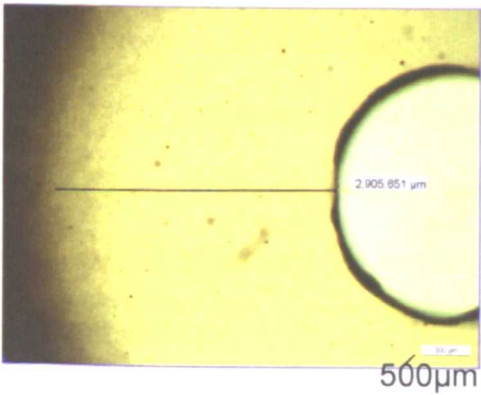
e. Control sample- 600.6 CH100 units/ml
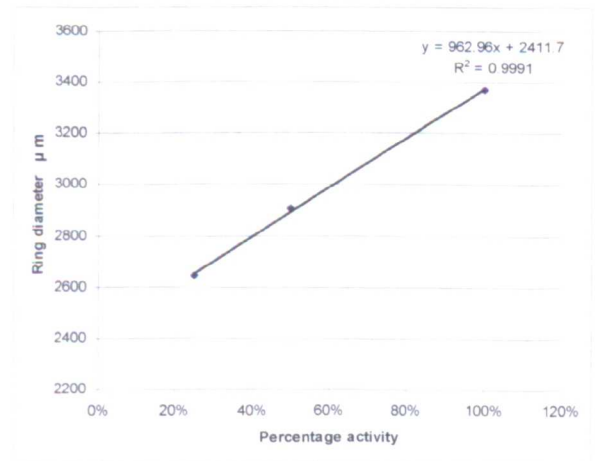
f. Standard curve

Figure 5.2.2.1 Total haemolytic complement activity in different complement sources.

(a) (b) and (c) Total haemolytic complement activity lysis rings measurable for assay standard curve. (d) Negative control PBS only sample and (e) positive control complement sample. (f) Standard curve used to derive the complement activity of the test samples. Test samples serum (g) heparin (h) or lepirudin (i).
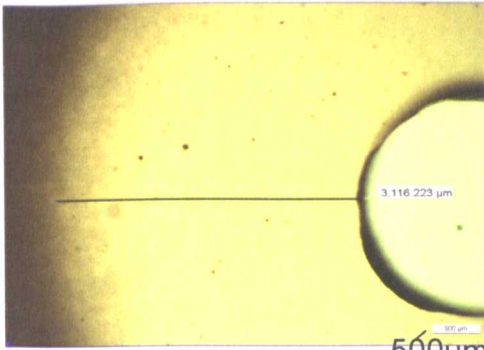
g.Serum
856.6 CH100 units/ml
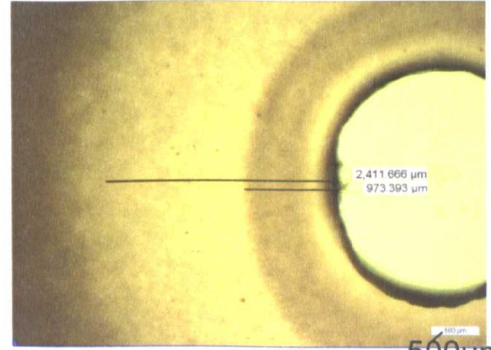
h.Heparin
0 CH100 units/ml (this activity
was below the detection limit of
the assay)
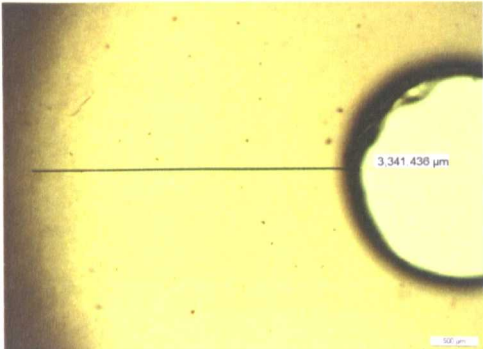
i.Lepirudin-1130 CH100 units/ml

i.

5.3 Optimisation of antibody depletion of plasma - ELISA assessment of efficiency and reproducibility of antibody depletion.

The antibody-mediated immunity to meningococcal disease is primarily IgG-dependent but there is also a role for IgA and IgM. IgG is able to initiate a range of effector functions including MAC deposition and bacteriolysis, and also C3b/iC3b deposition and OPA (Naess *et al.* 1999). Studies investigating the contribution of IgG subclass to activate human complement show IgG1= IgG3 > IgG2 >> IgG4 (Naess *et al.* 1999). Effective complement activation can also be mediated by IgM. However, IgM has been shown not to interact with phagocytes, whereas IgA does not bind C1q and is therefore not able to activate the classical complement pathway. However, IgA has been shown to activate the alternative pathway (Vidarsson *et al.* 2001). In addition, IgA is not able to mediate complement bactericidal activity. However, it is able to induce some phagocytic activity and respiratory burst response (Vidarsson *et al.* 2001). The contribution of the different antibody classes and subclasses to complement activation varies, thus it was important to assess if any were removed following depletion with different matrixes.

Native plasma was compared with antibody depleted plasma anti-coagulated with lepirudin depleted by either: protein G Sepharose which binds IgG1, IgG2, IgG3, IgG4 (high affinity), protein L Sepharose which removes IgG, IgM, IgA, IgE, IgD (all high affinity), and protein A Sepharose which removes IgG1, IgG2, IgG4 (high affinity) IgA, IgD, IgM, IgE (low affinity) (Thermo technical information sheet).

No significant ($P<0.01$) reduction in IgA was observed between native plasma and protein A-depleted plasma. This could be explained by the low affinity binding of

protein A Sepharose with IgA (table 5.3.1). The greatest reduction in IgA was observed following protein L depletion, and was found to be significant ($P<0.01$), and was consistent with the expected high affinity binding of IgA by protein L (table 5.3.1). A significant ($P<0.01$) reduction was also observed in IgA following depletion with protein G (table 5.3.1). This was unexpected as protein G is reported to remove all IgG subclasses but not IgA (Thermo technical information sheet).

A significant ($P<0.01$) decrease in IgM was observed between the native plasma and plasma antibody-depleted with protein L, protein A or Protein G (table 5.3.1). This reduction was expected following depletion with protein L or A as they are reported to bind IgM with high and low affinity respectively. The significant ($P<0.01$) reduction in IgM observed following depletion with protein G was unexpected as this is not reported to bind IgM (Thermo technical information sheet).

Following depletion with protein G there was a significant ($P<0.01$) reduction in IgG (table 5.3.1). This resulted in almost no detectable IgG by ELISA. Significant ($P<0.01$) falls in IgG were also observed following depletion with protein A or L (table 5.3.1). The drop in IgG was minimal for both these depletion methods. This was expected for depletion with protein A, however protein L binds IgG to high affinity and only low levels of IgG were removed. This would perhaps suggest that the binding sites available were saturated by binding IgM and IgA.

The removal of IgG was further investigated using an ELISA to assess the depletion of each subclass. Significant ($P<0.01$) reductions in IgG1 were observed between native plasma, protein G depleted plasma and protein A depleted plasma

(table 5.3.1). Depletion of IgG1 using protein G was complete with no detectable IgG1, which was consistent with the predicted outcome. No significant depletion of IgG1 was observed with protein L-depleted plasma (table 5.3.1). This was an unexpected finding as protein L is reported to bind all antibody to high affinity, although this was consistent with the total IgG ELISA result which indicated a significant but low level reduction in IgG (Thermo technical information sheet). Significant reductions ($P<0.01$) in IgG2 were observed between native plasma and protein G and L depleted plasma. Depletion of IgG2 using protein G was complete (table 5.3.1). There were significant ($P<0.01$) reductions in IgG3 following depletion with protein A and protein L, with no detectable levels of IgG3 seen following depletion with protein G (table 5.3.1). This result was unexpected for protein A depletion as this does not bind IgG3 (Thermo technical information sheet). Protein G and protein L depleted plasma showed a significant ($P<0.01$) reduction in IgG4 (table 5.3.1).

Thus protein G depletion was selected as the optimum method which provided the lowest assay background due to the complete removal of IgG.

Mean mg/ml of Immunoglobulin

	Native	ProteinG depleted plasma	Protein A depleted plasma	ProteinL depleted plasma
IgA	2.0	1.5 **	2.0	0.5 **
IgM	2.8	0.6 **	1.2 **	0 **
IgG	3.4	0.04 **	3.0 **	2.8 **
IgG1	6.5	0 **	2.4 **	6.1
IgG2	3.0	0 **	3.1	1.6 **
IgG3	3.8	0 **	1.4 **	1.2 **
IgG4	0.9	0 **	0.8	0.4 **

Table 5.3.1 Antibody ELISAs comparing antibody depletion methods. Significance was measured by t test (**$P<0.01$).

5.4 Optimisation of antibody depletion of plasma - Assessment of complement function using radial immunodiffusion assays.

As described earlier, using human complement in immunoassays is problematic, in order to achieve a human complement source which could be used for assays using all strains, the depletion of antibody was investigated. Native plasma was compared with antibody-depleted plasma, depleted using either: protein G Sepharose which removes IgG1, IgG2, IgG3, IgG4 (high affinity), protein L Sepharose which removes IgG, IgM, IgA, IgE, IgD (all high affinity), or protein A which removes IgG1, IgG2, IgG4 (high affinity) IgA, IgD, IgM, IgE (low affinity).

Total haemolytic complement activity was significantly ($P<0.01$) reduced following removal of antibody with all depletion methods, with the greatest reduction observed following depletion with protein G (figure 5.4.1). The alternative pathway also showed significant ($P<0.01$) reduction in activity (figure 5.4.2), however there was no significant ($P<0.01$) difference observed between the depletion methods. This observed loss in function was attributable to a dilution effect resulting from the affinity chromatography.

Overlay plots comparing the complement-only control measuring C3b/iC3b deposition, showed that the protein G-depleted complement had the lowest background complement deposition (figure 5.4.3a). Antibody-dependent C3b/iC3b deposition measured using homologous rabbit sera, showed only small differences between depletion methods (figure 5.4.3b). Overlay plots comparing the complement only control measuring C5b-9 deposition, showed that protein G-depleted complement had the lowest background control (figure 5.4.4a). Antibody dependent C5b-9 deposition measured using homologous strain OMV rabbit sera showed only small differences between depletion methods (figure 5.4.4b). A low

assay background from protein G-depleted plasma, and a high level of antibody-mediated C3b/iC3b and C5b-9 deposition with homologous strain OMV rabbit sera, similar to that observed with all other depletion methods and also native plasma, lead to the conclusion that protein G depletion was optimal.

These results indicated that the best reduction of assay background without reduction in positive response was achieved by protein G depletion. This was surprising as protein L depletion was expected to remove all antibody types with high affinity and although IgG is considered to be the most significant contributor to initiation of the classical complement cascade, IgM and IgA are considered to play a smaller role (Naess *et al.* 1999; Vidarsson *et al.* 2001). It was possible that protein L produced poorer depletion results due to saturation of binding to the column. A possible way of improving the depletion results obtained with protein L would be to dilute the plasma being used with the column, but it was considered that alteration of plasma concentration used in the depletion would significantly change the concentration used in the assay, altering the assay parameters.
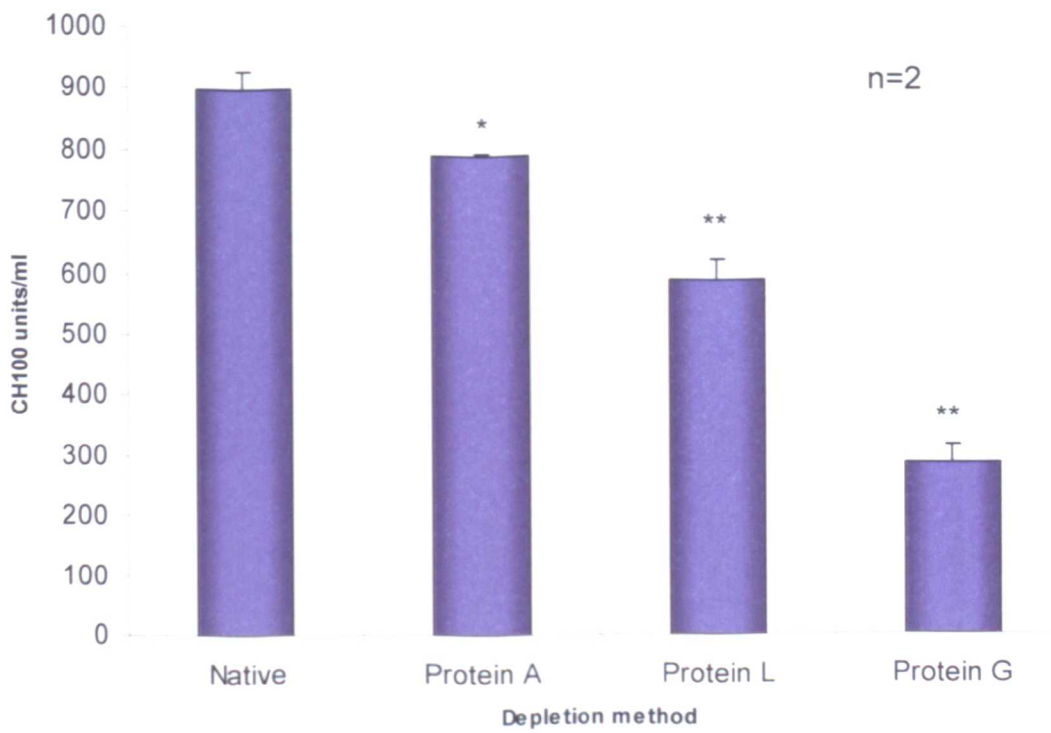
Figure 5.4.1 Comparison of total haemolytic function following depletion methods using either protein A, protein L or protein G Sepharose.

Significance was measured by t-test (**P<0.01, * P<0.05), and error bars denote standard deviation.
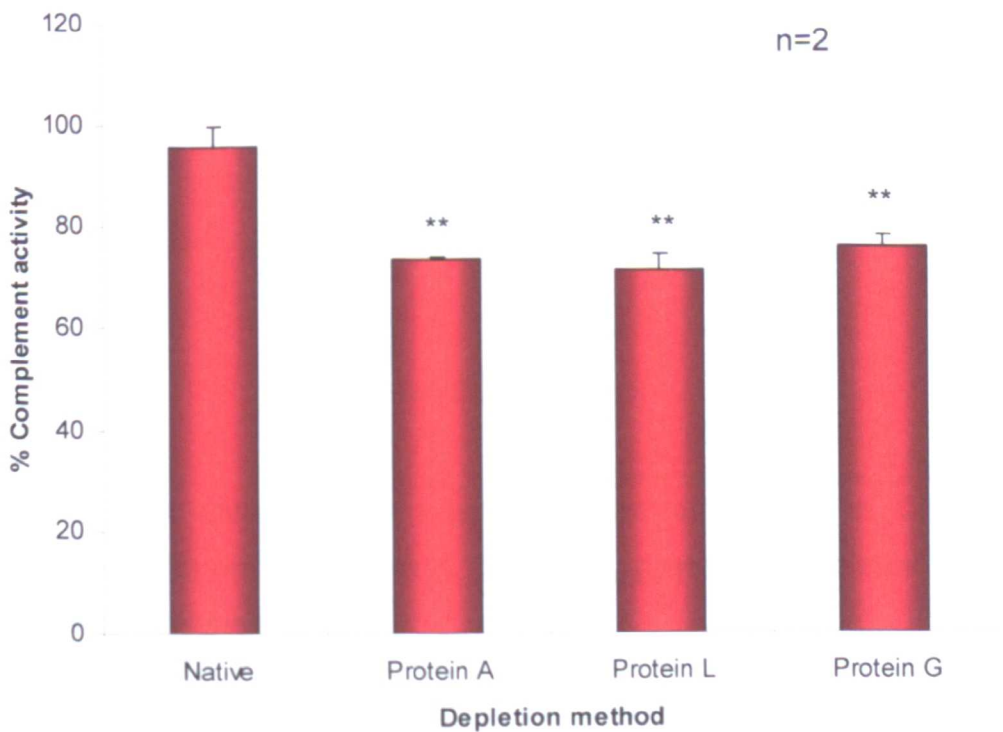
Figure 5.4.2 Comparison of alternative complement cascade haemolytic function following depletion methods using either protein A, protein L or protein G Sepharose.

Significance was measured by t test (**P<0.01), and error bars denote standard deviation.
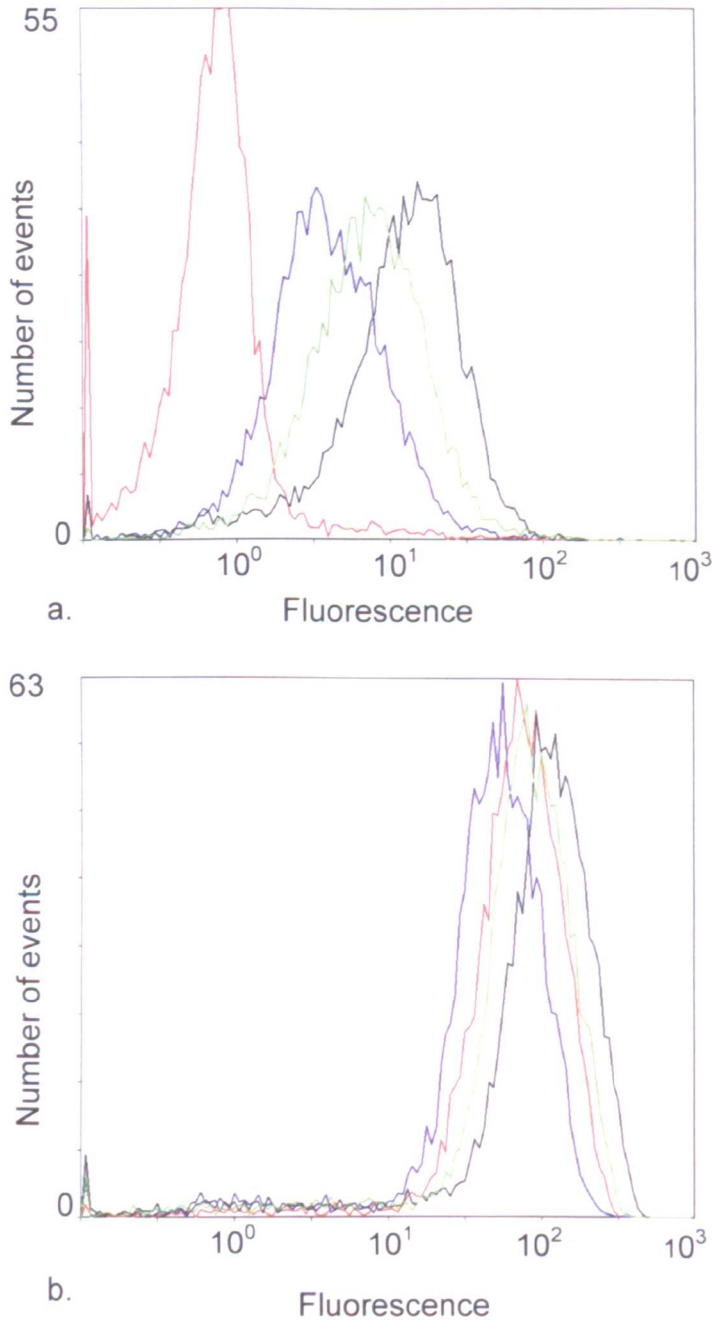
Figure 5.4.3 Overlay plot illustrating the difference in C3b/iC3b deposition achieved with either protein A, protein L, protein G depleted complement or native complement.

Overlay plot illustrating the difference in C3b/iC3b deposition in an (a.) complement only control and (b.) homologous strain OMV rabbit sera when using complement depleted with either protein A (green), protein L (blue) or protein G (red) Sepharose or native complement (black). Assay performed using *N. meningitidis* M01240149.
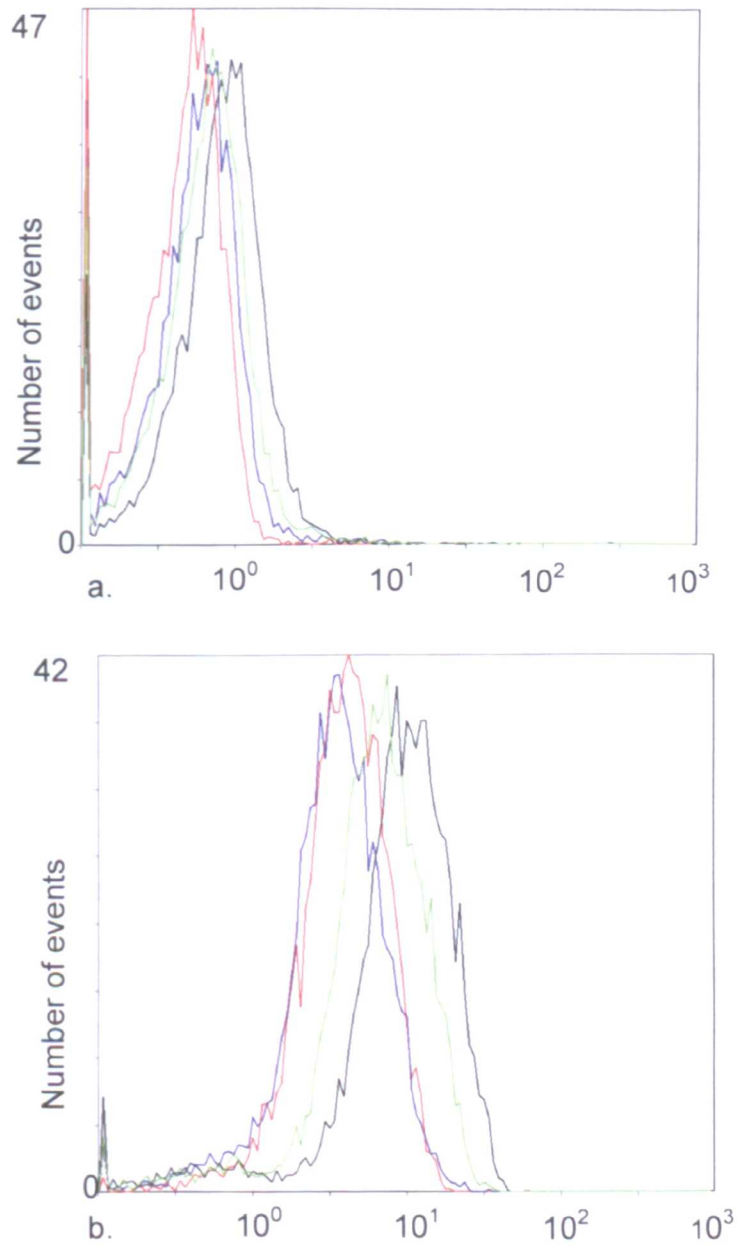
Figure 5.4.4 Overlay plot illustrating the difference in C5b-9 deposition achieved with either protein A, protein L, protein G depleted complement or native complement.

Overlay plot illustrating the difference in C5b-9 deposition in an (a.) complement only control and (b.) homologous strain OMV rabbit sera using complement-depleted with either protein A (green), protein L (blue) or protein G (red) Sepharose or native complement (black). Assay performed using *N. meningitidis* M01240149.

5.5 Effect of addition of C1q on complement activity

Removal of antibody using protein G provided the optimal reduction in IgG (table 5.3.1), and produced the lowest CDA background (figure 5.4.3 and figure 5.4.4). However, when the total haemolytic complement activity was assessed, a significant reduction was observed with the lowest activity seen in protein G-depleted plasma compared with the other depletion methods tested (figure 5.4.1). Protein G has been used for the purification of C1q (Kolb *et al.* 1979) and it was hypothesised that following antibody binding to the column Fc receptors were left exposed and this subsequently leads to the binding of C1q, resulting in removal of both antibody and C1q from the plasma. C1q is a vital part of the initiation of the classical pathway, a reduction in C1q concentration would reduce the classical haemolytic activity of complement (Kolb *et al.* 1979).

ELISA confirmed the low levels of C1q present following antibody depletion of plasma (figure 5.5.1). Addition of C1q (Quidel) back into the antibody-depleted complement was investigated to determine if this would restore the lytic activity of complement. A range of C1q concentrations was used around that present in normal human plasma (approximately 77 µg/ml) (Kolb *et al.* 1979) and the concentration observed prior to antibody depletion (40 µg/ml). C1q was added back into the protein G-depleted complement source and a CDA was performed to look at the effect on C5b-9 and C3b/iC3b deposition. This showed significant ($P<0.01$) increases in the antibody-mediated C5b-9 deposition (figure 5.5.2a), whilst C3b/iC3b deposition decreased (figure 5.5.2b). Total haemolytic activity was measured and compared with the complement prior to IgG depletion. The levels of total haemolytic complement activity were restored to 72% of the original activity before IgG depletion (figure 5.5.3). Addition of C1q restored the antibody-depleted complement source and would therefore be used in future assays.
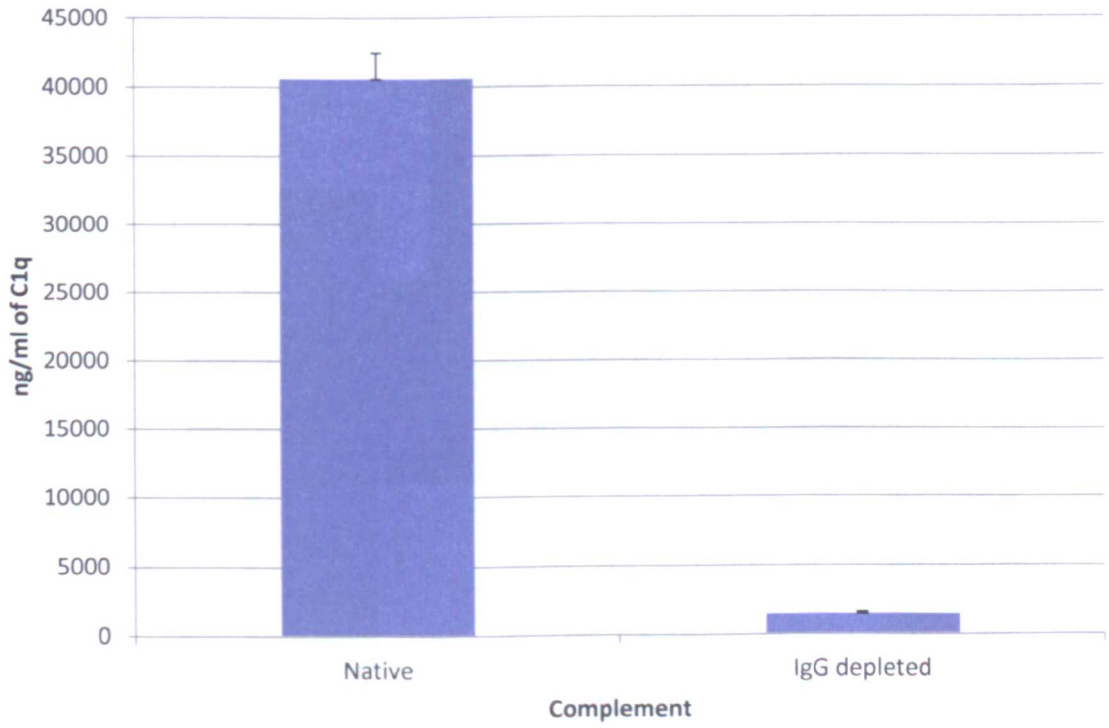
Figure 5.5.1 C1q ELISA comparing C1q concentration before and after IgG depletion of complement.

Error bars denote standard deviation.
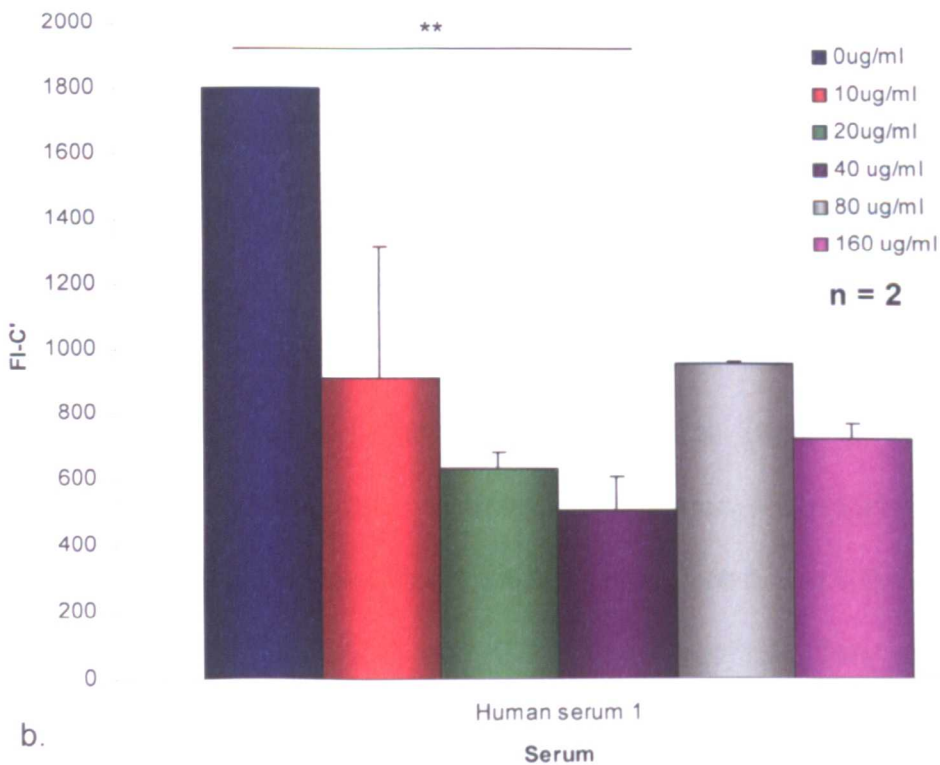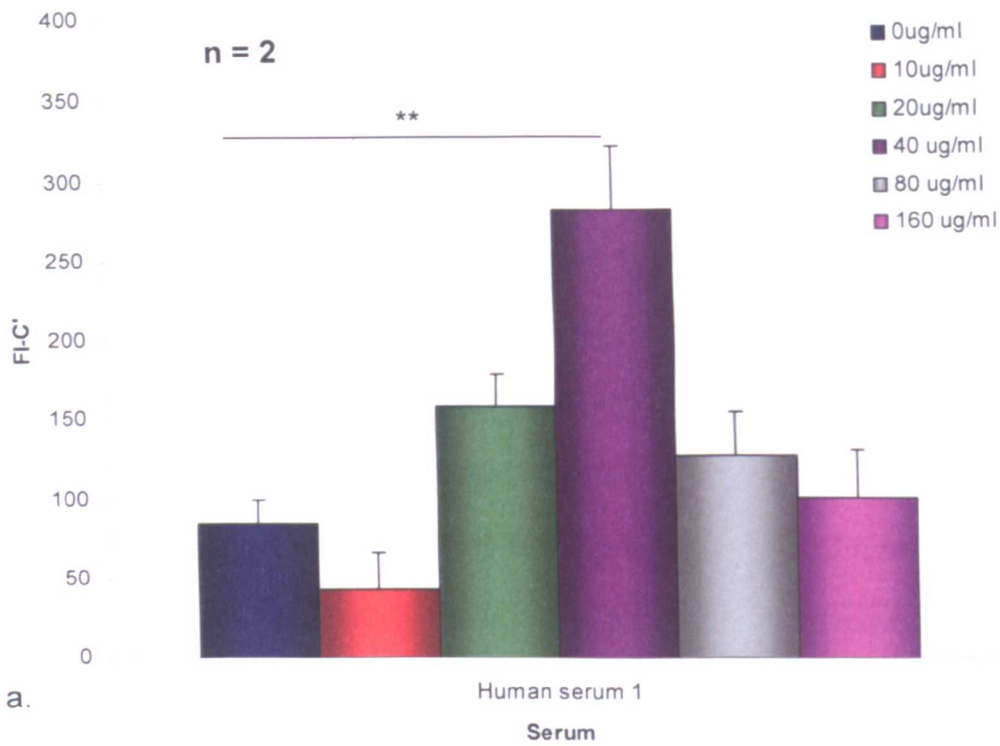
Figure 5.5.2 Addition of increasing concentrations of C1q to dCDA.

Antibody-mediated complement deposition of C5b-9 (a) or C3b/iC3b (b) on the surface of *N. meningtidis* NZ98/254 with a high responding human serum following addition of various concentrations of C1q (Quidel). Significance was measured by z test (**$P<0.01$), and error bars denote standard deviation.
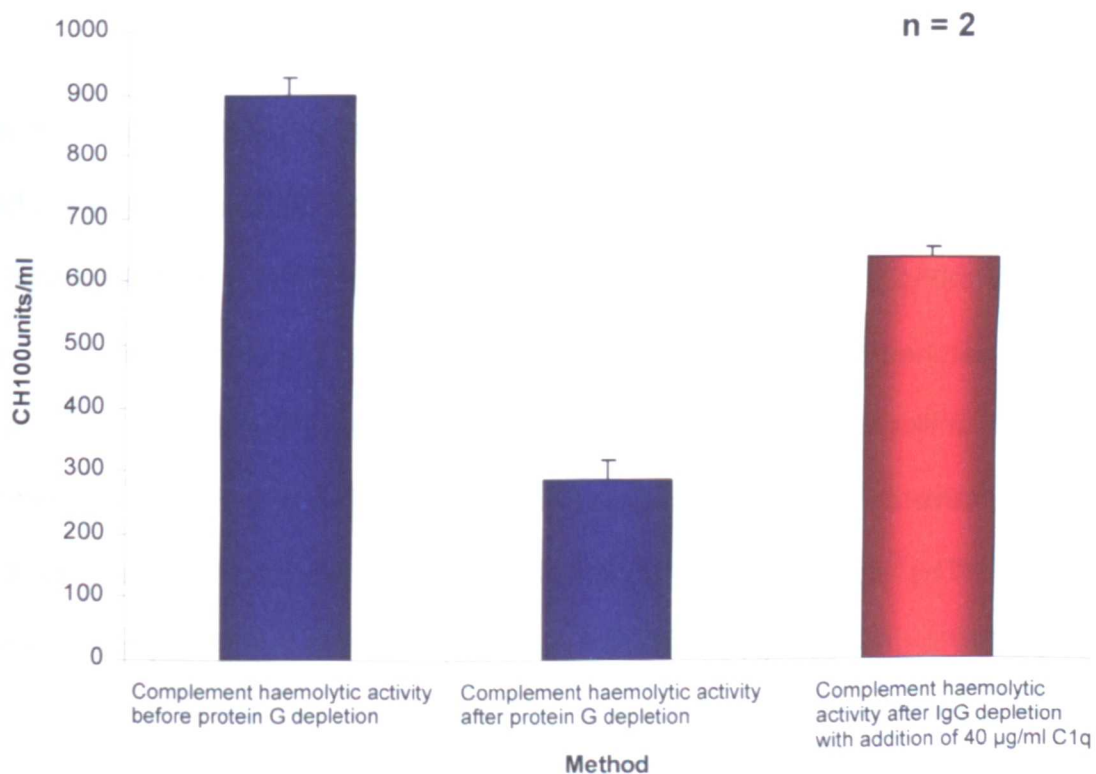
Figure 5.5.3 Total haemolytic complement activity of complement anti-coagulated with lepirudin before and after IgG depletion, or after IgG depletion with addition of 40 µg/ml purified C1q (Quidel).

Error bars denote standard deviation.

5.6 Large scale antibody depletion from plasma.

To test sera from large clinical trials it is important to use the same complement source for comparability. The production of large scale batches of plasma which had been antibody-depleted was therefore investigated.

The optimised IgG depletion method was scaled up to process upto 250 ml of plasma in a single batch. Initially blood was taken from 10 volunteers with plasma obtained using Lepirudin as an anti-coagulant (2.10.1). This was then split into several batches to be processed on a 250 ml protein G column. The large scale process, unlike the rapid small scale process, required the plasma to remain at 4°C for several hours, which could have resulted in the loss of complement function. The reproducibility of the large scale process was assessed, by comparing large scale depletion batches all derived from the same pooled plasma.

IgG-depleted plasma from 2 x 200 ml batches was prepared. 200 ml of plasma was thawed and passed through the protein G column. Then flow through fractions were collected (figure 5.6.1), aliquoted and frozen at -80°C. Following this, antibody was eluted from the column using 0.1M glycine HCl (pH 2.7) to clean the column. Each depletion batch had a very similar chromatogram (figure 5.6.1).

Batches of protein G-depleted plasma were assessed using ELISA to compare the consistency of antibody depletion. Significant ($P<0.01$) reduction in IgA was observed between native plasma and both batches of protein G-depleted plasma. A significant ($P<0.01$) difference was observed in the amount of IgA removed in each batch (table 5.6.1). A significant ($P<0.01$) reduction in IgM was observed between native plasma and both batches of protein G-depleted plasma, with no significant difference observed between batches (table 5.6.1). Following depletion

with protein G a significant (P<0.01) reduction in IgG was observed and this was consistent between the batches tested (table 5.6.1), this was also the case for IgG1, IgG2, IgG3 and IgG4 with no detectable IgG in either batch (data not shown).

A comparison of total haemolytic activity between the two batches of depleted plasma showed no significant difference in either classical complement haemolytic activity or alternative pathway haemolytic activity (figure 5.6.2 and figure 5.6.3).

Complement batches showed highly similar overlay plots for using antibody-mediated C3b/iC3b and C5b-9 deposition (figure 5.6.4). Batch variation was investigated further by comparing antibody-mediated C3b/iC3b and C5b-9 deposition. Significant (P<0.01) variation between complement batches was observed with 1 of 4 sera in both the antibody-mediated C3b/iC3b (figure 5.6.5) or C5b-9 (figure 5.6.6) deposition, with all other sera tested showing no significant (P<0.05) differences. Batch variation was also compared in the opsonophagocytosis assay. A significant (P<0.05) difference between complement batches was again observed in one of the four sera tested (figure 5.6.7).
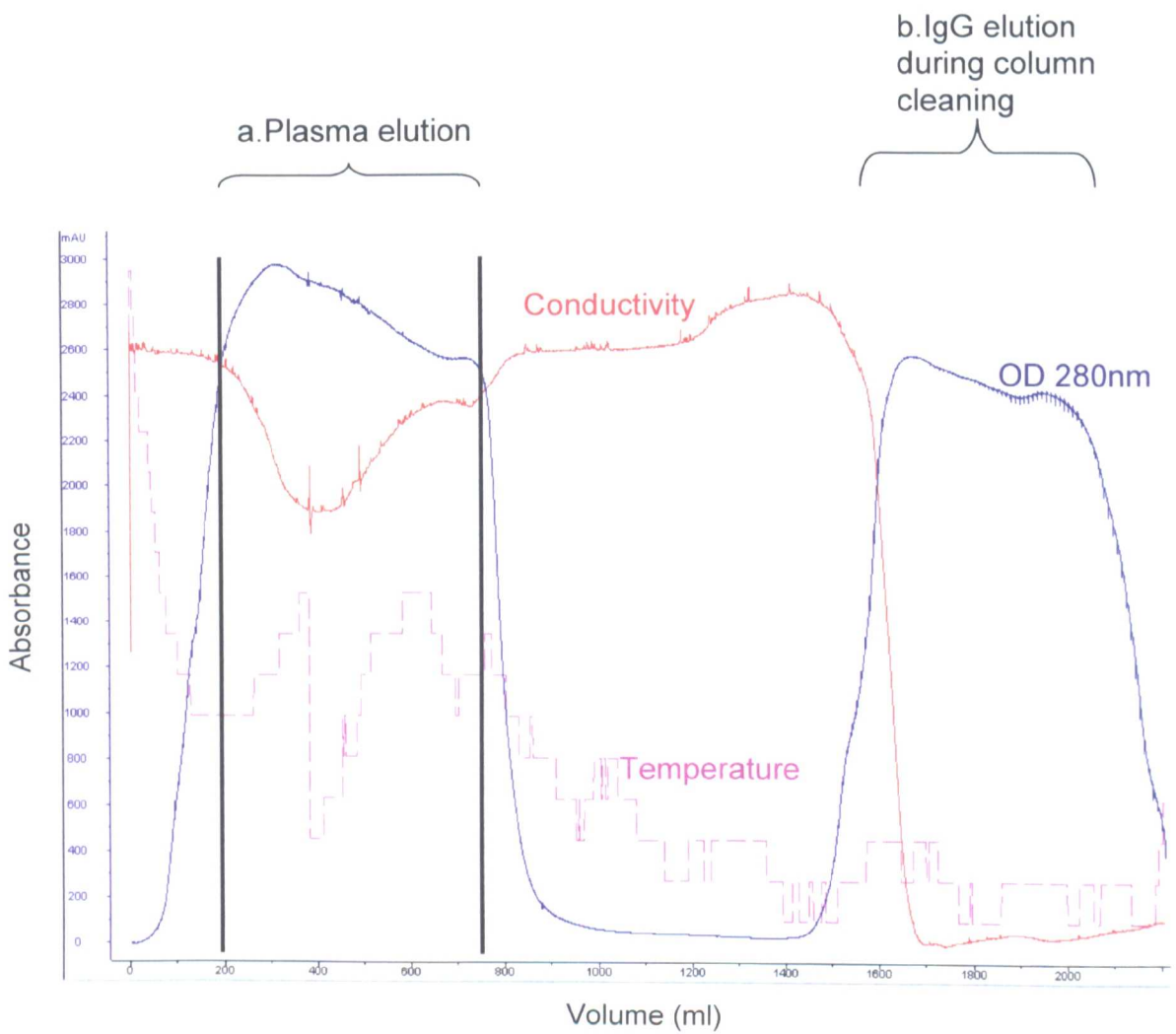
Figure 5.6.1 Chromatogram illustrating IgG depletion of lepirudin-treated plasma using protein G.

Chromotagram indicates the fractions of depleted complement collected for snap freezing (a.), and the IgG removed and eluted from the plasma with 0.2M Glycine HCL (b.).

	Mean mg/ml of Immunoglobulin			
	Native	ProteinG Batch 1	Protein G Batch 2	Significance between batches
IgA	2.0	1.5 **	1.00**	Significant difference between batches ($P<0.01$).
IgM	2.8	0.6 **	1.07**	No significant difference between batches.
IgG	3.4	0.04 **	0.08**	No significant difference between batches.

Table 5.6.1 Comparison of antibody depletion between batches of protein depleted plasma.

Significance was measured by t-test (**$P<0.01$).
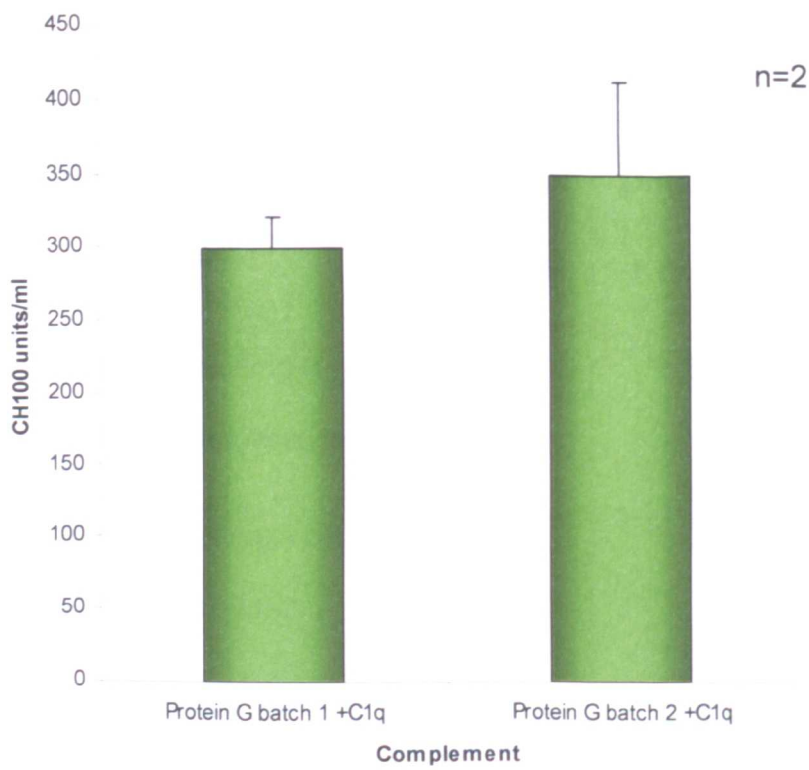
Figure 5.6.2 Comparison of total haemolytic activity of two batches of plasma following depletion with protein G adding 40 µg/ml of C1q.

Error bars denote standard deviation.
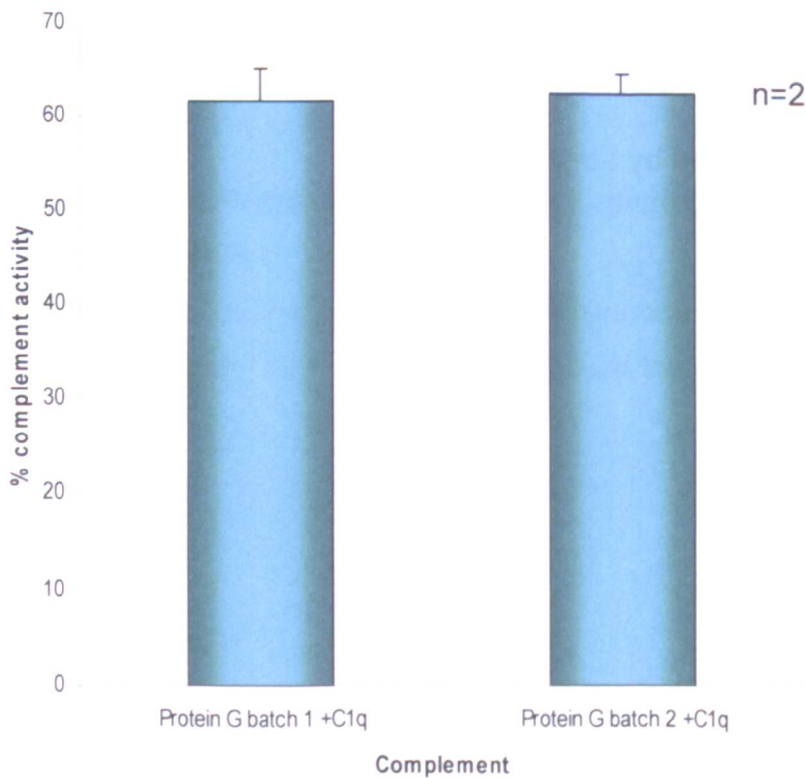
Figure 5.6.3 Comparison of alternative complement pathway of two batches of plasma following depletion with protein G adding 40 µg/ml of C1q.

Error bars denote standard deviation.
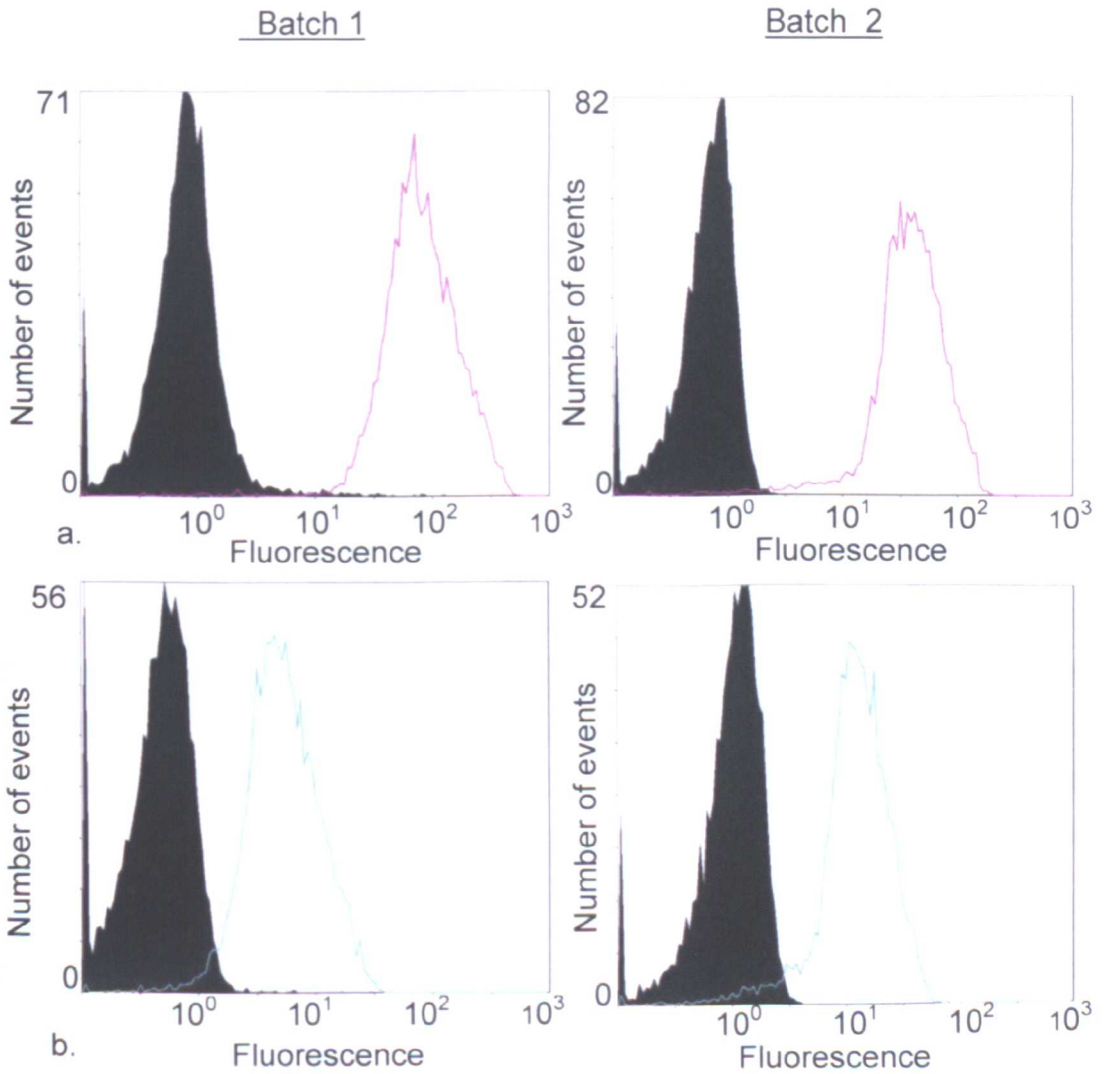
Figure 5.6.4 Overlay plot illustrating consistency between depleted plasma batches in a dCDA.

Overlay plot illustrating antibody-mediated (a) C3b/iC3b and (b) C5b-9 deposition on *N. meningitidis* NZ98/254 for a homologous strain OMV rabbit control serum using antibody-depleted plasma from batch 1 and batch 2.
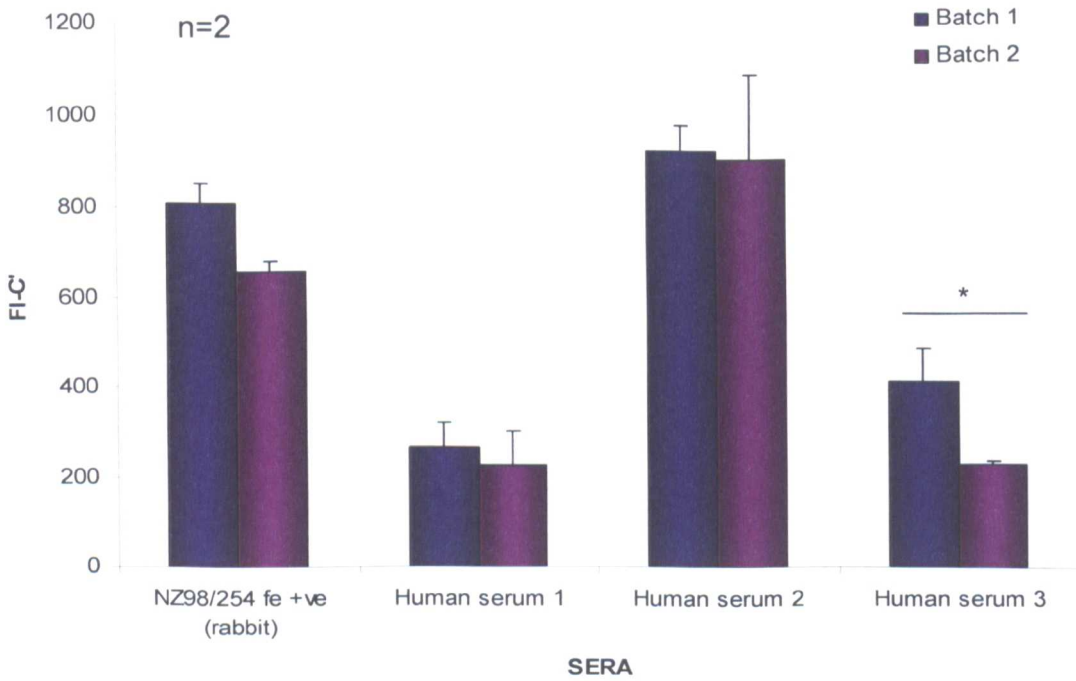
Figure 5.6.5 Antibody-mediated C3b/iC3b deposition on *N. meningitidis* NZ98/254 comparing antibody-depleted plasma from batch 1 and batch 2. Significance was determined by z-test (*$P<0.05$), and error bars denote standard deviation.
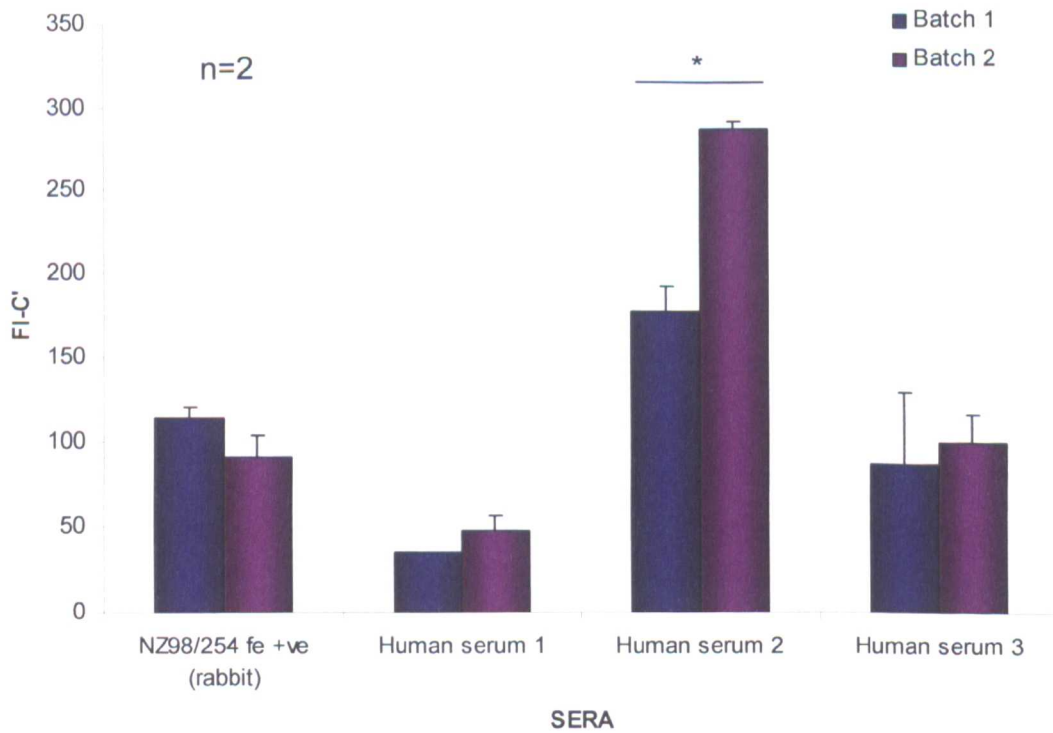
Figure 5.6.6 Antibody-mediated C5b-9 deposition on *N. meningitidis* NZ98/254 comparing antibody-depleted plasma from batch 1 and batch 2. Significance was determined by z-test (*$P<0.05$), and error bars denote standard deviation.
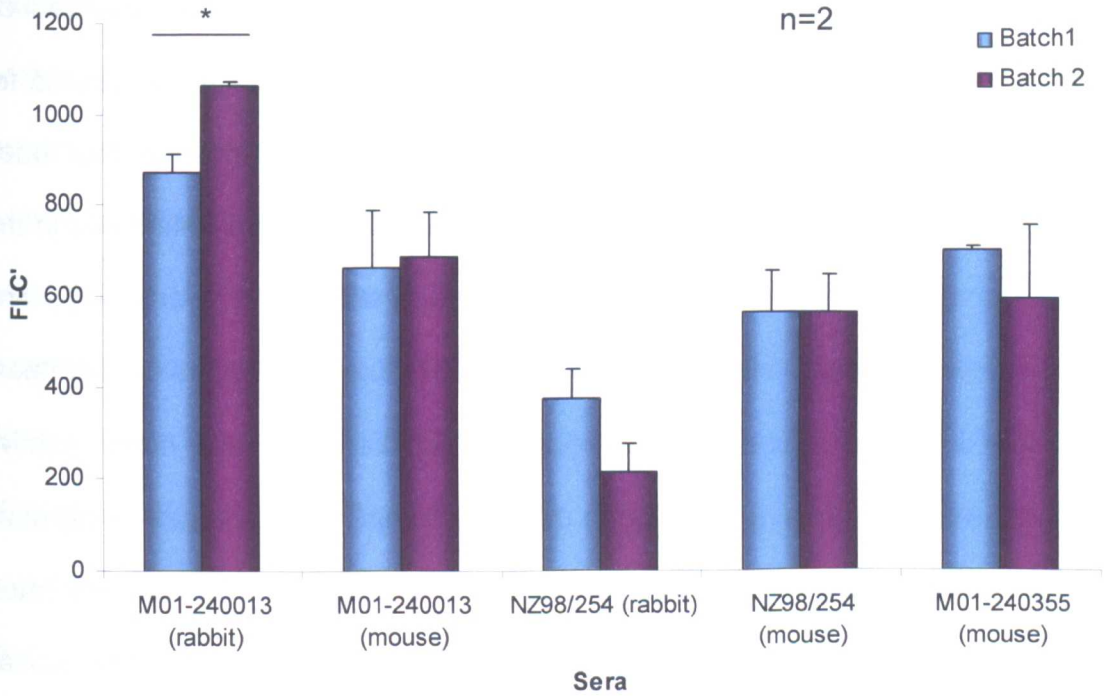
Figure 5.6.7 Opsonophagocytosis assay using *N. meningitidis* M01240013 comparing antibody-depleted plasma from batch 1 and batch 2.

Significance was determined by z-test (*$P<0.05$), and error bars denote standard deviation.

5.7 Discussion

Complement source is a key component used in the CDA and other functional assays used to assess antibody-induced bacterial lysis and opsonophagocytosis of *N. meningitidis*, and the optimisation of the complement used and the extraction method are important to allow the use of a single human complement source for all strains. Use of a human complement source in these assays has been problematic, complicated by the large amounts of cross-reactive antibodies in the population resulting from nasopharyngeal carriage of different *N. meningitidis* and commensal *Neisseria* strains (Cartwright *et al.* 1987). Large numbers of volunteers are screened to find a complement source which contains little cross-reactive antibody to a specific strain, resulting in different complement sources being used in assays against different strains and leading to poor inter-strain and inter-laboratory comparability. The antibody-mediated C3b/iC3b deposition assay initially used antibody-depleted human heparinised plasma, which was routinely antibody-depleted using a 1 ml protein G column immediately before use (2.10.2). Other assays have successfully used baby rabbit complement to assess the antibody induced bacterial lysis and opsonophagocytosis for *N. meningitidis*. Baby rabbit complement has been used in SBAs to assess serogroup A, C, Y and W135 vaccine responses (Maslanka *et al.* 1997; Andrews *et al.* 2003; Keyserling *et al.* 2005; Kshirsagar *et al.* 2007). Baby rabbit complement has been used as it is easily standardisable and can be purchased in bulk, reducing assay variation and it had no cross-reactive antibodies to cause high assay backgrounds. However, serum bactericidal assays using baby rabbit complement have been shown to give higher responses than that achieved using human complement, (Zollinger and Mandrell 1983; Santos *et al.* 2001; Granoff 2009) potentially overestimating the protection afforded. Correlation between the rSBA and the hSBA has been shown using serogroup C *N. meningitidis*, but weaker correlations have been shown with

serogroups A, W135 and Y (Gill *et al.* 2011). In addition, investigations have indicated that the high levels of IgM in vaccinee sera could cause increasing levels of bactericidal activity in the rSBA whereas this was not shown to affect the bactericidal titre measured with the hSBA, thus resulting in false assessment of protective efficacy (Santos 2010). Some of the differences between rabbit and human complement could be attributed to the specificity of meningococcal fHbp with human factor H (Granoff 2009). Factor H is able to cleave C3 and turn C3b (a part of the C5 convertase) into iC3b, a component able to contribute to opsonophagocytosis but not in the formation of C5b-9. Thus, lower bactericidal titres (1-2 log drop) are observed using human compared with rabbit complement (Granoff 2009). In addition to factor H, meningococci have other surface expressed proteins which contribute to resistance to complement mediated killing. Both PorA and type IV pili are able to bind C4 binding protein (Jarva *et al.* 2005), a protein which plays a role in proteolysis of the C4 classical complement cascade component. The recruitment of these negative regulators of the complement cascade could significantly increase survival of bacteria in serum (Schneider *et al.* 2006; Schneider *et al.* 2007). Human complement is therefore the optimal complement source for use in these assays and has been widely used as a complement source in SBA against *N. meningitidis* serogroup B.

This study optimised the complement source used, comparing plasma anti-coagulated with lepirudin, EDTA, heparin or serum. Lepirudin anti-coagulated plasma has significantly ($P<0.01$) higher levels of C3b/iC3b and C5b-9 deposition and OP activity. This result is explained as lepirudin is an anti-thrombic drug which has been reported as an anti-coagulant which does not activate complement (Mollnes *et al.* 2002), whereas the use of both EDTA and heparin as anticoagulants in plasma isolation or clot activators in serum isolation can

negatively affect the activation of complement. It is generally accepted that complement activation cannot occur with EDTA present as this chelates Ca^{2+} and Mg^{2+} (James 1982) and heparin also inhibits complement activation (Mollnes *et al.* 2002). The mechanism of complement dysfunction is unknown, but heparin has been shown to have two binding sites for fH (Blackmore *et al.* 1996; Blackmore *et al.* 1998) and this could have contributed to the dysfunction in the complement cascade observed in the total haemolytic radial immunodiffusion assay (5.2.2). In addition to this *N. meningitidis* can express neisserial heparin binding antigen (NHBA) (Findlow *et al.* 2010) a potential route of additional fH binding and complement regulation. Therefore a complement source with higher concentrations of heparin than is found in normal human plasma (as added during the plasma processing) could also affect assay results.

It was observed that samples using serum as a complement source became gel-like in the OPA and therefore could not be analysed by flow cytometry, perhaps a result of incomplete removal of clotting factors, rendering serum unusable as a complement source in this assay. Higher OPA values ($P<0.01$) were obtained with a rabbit complement source when compared with the IgG-depleted human complement source, possibly because of the specificity of negative complement regulators for human complement.

Aase *et al.* 2007 used human serum as a complement source with a *Bordetella pertussis* OPA and depleted antibody from the human serum using a protein G column, this method was also used in Taylor 2010. No comparison has been made between the functional affect of antibody depletion from plasma using other available matrices. Thus, this study compared antibody depletion by affinity chromatography using either protein G, A, or L (2.10.1), and also investigated the

functional total haemolytic activity and the alternative haemolytic activity of the complements.

Plasma was compared before and after antibody depletion using a radial immunodiffusion assay able to detect either the function of the classical complement cascade or the alternative complement cascade. Following antibody depletion with protein A, G and L, a significant ($P<0.01$) fall in total haemolytic activity was observed. The largest fall in haemolytic activity in comparison to the native plasma was observed following depletion with protein G, which was attributed to the removal of C1q (figure 5.5.1), possibly caused by binding to exposed heavy chain constant region 2 in the Fc portion of IgG which had bound to protein G (Kolb *et al.* 1979; Hombach *et al.* 2005). This is significant as C1q is an important component in the initiation of the classical pathway and thus affects the haemolytic function of the complement (Kolb *et al.* 1979). C1q was added back into the antibody-depleted complement in a range of concentrations near to that of the native levels found in serum (77 µg/ml) (Kolb *et al.* 1979). This caused significant ($P<0.01$) increases in the antibody-mediated C5b-9 deposition and the total haemolytic activity was restored to 75% of the original haemolytic level. Unexpectedly, the C3b/iC3b deposition levels were shown to significantly ($P<0.01$) fall as the C5b-9 deposition levels increased. The reason for this requires further investigation.

To further this work, it would be interesting to purify native C1q from the protein G following depletion of antibody from plasma and reintroduce this to the IgG-depleted plasma. It is possible that the endogenous C1q may improve the total haemolytic pathway function to a greater degree than that observed when purchased C1q is added.

Further work should also include investigations into whether antibody depletion removes other components of the complement cascade from the plasma. For instance properdin, C2, C4 and the late complement components C5- C9, as deficiency in any of these components will also affect the function of the complement.

Significant ($P<0.01$) reductions in alternative haemolytic activity were seen following depletion with protein A, G and L. The fall in alternative pathway activity was consistent between all the depletion methods used, possibly a result of a small dilution effect of processing the plasma on the column.

Plasma depleted using protein A, G or L was then compared in the antibody-mediated C3b/iC3b and C5b-9 deposition assay. Overlay plots demonstrated the lowest assay background (bacteria, complement and conjugate without test serum) following depletion with protein G, with all the depleted and native plasma showing comparable antibody-mediated deposition of C3b/iC3b with homologous OMV rabbit sera against M01240149. This same pattern was repeated with antibody-mediated deposition of C5b-9, and suggested that the use of protein G produced the best complement source. However the reduction in antibody was also linked to the reduction in total pathway function.

Following depletion of antibody using either Protein A, L or G the IgA, IgM, and IgG content was compared with native plasma. Protein G removed IgA, IgM and all subclasses of IgG, and produced plasma which had a low assay background when tested in the antibody-mediated C3b/iC3b and C5b-9 deposition assay,

whilst retaining the similar measurable deposition levels as the native plasma. Protein G is reported to bind only IgG1, IgG2, IgG3 and IgG4. Therefore the removal of IgM and IgA although to a lesser extent, was unexpected. Some of this reduction in IgM and IgA could have been a result of a dilution effect observed following processing the plasma in this way. Protein A affinity chromatography also removed a significant proportion of IgG3, a protein it reported not to bind, however significant reductions in IgM and IgG1 were observed. Protein L removed significant levels of IgM and IgA and significant reductions in IgG2, IgG3 and IgG4 were also observed. However the level of reduction was much less in comparison to protein G depletion. This was an interesting result as protein L is reported to bind all antibody to high affinity, but in this experiment did not remove IgG as effectively as protein G. This may have been because the protein L column had reached its binding capacity (20 mg/ml). Diluting the plasma would have perhaps improved the antibody removal achieved using this matrix, however this would have impacted on its use in the various assays. In addition to this antibody binding to protein L will only occur if the immunoglobulin has the appropriate kappa light chain. Lambda light chains and some kappa light chains are unable to bind, thus making this a less favourable method of depletion as there is an approximate 2:1 ratio of kappa : lambda light chain in humans and thus a third of antibody will not be able to bind (thermo technical information sheet). The results from protein G depletion confirmed almost complete depletion of IgG and the lowest assay background without significant effect on the assay response. This was therefore the optimal complement source.

Large scale depletion was completed for two batches derived from the same pooled sample of lepirudin-anti-coagulated plasma. The batches were compared for classical and alternative pathway function in a haemolytic assay, antibody

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ELISA and in both the antibody-mediated C5b-9 and C3b/iC3b deposition assay and the OPA. Large scale depletion produced a complement source which was functional and showed no significant difference between batches in either the total or alternative haemolytic assays. This batch consistency was also confirmed in the IgM, total IgG, with a significant but small difference in IgA observed. Similar levels of complement activity in the CDA and OPA were confirmed. This illustrated that the method of depletion was reproducible when using the same plasma (mixture of 10 volunteers). Further work should investigate reproducibility of a larger number of complement batches from different volunteers as this could potentially cause significant variability between batches. In addition to this it would be important to compare sero-negative (for the test strain) normal human complement with IgG depleted complement in an SBA against a panel of high, medium and low responding human sera.

The optimal complement source was found to be lepirudin anti-coagulated plasma which had been antibody-depleted using protein G with C1q (Quidel) added at 40 µg/ml prior to use in an assay. Further work could investigate whether by replacing the C1q (Quidel) with endogenous C1q purified from the protein G column, could improve the functionality of the complement. Further work could also investigate the function of the other components of the complement system. The development of an antibody-depleted complement source which is functional in OPA and SBA is important as it will improve assay reproducibility, as the same complement source can be used across all strains and could be used in inter-laboratory standardisation of these assays.

Chapter 6

Chapter 6: General discussion

6.1 General discussion

A correlate of protection is 'a specific immune response to a vaccine that is closely related to protection against infection or disease' (Plotkin 2010). The development of a correlate requires knowledge of the immune mechanisms involved in protection. Often there are multiple mechanisms involved and therefore co-correlates are required to assess protection. Alternatively, a true correlate may not be achievable either because the mechanism of protection is unknown or difficult to measure, and in this case a surrogate of protection can be used which correlates with the true correlate of protection.

Measurement of immune responses responsible for protection, and identification of correlates of protection, are a requirement for the development of effective vaccines. Correlates can be used for licensure of potential vaccines where demonstration of efficacy is not possible via clinical trials; for example when disease levels are too low to assess efficacy, such as meningococcal disease. Correlates are also important in the identification, development and assessment of pre-clinical novel vaccine candidates. There are many adaptive immune responses which can correlate with protection. Correlates of protection for vaccines mediated by antibody production are the most understood, although multiple mechanisms are likely to protect against infection, and thus for some diseases multiple co-correlates might be required for vaccine assessment.

Vaccination-induced memory B and T cells are likely to be important in diseases with long incubation periods, for example Hepatitis B, where B cell memory measured by ELISPOT is a surrogate of protection. Antibody levels of >10 mIU/ml (measured by ELISA) are protective after vaccination but after several years 50%

of vacinees are found to be seronegative. Despite this the established B cell memory is able to produce antibodies following stimulation with antigen and confer protection (Chen *et al.* 1990). For infections with a very rapid disease onset (for example *N. meningitidis* or *H. influenzae*), loss of circulating antibody could result in a vaccinee becoming susceptible where the invasion of the organism is faster than the generation of antibody by memory B cells (Anderson *et al.* 2000). When a 3 dose Hib vaccine schedule was introduced in the UK with children <1 year, the vaccine efficacy was observed to be lower than in countries which had introduced a booster dose. Although a memory response to Hib polysaccharide had been demonstrated in vacinees with no circulating antibody, due to the rapid disease onset vaccine effectiveness only increased following the introduction of a booster dose and catch up campaign, and thus circulating antibody is required at the time of infection, to provide protection (Ladhani *et al.* 2008).

For some vaccines, correlates of protection are well defined, for example tetanus and diphtheria vaccination antitoxin levels of 0.1 µg/ml have been shown to be protective. There are some rare exceptions to this where high levels of antibody are detected, but this is thought to be a result of poor diffusion of antibody into areas of toxin production (Bjorkholm *et al.* 1986; Livorsi *et al.* 2010). In addition, neutralizing antibody levels of 0.7 neutralisation units have been shown to be protective for yellow fever (Mason *et al.* 1973) and a neutralisation antibody titre of 1:10 is protective for Japanese encephalitis (Hombach *et al.* 2005). Following measles vaccination, antibody plays an important role in protection and an antibody microneutralisation titre of ≥200 mIU/ml is sufficient to protect from disease, but titres of ≥1000 mIU/ml are required for protection from both infection and disease (Chen *et al.* 1990). In the case of measles, the role of antibody is accepted as a correlate of protection. However, it is clear that cellular immunity is

important in immunocompromised individuals, where protection has been observed in the absence of antibody. Conversely, it has also been observed that in the absence of cellular immunity, vacinees do not become susceptible when antibody levels are sufficient (Zolopa *et al.* 1994).

For many other vaccines the correlate(s) of protection are not so clear. The development of new smallpox vaccines such as modified vaccinia Ankara (MVA) which is delivered by injection rather than by scarification, cannot use the accepted correlate of protection, which is vesicle development. For these vaccines neutralisation antibody levels of 1:20 are considered to be required for protection (Sarkar *et al.* 1975). However, it has become clear that CD8+ T cells are important in reducing the severity of disease as without CD8+ T cells, vaccinees with neutralising antibody were susceptible to mild disease (Amanna *et al.* 2008).

Vaccination with the pneumococcal 13 valent conjugate vaccine (Prevnar 13), induces serotype-specific protective pneumococcal antibodies. ELISA has been used to measure vaccine induced protection, with protective IgG levels accepted at 0.35 µg/ml. This was established in a study comparing data in a meta-analysis from three clinical trials in South African, American Indian and Northern Californian infants, vaccinated with the 7 valent Prevnar vaccine (Siber *et al.* 2007). Opsonophagocytosis is thought to be the most important mechanism of pneumococcal vaccine induced-immunity (Musher *et al.* 1986) and it has been shown that IgG in infants and elderly are not always able to induce opsonophagocytosis (Romero-Steiner *et al.* 1999). In addition, the relationship between ELISA titre and functional opsonic antibody is also thought to vary with serotype (Henckaerts *et al.* 2006). Measurement of opsonophagocytosis is

therefore increasingly important in the evaluation of pneumococcal vaccines (Goldblatt *et al.* 2010).

A defined correlate of protection for *B. pertussis* vaccines has not yet been identified. *B. pertussis* is the causative agent of whooping cough, which colonises the respiratory epithelium and has a number of virulence factors which have shown immunomodulatory effects. ELISA has been used to measure the antibodies against these virulence factors. Protection provided by acellular pertussis vaccines has been shown to correlate with levels of antibody to pertussis toxin, pertactin and fimbriae (Storsaeter *et al.* 1998). The protection induced by these multi component vaccines is complex, and the role of antibodies to each of these antigens is controversial as is the threshold level of protection. However, measurement of functional antibody responses is increasingly important in vaccine licensure and evaluation. Complement-mediated serum bactericidal activity has been shown to be of low importance in protection to *B. pertussis* (Fernandez et al. 1999), whereas some studies have suggested a role for opsonophagocytosis (Aase *et al.* 2007). It has been observed that neutrophils do not contribute in clearing *B. pertussis* in naïve mice, but play a significant role in mice which have received either a previous infection, or have been passively protected, suggesting a significant role for antibody-mediated opsonic protection (Andreasen and Carbonetti 2009). In addition to this, pertussis toxin (PT) has been observed to inhibit neutrophil recruitment (Kirimanjeswara *et al.* 2005). *B. pertussis* is able to survive intracellularly within phagocytes via entry through FHA-CR3 interaction, and bacteria have been recovered from mouse macrophages after 21 days of infection (Hellwig *et al.* 1999), suggesting the importance of measuring killing or induction of a respiratory burst response in measuring protective immunity. The

complexity of vaccine induced immunity to *B. pertussis* makes it likely that several factors may correlate with protection.

The role of antibody in protection from meningococcal disease was first identified in 1913 following the treatment of patients with meningitis with immune serum produced in a horse (Flexner 1913). The opsonic and bactericidal role of antibody was first described in 1918 (Kolmer 1918; Matsunami 1918) and *N. meningitidis* serum bactericidal activity is now accepted as the correlate of protection for vaccines against serogroups A,B,C,Y and W135, reviewed in (Frasch *et al.* 2009). Goldschneider *et al.* (1969) demonstrated an inverse relationship between serogroup B meningococcal disease incidence by age and SBA titres of $\geq$1:4 (Goldschneider *et al.* 1969). However, data from a more recent UK seroprevalence study have shown a different relationship. Whilst maternal antibodies persist disease incidence is low, when these wane disease incidence increases, and then declines during childhood peaking again in late teenagers. SBA titres showed no significant change until approximately 12 years old when carriage rates increase, resulting in a low disease rate in the absence of bactericidal antibody titres in 2-12 year olds (Trotter *et al.* 2007).

The meningococcal C conjugate vaccine was introduced into the UK childhood vaccination schedule without direct efficacy studies, with vaccine efficacy measured after the introduction of the vaccine, using rSBA as the surrogate of protection (Miller *et al.* 2001; Andrews *et al.* 2003). The introduction of this vaccine has been successful in not only inducing protection for serogroup C meningococcal disease, but also reducing carriage of this serogroup and thus also inducing herd protection (Trotter *et al.* 2007). The ability of rSBA to confer

protection to meningococcal disease is accepted and is used for conjugate vaccine licensure.

Serogroup B OMV vaccines have been used in clonal epidemics in Norway, Brazil, NewZealand, Cuba and Chile, with good correlations with SBA titre and vaccine efficacy. However, protection is serosubtype-specific, providing little protection against a diverse panel of serogroup B strains.

The Goldschneider *et al.* (1969) study also found that a negative titre of <1:4 did not predict disease susceptibility as many individuals with titres below 1:4 did not develop disease. This suggests that SBA titres may underestimate protection, an observation which has been reported on many occasions (Ison *et al.* 1999; Welsch and Granoff 2004; Welsch and Granoff 2007; Finney *et al.* 2008; Plested *et al.* 2009). In addition, evidence for the involvement of other protective mechanisms has been demonstrated in the infant rat model of meningococcal bacteriemia with passive protection achieved with serum which has an SBA titre of <1:4 (Welsch and Granoff 2004) and passive protection has also been achieved with IgG2a B polysaccharide mouse serum in a C6 deficient rat strain (Toropainen *et al.* 2006).

To fully understand the role of assay results as surrogates of protection the assay must be fully characterised, including the target bacteria, antibodies and source of complement.

The use of live or killed bacteria, or OMV coated fluorescent beads, is an important consideration in the development of these assays and their relevance with *in vivo* responses. The use of live bacteria presents problems with reproducibility and throughput and assays using live bacteria can be laborious and

in the case of *N. meningitidis* also present a safety concern. Due to the respiratory hazard associated with *N. meningitidis* any potential aerosol must be contained. Therefore the use of killed bacteria not only improves assay reproducibility as it removes the growth to growth variation, but also allows an *in vitro* assay to be performed without containment and greatly improves assay through-put as it removes the growth of bacteria and colony counting; both time consuming activities.

In this study, the use of killed bacteria as targets for both the duplexed CDA and RBA was investigated and, total antibody binding between live and Azide/PMSF killed bacteria, C5b-9 deposition on Azide/PMSF killed bacteria and SBA, and C3b/iC3b and OPA all demonstrated good correlations (3.2.5, 3.2.6). These correlations show that killed bacteria are suitable for use in the *in vitro* assays developed in chapters 3 and 4.

Determination of vaccine coverage from new vaccines is important when considering implementation, and there is a requirement for alternative methods as the SBA requires a large volume of serum to assess bactericidal activity against multiple strains. It is not straight forward to establish vaccine coverage with the leading *N. meningitidis* serogroup B vaccine candidates (Giuliani *et al.* 2006; Jiang *et al.* 2010), as this depends on antigen expression levels as well as the cross reactivity of induced antibody to the different antigen variants. Assays have been developed to inform on the vaccine coverage including the meningococcal antigen typing system (MATS) and the meningococcal antigen surface expression assay (MeASurE). The MATS assay has been used to measure vaccine coverage of the Bexsero Novatis vaccine (Donnelly *et al.* 2010). However, this assay has been designed for assessment of Bexsero vaccine coverage and is therefore unsuitable

for the assessment of other vaccines. In addition, this assay uses pooled sera from 13-months-old children to establish a positive bactericidal threshold for each antigen, where the relative potency above this threshold represents where 80% of Men B strains are killed in SBA. However, this threshold level could vary markedly with different age groups and the reliance on pooled sera may inflate the protective threshold due to the action of synergistic antibodies (Vu *et al.* 2011). The MeASure assay measures the expression of all fHbp variants using a mAb, this has been designed for use with the bivalent fHbp vaccine (McNeil *et al.* 2011) (1.7.6). However, this assay is only able to assess expression of fHbp. The developed dCDA could be used to assess antibody-mediated C3b/iC3b and C5b-9 deposition with a large panel of bacteria, to indicate the potential vaccine coverage. This assay is not limited to the measurement of expression of specific antigens and, as it utilises very low serum volumes, this maximises the number of strains which can be assessed with this assay.

The developed dCDA and the RBA both use very small volumes of serum, this aspect is particularly important for pediatric clinical trials where serum volumes obtained may be much lower. SBA and WBA require a minimum of 40 µl serum per test, and as it is becoming increasingly important to evaluate novel serogroup B vaccine candidates against large panels of strains to assess the coverage that a vaccine can provide, assays which use low sample volumes are desirable. The described dCDA and RBA require a small serum volume (5 µl and 2 µl respectively) and would enable the analysis of larger panels of bacteria with the limited serum volumes available in paediatric trials. The dCDA also measures two parameters, thus increasing the amount of information measured by the serum sample.

Previously described antibody-mediated complement deposition assays have not been designed for high throughput use or only measure the single C3b/iC3b parameter (Welsch *et al.* 2003; Taylor 2010). Additionally, assays designed to measure C5b-9 deposition were unable to demonstrate a correlation between SBA and C5b-9 deposition (Drogari-Apiranthitou *et al.* 2002). The development of an antibody mediated C3b/iC3b and C5b-9 deposition assay, able to measure two complement components is a valuable tool in the assessment of large scale clinical trial sera.

In addition to the number of tests which can be performed, the number of samples which can be analysed in a day is also important. The assays developed in chapter 3 and 4 have been developed for high throughput. Most assays are titre-based and require analysis of serial dilutions whilst the dCDA and RBA use single point dilutions and this greatly increases the sample numbers which can be assessed in a single day.

The dCDA has been used in a study investigating immune responses induced by protein complexes. Five different complexes were assessed: CxABR (PorA/PorB/RmpM), CxAB (PorA/PorB), CxBR (PorB/RmpM), CxB (PorB) and CxChap (Chaperonin MSP63). Anti-CxABR mouse sera were able to induce antibody-mediated C3b/iC3b and C5b-9 deposition, opsonophagocytosis and bactericidal activity against the homologous strain, showing the greatest activity of the porin complexes. Anti-CxChap mouse sera showed lower levels of antibody mediated C3b/iC3b and C5b-9 deposition in comparison with anti-CxABR but was found to be cross-reactive; with heterologous strains inducing high levels of C3b/iC3b and C5b-9 deposition across a panel of six strains (Marzoa *et al.* 2011). In addition, the dCDA has also been used in a study investigating the structural

characteristics and stability of NHBA-GNA1030, a component of the 4CMenB vaccine. Antibody-mediated C3b/iC3b and C5b-9 deposition was used to assess the effect of thermal stability on the immunogenicity of the fusion protein. Both antibody-mediated C3b/iC3b and C5b-9 deposition were reduced with mouse sera raised against heat stressed NHBA-GNA1030, and the same trend was seen with the avidity ELISA measuring the total IgG (Martino *et al.* 2011).

During the development of the duplexed CDA, antibody mediated C3b/iC3b deposition performed as a singleplex assay was used to identify immunity induced by immunisation with DNA expression library pools. A DNA expression library was developed by Yero *et al.* (2007), this library was split into 10 pools of which antibodies induced by one of these pools was able to confer partial protection in the infant rat model. This pool was then split into further subgroups and analysed by SBA, whole cell ELISA and CDA. Significant ($p<0.05$) increases in C3b/iC3b deposition were observed between pre and post vaccination groups for pool L8-D. This data was consistent with SBA (Yero *et al.* 2007). Recombinant proteins were generated from the expression library and used to immunise mice. Sera generated from protein NMB0938 were able to induce significant increases in C3b/iC3b deposition following vaccination and NMB0938 is able to induce cross-reactive bactericidal activity against a panel of strains (Pajon *et al.* 2009; Sardinas *et al.* 2009).

The dCDA could be used to assess antibody-mediated C3b/iC3b and or C5b-9 deposition in other bacteria. This could apply to *S. pneumoniae* where the deposition of C3b/iC3b could be used to indicate the opsonic activity of antibodies, useful for the assessment of large serum numbers with large panels of *S. pneumoniae* strains. This assay could also be applied to *Streptococcus agalactiae*,

H. influenzae, B. pertussis or other bacterial pathogens where the role of antibody and complement is thought to be important. The suitability of using the dCDA with other bacterial targets would require investigation for each application.

The importance of SBA and complement in immunity to meningococci is emphasised by the observation that individuals deficient in complement not only have an increased susceptibility to meningococcal disease but to also to recurrent infections. Individuals deficient in late complement components (C5b-9) are at 1000 fold greater risk of developing meningococcal disease and also suffer recurrent attacks, often of less common capsule types (Fijen *et al.* 1989; Lehner *et al.* 1992). This supports the role of SBA in immunity to meningococcal disease. However LCCD individuals present with meningococcal disease usually during the teenage years, which indicates a role for another mechanism in protection. Even though LCCD individuals are unable to clear bacteria via SBA, vaccination of these individuals with A, C, W135, Y polysaccharide vaccines showed a reduced risk of developing meningococcal disease, indicating a role for a different mechanism, perhaps an opsonic response (Platonov *et al.* 2003). Individuals who are deficient in properdin (an important stabiliser of the alternative pathway C3 convertase) also more frequently present with meningococcal disease, and it has been observed that this is associated with a poorer prognosis than disease in LCCD individuals. The reason for this is unknown, but some suggestions are that this results from an inability to clear bacteria via either opsonophagocytosis or serum bactericidal activity (Schneider *et al.* 2007).

The source of complement is very important for *in vitro* assays measuring antibody functions that depend on complement components. Baby rabbit complement has been used in SBAs for *N. meningitidis* serogroup A,C,Y, W135 polysaccharide

vaccines (Maslanka *et al.* 1997) but also in the assessment of opsonic responses (Martinez *et al.* 2002; Newcombe *et al.* 2004). Baby rabbit complement is also used in opsonophagocytosis assays for group B streptococcus (Guttormsen *et al.* 2008), and also *S. pneumoniae* (Henckaerts *et al.* 2006). It is used as it provides a complement source which contains no cross reactive antibody and is readily available, allowing inter-laboratory standardisation. It is now considered that human complement is the most appropriate complement source for the evaluation of serogroup B meningococcal vaccines, due to the presence of meningococcal complement binding proteins specific for human complement components (Granoff 2009). Binding of human factor H to fHbp on the surface of meningococci has been shown to reduce SBA titres by 1-2 logs when compared with the titres achieved in an SBA using baby rabbit complement (Granoff 2009). It has also been observed that the change in titre between rSBA and hSBA is most variable with high levels of *N. meningitidis* serogroup C anti-caspular IgM antibodies, whereas the difference in response between rSBA and hSBA for IgG2 was found to be mimimal when this was investigated with *H. influenzae* (Mandrell *et al.* 1995; Santos *et al.* 2001). In addition to this, an SBA titre may be measurable in an rSBA but no titre may be detectable with the hSBA. This is an important observation as this may lead to a false assessment of protective immunity when using rabbit complement in the SBA. Gill et al. (2011) demonstrated correlation with rSBA and hSBA and serogroup C meningococci, however also showed weaker correlations with A, W135 and Y. A moderate correlation with rSBA and hSBA with serogroup A meningococci were also reported by (Findlow *et al.* 2009), however it was also observed that in this study the percentage of subjects with putatively protective titres were similar with either rSBA or hSBA.

As discussed in chapter 5, generating a human complement source is firstly reliant on volunteer donation. Also the donated plasma/serum has to be screened for cross reactive antibody acquired by nasopharyngeal carriage of different *N. meningitidis* and commensal *Neisseria* strains, which can mediate meningococcal bacteriolysis in the absence of test antiserum and cause interference in the results of the assays. These complement sources, which are low in cross reactive antibodies against the strain of interest, may be high against another and therefore different complement sources from different individuals are often used with different strains, resulting in poor assay comparibility.

The development of a large scale method of IgG-depleting human plasma, described in chapter 5, provides a functional complement source which is able to be utilised with all meningococcal strains in SBA, OPA and CDA. The use of a single complement source in a clinical trial, comparing responses across panels of strains, is desirable and would allow comparisons of titres between strains and improve assay reproducibility. Development of a large scale process able to produce a batch of antibody-depleted complement sufficient to assess a large study is important as previous studies have only depleted small volumes of complement (Santos 2010). Although the work described in chapter 5 discusses the utility of this complement source for evaluation of *N. meningitidis* protective immunity, an antibody-depleted complement source would also be a useful reagent for use in investigations with other pathogens.

Another important consideration for the complement source is whether to use serum or plasma, and then subsequently which anticoagulant. The whole blood killing assay is a measure of both opsonic and bactericidal response. For this assay either citrate or heparin are used as anticoagulants which has been

highlighted as a potential problem, due to the possible interference of these anticoagulants with the complement cascade (Mollnes *et al.* 2002). Heparin has been shown to bind 13 complement proteins and has two binding sites for factor H, potentially able to alter assay results (Ison *et al.* 1995). This concern is also applicable to the plasma used as a complement source in *in vitro* assays, as interference of the chosen anticoagulant could alter assay results. Lepirudin was found to be the most appropriate anticoagulant as this has previously been described to not activate the complement system and was shown to provide plasma with the greatest complement lytic activity (Mollnes *et al.* 2002).

Further investigations

The dCDA has been shown to be sensitive and reproducible. However, to transfer the dCDA for use in a clinical trial setting, further assay qualification would be required to demonstrate the utility of this assay as a surrogate for OPA and SBA. An important development in the dCDA would be the investigation of the relationship between measured FI-C' and the SBA titre, to determine at what level of complement deposition a bactericidal or opsonophagocytic response is detectable. As the dCDA is sensitive in comparison with the SBA, at low levels of deposition of C5b-9, it is likely that a threshold of deposition needs to occur before a bactericidal response is achieved. This could be investigated by comparison of FI-C' from a panel of sera, with the SBA response detected at different dilutions. This work would require a detailed investigation with lower responding sera and a diverse panel of strains.

Responses determined with human sera using the dCDA have been correlated with OPA and SBA, but to develop this as a reliable tool in assessment of pre-clinical vaccines it would also be important to investigate the correlation of the

SBA with the dCDA response with a range of high medium and low responding mouse serum, to assess whether a correlation is achieved also with mouse serum.

Investigation of assay robustness would help to identify the capacity of the assay to remain unaffected by small alterations to the assay method, which would help to inform on the assay reliability, an important aspect of any assay used in a clinical trial setting. In addition, it would be important to establish the upper and lower limits of detection for the assay and to also establish assay acceptance criteria. This ensures that results are only reported from assays which have worked to the specified criteria and that any assay problems can be identified and corrected as they appear. Importantly the assay positive and negative controls should be within a defined range, which can be established by monitoring the assay over time.

The developed dRBA was demonstrated to be suitable for small sample sizes, but poor assay reproducibility was demonstrated with larger sample numbers as the respiratory burst reagent demonstrated drift in signal during the time taken for flow cytometric analysis.

A new more stable respiratory burst detecting reagent is required to allow increased numbers of sera to be analysed in a single assay. This may be possible with the increasing availability of flow cytometers which are able to detect a much wider variety of reagents.

If a duplexed uptake and respiratory burst assay could be developed then it would be essential to investigate the responses generated by this assay in comparison with functional killing assays, to ascertain whether the duplexed response correlates more highly than the single parameter measurements. In addition to

this, assay qualification would be required to ascertain assay precision, linearity, robustness and upper and lower detection limits.

Development of the dCDA has shown the assay to be reproducible, sensitive and a potential surrogate of protection for assessment of responses to meningococcal vaccines in both a preclinical and clinical setting. Although the dRBA has shown some reproducibility problems when dealing with very large numbers of sera, it is still useful for the assessment of small panels of serum. However with further work and the availability of new reagents and equipment this may become a useful surrogate for the assessment of meningococcal vaccines. The correlations performed with both of these assays with the functional OPA and SBA was performed against three strain, it would be important to continue this work to assess the correlations against a panel of disease isolates that represents the diversity of the meningococcal population.

Complement is an important component of a range of functional antibody assays, and the IgG-depletion method developed in this study shows great promise to provide complement suitable for use in any assay. Further studies are required to characterise the complement components before and after IgG depletion and to compare SBA and OPA responses obtained with a panel of sera against a panel of representative strains. Investigations will be needed to establish the dilution effect on the plasma following processing, which could be done using an albumin ELISA to evaluate the albumin concentrations before and after. The IgG-depleted complement could allow inter-laboratory standardisation for assays with a common complement source and different laboratories could calibrate their results obtained with their complement source against a recognised standard.

The variability of the depleted batches still requires further investigation. In chapter 5, only 2 batches from the same pooled plasma were compared to assess the reproducibility of the depletion method. It would be important to examine the variation observed across more batches of plasma collected from different groups of volunteers, as it would be anticipated that this could introduce differences in complement function in a range of assays.

This study has developed a duplexed antibody-mediated C3b/iC3b and C5b-9 deposition assay, which has the potential for assessment of meningococcal disease and other vaccines. This is particularly important for the assessment of strain coverage afforded by a vaccine, and this assay has the potential to be an alternative to the MATS or MeASure assays. In addition, this study has also begun the development of a universal complement source, developing a large scale method of antibody depletion able to produce functional complement. A universal complement source is of huge value for vaccine assessment as complement-mediated mechanisms of protection apply to a wide range of pathogens.

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