NOVEL COMPLEMENT BLOCKING ANTIBODIES AGAINST SEROGROUP B *N. meningitidis*

A Dissertation Presented

By

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Submitted to the Faculty of Graduate School of Biomedical Sciences, University of Massachusetts Medcical School, Worcester in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

JULY 23, 2010

IMMUNOLOGY AND VIROLOGY PROGRAM

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Acknowledgements

This very eventful journey through Graduate School would never have been possible without the support, guidance and contributions of a lot of people. Foremost, I would like to thank Dr. Peter A. Rice for giving me this oppurtunity to work in his lab and on this project. Dr. Rice had shown a lot of faith on me at a very turbulent time in my career. I shall be indebted to him forever for the kindness, faith and support he has shown towards me. As a mentor, Peter has always provided me with his valuable guidance and advise at all stages of this dissertation. I would like to thank Dr. Sanjay Ram, who has mentored me closely and constantly throughout this project. Sanjay has been an excellent mentor and I have learned a lot from him. His valuable scientific suggestions and technical guidance has seen me through lots of ups and downs of the project. I am grateful to Sanjay for being so patient with me, for never letting go of his confidence in my abilities and allowing me the freedom to work in his lab.

It was an honor to have Drs, Leslie Berg, Neal Silverman, Stuart Levitz and Egil Lien on my thesis committee. Their comments and suggestions were highly valuable and I thank them for their time. I am also grateful to Dr. Robyn Ingalls for taking the time to evaluate my dissertation.

I was extremely fortunate to be able to train with scholars and scientists with diverse interest and expertise in their field. I would especially such as to thanks the past and present members of the Rice and Ram labs. In particular I would like to thank Lisa Lewis and Sunita Gulati for teaching me various techniques in the lab and also for providing me with their scientific and moral

support. I would also such as to thank all the present and past members of the lab Sarika, Juntamas, Xiahong, Liz, Nancy, Bo, Matt, and Joanna for providing a great working environment.

The journey through these seven years of graduate school was made easier because of the constant support of all my friends Suvi ,Vipul, Manjari, Florentina, Sujan Rafiul Kabir, Sameer, Hema Bashyam, Anjan, Agnita and Rinty and the entire WCBA family.

My family especially my parents have sacrificed a lot all throughout their lives to get me where I am today. They have been my pillars of strength and inspiration all my life. I am indebted to them for supporting me in all my dreams and aspirations to the extent of having to sacrifice many of their own desires. I am also thankful to my parent in laws for letting me marry their daughter and for loving me such as their son.

Last but not the least, I would such as to thank my wife Kamna for being there with in times both good and bad. I cannot express my love and affection for her in mere words. Kamna has been by my side through some of the hardest times of my career and it would have been impossible to go through them without her support and love. I am extremely fortunate to have her as my wife.

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Abstract

N. meningitidis, is a common commensal of the human upper respiratory tract and a leading cause of bacterial meningitis and septicemia worldwide. The classical pathway of complement (C) is essential for both naturally acquired and vaccine induced immunity against *N. meningitidis*. Qualitative and/or quantitative differences in anti-meningococcal antibodies (Abs) in serum is one reason for variations in C-dependent bactericidal Ab activity among individuals. I showed that IgG isolated from select individuals could block killing of group B meningococci by Abs that were otherwise bactericidal. Ligand overlay immunoblots revealed that these blocking IgG Abs were directed against a meningococcal antigen called H.8, Killing of meningococci in reactions containing bactericidal mAbs and human blocking Abs was restored when blocking Ab binding to meningococci was inhibited (or competed for) using either synthetic peptides corresponding to H.8 or a non-blocking mAb against H.8. Further, genetic deletion of H.8 from target organisms abrogated blocking. The Fc region of the blocking IgG was required for blocking because $F(ab)_2$ fragments alone generated by pepsin treatment were ineffective. Blocking required IgG glycosylation; deglycosylation of blocking IgG with peptide:*N*-glycanase (PNGase) eliminated blocking. C4 deposition mediated by a bactericidal mAb directed against a meningococcal vaccine candidate, called factor H-binding protein (fHbp), was reduced by blocking Ab. Anti-fHbp-mediated C4 deposition was unaffected, however, by deglycosylated blocking IgG. Although preliminary, our data suggests blocking of serum bactericidal activity by human anti-H.8

blocking antibody may require mannan-binding lectin (MBL), which itself is a complement activator. Also, whether MBL recruits a complement inhibitor(s) that facilitates blocking remains to be determined. In conclusion, we have identified H.8 as a meningococcal target for novel blocking antibodies that are commonly found in human serum. Blocking Ab may reduce the efficacy of meningococcal vaccines. We propose that outer membrane vesicle-containing meningococcal vaccines may be more efficacious if purged of subversive immunogens such as H.8.

Chapter I

Introduction

1.1 Bacteriology

Neisseria meningitidis is one of the leading causes of meningitis and septicemia worldwide. It is commonly found as a commensal organism in the human upper respiratory tract. *N. meningitdis* is a gram-negative diplococcus that belongs to the family Neisseriaceae (1). It has a genome size of 2.0-2.2 million base pairs (approx. 2000 genes). The complete genome sequences of meningococcal strains Z2491 (NC_003116.1), MC58 (NC_003112.2), FAM18 (NC_008767.1) and 053442 (NC_010120.1) and incomplete sequences of strains α153 (GenBank: AM889137.1) and α275 (GenBank: AM889138.1) are available (2-6). Structurally, meningococcal cell walls are similar to those of most gram-negative organisms (Figure 1.1). The cell wall is comprised of outer and inner membranes with an intervening periplasmic space. The outer membrane components include outer membrane proteins and lipopolysaccharide (LPS) and may also include one of several different capsular polysaccharides. The LPS of *N. meningitidis* does not possess O antigenic repeating units that characterizes the LPS of most gram-negative bacteria and is therefore frequently referred to as lipooligosaccharide (LOS). Akin to LPS, LOS also possesses endotoxic properties and is linked with bacterial pathogenesis (7).

1.2 Epidemiology

Meningococcal disease is a major public health problem in most parts of the world because of the rapid onset of disease (both meningitis and septicemia), high mortality and serious sequelae. *N. meningitidis* has been classified into 13 different serogroups based upon the chemical composition

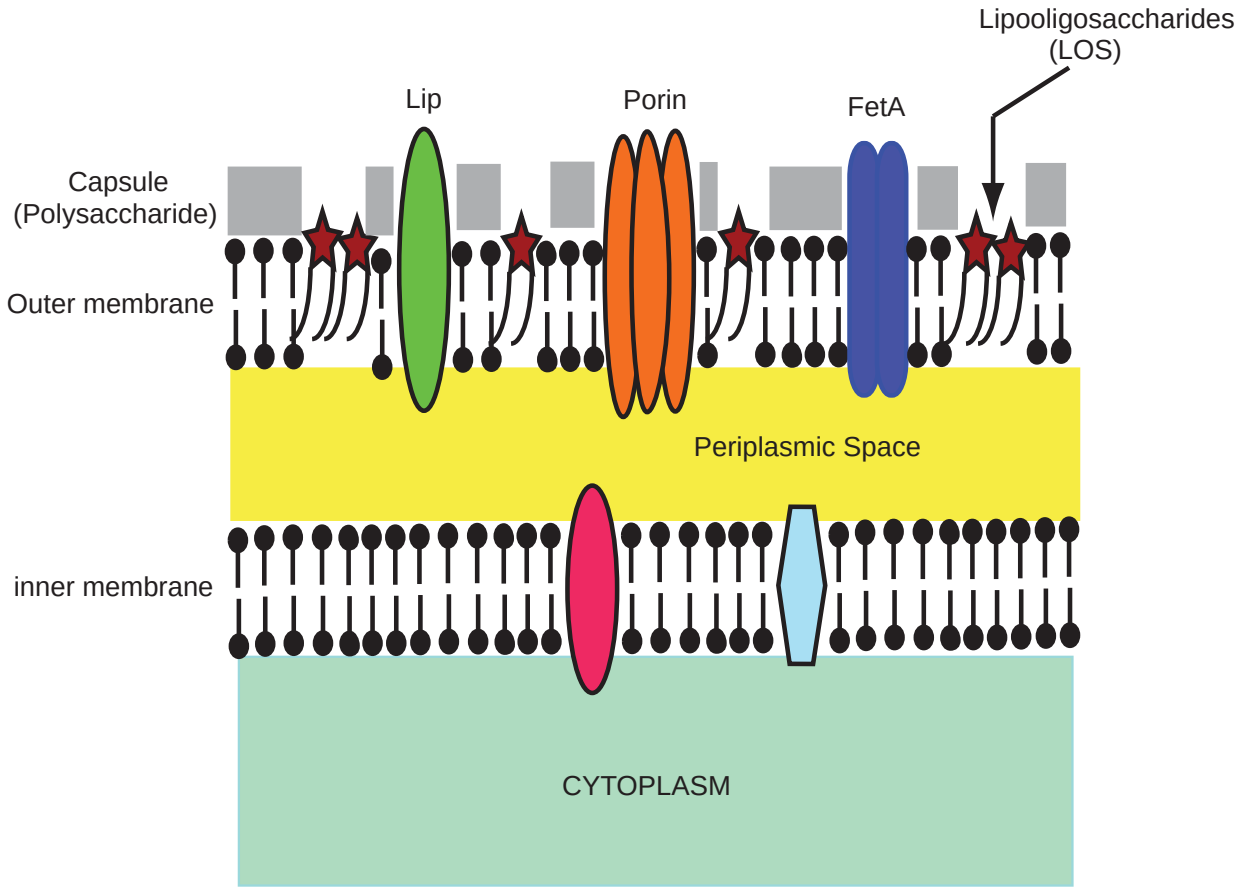


Figure 1.1: Structure of *N. meningitidis* membrane.

of its capsular polysaccharide. Of these, serogroups A, B, C, W-135 and Y are responsible for most cases of epidemic meningitis (Figure 1.2). The incidence of meningococcal disease is highest in children under 2 years of age; a second peak of incidence occurs in adolescents (14-19 years) and young adults who live in college dormitories and military barracks (Figure 1.3). Epidemics caused by the different serogroups of *N. meningitidis* are localized to distinct geographic locations (Figure 1.4). The African meningitis belt (located in sub-Saharan Africa stretching from Senegal to Ethiopia) has been one of the most severly striken areas in recent times (8, 9), where recurring epidemics of serogroup A *N. meningitidis* have been prevalent. The high prevalence of meningitis in this region is not fully understood; climatic factors (dusty winds in dry seasons when incidence of infection is higher) (10), low levels of natural immunity in the population and perhaps, certain characteristics unique to the pathogen have been cited as reasons (11). Serogroup C epidemics (12) and more recently, a serogroup W-135 epidemic was reported in pilgrims to the Hajj (13). Serogroup C epidemics are common in many parts of the world, including Europe; currently, serogroup Y strains are responsible for about a third of cases of meningococcal disease in the United States. Serogroup X epidemics are emerging as the cause of epidemics in certain African countries including Nigeria, Togo and western Kenya. In Europe and the majority of the developed world, serogroup B disease is most prevalent. Endemic disease incidence averages about 1.1 per 100,000, which can rise to as high as 1 in 100 per 100,000 during epidemics. In Australia, the incidence of meningococcal disease averages about 1.5-4 per 100,000; most disease is caused by serogroups B and C. In

Serogroup	Capsular Polysaccharide Structure
A	- 6) - α - D - ManNAc - (1 - OPO_3 -
B	- 8) - α - D - Neu5Ac - (2 -
C	- 9) - α - D - Neu5Ac - (2 -
W-135	- 4) - α - D - Neu5Ac - α – (2--- 6) - α - D - Gal - (1 -
X	- 4) - α - D - GlcNAc - (1 - OPO_3 -
Y	- 4) - α - D - Neu5Ac - α – (2--- 6) - α - D - Glc - (1 -
Z	- 3) - α - D - Gal5Ac - (1--- 1) - Gro - (3 - OPO_3 -

Figure 1.2: Composition of the capsular polysaccharides of the major disease causing serogroups of *N. meningitidis*.

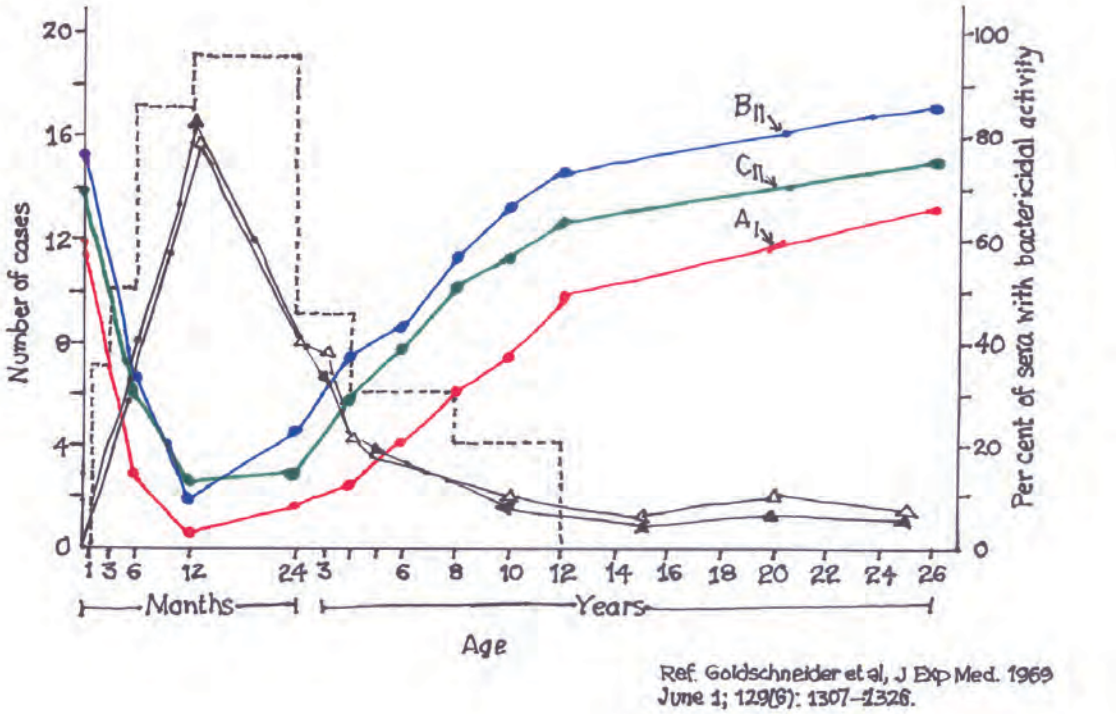


Figure 1.3: Age related incidence of meningococcal disease in the United States prevelance of serum bactericidal activity against three pathogenic strains of *N. meningitidis* (reproduced from: Goldschneider I et al, J Exp Med. 1969 Jun 1;129 (6):1307-26) .Number of cases/100,000 age-specific population (black triangle), 1965; number of cases/100,000 age-specific population, 1966 (white triangle); [-----] age distribution of 72 cases admitted to Los Angeles Children's Hospital 1944-1953; percent of sera in each age group having a bactericidal titer of 1:4 or greater against meningococcal strains AI (red line), BII, (blue line) and CII (green line). Sera from 282 children (at least 20 in each age group) and 567 army recruits (ages 19 to 26 yr) were tested in the presence of exogenous 'human' complement. Each point in the figure represents the incidence of disease or prevalence of bactericidal activity among subjects in the age range encompassed by it and the previous point (e.g., 2.5% of children 6-12 months of age had serum bactericidal activity against meningococcal strain A1. .

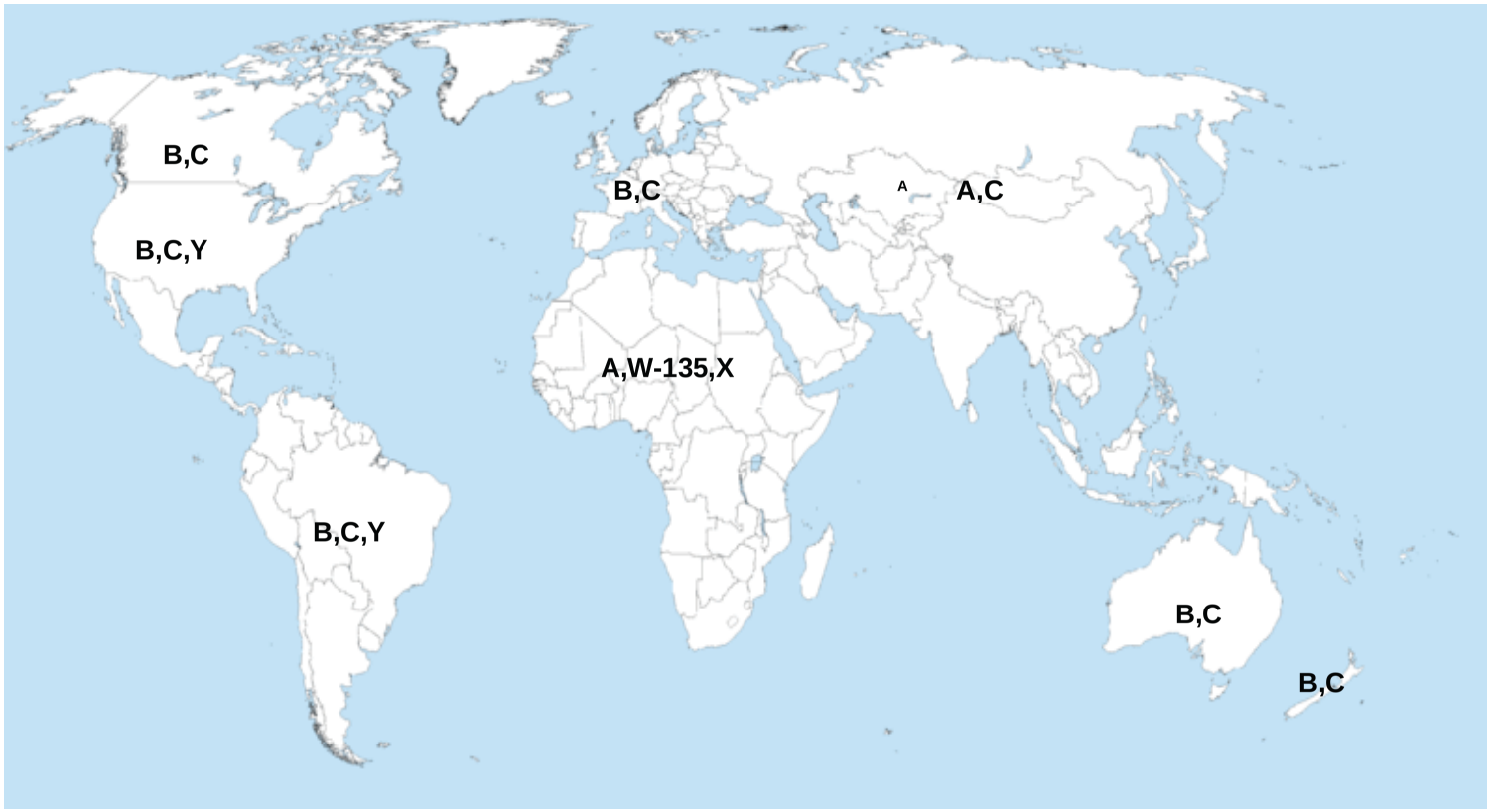


Figure 1.4. Global distribution of *N. meningitidis* serogroups. Worldwide distribution of the different disease causing serogroups (A, B, C, W-135 and Y) of *N. meningitidis* is depicted with the alphabet corresponding to the serogroup.

New Zealand the incidence of serogroup B infections rose to 17.4 per 100,000 in 2001, following the epidemic of serogroup B disease that began in the 1990s. Outbreaks of serogroup B disease epidemics have also been reported in Norway (14), Brazil (15), Cuba (16) and Chile (17). Epidemics of serogroup B meningococcal disease in these countries have led to efforts to produce "tailor-made" outer membrane vesicle vaccines (discussed below). Lack of an effective vaccine against serogroup B disease that can be used universally has made prevention of this disease difficult.

1.3 Epidemiology of meningococcal meningitis in the United States

The epidemiology of meningococcal disease in the United States is highly dynamic. The annual incidence of meningococcal meningitis in the US overall has remained at 0.5 to 1.5 cases per 100,000 since the Second World War. However, the incidence of meningitis has shown cyclical variations over the last 30 years. Following a recent peak in incidence in the mid 1990s, the incidence rate decreased to a low of 0.35 per 100,000 in 2007, which followed the licensure of a quadrivalent meningococcal conjugate vaccine (MCV4) in 2000. MCV4 contains capsular polysaccharide antigens of *N. meningitidis* serogroups A, C, Y, and W-135 strains individually conjugated to diphtheria toxoid protein. As expected, MCV4 vaccine has not affected the incidence of serogroup B disease. In the U.S between 1998 and 2007, the highest incidence of serogroup B meningococcal disease has been recorded in infants (<1 year, 5.4/100,000), toddlers (1-4 years, 1.04/100,000), young adults (15-24years, 0.78/100,000) and senior citizens (>65 years, 0.69/100,000).

1.4 Pathogenesis and route of infection

1.4.1 Initial Colonization: Because *N. meningitidis* commonly colonizes the human upper respiratory tract it is transmitted from person-to-person by droplets. In the nasopharynx, meningococci are believed to interact with CD46 or membrane cofactor protein (MCP) through bacterial type IV Pili (TFP) (18). The role of CD46 in facilitating adherence of meningococci to eukaryotic cells remains controversial because CD46 is believed to be localized primarily at the basolateral surfaces of polarized cells. TFP mediated adhesion of meningococci to the epithelial surface of the nasopharynx occurs in two sequential steps: 1) bacteria attach to the apical surface of the host cells and 2) bacteria divide and form a monolayer on the apical surface. Monolayer formation is aided by bacteria-bacteria interactions that is mediated by pilin (19, 20).

1.4.2 Invasion of the epithelial layer and transcytosis: Adherence of meningococci to epithelial cell is followed by bacteria-induced formation of local elongation of microvilli around the bacteria that leads to engulfment and internalization (21, 22). Several hours after interaction of meningococci with epithelial cells, bacteria disperse on the epithelial surface and form tight contacts with the cells, a process called diffuse adhesion (22, 23). This process is coordinated by regulated expression of several bacterial genes that contain the promoter element called CREN (contact regulatory element of Neisseria) (24). CrgA, a LysR-type transcriptional regulator involved in the intimate adhesion of *N. meningitidis* to target human epithelial cells, is one such protein. Upregulaion of CrgA results in downregulation of *sia* and *pilE*

gene expression, thereby inhibiting synthesis of the capsule and pilin E respectively. These two structures inhibit intimate bacterial adhesion and invasion of host cells (24, 25). Meningococcal outer membrane opacity proteins, Opa and Opc, are also involved in intimate adhesion of meningococci to epithelial cells (26-28). The Opa family of proteins contains 8 transmembrane beta-barrel strands and four surface exposed loops. The first three loops are antigenically variable. Because meningococci possess up to 4 *opa* genes, a single bacterium can express more than one Opa protein simultaneously (26). Differential Opa expression may also determine tissue tropism (29, 30). Opa proteins bind the CD66/CEACAM family of proteins on host cells. Different members of the CD66 family of proteins are expressed on different host cells, which determines the cell specificity of the interaction of meningococci that express a certain type of Opa(s) (31). Opc shares weak amino acid homology with Opa but is structurally different (32). One of the Opc proteins, OpcA, adopts a 10-stranded beta-barrel structure with extensive loop regions that protrude above the predicted surface of the membrane (33). Once it enters the blood stream, meningococci may traverse the blood-brain barrier to cause meningitis or disseminate to various organs to cause meningococcemia, or both. Vitronectin forms a bridge between Opc protein and $\alpha_{\upsilon}\beta_{3}$ integrins on the endothelial cell surface independent of pili or Opa (34-38).

1.5 Clinical manifestations

In most instances pharyngeal acquisition of *N. meningitidis* results in asymptomatic carriage. Colonizing stains spread from individual to individual

via respiratory droplets. The carriage rate and disease rate of meningococci are not directly proportional (39). At any time, about 10% of the population at large are colonized with meningococci. Carriage rates are low in infants and children but higher in teenagers and young adults (40, 41). Progression from carriage to disease is not fully understood, but is likely determined both by host and bacterial factors. Typically, disease when it occurs, takes place 10 days following colonization of a susceptible individual (42).

Meningococcal disease may present either as meningitis (infection and inflammation of the meninges) or bloodborne infection accompanied by septicemia (meningococcemia). Sometimes, menigococcal bacteremia occurs without symptoms but may progess to meningitis or septicemia. Of all cases of meningococcal disease, meningitis alone occurs in 30-50% and results in a 5% mortality; septicemia, without meningitis, occurs 7-10% of the time and results in a higher (5-40%) mortality. Meningitis and sepsis occur together in ~40% of cases (43-45). Patients with meningitis may have one or more of the following symptoms and signs: headache, fever, vomiting, photophobia, stiff neck (nuchal rigidity) and a positive Kernig's or Brudzinski's sign. Patients with meningococcemia may present with fever, purpuric rash, headache, flu-like symptoms, vomitting and abdominal pain.

1.6 Principles of treatment

Early diagnosis and treatment is essential in managing meningococcal disease. Delay in the initiation of treatment is associated with adverse outcomes including death. Third-generation cephalosporins currently are the mainstay of treatment. Prevention of disease in close contacts of the index

case is important; individuals living in the same household are at higher risk for developing disease in the first week following diagnosis of the primary case (46) and chemoprophylaxis of close contacts is recommended. It should be noted that resistance of meningococci to quinolones, one of the agents used in chemoprophylaxis, has been reported.

1.7 Vaccines against meningococcal disease

Most cases of meningococcal disease are vaccine-preventable. Several different vaccine formulations against meningococcal disease have been developed and are discussed below.

1.7.1 Capsular polysaccharide-based vaccines

A. Unconjugated capsular polysaccharide vaccines: Groups A and C polysaccharides were used as vaccines in the 1960s (47). A quadrivalent purified polysaccharide vaccine that provided protection against groups A, C, W-135 and Y was licensed for use in the U.S in 1981 (48). With the exception of the group A polysaccharide, this vaccine is poorly immunogenic in children < 2years of age. Capsular polysaccharides are T cell-independent antigens and as a result provide only short-lived immunity. Repeated doses of polysaccharide vaccine may be required every 3-5 years because the immune B cell pool becomes depleted (49). Furthermore, repeated doses of purified polysaccharide vaccines may induce antibody hyporesponsiveness due to tolerance.

B. Polysaccharide-Protein conjugate vaccines: To circumvent the limitation of purified capsular vaccines, several protein-polysaccharide conjugate vaccines have been developed. Serogroup C polysaccharide

conjugate vaccine (MenC) was first introduced in the United Kingdom in 1999 in response to an epidemic of serogoup C disease, which resulted in substantial reduction in the number of disease cases (50) and carriage rates (51, 52). Although, MenC administered at 2, 3 and 4 months of age was effective in providing protection up to 1 year of age, significant protection did not extend beyond one year (53). The revised schedule in the U.K now recommends doses at 3 and 4 months, followed by a booster dose of Men C vaccine at 12 months. Because typical symptoms of meningococcal infection manifest 24-72 h following invasion (54), progression of disease cannot be countered in time by a memory response (to the infection) that had been initiated by a conjugate vaccine (55). Such protection requires significant levels of circulating antibodies that usually are not induced following vaccination in infancy because of the low survival rate of plasma cells in the first year of life (56).

In 2000, a tetravalent polysaccharide vaccine conjugated to diphtheria toxoid (Menactra, or MCV4; Sanofi-Pasteur) was licensed in the U.S for use in people between the ages of 2 and 55 years. Serogroup Y had accounted for 37% of all meningococcal disease in USA between 1997 and 2002) (48). MCV4 is now universally recommended for college freshmen, travelers to meningococcal disease–endemic areas, military recruits and persons with immune deficiencies that diminish antibody production or compromise the complement system. Again, the immunogenicity of this vaccine in infants is poor. A second polysaccharide conjugate vaccine (Menveo; Novartis) conjugated to CRM_{197} (a mutant diphtheria toxin) was recently licensed for use in the U.S (57). Presently, a combined vaccine comprising *Haemophilus*

influenzae type B and meningococcal C and Y capsules, each conjugated to tetanus toxoid, is undergoing clinical trials (58). A conjugate vaccine (MenAfriVac) against serogroup A, which is a major cause of meningococcal disease in sub-Saharan Africa, is being developed by the Serum institute of India and is currently undergoing evaluation in Africa and India (59).

1.8 Vaccines against Serogroup B *N. meningitidis.*

There is no licensed polysaccharide based vaccine against serogroup B disease. The polysialic acid capsule (α [2,8]-linked polysialic acid) of serogroup B *N. meningitidis* is a poor immunogen because it mimics certain sialic acid containing glycoproteins on mammalian cells and tissues (self-antigens). This form of sialic acid is found as a component of neural cell adhesion molecule (N-CAM) which is involved in axonal growth and synaptic plasticity (60). Purified serogroup B polysaccharide has been used as a vaccine in adult volunteers but it failed to elicit a significant anti-capsular antibody response (61). To circumvent the problem of poor immunogenicity and concerns surrounding the production of auto antibodies following immunization with group B polysaccharide, alternative strategies have been attempted and are discussed below.

1.8.1 Modified serogroup B capsule polysaccharide-protein conjugate:

A group B polysaccharide vaccine has been designed whereby the N-acetyl group of polysialic acid was substituted with an N-propionyl group, followed by conjugation to tetanus toxoid (62). This vaccine was highly immunogenic in mice. Although also immunogenic, producing antibodies in human volunteers, no significant serogroup B specific serum bactericidal antibody activity was

elicited (63). The generation of autoreactive antibodies against human polysialic acid residues may remain as a concern with these serogroup B vaccines (64) although no evidence of binding or deleterious effects of these potential auto-reactive antibodies to human cells were seen in early human and animal trials. In addition, no bactericidal or opsonophagocytic antibody responses were elicited in the human trial (65). Safety and Immunogenicity of an N-propionylated group B meningococcal polysaccharide conjugate vaccine in adult volunteers (In Proceedings of the 10th International Pathogenic Neisseria Conference (IPNC), September 8-13, 1996, Baltimore, MD. p.225). However, Granoff et al. have shown that certain epitopes of the N-propionyl sialic acid polysaccharide generate bactericidal antibodies in mice that do not cross react with human polysialic acid residues, thus extending the case for further consideration of this vaccine strategy (66).

1.8.2 Outer membrane vescicle vaccines: Outer membrane vesicles (OMVs) can be generated from meningococcal culture supernatant fluids and comprise a lipid bilayer that contains several outer membrane proteins (OMPs) and lipooligosaccharides (LOS). OMVs prepared by detergent extraction of whole organisms reduce their LOS content significantly. Such OMV vaccines have been administered with varying results in Chile (67), Cuba (16), Brazil (68) and Norway (14). Recently, one such “tailor made” OMV vaccine was successful in containing an epidemic in New Zealand (69). However, the main obstacle to the efficacy of these OMV vaccines is the generation of a strain-specific antibody response because the majority of elicited antibodies are directed against PorA, an outer membrane protein that is highly variable across different serogroup B strains. Therefore, an OMV

vaccine made from a particular sergroup B strain affords protection only against that particular strain but not against diverse strains that cause endemic disease and possess heterologous PorA molecules (70). Another, potential problem may be the induction of antigenic shift in PorA or deletion of *porA* genes from infecting strains, which would render the vaccine potentially ineffective (71). An approach under consideration currently is the production of OMV vaccines that express several PorA variants that may confer protection against a wider array of circulating serogroup B strains (70).

1.8.3 Protein vaccines: In 2000, a method called “reverse vaccinology” was introduced that was based on genome mining that used computer based algorithms to identify genes that encoded the entire genome sequence of *N. meningitidis* (strain MC58) for potential surface exposed protein antigens (72, 73). A total of 350 candidate antigens were expressed in *Escherichia coli*, purified, and used to immunize mice. The sera allowed the identification of proteins that were surface exposed, that were conserved in sequence across a range of strains and that induced a bactericidal antibody response. Experimental evaluation of vaccine potential of most of these antigens identified five promising surface antigens: *N. meningitidis* adhesin A (NadA), factor H binding protein (fHbp), Genome-derived Nesseria Antigen (GNA) 2091, Neisseria heprin binding antigen (NHBA) and GNA1030. Novartis Vaccines formulated a vaccine formulation that incorporates these 5 antigens into OMVs, called rMenB. Mice immunized with this formulation generated bactericidal antibodies against 78% of a panel of selected serogroup B strains (74, 75). rMenB is currently undergoing clinical trial. Of note, fHbp was also identified as a vaccine candidate by Wyeth (now Pfizer) using an independent

approach, which involved fractionation of meningococcal outer membrane proteins to identify fractions that yielded bactericidal responses following immunization of mice (76).

Two of the vaccine candidates included in rMenB, called NadA and fHbp, have been functionally characterized and may play an important role in bacterial pathogenesis. Hypervirulent meningococcal strains that do not express NadA are unable to invade human epithelial cells (77). fHbp has been shown to bind the alternative complement pathway inhibitor, factor H, which enbles meningococci to escape complement-mediated direct killing (78, 79).

1.9 Natural immunity and susceptibility to *N. meningitidis*

Most individuals are asymptomatic carriers of *N. meningitidis*. Factors implicated in the predisposition to meningococcal disease include age (highest in infants and adolescents), socio-economic status, climate (more commonly seen in winter and in the dry months) and social behavior (associated with kissing and cigarette smoking). Natutral immunity plays a key role in protection against meningococcal disease; individuals who lack bactericidal antibodies are at risk for invasive disease.

The importance of complement dependent bacterial killing in protection against meningococcal is illustrated by the observation that persons with congenital or acquired deficiencies of the complement system and in particular, defects of the alternative or terminal pathways are at a significantly higher risk for invasive meningococcal disease (discussed below). Complement deficiencies are very rare; lack of protective antibodies is more common and probably constitutes a more important risk factor.

Most individuals clear invading meningococci either by serum-mediated bactericidal activity (SBA) or by opsonophagocytosis. The small fraction of normal healthy individuals who lack functional antibodies against meningococci are more susceptible to infection. Colonization with meningococci can be an 'immunizing' process. Bactericidal antibodies that are elicited against epitopes shared by heterologous strains may also contribute to cross-protection. Landmark sero-epidemiologic studies performed by Goldschneider and colleagues in the 1960s that involved 282 children (age 0 to 12 years) and military recruits (age 19-26) showed that individuals who possessed a serum bactericidal titer (using human complement) of 1:4 or greater were likely to be protected against invasive disease by serogroups of the test strains (80). I review several hypotheses below (1.9.1-4) that might explain acquisition of natural immunity to meningococcal disease.

1.9.1 Exposure to non-Neisserial organisms: Anti-meningococcal antibodies may be elicited following exposure to non-Neisserial microorganisms that share surface antigenic features with *N. meningitidis*. The development of IgM antibodies to serogroup B capsular polysaccharide in adults (81, 82) may be secondary to repeated exposure to *E. coli* K1 and *Moraxella nonliquefaciens*. Both of these organisms can colonize humans and have polysaccharide capsules that are chemically identical to group B meningococcal capsule (83). Nevertheless, the significance of cross-reacting anti-meningococcal antibodies in protecting against meningococcal disease is questionable (84, 85). For example, anti-serogroup B polysaccharide IgG and

IgM show low bactericidal activity in the presence of human complement (86, 87).

1.9.2 Carriage of non-pathogenic Neisserial species, for example *N. lactamica:* The prevalence of *N. lactamica* in the nasopharynx is believed to be highest during infancy and early childhood (<4 years of age) but decreases thereafter. In contrast, colonization with *N. meningitidis* is less than 1% between infancy to 5 years of age. It increases thereafter and peaks at 15-19 years (41, 88-91). Most of the data on *N. lactamica* vs *N. meningitidis* carriage in terms of age is derived from a nasopharyngeal swab survey conducted in Stonehouse, Gloucestershire in November 1986 (90). Because most cases of meningococcal meningitis occur in children between 6 months (when maternal antibodies wane) and 2 years of age (92), and the meningococcal carriage rate in this age group is less that 1% (41), the immunizing effect of meningococcal colonization is diminished. However, higher rates of colonization with *N. lactamica* in this age group and resultant antibodies elicited against *N. lactamica* may cross react with *N. meningitidis*, are believed to provide protection against what otherwise could be an even higher rate of meningococcal disease in this age group. Unlike *N. meningitidis*, *N. lactamica* lacks a capsule; therefore antibodies elicited against *N. lactamica* that cross-react with *N. meningitidis* target membrane proteins and LOS (93-95)

1.9.3 Asymptomatic carriage of *N. meningitdis*: Asymptomatic carriage rates of meningococci range from 10-35% in young adults and approach 100% in closed populations during an epidemic (96, 97). Hence, asymptomatic carriage of *N. meningitides* is a major contributor to protection

against meningococcal disease. Following carriage, levels of IgG and IgA antibodies increase against the colonizing strain, which may provide systemic and mucosal protection, respectively (98-103). Protection induced by asymptomatic carriage is usually strain specific as evidenced by a rise in antibody titers against noncapsular antigens of the homologous strain.

Antibodies that develop against capsule during carriage may also provide some degree of cross protection against other strains of the same serogroup (84, 98, 99, 104, 105). In addition to protection against the homologous strain, a recent study in young adults has shown that colonization elicits serum bactericidal antibody activity against heterologous meningococcal strains, albeit at lower titers. Serum bactericidal activity in this study was attributed to antibodies directed against outer membrane proteins that were conserved across different strains of the same serogroup (106).

1.9.4. Meningococcal disease: Although meningococcal disease itself may protect against future bouts of infection, low incidence rates of meningococcal disease (1-3 per 100,000 population in western countries) preclude this mode of protection to most people. An increased level of antibodies directed against the infecting meningococcal strain occurs in 45-90% of patients in the second week following infection (107-113). Protective IgG and IgM antibodies follow meningococcal disease (84, 109, 112, 114, 115). These antibodies develop against both capsular (81, 111, 116, 117) and noncapsular antigens such as outer membrane proteins and LOS (118-123). In the case of serogroup B meningococcal disease most of the resultant protective antibodies are directed against subcapsular antigens because the group B polysialic acid capsule is poorly immunogenic (124).

1.10 The complement system

The complement system is an important arm of the innate immune system against invading pathogens including *N. meningitidis*. Almost every blood borne bacterial pathogen must have mechanisms to subvert complement-dependent killing; four decades ago Roantree and Rantz reported that almost every bacterial isolate that caused bloodstream infection was resistant to antibody mediated complement-dependent killing (125). The complement cascade consists of three pathways that converge at the level of C3 (Figure 1.5).

1.10.1 Classical Pathway: The classical pathway is most commonly activated when an antibody binds to an antigen, as may occur on the surface of a microbe. Upon binding to the surface of a pathogen, the Fc portion of the antibody undergoes conformational change and binds the $C1q(C1r)_2(C1s)_2$ complex through the C1q subunit. Antibodies vary in their ability to activate complement. In general, IgM is the most potent activator of the classical pathway. Human IgG3, IgG1 and IgG2 activate complement in decreasing order of efficiency; IgG4 does not activate complement. Binding of the C1q subunit induces a change in the confirmation of C1r and subsequently C1s. C1s, a serine protease, then cleaves C4 to C4a (released into solution) and C4b, which binds covalently to the surface of the pathogen. C4b possesses a highly labile internal thioester residue, which becomes exposed after cleavage of C4. The labile thioester must react with electron-donating –OH groups (or $–NH_2$) on surfaces within a very short period (<100 microseconds) or the C4b molecule is hydrolyzed and remains in solution. C1s cleaves C2 into C2a and C2b. C2a binds noncovalently to surface bound C4b, to form the C4b,C2a

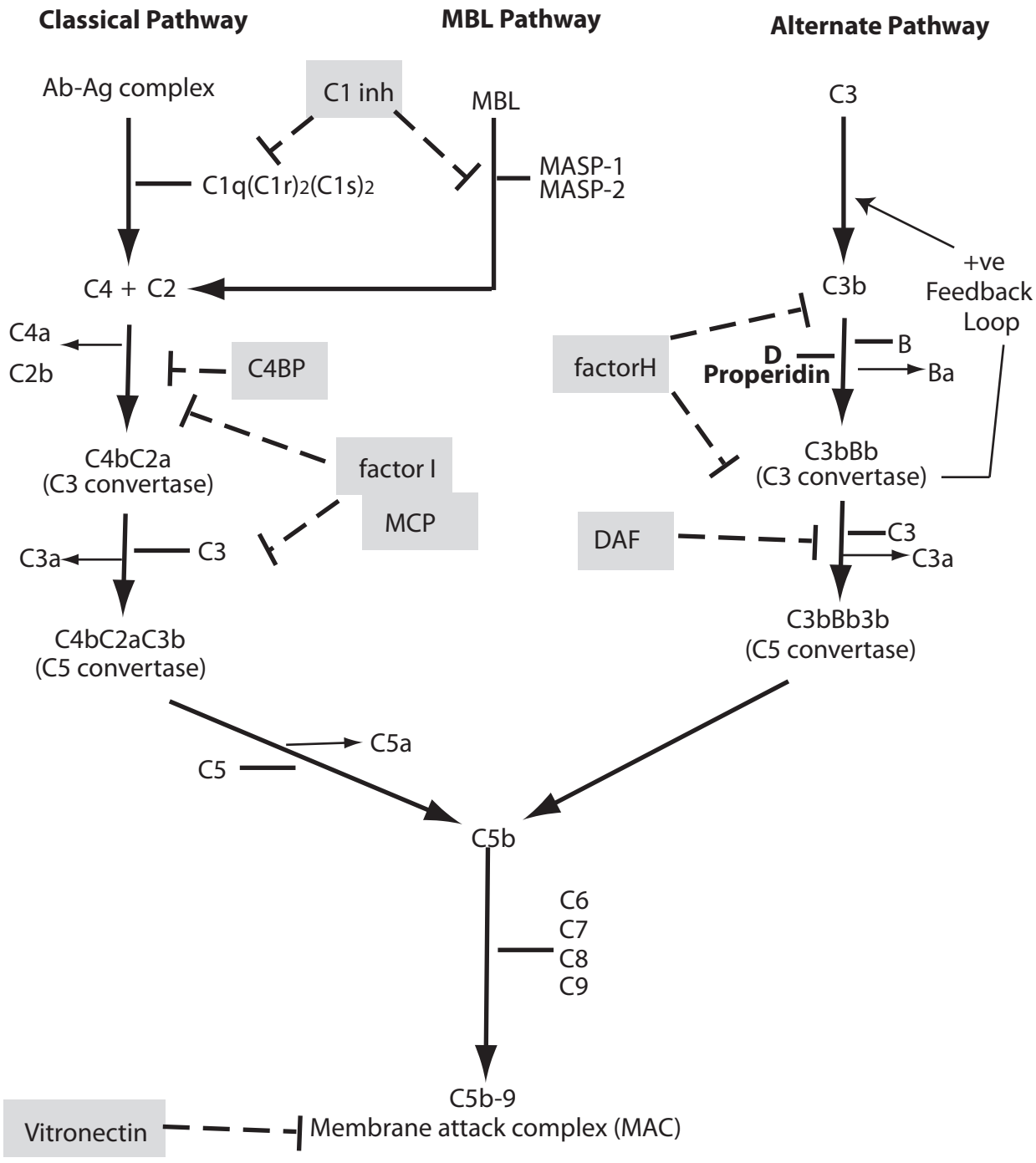


Figure 1.5: Schematic representation of the three pathways of complement. Selected (negative) complement regulators are shown in grey shaded areas with dashed arrows indicating where they inhibit.

complex, the C3 convertase of the classical pathway. As its name suggests, the C3 convertase cleaves C3 to C3b. Similar to C4, C3 also possesses an internal thioester residue. Cleavage to C3b exposes the thioester and progression to binding (or hydrolysis) occurs exactly as it does with C4b (described above). C3b that is bound to surfaces forms a complex with C4b,C2a. The resulting C3bC4bC2a complex functions as a C5 convertase that cleaves free C5 to form C5b. C5b binds to the surface of the pathogen and C6, 7, 8 and 9 are successively recruited to form a scaffold that is the membrane attack complex (MAC or C5b-9). The MAC forms a hollow tubular structure (a pore) that can insert into the membrane of a pathogen and in the case of gram-negative bacteria, can initiate lysis and death.

1.10.2 Mannan binding lectin (MBL) pathway: The mannan binding lectin (MBL) pathway is a more recently described pathway. MBL is similar in structure to C1q and binds to select carbohydrates on pathogenic surfaces, which results in the activation of MBL associated serine proteases (MASPs). MASP-2 is the best characterized of the MASPs. MASP-2 cleaves C4 and C2 to form the C3 convertase that then generates C3b. Subsequent activation proceeds as described for the classical pathway. MASP-1 possesses marginal C3 cleavage activity and the function of MASP-3 is not well understood. The MBL pathway also facilitates opsonophagocytosis of bacteria by engaging complement receptor (CR) 1 (also called CD35). MBL also binds to meningococcal Opa and PorB through protein-protein interactions (126) but the contribution of this interaction to complement activation and pathogenesis is not known.

1.10.3 Alternative Pathway: The alternative pathway is activated by spontaneous hydrolysis of C3 that leads to the generation of C3b. Newly generated C3b has a short half-life and must quickly attach to the surface of a nearby pathogen or it is hydrolyzed. Factor B then binds to attached (or deposited) C3b to form the C3bB complex; cleavage of B in this complex by factor D to Bb results in formation of C3bBb, also known as the C3 convertase of the alternative pathway. C3bBb then cleaves additional C3 molecules to C3b, forming a positive feedback loop that perpetuates the process; this is a unique feature of the alternative pathway. Some of the activated C3b escapes from the loop and binds to the C3 convertase to form C3bC3bBb, also known as C5 convertase, which cleaves C5 to form C5b. C5b initiates the formation of the C5b-9 complex (also called the membrane attack complex [MAC]) that leads to lysis and death of gram-negative organisms.

1.10.4 Regulators of the complement pathway: Activation of the complement system can occur spontaneously in the fluid phase and may be amplified rapidly, which can be detrimental to host cells. To limit complement activation on foreign surfaces and limit unwanted activation on host cells, the complement cascade is tightly regulated by a series of soluble and membrane-bound regulators. Some of the important regulators of the complement system are shown in Table I.

1.10.5 Role of complement pathway in immunity against *N. meningitidis*: Deficiency in terminal complement components C5 through C9 and in components of the alternative pathway (properdin, factor D and C3) predisposes individuals to meningococcal infections (127, 128). In fact, the only reported disease associated with defects in the terminal pathway and

Table 1. Description of Key complement regulators in humans.

Complement regulator	MW (kDa)	Serum level	Function	Deficiency
C1 inhibitor	160	200 mg/ml	Binds to C1rC1s and dissociates it from C1q. Prevents spontaneous activation of C1 in circulation. Also inhibits MBL-MASP2 activation on host cell surface.	Autosomal dominant disorder called Hereditary Angioedema. Leads to uncontrolled activation of C2 and C4 leading to deficiency these complement components (256)
Factor I	Heavy chain: 35.4 Light chain: 27.6	35 mg/ml	A circulating serine proteae that can cleave C3b into smaller fragments iC3b and then into an even smaller C3dg once it binds to host cells. Similarly, it cleaves C4b to C4c and C4d. Factor I requires several cofactors for its activity.	Autosomal recessive.Uncontrolled activation of alternate pathway results in loss of C3 due to consumption. Patients are susceptible to recurrent infections, glomerulonephritis and SLE like illness ((126); (257))
C4 binding protein (C4BP)	570	150-300 mg/ml	Binds to C4b and accelerates the decay of C4b containing C3 and C5 convertase by acting as a cofactor for factor I ((258); (259). In *Neisseriae* C4BP binds to LOS and gonococcal Porin.	Only one case reported so far. Excess C3 consumption and angioedema like syndrome (Trapp RG et al, 1987)
Complement Receptor 1(CR1)/ CD35	205-250	Expressed on RBCs, PMNs, DCs. 30-60 ng/ml	Cofactor for factor I mediated cleavage and inactivation of C3b and C4b (261); (262); (263). It is also a receptor for C1q ((264))	Total CR1 deficiency is unknown.
Membrane Cofactor of Proteolysis (MCP/CD46)	51-68	Expressed on most cells except RBCs	is an integral membrane glycoprotein of most mammalian cell membranes and is a cofactor for factor I mediated cleavage of C4b and C3b on self tissue ((265))	Not known
Decay accelerating factor (DAF/CD55	75	Expressed on most cells	It competes with factor B for binding to C3b and thereby prevents the formation of a convertase. It can also	Deficiency leads to hightened uptake of C3b (269)

			dissociate C3b from an already formed complex with factor B (Nicholson-Weller A et al, 1982; (266); (267); (268)	
Properidin	55	4.3-5.7 mg/ml	Binds to surface bound C3b and stabilizes the C3bBb complex and prevents cleavage of C3b by factor I (270); (271)	X-linked. Defective aternate pathway and increased susceptibility to meningococcal infections ((272); (273))
S Protein (Vitronectin)	65-75	0.14-0.7 mg/ml	Competes with C5b-7 for binding to membrane lipids and prevents anchoring and C9 polymerization ((274))	Hereditary disorder is autosomal dominant Causes thrombosis (275)(275).

properdin is invasive meningococcal infection. An effective classical pathway is essential for bactericidal activity against Neisseriae (129). Defects in regulation of the alternative pathway results from deficiency of factor H or factor I that leads to uninhibited activation of the alternative pathway and consumption of C3; these deficiencies predispose individuals to invasive meningococcal disease (130, 131). A single nucleotide polymorphism in the promoter region of the factor H (C-496T) gene that results in higher levels of circulating factor H has been associated with increased susceptibility to invasive meningococcal disease (131); relatively high factor H levels may shift the balance of the complement system in favor of inhibition and could interfere with effective killing or opsonophagocytosis of meningococci. Deficiency of the MBL pathway also results in predisposition to a variety of pathogens including Neisseriae (132). MBL presumably plays an important role in infants who have an immature adaptive immune system and lack sufficient protective anti-meningococcal antibodies.

Persons deficient in terminal complement components C5 through C8 are ~10,000-fold more susceptible to invasive disease than normal individuals; C9-deficient persons are at 10-fold lower risk than persons with C5 through C8 deficiencies. In contrast to sera deficient in C5, C6, C7 or C8 that does not support bactericidal activity at all, C9 deficient serum supports weak antibody-dependent complement-mediated killing, albeit at a rate much slower than complement-sufficient serum (133-135).

Fortunately, inherited complement deficiencies are extremely rare. Also, only a small number of patients with meningococcal infections are deficient in complement. Antibody-mediated classical pathway activation

remains the most important criterion for protection against meningococci. In infants up to the age of 3 to 6 months, protective immunity against invading pathogens is mediated by maternal antibodies. Susceptibility to meningococcal infections increases around 6-12 months when maternal antibodies wane. Susceptibility to meningococcal infection decreases with progression from early childhood to adulthood possibly because of intermittent carriage with *N. meningitidis* itself or increased colonization with non-pathogenic strains such as *N. lactamica* that generates cross-reactive antibodies against pathogenic Neisseriae strains (41).

A detailed understanding of the interaction between the complement system and meningococci is required to understand the reasons for increased susceptibility to meningococcal infections and to design effective vaccines against the pathogen.

1.10.6 Evasion of complement pathway by Neisseriae: The pathogenic Neisseriae, *N. meningitidis* and *N. gonorrhoeae*, have evolved several mechanisms to evade the detrimental effects of complement to these organisms. These pathogens target all arms of the complement cascade utilizing several and often redundant mechanisms. The mechanisms used by *N. meningitidis* to subvert the human complement system are discussed below.

A. Capsular polysaccharide-mediated serum resistance: With rare exceptions, all invasive *N. meningitidis* isolates possesses a polysaccharide capsule. Encapsulated group B and group C meningococci are more resistant to killing by normal human serum (serum resistance) than

unencapsulated isogenic mutants (136-138). Earlier work hypothesized that that sialic acid residues of the serogroup B capsule recruited factor H, which regulated the alternative pathway (136). However, studies performed with *E. coli* encapsulated with colominic acid (similar in structure to serogroup B capsule) (139, 140) and subsequently with serogroup B *N. meningitidis* directly (78, 79) did not invoke a role for factor H binding to capsule. Unpublished work in our laboratory indicates that group B and group C capsular polysaccharides regulate the alternative pathway by a mechanism independent of factor H binding. The molecular basis of serum resistance mediated by the other capsular groups (A, W-135 and Y) remains an area of investigation in our laboratory.

B. Lipooligosaccharide modification and serum resistance: *N. gonorrhoeae* sialylates LOS (in vitro) by using 5'-cytidinemonophospho-N-acetylneuraminic acid (CMP-NANA) as a donor molecule, which leads to serum resistance (141-143). Sialylation of gonococcal LOS enhances factor H binding, which results in downregulation of the alternative pathway and serum resistance. Enhanced factor H binding to sialylated gonococci occurs through binding to porin rather than to sialylated LOS (144). LOS sialylation enhances factor H binding to meningococci in vitro in select strains only and the development of serum resistance is less prominent than with gonococci. The role of LOS sialylation in serum resistance is as not well defined in *N. meningitidis* as it is in *N. gonorrhoeae*. Wild type and sialyltransferase deficient (*lst*) mutants (lacking LOS sialylation) of *N. meningitidis* are more resistant to normal human serum killing but are not different in causing disseminated disease in infant rats (137). Nevertheless, most meningococcal

isolates that are invasive in humans express sialylated LOS species, suggesting that this LOS structure may confer a survival advantage to this bacterium in vivo

Upon activation, C4 and C3 bind to their targets through covalent ester or amide bonds. In *N. gonorrhoeae*, C3b and C4b bind to LOS glycose residues, gonococcal PorB1.1B, Opa proteins (145) and with certain phosphoethanolamine residues on Hep II of LOS (unpublished oservations). Edwards and Apicella have shown that C3b also interacts with gonococcal lipid A (146). In *N. meningitidis,* C4b preferentially forms amide linkages with the 6-phosphoethanolamine residue (6-PEA) residues on Hep II of LOS preferentially over 3-PEA residues on Hep II (147); reduction in C4b deposition on 3-PEA residues may explain why most clinical isolates of *N. meningitidis* express 3-, but not 6-PEA residues on HepII of LOS (148).

C. Binding to host complement inhibitors: Binding to host complement inhibitory molecules such as C4bp and factor H constitutes an important mechanism of complement evasion by many microbes. C4bp binds to meningococcal PorA and acts as a cofactor for factor I mediated cleavage and inactivation of C4b. PorA positive strains are more serum resistant than PorA negative strains (149). Because the binding of C4bp to meningococci and enhanced serum resistance mediated by PorA are most evident under hypotonic conditions and in absence of the capsule, the true physiological significance of this interaction in vivo remains to be determined.

Factor H, a key inhibitor of the alternative pathway of complement, binds to a 29 kDa meningococcal outer membrane protein designated as

factor H binding protein (fHbp) (78, 79), previously called either Genome-derived Neisserial Antigen (GNA) 1870 (75) or LP2086 (150). The amount of factor H binding is proportional to the level of fHbp expression. Loss of fHbp expression increases the sensitivity of meningococci to complement-dependent killing. Antibodies against fHbp are bactericidal and fHbp is an important component of two group B meningococcal protein vaccines undergoing clinical trials (151, 152).

D. Blocking Antibodies-possible role in Complement pathway regulation: As their name suggests, these antibodies bind to pathogens and prevent complement mediated lysis by antibodies that are otherwise bactericidal. Blocking antibodies have also been shown to play a role in complement evasion by other pathogens including *Brucella abortus* (153), *B. melitensis* (154) and *Pseudomonas aeruginosa* (155, 156) and pathogenic Neisserial species e.g. *N. gonorrhoeae* (157, 158). Gonococcal strains isolated from patients with disseminated gonococcal disease (DGI strains) are resistant to killing by normal human serum (serum-resistance) (157). Nonbactericidal human serum and more specifically IgG from nonbactericidal human serum blocks killing of (human) serum-resistant gonococcal strains by bactericidal rabbit sera (162). Specificity of blocking IgG is directed against gonococcal reduction modifiable protein (Rmp) (158). Human blocking antibody (both whole IgG and $F(ab)_2$ fragments) directed against Rmp also block killing of gonococci by human killing antibodies that are sometimes elicited by patients with DGI (159). While the mechanism of blocking has not been fully elucidated, Joiner *et al*, suggested that blocking antibodies against Rmp in *N. gonorrhoeae* may divert C3 to non-bactericidal targets (159).

The role of Rmp (called Class 4 protein in *N. meningitidis*) as a blocking target in *N. meningitidis* is also not clear (160, 161). While Munkley et al, have shown that a murine monoclonal antibody against Class 4 protein could block killing by a bactericidal monoclonal antibody or bactericidal normal human serum (160), another group using the same monoclonal antibody observed no blocking (161).

Neisser and Wechsberg gave the first account of blocking activity against gram-negative microorganims including Neisseriae in 1901 (196). Further evidence of blocking activity directed against *N. meningitidis* was later provided by Lewis Thomas in 1943 (162) who reported that serum obtained from rabbits following challenge with high doses of bacteria were less bactericidal than preinfection serum. Furthermore, rabbits re-challenged with live *N. meningitidis* following survival of previous meningococcal infection were less likely to survive than normal (control) rabbits. These findings suggested that blocking activity may be an important risk factor in surviving meningococcal infection, although the study did not address whether blocking activity was a risk factor for acquiring infection and developing disease. A study by Jarvis GA and Griffiss JM, showed that IgA purified from convalescent sera from patients with serogroup C meningococcal disease and from a recipient of a tetravalent (A, C, W-135 and Y) polysaccharide vaccine blocked the bactericidal action of IgG purified from sera of patients convalescing from serogroup C meningococcal disease (163). This IgA1 was shown to block the bactericidal activity of serum IgG and IgM against serogroup C meningococci distinctly because of relative differences in the affinities of IgG and IgM antibodies for their capsular polysaccharide targets

(164, 165). Inhibition of IgG mediated lysis by IgA1 was attributed to competition for antibody-binding sites; IgA1 and IgG had similar affinities for their targets. However, IgA1 blocked of lysis by IgM in a non-competive manner because of the greater affinity of IgA1 for their targets than IgM. Another study done by Selander et al, showed that IgG purified from the serum of a healthy sister of a patient (both C2 deficient) suffering from recurrent meningococcal infection could block killing of Serogroup W-135 meningococci by their C2 deficient serum. The blocking IgG was serogroup W-135 specific and reduced C3 deposition on ELISA plates coated with serogroup W-135 capsular polysaccharide.

Both these studies identified blocking anti-meningococcal antibodies in convalescent or post-vaccinee human sera (163, 166). So far there is no evidence of such complement blocking antibodies in normal human serum. Also, the mechanism(s) of blocking has not been fully characterized.

Thesis Objective

The objective of this thesis is to determine whether blocking antibodies can account for differences in killing of serogroup B *N. meningitides* among sera from normal individuals Having defined the antigenic targets for these naturally occurring blocking antibodies, I also characterized the molecular basis for blocking.

Chapter II

Identification of novel blocking human IgG directed against the pentapeptide repeat motifs of *Neisseria meningitidis* Lip/H.8 and Laz lipoproteins

Abstract

Classical pathway activation initiated by natural antibodies that results in bactericidal activity is important for acquired immunity against invasive meningococcal disease. Sera from non-immunized individuals vary in their bactericidal activity directed against meningococci; in this study, 6/19 sera tested lacked bactericidal activity (defined as less than 50% killing of 2.5 $X10^3$ colony forming units [cfus]/ml. of a test strain, in vitro); the remaining 13 sera were bactericidal. Despite possessing high levels of meningococcal-specific IgG antibodies, 2 of the 4 sera with the lowest bactericidal activity blocked killing of meningococci by 2 of the 4 sera with the highest activity. Blocking was mediated by IgG directed against lipoprotein H.8 that is composed almost entirely of pentapeptide AAEAP repeats. Microbial expression of H.8 and another distinct meningococcal lipoprotein, called Laz, which contains (imperfect) AAEAP repeats, were both required for maximal display of blocking. The name H.8 was originally assigned to a mAb that identified a common surface protein antigen on *N. gonorrhoeae*; the name H.8 was based on the mAb's location in a 96 well plate (167). The bactericidal activity of a mAb against factor H-binding protein (fHbp; a meningococcal protein vaccine candidate), but not an anti-PorA mAb, was reduced by human serum that contained anti-H.8 IgG. Diverting anti-H.8 human IgG away from the bacterial surface using synthetic peptides corresponding to H.8, recombinant soluble Laz or a non-blocking/non-bactericidal murine mAb against H.8, each restored bactericidal activity of the anti-fHbp mAb. Meningococci use blocking antibodies present in normal human serum to subvert naturally acquired

protective immunity. These findings have important implications for the development of meningococcal vaccines, which ideally should overcome natural blocking antibody activity and do not themselves stimulate the development of blocking antibodies.

2.1 Introduction

N. meningitidis is one of the leading causes of sepsis and bacterial meningitis worldwide (15, 168, 169), however, it most often resides as a commensal in the human upper respiratory tract and does not elicit symptoms. The incidence of invasive meningococcal disease (about 1 in 100,000 in non-epidemic settings) is rare relative to the rates of colonization (population prevalence of ~10%) (90, 97, 170). During epidemics and in closed populations such as military recruits, the rates of colonization can exceed 50% (171). The rates of invasive disease during epidemics in sub-Saharan Africa can approach 1% (172). The incidence of meningococcal carriage is higher in infants >3 years old and in persons over 30 (173).

Colonization with meningococci can be an 'immunizing' process (100). The studies of Goldschneider and colleagues showed that individuals who possessed a serum bactericidal titer of 1:4 or greater using human complement in the reaction mixtures were likely to be protected against invasive disease by the test strain. Individuals who lacked protective antibodies were at higher risk of invasive meningococcal disease (80). Bactericidal antibodies elicited against epitopes shared by heterologous strains may contribute to cross-protection.

I sought to determine the reason for the variation among individuals in serum bactericidal activity (SBA) against serogroup B meningococci. Lack of antibodies against a test strain would seem the most likely explanation; alternatively select bacterial antigens or epitopes may preferentially elicit antibody responses that are effete or nonbactericidal. Another possibility is that a subset of nonbactericidal antibodies may interfere with killing by

bactericidal antibodies. Such antibodies are known as 'blocking' (or subversive) antibodies and have been identified previously in individuals recovering from meningococcal disease (163, 165). The identification of subversive antibodies that prevent killing by bactericidal antibodies is important because it could represent a strategy that meningococci use to evade host immune defenses. Individuals who possess high titers of blocking antibodies may be at greater risk for developing invasive meningococcal disease. In addition, blocking antibodies may also undermine the effectiveness of meningococcal vaccines.

2.2 Results

Sera from individuals vary in their ability to mediate complement-dependent killing against serogroup B *N. meningitidis.*

Sera obtained from 19 normal individuals were screened for killing of serogroup B *N. meningitidis* strain H44/76 (Figure 2.1). The final concentration of serum in each bactericidal reaction mixture was 20%. Sera that resulted in ≤50% survival were designated as "killing" (K) sera, while those that resulted in >50% survival of bacteria were designated "non-killing" (NK) sera. Heat inactivated 'K' sera did not reduce colony counts at t_{30}. As expected, individual sera varied widely in their ability to kill meningococci; < 50% of bacteria survived (>50% killing) in 13 of the 19 (68%) individual sera (labeled as K serum, K1 through K13 in Figure 2.1). > 50% of bacteria survived (< 50% killing) when tested against H44/76 in the remaining 6 sera (labeled as NK serum, NK1 through NK6 in Figure 2.1).

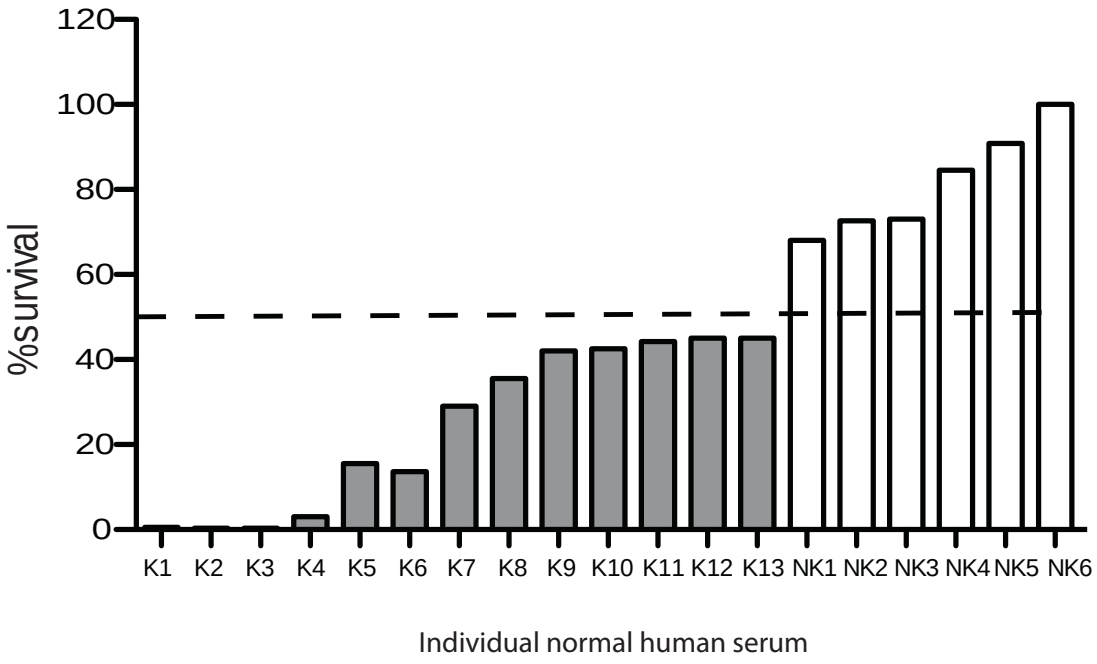


Figure 2.1. Bactericidal activity of individual human sera against strain H44/76. Sera obtained from 19 normal individuals were tested for complement dependent bactericidal activity against wild-type *N. meningitidis* serogroup B strain H44/76. The final concentration of each serum in the bactericidal reaction mixture was 20%. Sera that killed ≥ 50% (% survival ≤ 50%) of bacteria were designated killing (K) (grey bars) and those that killed <50% (% survival > 50%) as non-killing (NK) sera (blank boxes). All sera displayed normal total hemolytic (CH50) activity.

To examine if lack of anti-meningococcal antibody explained the deficiency in bactericidal activity, four of the sera that displayed the highest bactericidal activity (K1 through K4) and four sera with the lowest bactericidal activity (NK3 through NK6) were selected to examine binding of IgG and IgM to strain H44/76 by flow cytometry. There was no correlation between the amount of IgG or IgM that bound to bacteria (Figure 2.2) and the amount of killing mediated by the sera (Figure 2.1). These data suggested that differences in bactericidal activity were likely the result of qualitative differences in the antibodies present in the killing and non-killing sera. We hypothesized that antibodies present in NK sera were directed against non-bactericidal targets on meningococci. In some instances, antibodies directed against non-bactericidal epitopes could have possessed blocking activity (i.e., antibodies that subvert bactericidal antibody activity (163, 165, 174); I addressed this possibility next.

Select non-killing sera block killing by bactericidal sera

The four sera that possessed the least amount of bactericidal activity (NK3, NK4, NK5 and NK6) were screened for their ability to block killing of H44/76 by killing sera K1, K2, K3 and K4 (containing the highest bactericidal activity). In this experiment, each of the heat-inactivated (heat treatment destroys complement activity but leaves Ab intact) NK sera were incubated with H44/76 (20% NK serum in the final reaction mixture) followed by the addition (separately) of each of the thawed sera that had originally been freshly frozen--killing sera K1 through K4 (10% K serum in the final reaction mixture) that contained active complement. Blocking was defined as ≥ 50%

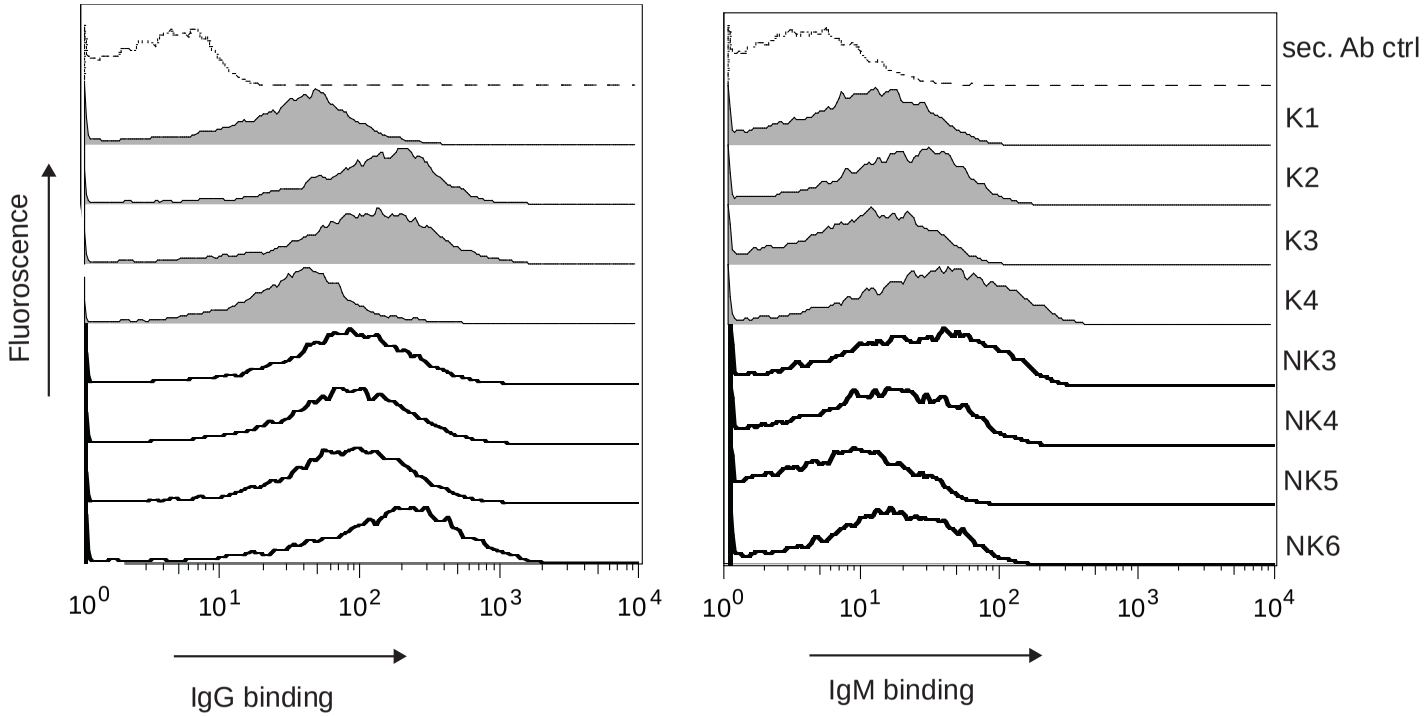


Figure 2.2. Binding of IgG and IgM binding to strain H44/76 in select killing (K) and non-killing (NK) sera. Serogroup B strain H44/76 was incubated with each of four sera that had displayed maximal bactericidal activity (K1 through K4; Figure 1; grey plots) or each of four sera with the least bactericidal activity (NK3 through NK6; white plots). The amount of IgG and IgM binding was quantified by flow cytometry. The x-axis represents fluorescence on a log10 scale and the y-axis the number of events. The secondary antibody control (sec. Ab ctrl; dashed plot) reaction did not contain serum.

reduction in killing of bacteria in the presence of K serum mixed with the heat inactivated NK serum compared to killing mediated by the same killing serum alone. Of the 16 reaction mixtures that contained a combination of active K and heat-inactivated NK serum, blocking was seen in 3 instances (Figure 2.3 A; dark grey shaded boxes). NK3 blocked killing that was mediated by K1 and K2. NK4 blocked killing of K1 only. Four combinations: NK4 plus K2, NK5 plus K1, NK5 plus K2 and NK3 plus K3 yielded 25% to 50% blocking compared to baseline bactericidal activity in killing serum alone (light grey shaded boxes in Figure 2.3 A). Killing of H44/76 by sera K3 and K4 were not blocked by any of the NK sera used. Thus, select NK sera possessed blocking activity and conversely, the bactericidal activity of certain K sera could be blocked by only select NK sera.

Killing of wild-type strain H44/76 by K1 (containing endogenous active complement) was blocked incrementally (dose responsive) by increasing amounts of heat inactivated NK3 added to the reaction mixtures (Fig, 2.3 B). In the converse experiment, to examine if antibody in killing serum could overcome blocking, heat inactivated K1 (final concentration of 20%) was incubated with the organisms, followed by addition of 10% NK3 (complement active). The survival seen with heat inactivated K1 was not statistically significant when compared with survival in NK3 alone (Figure 2.3 B). These data provided evidence that a heat-stable component in NK3, likely Ab, could prevent killing by K1 and that in the “competition” between killing and blocking in human serum, blocking was favored at the antibody concentrations tested.

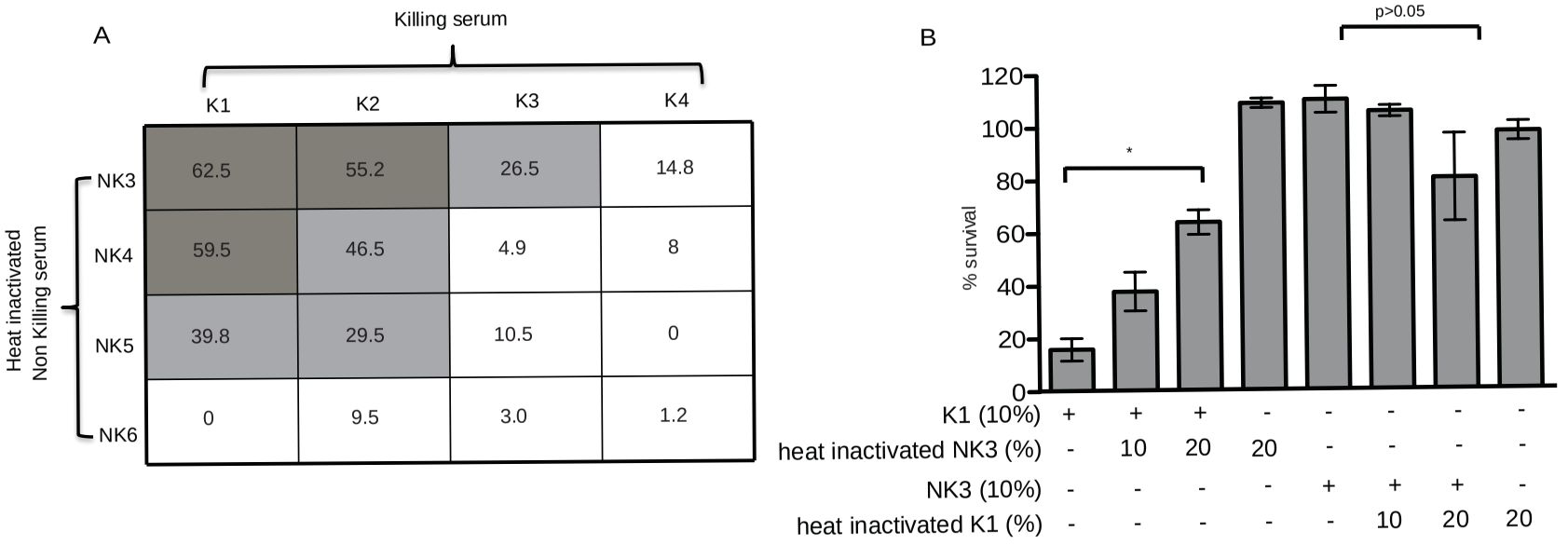


Figure 2.3. Select non-killing (NK) sera possess blocking activity and bactericidal activity of certain sera can be blocked. A) Four non-killing (NK) sera were each tested for blocking. The final concentration of heat-inactivated NK sera in the reaction mixture was 20% (v/v); concentration of K sera (complement active) was 10%. Blocking (≥ 50% decrease in killing compared to killing serum alone using formula: [%killing (K1)-%killing (K1+heat inactive NK3)]/ %killing (K1)] x100) is indicated by dark gray boxes. Combination of sera that yielded 25-50% decrease in killing compared to baseline are indicated by the light grey shaded boxes. B) Blocking of bacterial killing is dose responsive. Wild-type serogroup B strain H44/76 was incubated with 10% K1 serum in the presence (or absence) of 10% and 20% heat inactivated NK3 serum. In the converse experiment, 10% NK3 serum (complement active) was incubated with 10% and 20% heat-inactivated K1 serum. The y-axis indicates percent survival and each column represents the mean (±SEM) of 3 separate experiments.

IgG is responsible for blocking

Prior studies have shown that immunoglobulin (Ig) in serum is responsible for blocking killing of Neisseriae (158, 163, 165, 174) The experiments above indicated that blocking was mediated by a heat-stable molecule in serum. We hypothesized that Ig in NK3 mediated blocking. IgG and IgM from NK3 were purified and each was tested for its ability to block killing of H44/76 by K1. IgG purified from NK3 (NK3 IgG) blocked killing of H44/76 by K1 in a dose-responsive manner (Figure 2.4 A; 2 mg/ml of NK3 IgG in the reaction mixture corresponds to the amount of IgG contained in 20% (v/v) serum). The converse experiment, using IgG depleted NK3 serum, showed that depleted serum was no longer effective as a blocking agent (Figure 2.4 B). Only a small increase in survival (~15%) of bacteria was seen when 0.2 mg/ml of NK3 IgM (corresponding to the amount of IgM in a reaction mixture that contains ~20% (v/v) serum) was added to K1 (10%) serum in the bactericidal reaction mixture (Figure 2.4 C; 5th column). These experiments showed that IgG in NK3 was largely responsible for blocking killing by K1.

The bactericidal activity of mAbs directed against selected meningococcal vaccine candidates is attenuated by blocking IgG

Serogroup B meningococcal capsule is not immunogenic; therefore, several outer membrane proteins are being investigated for their vaccine potential (175). *N. meningitidis* outer membrane protein fHbp is one of the proteins being investigated as a potential vaccine candidate against group B disease (75, 78, 176). According to one classification scheme, fHbp is divided into 3 variant families (75). Strain H44/76 expresses variant 1 fHbp; an anti-

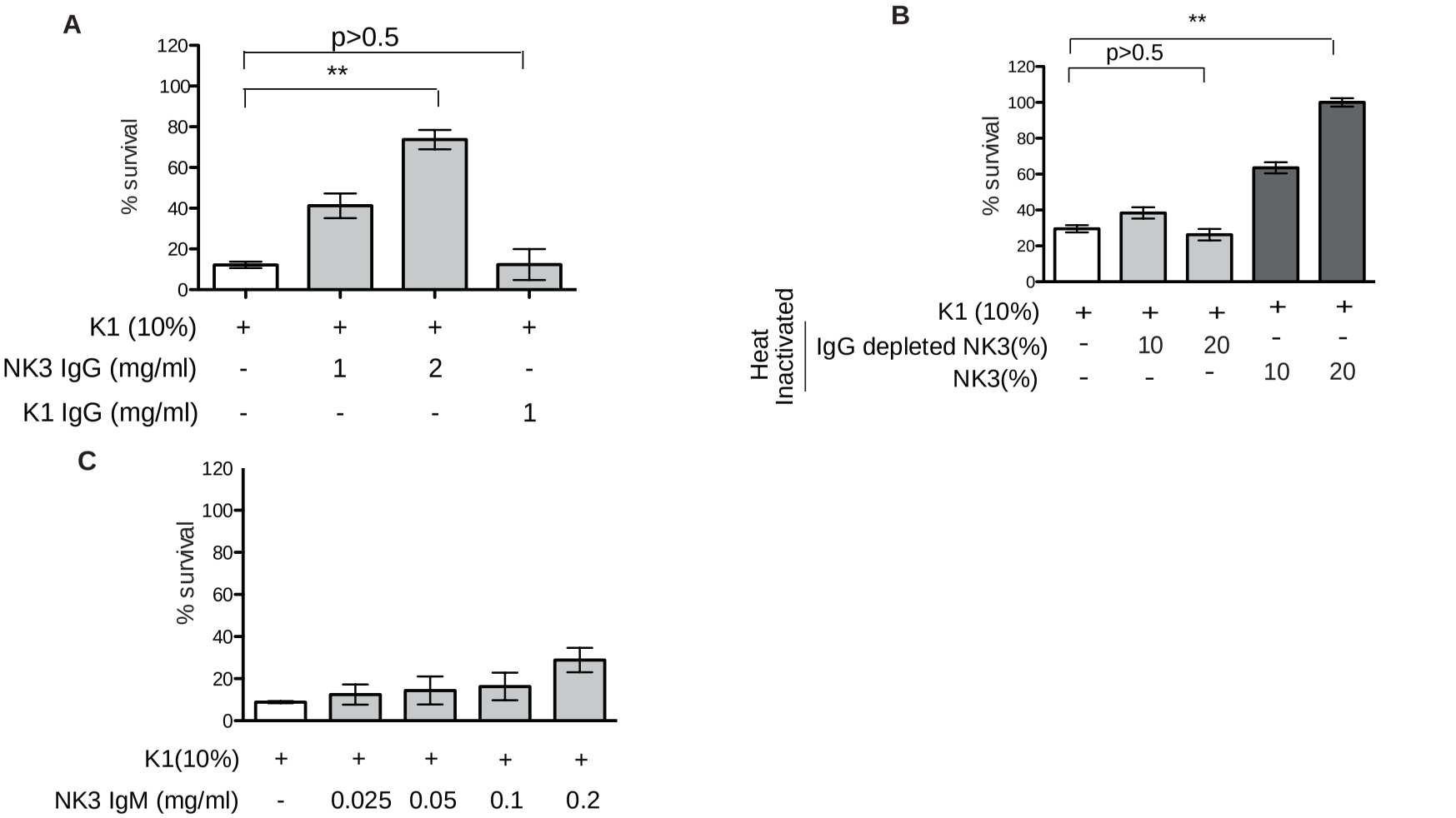


Figure 2.4. IgG in NK3 mediates blocking. A) IgG purified from NK3 blocks killing by K1 serum. NK3 IgG was added incrementally (final concentration, 1 and 2 mg/ml) to a bactericidal reaction mixture containing strain H44/76 and serum K1 (10% (v/v)). K1 IgG served as a control. B) Depletion of IgG from NK3 abrogates blocking activity. Heat inactivated IgG-depleted NK3 was added to the bactericidal reaction mixture described in A. The control used heat inactivated NK3 serum in the bactericidal reaction mixture. C) IgM purified from NK3 does not block. Incremental doses of NK3 IgM failed to block killing by K1. In all experiments (A, B and C) the y-axis indicates percent survival and each column represents the mean (±SEM) of 3 separate experiments

variant 1 fHbp mAb called JAR 3 is bactericidal against H44/76 (177). Another important component of outer membrane vesicle vaccines is PorA that evokes a bactericidal antibody response (106, 178). mAbs against PorA also form the basis for serosubtyping of meningococci; one of these, mAb P1.7, recognizes PorA of strain H44/76 and is also bactericidal (179).

We examined blocking activity of NK3 IgG upon JAR 3 mediated killing of H44/76. Bacterial survival in the presence of JAR 3 at concentrations of 0.25 and 0.5 μg/ml was higher in reaction mixtures that contained NK3 IgG than in reactions containing IgG-depleted NK3 (Figure 2.5 A). Increasing the concentrations of JAR 3 overcame blocking by NK3 and resulted in progressively increased bacterial killing; the presence of NK3 IgG did not increase bacterial survival at JAR 3 concentrations of 1.0 and 2.0 μg/ml compared to the corresponding reactions that lacked NK3 IgG. The shift of the survival curve to the left when IgG was depleted from NK3 provided evidence that IgG in NK3 blocked the bactericidal activity of JAR 3.

In contrast, depleting IgG from NK3 had no effect on the bactericidal activity of anti-PorA mAb P1.7 (Figure 2.5 B). At any of the dilutions of P1.7 used, there was no significant difference in the survival rates due to presence or absence of the NK3 IgG in the reaction. These data indicate that blocking IgG in NK3 selectively blocks the function of some, but not all, bactericidal antibodies.

Identification of lipoprotein H.8 as a target for IgG in blocking serum

In order to gain a better understanding of why some sera possessed blocking activity and why the bactericidal activity of only certain killing sera or

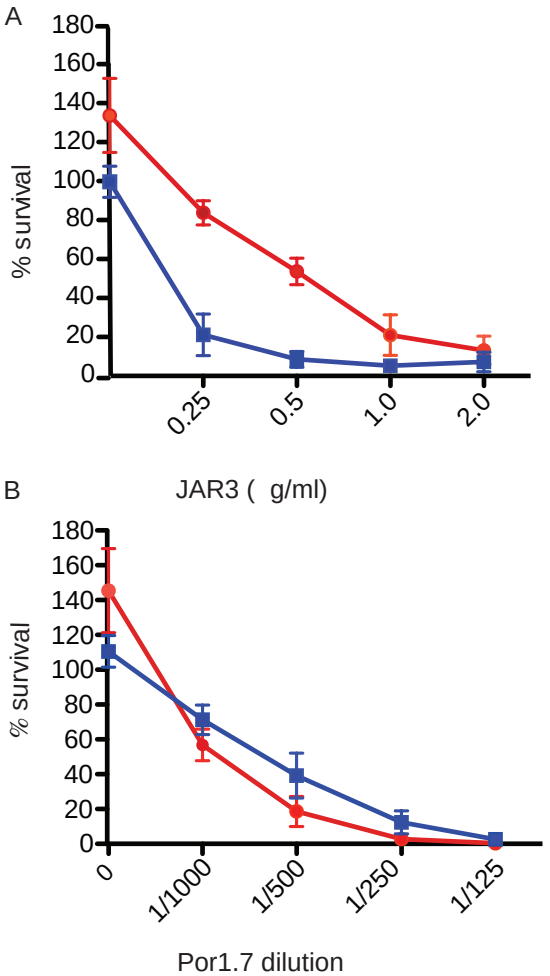


Figure 2.5. Blocking potential depends on the specificity of killing antibody. A) Bactericidal activity of JAR3 (anti-fHbp mAb), but not P1.7 (anti-PorA mAb) is attenuated by IgG in NK3. *N. meningitidis* strain H44/76 was incubated with increasing concentrations (0.25, 0.5, 1, and 2 mg/ml) of JAR 3 in a reaction mixture that contained either 20% (v/v) of blocking serum NK3 (Ab and complement intact; red line and solid circle) or 40% (v/v) IgG-depleted NK3 (blue line and solid box). The higher concentration of IgG-depleted NK3 was used to normalize for the amount of hemolytic complement activity in the bactericidal reaction mixture (the process of IgG depletion resulted in loss of ~50% of hemolytic activity). B) IgG depletion of NK3 had no effect on the bactericidal action of P1.7 (anti-PorA mAb). Serial dilutions (1/1000, 1/500, 1/250 and 1/125) of ascitic fluid containing murine mAb P1.7 were each incubated with strain H44/76 and the bactericidal assay performed as described above in A. In all experiments (A and B) the y-axis represents percent survival and each data point represents the mean (±SEM) of 3 separate experiments.

mAbs could be blocked, I examined the specificity of IgG binding to meningococcal proteins by western blot using the following representative sera; i) blocking serum NK3 ii) non-killing and non-blocking serum NK6, iii) killing serum K1 that could be blocked and iv) killing serum K3 that was not blocked (Figure 2.6). To determine the target(s) of blocking IgG, whole bacterial lysates of H44/76 were separated on a 4-12% Bis-Tris gel and transferred to a PVDF membrane for western blot analysis.

Whole bacterial lysates of H44/76 probed with NK3 showed a prominent IgG-reactive band at ~21 kDa (marked with a black asterisk in the 2nd of 3 lanes in the NK3 grouping on the left in Figure 2.6) that was not visible in the NK6 or K1 groupings and was faint in K3. This band did not correspond to the location of other known outer membrane proteins such as Opa, Opc or Class 4 protein; the latter is the homologue of Rmp in *N. gonorrhoeae*, which is the target of blocking antibody in that organism (158). An important Neisserial outer membrane antigen that migrates at ~21 kD and does not stain with coomassie blue is the lipoprotein H.8 (180-182), also called Lip.

In preliminary experiments, an anti-H.8 mAb had also shown reactivity at the ~21 kD band location (Figure 2.6; lanes marked “H.8/Lip knockout” in blots probed with NK3 and K3). The identity of this band was confirmed as Lip/H.8 by loss of reactivity upon deleting *lip* (Figure 2.6; lanes marked “H.8/Lip knockout” in blots probed with NK3 and K3). The IgG binding pattern of blocking serum NK4 was similar to that of blocking serum NK3. Blots done using NK4 also shows a H.8 specific band like NK3 (Figure 2.6; last blot labeled NK4).

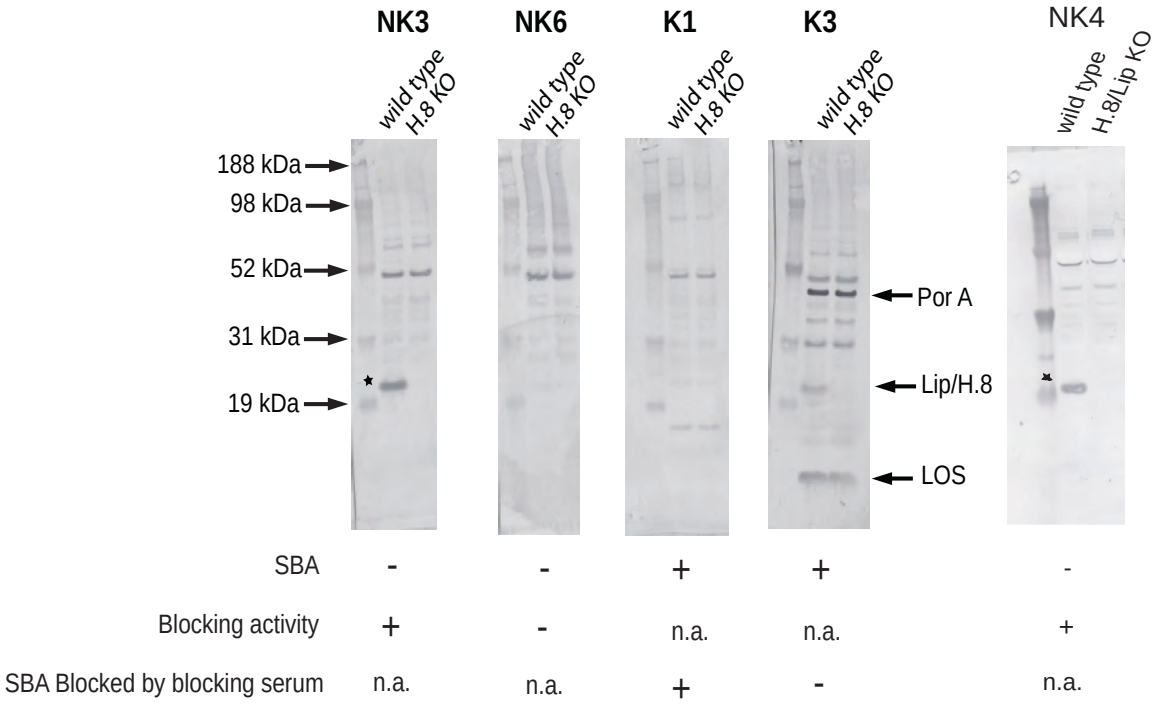


Figure 2.6. H.8 is a meningococcal target for IgG in blocking NK3 serum. Bacterial lysates of strain MC58 and its isogenic lipoprotein H.8 knockout (KO) mutant were electrophoresed on a 12% Bis-Tris gel followed by western blotting. Parallel blots were incubated with a 1:100 dilution of NK3 (extreme left) and NK4 serum (extreme right), (Both nonbactericidal and possessing blocking activity), NK6 (nonbactericidal and without blocking activity), K1 (bactericidal activity that could be blocked by NK3) and K3 (bactericidal activity that could not be blocked by NK3). Human IgG-reactive bands were disclosed with alkaline phosphatase conjugated anti-human IgG. The black asterisk (on the left) indicates the Lip/H.8-reactive band (indicated by a solid arrow on the right). The location of PorA and lipooligosaccharide (LOS) reactive bands are shown with arrows on the right. The identity of each serum in terms of its bactericidal activity (SBA) and blocking activity is indicated in the labels below the gel pictures. Sera that lack SBA or blocking activity are lablled as n.a. (not applicable) for that particular category.

Interestingly, the most obvious difference between K1 and NK3 was the ~21 kD Lip/H.8-reactive band (depicted by a black asterisk in Figure 2.6 [1st blot]), which suggested that IgG directed against this antigen may have contributed to the blocking activity of NK3. Serum K3, whose bactericidal activity was not blocked by NK3 (Figure 2.3 A), showed a strongly reactive band at ~45 kD that likely corresponds to PorA in this strain. In addition a band at ~5 kD (likely lipooligosaccharide; LOS) and other undefined fainter bands in the 30-40 kDa range were noted. The presence of PorA antibodies in K3 and the inability of NK3 to block its bactericidal activity are consistent with the data in Figure 2.5 B where the bactericidal activity of anti-PorA mAb was not affected by the presence of the IgG in NK3. Subsequent experiments were directed at defining the role and specificities of Lip/H.8 and a related protein called Laz that shares a region of sequence homology with Lip/H.8 as targets for blocking IgG.

Lip/H.8 and Laz are the targets of the human complement blocking antibody against *N. meningitidis*.

Lip/H.8 is composed entirely of 13-14 tandem repeats of pentameric sequences, most (9 to 10) of which are identical “AAEAP” sequences (181). The N-terminal ~40 amino acids of another Neisserial lipoprotein called Lipid-modified azurin (Laz gene of the following neisseriae strains: Z2491 (NC_003116.1; gene id 908122), MC58 (NC_003112; Gene id 904058), alpha 14 (NC_013016.1; gene id 8221802), FAM18 (NC_008767.1; gene id 4676614), FA 1090 (NC_002946.2; gene id 3281780) among others is misannotated and referred to as “H.8” in the sequenced meningococcal and

gonococcal genomes) form a domain composed of imperfect AAEAP repeats; the remainder of Laz bears similarity to the azurin protein found in organisms such as Pseudomonas, Alcaligenes and Bordetella (180, 183). A sequence alignment of these two AAEAP-motif bearing proteins is provided in Figure 2.7.

In order to determine the relative contribution of these two lipoproteins in blocking, we created Lip/H.8 and Laz knockouts in the background of serogroup B strain MC58 (Figure 2.8). The ability of heat-inactivated NK3 to block killing of the mutant strains by K1 was also examined. Loss of either Lip or Laz (Figure 2.9 A) restored killing by serum K1 even in the presence of heat-inactivated NK3. Thus, maximal blocking required expression of both the lipoproteins.

I next determined whether loss of Lip/H.8 or Laz expression would increase bactericidal activity of anti-fHbp mAb JAR3 in the presence of NK3. Akin to observations with strain H44/76 (Figure. 2.5 A), ~95% survival of wild-type strain MC58 was seen when JAR 3 was added to NK3. Deleting either H.8/Lip or Laz decreased survival by ~25% and ~50%, respectively (Figure, 2.9 B). To study the effects of loss of both Lip and Laz on blocking I created a ‘double’ H.8/Lip and Laz knockout strain but for reasons not fully understood this double mutant was highly resistant to complement-dependent killing; no killing was seen when this mutant was incubated with 2 µg/ml JAR 3 plus IgG-depleted NK3 (complement sufficient), or with serum K1, usually our most bactericidal serum. Taken together, these data show that expression of both H.8/Lip and Laz were required for maximal blocking by human IgG in NK3.

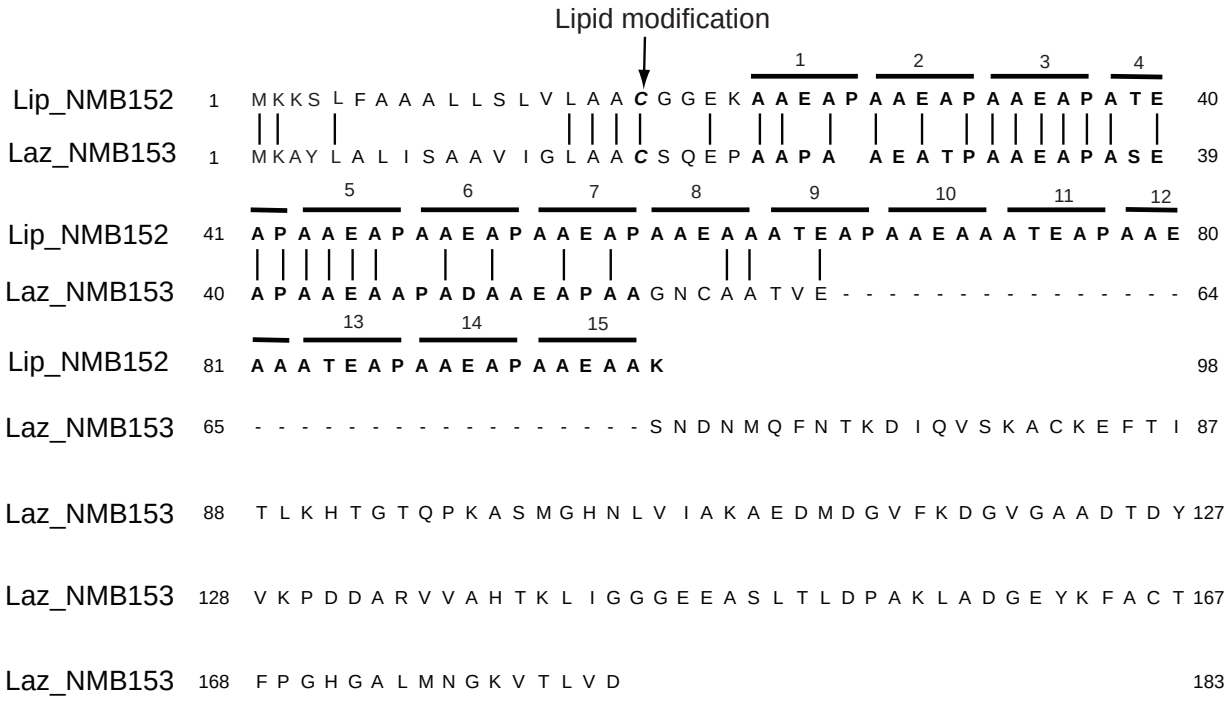


Figure 2.7. Sequence alignment of N. meningitidis Lip/H.8 and Laz. Alignment of amino acid sequences of Lip/H.8 and Laz of serogroup B N. meningitidis MC58. Lip/H.8 is a 98 amino acid protein composed almost entirely of the H.8 domain which has 12-15 conserved AAEAP pentameric repeats depending on the strain (15 in serogroup B N. meningitidis MC58). The AAEAP pentameric repeats are emboldened. Each pentameric repeat is marked with a bar overhead labeled with the repeat number. Laz is a 183 amino acid protein composed of 7 non-conserved, or imperfect, AAEAP pentameric repeats that comprises the H.8 domain at its N-terminus (the 7 imperfect repeats of Laz correspond in position to the first 7 conserved pentameric repeats of Lip/H8). The C-terminal portion of Laz (amino acid residues 57 to 183) is homologous to bacterial copper binding proteins called azurin. Both proteins are lipid modified at the cystine residue (marked by an italicized C at position18) following cleavage of the N-terminal 17 amino acid signal sequence. Conserved amino acids between the H.8 domain of both proteins are shown.

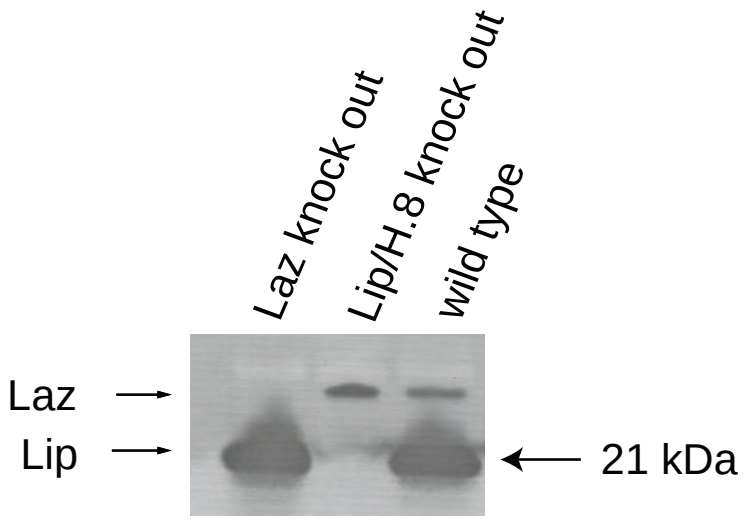


Figure 2.8. Western Blot analysis of strain wild type MC58 and its Lip/H.8 and Laz knock out isogenic mutants. Bacterial lysates of strain MC58 and its isogenic Lip/H.8 and Laz single knockout mutants (L to R) were electrophoresed on a 12% Bis-Tris gel followed by blotting and incubation with 2C3 (anti-H.8 mAb). The western blot was disclosed using alkaline phosphatase conjugated goat anti-mouse IgG. Bands specific for Lip/H.8 and Laz are indicated.

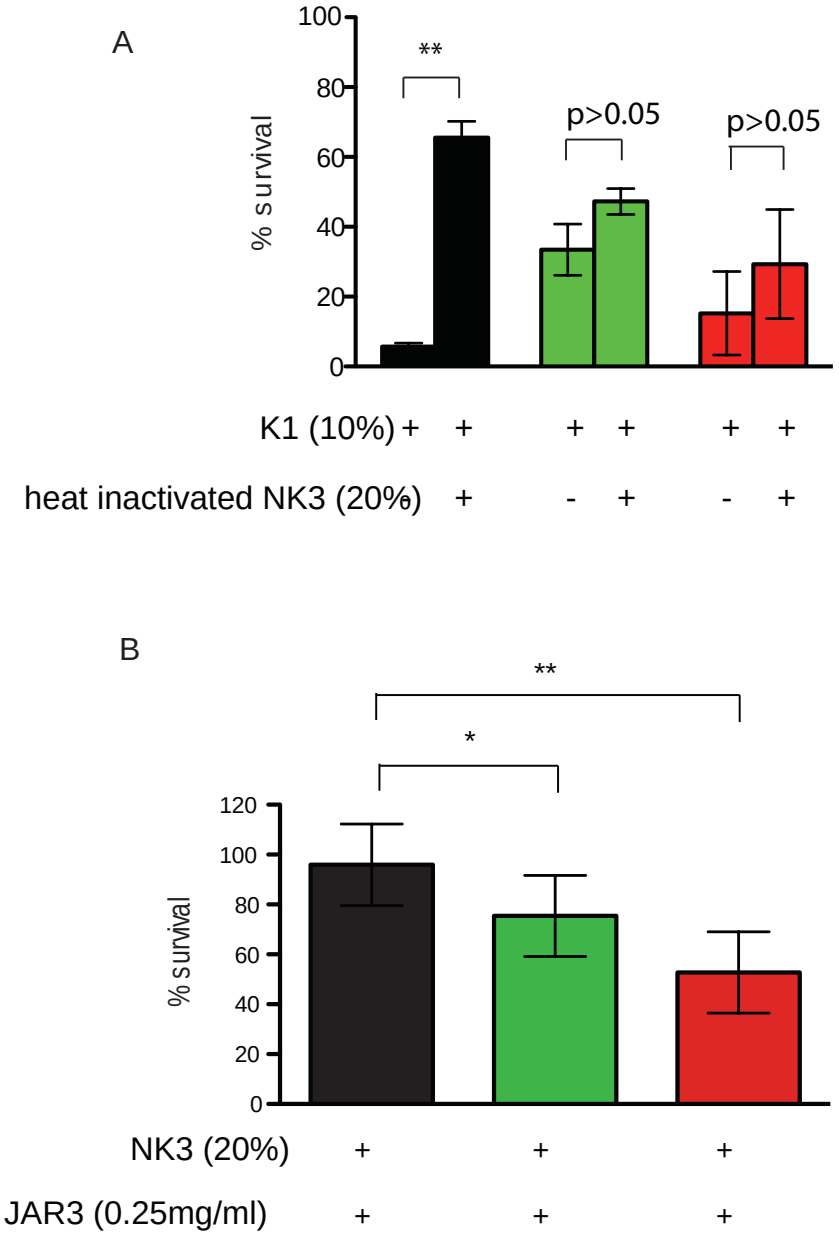


Figure 2.9. Expression of both Lip/H.8 and Laz are required for maximal blocking. A) Wild type strain MC58 (black bars) and its isogenic Lip/H.8 (green bars) and Laz deficient (red bars) mutants were incubated with 10% (v/v) killing serum K1 and 20% (v/v) heat inactivated blocking serum NK3. Percent survival is shown on the y-axis. Mean (±SEM) of 3 separate experiments is shown. B) Loss of Lip/H.8 or Laz expression increases killing by JAR 3 (anti-fHbp mAb) in the presence of NK3. Wild-type MC58 and its Lip/H.8 and Laz mutants were incubated with JAR 3 (0.25 µg/ml) and NK3 (20% (v/v)). In all experiments (A and B) percent survival is indicated on the y-axis and each column represents mean (±SEM) of 3 separate experiments.

Murine anti-H.8 mAb, 2C3 inhibits the blocking activity in human serum

mAb 2C3 binds to both Lip/H.8 and Laz (184). Unlike human IgG directed against H.8, the anti-H.8 mAb, 2C3, does not possess blocking activity (Figure 2.10 A).

I tested mAb 2C3 for its ability to inhibit binding of human blocking IgG that would result in restoration of killing (Figure 2.10 B). The ability of 2C3 to restore killing would provide evidence that the target for human blocking IgG against *N. meningitidis* is H.8. Strain H44/76 was incubated with 20% NK3, 0.25 µg/ml JAR 3 and increasing doses (2.5, 5 and 10 µg/ml) of mAb 2C3. The mAb 2C3 antibody restored killing by JAR3 in a dose-responsive manner similar to that produced by 0.25 µg/ml of JAR 3 plus antibody free human complement (~20% survival; see Figure 2.5 B). This restoration of killing is not due to a synergistic effect of 2C3 on the bactericidal activity of JAR3. $F(ab)_2$ fragments of mAb 2C3 restore killing by JAR3 in NK3 serum similar to whole 2C3 IgG (Figure 2.10 C). In addition to providing further proof that Lip/H.8 and Laz are targets for blocking IgG human serum, these data suggest that blocking function is unique to human IgG, but not murine mAb 2C3, despite recognition of both antibodies to similar or overlapping epitopes.

Killing of meningococci is restored by diverting blocking Ab from the bacterial surface with Lip/H.8 or Laz peptide fragments

I tested the ability of synthetic peptides that corresponded to the N- and C-termini of Lip/H.8 and contain perfect AAEAPs to inhibit or divert the binding (and therefore the function) of human blocking IgG. I used increasing concentrations of the two 30-mer synthetic peptides either individually or in an

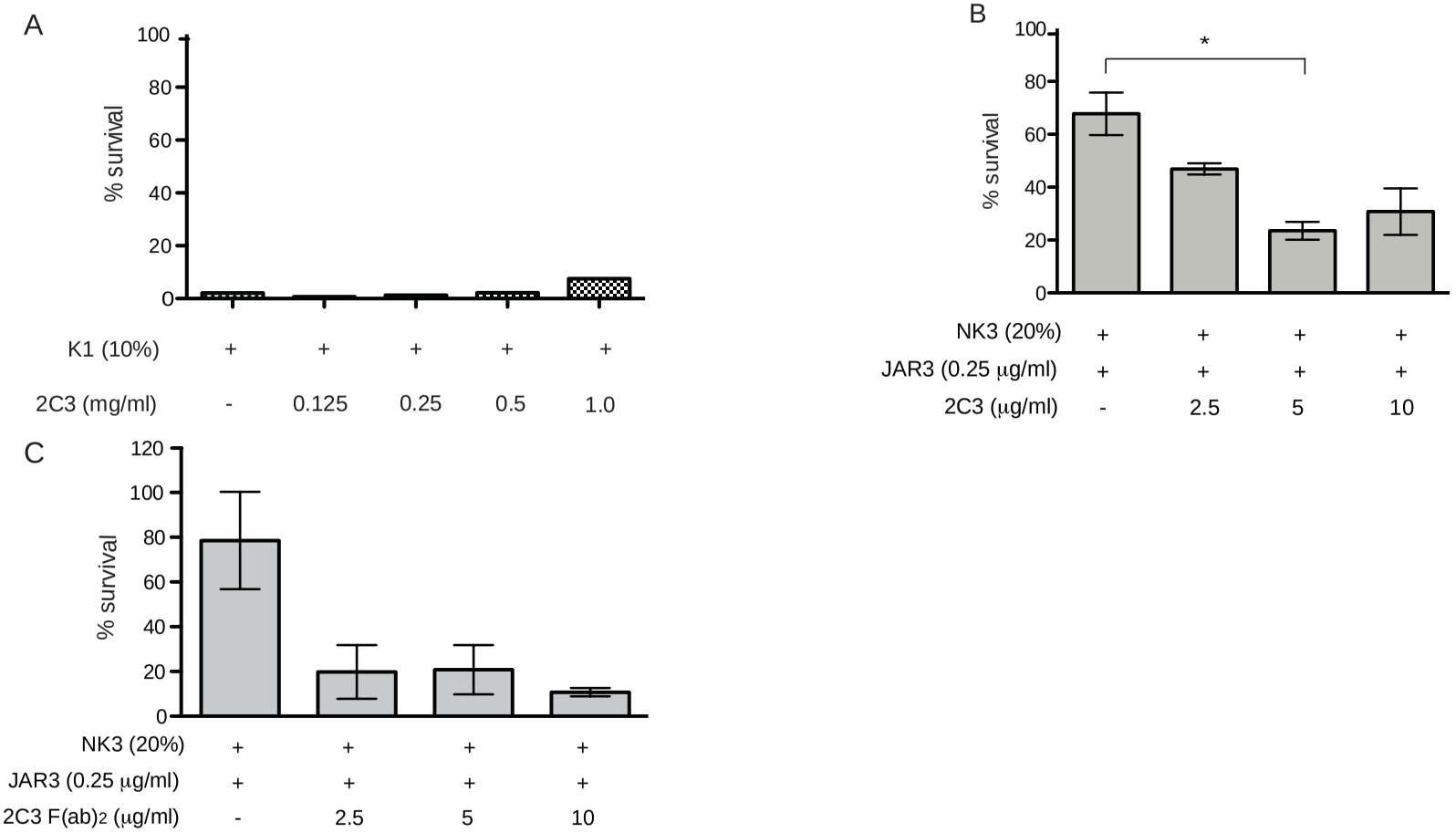


Figure 2.10. Murine anti-H.8 monoclonal antibody (2C3) does not block K1 bactericidal activity. Both whole IgG and F(ab)2 fragments of 2C3 competes with blocking human IgG (in NK3) and restores killing by anti-fHbp mAb. A) Monoclonal anti-H.8 IgG1 (2C3) was added incrementally (final concentration, 0.125, 0.25, 0.5 and 1 mg/ml) to a bactericidal reaction mixture containing strain H44/76 and serum K1 (10% (v/v)). The y-axis indicates percent survival and each column represents the mean of 2 separate experiments. B) Strain H44/76 was incubated with 0.25 µg/ml of JAR 3 (anti-fHbp mAb) in the presence of increasing doses (2.5, 5 and 10 mg/ml) of 2C3 (murine anti-H.8 mAb), followed by addition of 20% (v/v) of NK3 (complement active). Percent survival is indicated on the y-axis and each column represents mean (±SEM) of 3 separate experiments. C) Strain H44/76 was incubated with 0.25 µg/ml of JAR 3 in the presence of increasing doses (2.5, 5 and 10 mg/ml) of F(ab)2 fragments of 2C3, followed by addition of 20% (v/v) of NK3 (complement active). Each column represents mean of 2 separate experiments with range.

equimolar mixture. I added these to bactericidal reaction mixtures that contained NK3 (20%) and JAR 3 (0.25 µg/ml). Bacterial killing increased with the addition of increasing concentrations of the two peptides to the reaction both individually as well as in an equimolar mix (Figure 2.11 A). The peptide corresponding to the C-terminus of Lip/H.8 had a slightly greater effect on restoration of killing compared to the peptide corresponding to the N-terminus. Control reactions included JAR 3 plus IgG-depleted NK3 with or without the Lip/H.8 peptides.

I also tested the ability of recombinant Laz (rLaz; not lipidated, but containing the N-terminal imperfect AAEAP repeats) to inhibit blocking using the assay described above. Soluble rLaz also restored killing by JAR 3 in a dose-dependent manner (Figure 2.11 B). A His-tagged recombinant protein that contained only the Azurin-like domain and lacked the N-terminal AAEAP repeat did not restore killing by JAR 3 (Figure 2.11 C), thereby confirming blocking specificity to the N-terminal AAEAP domain of the protein Laz. Controls included the addition of recombinant proteins to reaction mixtures that contained JAR3 as the killing antibody and complement (IgG-depleted NK3); these had no effect on killing of the bacteria (Figure 2.11 A, B, C). Collectively, these data provide strong evidence that the H.8 pentapeptide motifs of Lip/H.8 and Laz contribute to bacterial survival by serving as targets for blocking antibodies in human serum.

2.3 Discussion

Serum bactericidal activity (SBA) is widely accepted as a surrogate of natural or vaccine induced protective immunity against *N. meningitidis* (80,

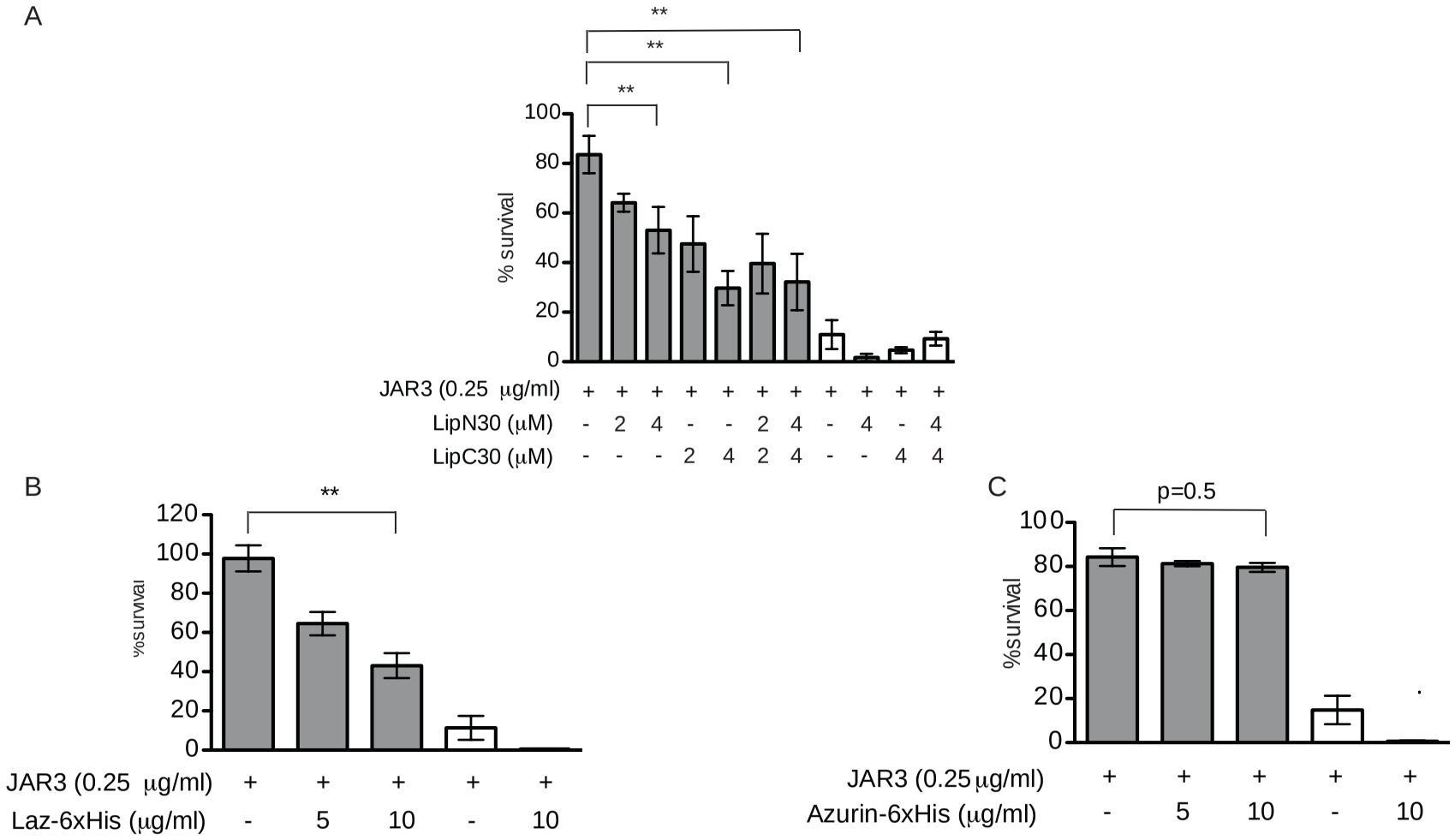


Figure 2.11. Synthetic peptides that correspond to Lip/H.8 or recombinant soluble Laz divert blocking human IgG (in NK3) and restore killing by anti-fHbp mAb. A) Strain H44/76 was incubated with increasing amounts (2 and 4μM) of synthetic 30-mer peptides corresponding to the N- and C-terminal regions of Lip/H.8, individually or as an equimolar mix, to divert blocking IgG (in NK3) from bacteria and restore killing by anti-fHbp mAb JAR3 (0.25 μg/ml). B) Recombinant His-tagged Laz (contains the N-terminal imperfect H.8-like repeats or C) recombinant His-tagged azurin (contains only the azurin domain of Laz), 5 and 10 μg/ml were used to divert blocking IgG and restore killing of JAR3. Grey bars and white bars represent samples using NK3 serum and 'IgG depleted NK3' serum as complement source. reThe y-axis represents survival and each column represents the mean (±SEM) of 3 separate experiments.

84). SBA titers against meningococci vary substantially among individuals. Most persons have been exposed to *N. meningitidis* and they usually mount an antibody response against the colonizing strain. The specificity of the antibody response elicited by colonization may be heterogeneous (106); the protective potential against a given strain depends on epitope specificity, and subclass distribution and titer, which are factors that combine to determine whether fixation of complement on the bacterial surface results in SBA. So far in this thesis, I have shown that antibodies in the sera from about 10% of individuals that I tested prevented serum bactericidal antibody function. Using several approaches, I have provided evidence that these antibodies are directed against the repeating pentapeptide 'AAEAP' sequences of H.8/Lip and the N-terminus of lipid modified azurin (Laz), which possesses 'imperfect' repeats; these antibodies block killing by select bactericidal antibodies.

H.8 was discovered when a series of monoclonal antibodies (mAbs) generated by immunizing Balb/c mice with outer membrane preparations of gonococcal strain FA1090 identified, by western blot, H.8 in all 48 gonococcal and 25 meningococcal strains (the latter represented serogroups A, B, C, X and Y). mAb to H.8 also bound to 4 of 5 *N. lactamica* strains but not to other commensal strains tested (*N. subflava, N. perflava, N. mucosa, N. flava, N. flavascens*). Further analysis of FA1090 outer membrane preparations using SDS PAGE and western blotting revealed that H.8 migrated at ~20 kDa in samples prepared at 37^{o}C; bands sizes of 30 kDa were also seen when samples were prepared at 100^{o}C. The H.8 antigen has unique properties. Because of its composition that lacks aromatic amino acids (181, 182, 185); H.8 cannot be stained by Coomassie blue or by silver and does not yield an

absorbance at 280 nm. Following the discovery of H.8 (167), the characterization of H.8 antigen was motivated by interest in identifying a surface exposed outer membrane protein antigen common to all disease causing Neisseriae spp. for potential use as a universal vaccine candidate (119, 181, 182, 185, 186). Antibody responses to H.8 were observed in subjects with disseminated gonococcal and meningococcal infections (119). However, a subsequent study refuted the possibility of H.8 mediating protection against Neisseriae (187).

Only pathogenic Neisseriae possess H.8/Lip (167); except for the commensal sp. *N. lactamica* which on occasion has been reported to cause disease (188, 189). H.8 has been found in all strains of pathogenic Neisseriae that have been examined for H.8/Lip (the *lip* gene has been misannotated in published meningococcal genomes), suggesting an important role for this molecule in pathogenesis. My data suggest that expression of this protein serves to elicit a separate antibody response that attenuates bactericidal antibody activity thereby providing a survival advantage to meningococci.

Azurin is involved in defense against oxidative stress and copper toxicity; Neisseria mutants that lack lipid modified azurin (Laz) are highly sensitive to hydrogen peroxide and copper (190). Azurin is also expressed by other bacteria such as *Pseudomonas aeruginosa* (183). However, the presence of the N-terminal “H.8-like” motif appears to be a feature unique to pathogenic Neisseriae. The peptapeptide repeats in Laz also contribute to blocking antibody targets; “replication” of an H.8-like motif in Laz could also serve to maximize the efficiency of blocking against Laz. Indeed, both Lip/H.8

and Laz expression are necessary to provide the maximal number of target sites for blocking antibody. It may be noteworthy that a double mutant in MC58 that lacked both lip/H.8 and Laz was more resistant to complement dependent killing. However, this may occur at the expense of the important functions of Laz as described above and might place the bacteria at a disadvantage in vivo.

Blocking antibodies in normal human serum prevented killing by an anti-fHbp mAb, but did not prevent killing by an anti-PorA mAb. As an example of supremacy of killing over blocking in a purely human system, serum specimen K3, contained both IgG anti-PorA and anti-H8 (Figure 2.6) and maintained killing even when additional H.8-specific human antibody (heat-inactivated NK3) was added (Figure 2.3 A). Although antibodies against other meningococcal antigens may also be bactericidal, nevertheless PorA is one of the most abundant protein targets for antibody in the meningococcal outer membrane. Antibodies directed against PorA are often present in normal sera that contain SBA (106) and this antigen elicits a significant proportion of SBA in humans vaccinated with outer membrane protein vaccines (191). Factor H binding protein (fHbp) is a relatively sparsely distributed 29 KDa outer membrane lipoprotein that is currently being investigated as a candidate for a group B meningococcal vaccine (75, 150). As its name suggests, this lipoprotein binds factor H, a major inhibitor of the alternative pathway of complement (78). Our findings may have important implications for vaccine development because bactericidal activity of antibodies directed against less abundant antigens may be blunted in persons with higher titers of anti-H.8 antibodies. Based on our experiments using

mAbs and human blocking IgG, killing was restored by increasing the concentration of anti-fHbp mAb in reaction mixtures.

Blocking antibodies directed against the pathogenic Neisseria vary in their target specificity. Antibodies against gonococcal reduction modifiable protein (Rmp) mediate blocking against *N. gonorrhoeae*. Rmp is an outer membrane structural protein of approximately 30 kDa whose function has not been fully elucidated (192). A monoclonal antibody against the class 4 protein of *N. meningitidis* (the Rmp counterpart in *N. meningitidis*) blocked the killing of meningococci by otherwise bactericidal antibody or serum in one study (160) but the same antibody showed little or no blocking activity against *N. meningitidis* in a separate study (161). Likewise, human anti-H.8 antibodies that block killing of *N. meningitidis* do not block killing of *N. gonorrhoeae* (my unpublished observations conducted with colleagues in the Rice/Ram laboratories). Anti-Rmp blocking antibodies activate complement and deposit C3 and C9 on the surface of *N. gonorrhoeae* (159). While not formally proven to occur as a result of blocking antibodies *per se*, serum-resistance of *N. gonorrhoeae* is associated with larger polymers or aggregates of C5b-9 (193) or as C5b-9 complexed to distinct bacterial outer membrane constituents (194). Qualitative differences in bactericidal versus nonbactericdial C5b-9 in *N. gonorrhoeae* was suggested by two-fold greater release of ^{125}I-C9 from C5b-9 inserted into serum-resistant strains (193). It has been hypothesized that anti-Rmp blocking antibodies divert C3 deposition from bactericidal sites on the bacteria to non-bactericidal sites (159). The effects of anti-H.8 antibodies on complement activation on meningococci and the molecular basis of blocking mediated by human anti-H.8 antibodies merit further study.

The phenomenon of blocking of bacterial killing was described as early as 1894 by Pfeiffer who noted that animals given excess immune serum may be more susceptible to challenge organisms (195). The first written account of blocking serum activity was provided by Neisser and Wechsberg, who showed blocking of normal fresh animal serum mediated killing of a range of gram negative micro-organisms by immune sera obtained from animals that were immunized with whole bacteria (196). Lewis Thomas *et al*. observed that antisera from rabbits immunized with larger doses of meningococci were less bactericidal than animals immunized with a lower dose. The bactericidal activity was restored upon diluting the antiserum obtained from the heavily immunized rabbits. When these sera were heat inactivated and mixed with normal rabbit serum, killing of meningococci was abolished. Furthermore, rabbits that were passively immunized with the heavily immunized antiserum showed impaired clearance of meningococci. This inhibitory effect of heavily immunized rabbit sera is specific for meningococci and is abrogated upon absorption of the serum against the immunized meningococcal strain (162). In a preceding publication, Lewis Thomas *et al*. also showed that sera from patients convalescing from meningococcal disease were sometimes less bactericidal than acute (non-immune) serum. This inhibitory effect of heavily immunized rabbit sera is specific observation made only in case of patients of meningococcal meningitis (197). This may have been the first recorded evidence of blocking antibodies against meningococci (162, 197).

Blocking IgA1 has also been identified in sera of patients following group C meningococcal disease and post-serogroup C capsular vaccinees (163, 165). Jarvis and Griffiss have shown that serum IgA derived from either convalescent sera from disseminated group C meningococcal meningitis cases or adult volunteers given tetravalent (A, C, W135, Y) vaccine could block IgG mediated lysis of serogroup C *N. meningitidis* (163). The blocking IgA1 was directed against the serogroup C capsular polysaccharide and did not inhibit binding of IgG to the bacteria and is therefore directed against a distinct capsular epitope(s). A novel type of blocking IgG against serogroup W-135 *N. meningitidis* was also demonstrated in the serum of a healthy C2-deficient sister of a forteen year old girl diagnosed with serogroup W-135 meningitis. Following vaccination of both the patient and her healthy sibling with tetravalent polysaccharide meningococcal vaccine, the healthy sister developed blocking IgG directed against group W-135 capsule (166). Blocking antibody that reduces bacterial killing has also been shown following *Brucella abortus* (153), *B. melitensis* (198) and *Pseudomonas aeruginosa* (155, 156) infections. Anti-LPS antibodies have been shown to block complement mediated killing of non-typhoid salmonella in convalescent sera of HIV-positive patients in Africa (199). Our data are the first to identify blocking anti-meningococcal antibodies in otherwise healthy persons who have not had preceding meningococcal disease or meningococcal vaccination.

Effective capsular polysaccharide-based vaccines are licensed against serogroup A, C, W-135 and Y *N. meningitidis*. However, there is no effective vaccine available against group B *N. meningitidis*. Several protein antigens,

including fHbp, have been identified as potential vaccine candidates against serogroup B *N. meningitidis* (74, 75, 78, 150, 175, 176, 179, 191). While I have shown that the bactericidal efficacy of an anti-fHbp mAb is disproportionately reduced in the presence of anti-H.8 human antibodies, compared do anti-Por mAb, the effect of these blocking antibodies on polyclonal anti-fHbp antibodies elicited by immunization of humans is not known. Outer membrane vesicles (OMVs) often used as vaccines (14, 104, 123, 200, 201) or as carriers for protein vaccine antigens contain Lip/H.8 and/or Laz and may therefore elicit blocking antibodies and attenuate bactericidal responses.

In conclusion, I have identified naturally occurring human blocking antibodies that are directed against the H.8-pentapeptide repeat motifs of the Lip and Laz lipoproteins of *N. meningitidis*. These results suggest an important role for these peptide repeats in meningococcal pathogenesis and may explain why these repeats occur in two distinct proteins that are ubiquitously expressed in pathogenic Neisseriae. Antibody-mediated complement-dependent killing is critical for host defense against Neisseriae. I have characterized an important strategy employed by this bacterium to subvert a key immune defense that may have important implications for vaccine development.

2.4 Materials and Methods

Bacterial strains

N. meningitidis strains H44/76 (B:15:P1.7,16: ST-32; invasive isolate from Norway (1976)) (202) and the serologically closely related strain MC58

(B:15:P1.7,16: ST-74; invasive isolate (United Kingdom (1985)) (6) have been described previously.

Human serum

Sera obtained from 19 healthy adult human volunteers without prior history of meningococcal disease were aliquoted and stored at –80^{o} C until used. Sera were used only once within 1 hour after thawing. Total hemolytic complement (CH_{50}) of sera was measured using the Total Haemolytic Complement kit (The Binding Site) or the EZ Complement CH_{50} assay (Diamedix Corporation) according to the manufacturers instructions. For some experiments, sera were heated to 56^{o} C for 30 min to destroy complement activity.

Ethics statement

This study was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts Medical School. All subjects who donated blood for this study provided written informed consent.

Monoclonal antibodies

mAb JAR 3 (IgG3) that recognizes variant 1 meningococcal factor H-binding protein (fHbp) that is expressed by strains H44/76 and MC58) was provided by Dr. Dan M. Granoff (Children's Hospital Oakland Research Institute) (203). Anti-PorA mAb P1.7 (ascites fluid) was purchased from the

National Institute of Biological Standards and Control (Potters Bar, Hertfordshire, U.K).

Serum bactericidal assays

Bacteria taken from an overnight culture on chocolate agar plates were re-passaged onto fresh chocolate agar and allowed to grow for 6 h at 37°C in an atmosphere containing 5% CO_2. Approximately, 2000 CFU of meningococci were incubated with serum (concentrations specified for each experiment), purified proteins, peptides or antibodies; the final reaction volume was maintained at 150 µl. Aliquots of 25 µl of the reaction mixtures were plated onto chocolate agar in duplicate at the beginning of the assay (t_0) and again after incubation at 37°C for 30 min (t_{30}). Survival was calculated as the number of viable colonies at t_{30} relative to t_0. Heat inactivated killing sera were tested separately for loss of killing activity when complement was destroyed (complement control).

Preparation of IgG depleted serum

All manipulations were performed at 4 °C. To deplete IgG, 2 ml of serum containing 10 mM EDTA to minimize complement activation, was passed over a 1 ml protein G sepharose column (Sigma Catalog No. P3296; binding capacity >20 mg human IgG/ml) equilibriated with Tris-buffered saline (TBS); pH **7**. The fall through material (absorbed serum) was spin-concentrated and dialyzed against TBS using a 10kDa cut off Amicon Ultra-15 centrifugal filter device (Millipore). The absorbed serum was concentrated to near its original volume and stored as single-use aliquots at -80º C. Depletion

of IgG was confirmed by flow cytometry, where complete loss of IgG binding to H44/76 was observed (Figure 2.12). The hemolytic activity of IgG depleted serum was quantified following reconstitution with 2 mM Ca^{++} and 2 mM Mg (resultant $C_{50}H$ ~50%). I used a two-fold higher concentration of depleted serum vs. the intact serum counterpart in functional bactericidal assays to account for loss of hemolytic activity during manipulation of serum.

IgG and IgM purification

IgG that had bound to protein G sepharose following passage of serum through the column (as described above) was eluted with 0.1 M glycine-HCl, pH 3.0, and neutralized immediately with 0.2 M Tris in 0.5 M NaCl, pH 8.0. Protein containing fractions were spin-concentrated and dialyzed against TBS, pH.7 (TBs) using an Amicon-15 Ultra device (30 kDa cutoff).

IgM was purified by passage of serum over an anti-human IgM agarose column (Sigma), followed by elution and neutralization as described above. Spin-concentration and dialysis against TBS was performed using an Amicon-15 Ultra device (100 kDa cutoff). IgG and IgM concentrations were estimated by the Bradford assay.

Flow cytometry analysis

Binding of human IgG and IgM to *N. meningitidis* was determined by flow cytometry as described previously (147). Briefly, 10 µl of serum was added to 10^8 bacteria suspended in 90 µl Hank's Balanced Salt Solution (HBSS) for 30 min at 37 °C. Bacteria were washed and bacteria-bound IgG and IgM were detected using FITC conjugated anti-human IgG and anti-

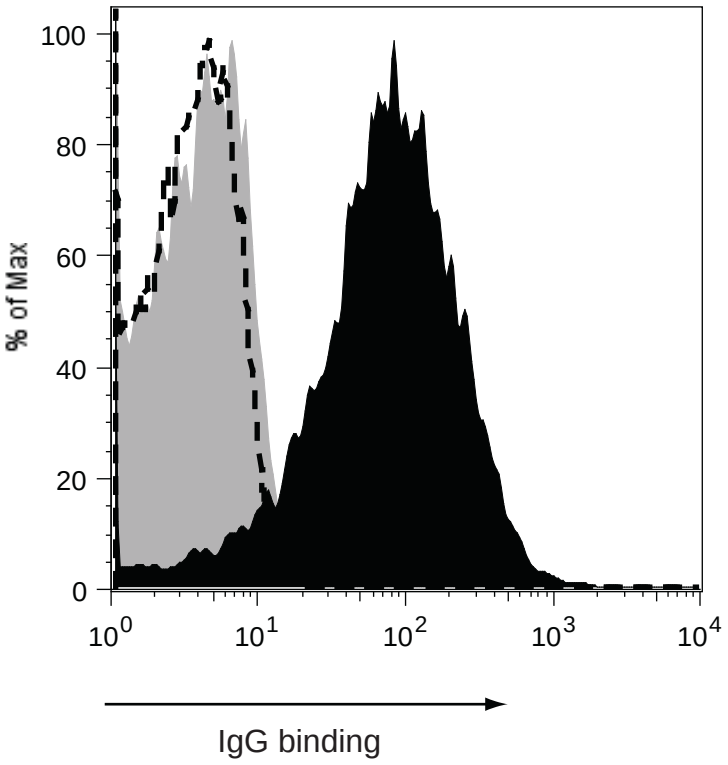


Figure 2.12. IgG depletion from NK3 serum. *N. meningitidis* serogroup B strain H44/76 was incubated with non-killing NK3 serum (solid black plot) or IgG-depleted NK3 (Dashed plot). IgG binding to bacteria determined by flow cytometry using anti-human IgG-FITC. The secondary antibody control (solid grey plot) represents bacterial incubation in the absence of serum.

human IgM (both from Sigma). Data were generated using a BD FACSCalibur (Becton Dickinson) flow-cytometer and analyzed using FlowJo software (Tree Star, Inc.).

Western blotting

Western blotting was used to determine binding of human IgG to meningococcal antigens. Bacterial lysates were separated on a 4–12% Bis-Tris gel (Invitrogen Life Technologies) using 2-(*N*-morpholino) ethanesulfonic acid (MES) running buffer, pH 7. Proteins were transferred to PVDF membranes (Millipore) and blocked with PBS-1% dry milk for 30 min at 24° C. Membranes were then incubated with heat-inactivated NHS (diluted 1:100 in PBS-0.05% Tween 20) for 15 h at 4°C. Membrane-bound IgG was detected using alkaline phosphatase conjugated anti-human IgG (Sigma) at a dilution of 1:1000 dilution in PBS-0.05% Tween 20 and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NPT) purple liquid substrate (Sigma; Cat No. B3679).

Lip/H.8 deletion (*lip*) mutants and insertional inactivation of *laz*

Chromosomal DNA isolated from an H.8 knockout mutant of *N. gonorrhoeae* strain F62 (204) was used to transform *N. meningitidis* strain MC58 and transformants were selected on GC agar plates containing chloramphenicol (5 µg/ml) and loss of Lip/H.8 expression was confirmed by western blotting (Figure 2.9).

To construct the *laz* deletion mutant of strain MC58, chromosomal DNA isolated from a Laz knock out gonococcal strain F62. In order to make the F62

Laz::erm mutant, bps 40-184 of the *laz* open reading frame (ORF), which includes the signal sequence, was replaced with an erythromycin resistance (*erm*) marker using overlap extension PCR described herein. DNA extracted from strain F62 was used as a template, and a 329 bp fragment upstream of bp 40 and a 359 bp fragment downstream of bp 184 of the *laz* ORF were amplified using primer pairs 5′ CGGCAGGGATGTTGTAAATATC 3′ (LAZ-UP-FOR) and 5′-**GTTCCTATTTTTTG**GATAACGGCGGCAGAAATCAGA-3′ (LAZ-UP-REV)and 5′-**TGA ACAAGATGAGT**CTGACGAATCCAACGACAATATGC-3′ (LAZ-DOWN-FOR) and 5′-GTTCATCAAAGCACCGTGACCC-3′, (LAZ-DOWN-REV) respectively (the emboldened underlined sequences overlap with the *erm* marker). The erythromycin resistance marker was amplified using genomic sequence of mutant gonococcal strain F62::*lgtE::erm* (unpublished) as a template with primers 5' **GCCGCCGTT ATC**CAAAAA ATAGGAACACGAAAAACAAG 3' (ERM-FOR) and 5' **GTTGGA TTCGTCAG** ACTCATCTTGTTCATATTTATCAG 3' (ERM-REV) (the emboldened underlined sequences overlap with *laz*). The three PCR products were gel purified and linked by overlap extension using primers LAZ-UP-FOR and LAZ-DOWN-REV. The resulting 1527 bp product was cloned into TOPO 2.1, linearized with *Sca*I and used to transform strain F62. Erm resistant colonies were selected on GC agar containing Erm (2.5 µg/ml). Interruption of *laz* was verified by PCR and loss of Laz expression by western blotting The strain MC58 was transformed with this DNA and the transformants were selected in GC agar plates containing erythromycin (5mg/ml) (Figure 2.9).

Generation of IgG F(ab)$_2$ fragments.

Anti-H.8 mAb 2C3 was digested using immobilized pepsin bound to agarose beads in Tris-Cl buffer, pH 6 for 4hrs at 37^{o}C while shaking. The beads were spun down at 2000rpm for 5min. The F(ab)$_2$ fragments in the supe was purified using sepharose G beads and spin-concentrated and dialyzed against Tris Buffered saline, pH. 7, using Amicon-15 Ultra device (30 kDa cutoff). Generation of F(ab)$_2$ was checked by running untreated and pepsin treated mAb 2C3 on a 4-12% Bis-Tris Gel followed by staining with coomassie stain.

Synthetic Lip/H.8 peptide fragments

The synthetic Lip/H.8 fragments Lip N30 (representing the N-terminal 30 amino acids of the Lip/H.8 from residues 18 to 47 with the Met residue in the signal peptide was designated as amino acid no. 1; peptide sequence CGGEKAAEA PAAEAPAAEAPATEAPAAEAP]) and Lip C30 (representing the C-terminal 30 amino acids of Lip/H.8 spanning residues 68 through 97 plus an added N-terminal Cys residue [peptide sequence CAEAAATEAPAAEAAATEAPAAEAPAAEAAK] were synthesized by Genway Biotech and used to inhibit binding of putative blocking IgG .

Cloning and expression of recombinant Laz-6xHis and Azurin-6xHis proteins

laz was amplified from MC58 chromosomal DNA using primer pair rLAZ-FOR (5′ GGAATTC**CATATG**TCTCAAGAACCTGCCGCGCC 3′; the *Nde*I site is emboldened and underlined) and rLAZ-REV (5′ CCG **CTCGAG**A

TCGACCAAAGTCACTTTGCCG 3′; the *Xho*I site is emboldened and underlined). The 496 bp amplicon encoded *laz* without the N-terminal Cys residue and also lacked the stop codon. The resulting PCR product was cloned into the *Nde*I-*Xho*I sites of pET-21a (EMD Biosciences, cat. no. 69740-3) that introduces a C-terminal 6xHis tag. The plasmid, called pET-21a-Laz-His, was transformed into *E. coli* strain BL-21 (DE3) pLysS. Laz expression was induced with 0.3 mM IPTG and the recombinant protein was purified from inclusion bodies following lysis of bacteria with B-PER lysis buffer (Pierce # 78243) supplemented with lysozyme (200 mg/ml) followed by nickel-affinity chromatography. The recombinant protein was analyzed for purity by coomasie staining and the presence of the His-tag was confirmed using western blot analysis with an anti-poly histidine mAb (Sigma; cat # H1029). The presence of the "H.8-like" domain of Laz (the N-terminal 35 amino acids that are organized into imperfect 'AAEAP' pentapeptide repeats) was revealed by western blotting with anti-H.8 mAb 2C3 (205).

Similarly, the azurin domain of Laz (lacks the"H.8-like" N-terminal 35 amino acid domain that contains imperfect AAEAP repeats) was also expressed as a recombinant protein in *E. coli*. The azurin domain of *laz* was amplified from the MC58 chromosomal DNA using primers: Azurin-FOR (5′ G GAATTC**CATATG**GGCAACTGTGCGGCAACTGTC 3′; the *Nde*I site is emboldened and underlined) and Azurin-REV (5′ CCGCTCGAGATCGACCAA AGTCACTT TGCCG 3′; the *Xho*I site is emboldened and underlined). Cloning into pET21a and purification of His-tagged recombinant azurin was performed as described for Laz.

Statistical Analysis

Percent of survival of bacteria with 95% confidence interval around the mean percentage is shown in the figures. Differences in percentage survival were assessed for significance using a two tailed paired student t test (p<than 0.05 are indicated as * and < 0.005 are indicated as **).

Chapter III

Mechanism of action of anti-H.8 Blocking IgG

Abstract

Serum mediated bactericidal activity (SBA) is an important arm of natural immunity against *N. meningitidis*. Complement deficiency, lack of specific bactericidal antibodies against meningococci and complement blocking antibodies constitute several factors that affect SBA. Although blocking antibodies have been identified against meningococci previously, their mechanism of action has not been well defined. Anti-Rmp antibodies against *N. gonorrhoeae* are believed to divert C3 to “ineffective” targets on the meningococcal surface (159), while anti-capsular blocking IgA1 against group C meningococci probably sterically hinder binding of otherwise bactericidal IgG or IgM. Anti-H.8 blocking IgG from select nonbactericidal sera blocked killing of meningococci by otherwise bactericidal mAbs against a meningococcal vaccine candidate called factor H-binding protein (fHbp). IgG from blocking serum that contained anti-H.8 did not hinder binding of anti-fHbp mAbs. Blocking anti-H.8 IgG downregulated classical pathway activation (C4 deposition) by anti-fHbp mAb. Blocking activity required N-linked oligosaccharides of the Fc region. Furthermore, blocking IgG also required participation of a heat stable serum factor for its activity. Our preliminary data indicates that this serum factor may be mannan binding lectin (MBL). These findings provide important insights into the mechanism of action of blocking IgG against *N. meningitidis*, emphasizing differences between blocking IgGs directed against different members of the Neisseriae family. This work also highlights a role for specific IgG glycosylation patterns in modulating complement activation on meningococci.

3.1 Introduction

In Chapter II, I have identified novel SBA blocking IgG in normal human serum directed against 'AAEAP' motifs in the H.8 outer membrane protein of serogroup B *N.meningitids*. In this chapter I propose to understand and partially characterize the mechanism of blocking activity.

Serum immunoglobulins (Ig) are critical components of the humoral immune system (206). IgG is the most abundant Ig molecule in human serum. Typically, an IgG molecule is composed of two heavy and two light chains linked to each other by disulfide bonds. Each heavy chain consists of three constant regions or domains (CH1, CH2 and CH3) and a variable domain (VH). Each light chain consists of a constant region/domain (CL) and a variable domain (VL). The constant and variable domains of the two light chains (CL and VL) together with the constant and variable domains of the two heavy chains (CH1 and VH) comprise the two Fab fragments. The constant regions/domains of the two heavy chains (CH2 and CH3) comprise the Fc fragment. (Figure 3.1 A) (206-208). The Fab portion dictates specificity of binding to antigens while the Fc portion is involved in numerous biological effector functions. Upon binding its cognate antigen, the antibody undergoes a conformational change that triggers Fc-mediated effector functions that aid in clearing invading pathogens. Two important Fc functions include: 1) Fcγ receptor mediated uptake of antibody-opsonized pathogens by immune cells such as neutrophils and macrophages; 2) activation of the classical complement pathway that leads to C3b (and then iC3b) deposition that promotes opsonophagocytosis through the CR1 and CR3 receptors and 3) in the case of gram-negative organisms, further activation of the complement

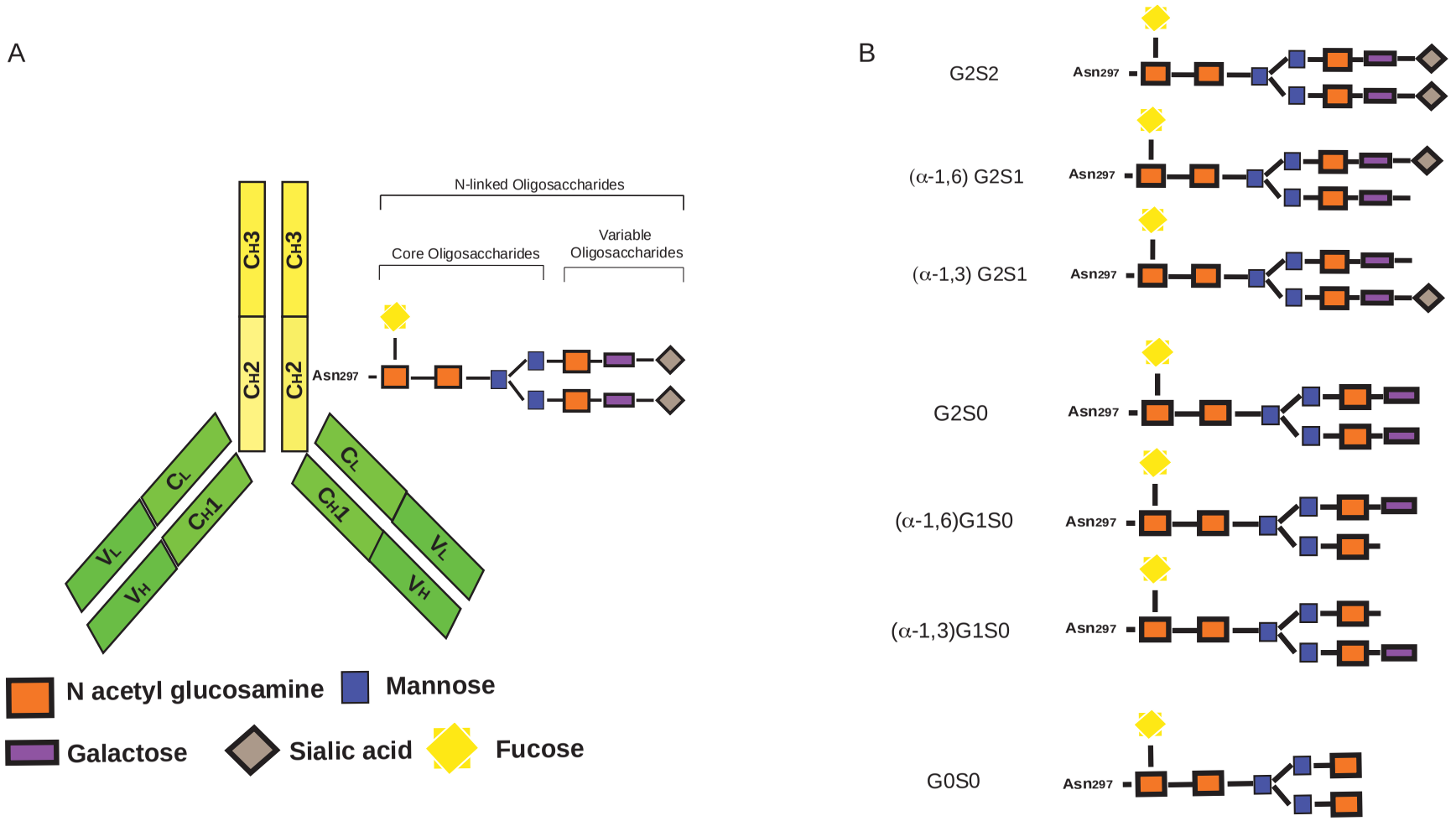


Figure 3.1. Schematic diagram depicting organization of the IgG molecule and the most commonly encountered N-linked Fc glycan structures. A) The Fc portion of the IgG is depicted in yellow (CH2 and CH3) and the 2 arms of F(ab)2 shown in green (CH1+VH) and (CL+VL). The asparagine residue at amino acid position 297 is substituted with a biantennary N-linked oligosaccharide with core and variable oligosaccharides indicated; shown here is the G2S2 structure shown in Figure 3.1B. B) Commonly encountered Fc N-glycan substitutions at the asparagine residue at amino acid position 297 and their glycose structural components.

cascade that leads to insertion of the membrane attack complex (C5b-9) and results in lysis and killing of orgnisms (208). Antibody mediated activation of the classical complement pathway (CP) is an important arm of the immune system that facilitates clearance of meningococci (127, 128).

Immunoglobulins (Igs) are glycoproteins and Ig glycosylation is essential for their biological effector functions. The Asn^{297} residue of the CH_2 domain of Fc region of IgG in serum is a site for substitution with a N-glycosyl residue (208, 209). This N-linked oligosaccharide substitution is critical for both Fcγ receptor and complement activation (208, 210). Typically, the oligosaccharide chain is made up of a core biantennary structure (Figure 3.1 A); heterogeneity of the glycans results from differences in the composition of the terminal sugar residues (Figure 3.1 B) (207, 208, 211). Glycan heterogeneity plays a critical role in modulating IgG effector functions (212).

In this chapter, I show that the N-linked oligosaccharide substitution of the Fc region is essential for the blocking activity of anti-meningococcal H.8 blocking IgGs present in normal human serum. I also show that blocking IgG requires Fc N-glycosylation to down-regulate classical complement pathway activation by antibodies that are otherwise bactericidal. Furthermore I show that downregulation of C4 deposition by blocking IgG may possibly require mannan binding lectin (MBL), a heat-stable serum factor. Contrary to its 'conventional' role as a complement activator, here I speculate a possible inhibitory role for MBL in complement activation on *N. meningitidis*. Futher experiments are required to validate this observation.

3.2 Results

Blocking by IgG requires the Fc domain.

To examine which parts of blocking antibody molecules are required for blocking, I first generated $F(ab)_2$ fragments from blocking IgG present in serum NK3 (Figure 3.2 A). I tested $F(ab)_2$ fragments for their ability to inhibit bactericidal activity of anti-fHbp mAb JAR 3 (intact blocking IgG served as a positive control) using IgG-depleted NK3 as the source of complement. Unlike gonococcal blocking, where $F(ab)_2$ derived from blocking IgG is stoichiometrically simlar in blocking potency (158, 159). I observed that loss of the Fc portion of the NK3 IgG abrogated its ability to block killing by JAR 3 (Figure 3.2 B).

The N-linked oligosaccharides of the blocking serum IgG Fc region are required for blocking activity.

Immunoglubulin isotype (or subclass) plays an important role in dictating which of the four IgG subclasses will activate complement. The hierarchy of complement activation by human IgG subclass is: IgG3 > IgG1 > 1gG2 >> IgG4 (213). Western blotting experiments revealed that blocking antibody in NK3 that was specific for H.8 was IgG3 (antibody disclosure used subclass specific conjugated anti-serum (Figure 3.3). Therefore, non-activation of complement by blocking IgG antibody (as might be expected with IgG4) was an unlikely explanation of the blocking phenomenon.

Having demonstrated that the Fc domain of IgG was required for blocking, I speculated that a glycan residue on Fc may play a role in blocking. The structure of Fc glycans modulates the effector functions of IgG (206-208).

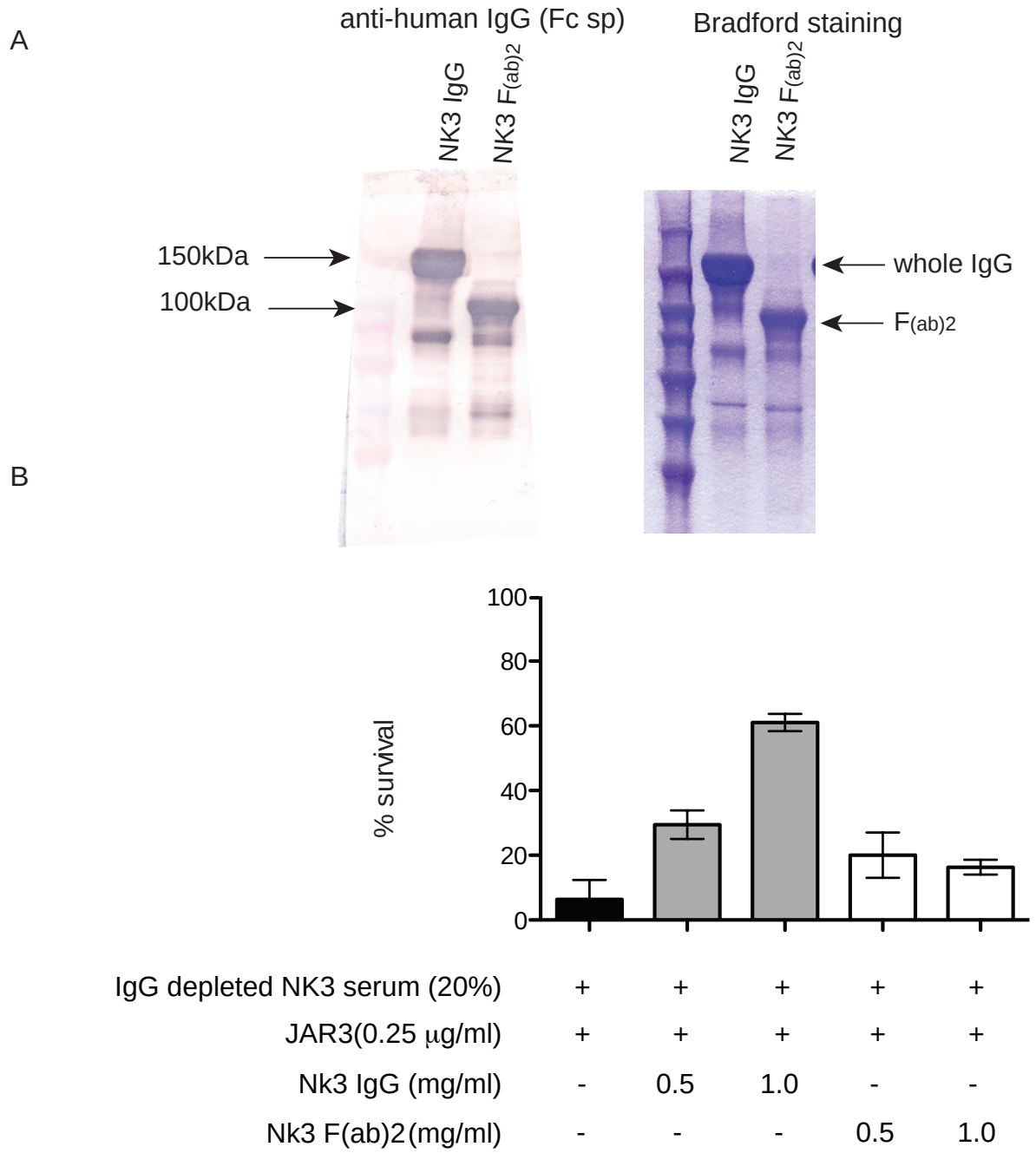


Figure 3.2. Blocking anti-H.8 IgG requires Fc for blocking. A) Pepsin and untreated NK3 IgG were electrophoresed on a 12% Bis-Tris gel followed by either coomassie staining (right panel) or western blotting with anti-human IgG (left panel) to confirm the separation of F(ab)2 (~110 kDa) from the rest of the IgG (~160 kDa) molecule. B) F(ab)2 recovered from digested NK3 IgG was added in increasing concentrations (white bars; final concentrations 0.5 and 0.1 mg/ml) to a bactericidal reaction mixture containing strain H44/76, mAb JAR3 and IgG depleted serum NK3, 20% (v/v) used as the complement source The corresponding doses of whole NK3 IgG (grey bars) served as a control. Each bar shows the mean percent survival (±range) of 2 separate experiments.

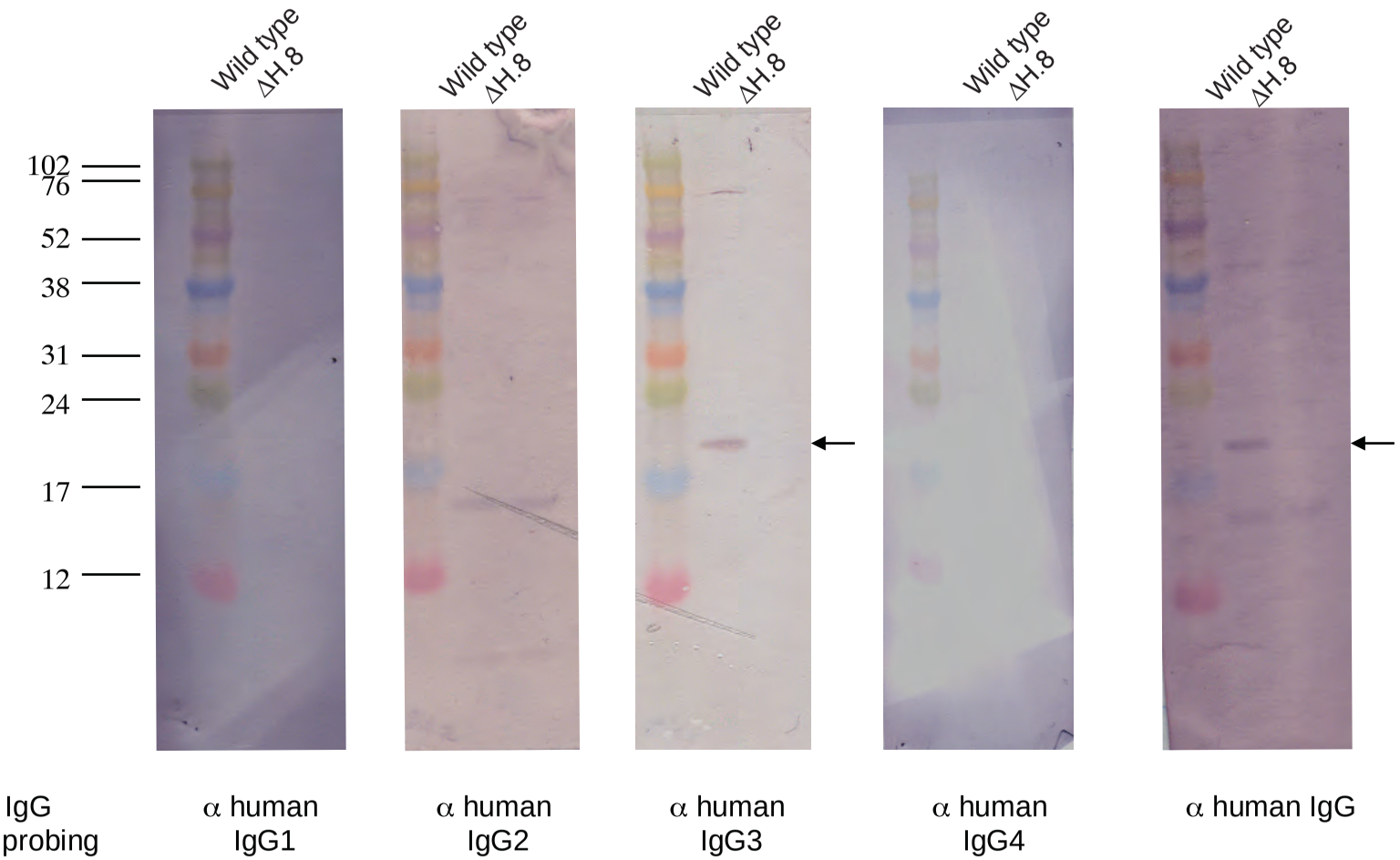


Figure 3.3. Anti-H.8 IgG in NK3 serum is IgG4 subclass. Bacterial lysates of strain MC58 and its isogenic lipoprotein H.8 knockout (KO) mutant were electrophoresed on a 12% Bis-Tris gel followed by western blotting. Parallel blots were incubated with a 1:100 dilution of NK3 serum. Specific human IgG subclass reactive bands were disclosed with alkaline phosphatase conjugated mouse anti-human IgG1, IgG2, IgG3 and IgG4. A separate blot was also probed with alkaline phosphatase conjugated mouse anti-human IgG as appositive control and labeled as such. Blots probed with alkaline phosphatase conjugated mouse anti-human IgG was used as appositive control. The Lip/H.8-reactive band is indicated by an arrow on the right. The identity of each IgG subclass that has been probed for is indicated in the labels below the gel pictures.

The IgG purified from blocking serum NK3 was deglycosylated using the endoglycosidase Peptide N-Glycanase, PNGase (Figure 3.4 A). Deglycosylation did not affect binding of NK3 IgG to H44/76 (Figure 3.4 B). A bactericidal assay was performed where untreated blocking IgG and deglycosylated blocking IgG were compared for their abilities to block the bactericidal effect of anti-fHbp mAb, JAR 3; IgG-depleted serum NK3 was used as the complement source. I observed that the deglycosylated NK3 IgG failed to block killing by JAR 3 (Figure 3.4 C).

Blocking serum IgG mediates blocking by reducing classical pathway activation by anti-fHbp monoclonal antibody.

Early events following binding of a complement-fixing (or bactericidal) Ab to a bacterial surface includes engagement of the C1 complex, activation of C4 and deposition of C4b on the bacterial surface. Surface-bound C4b then participates in the formation of classical pathway C3 and C5 convertases, which results in membrane attack complex insertion and bacterial lysis and killing. I asked whether human blocking IgG interfered with classical pathway activation by bactericidal antibody. In these experiments, strain H44/76 was incubated with the complement source (IgG-depleted NK3) and bactericidal mAb JAR 3, either alone or in the presence of intact purified NK3 IgG or PNGase-treated IgG. C4b deposition on bacteria was measured by flow cytometry. The presence of blocking IgG significantly decreased JAR 3-mediated deposition of C4b on wild-type serogroup B stain H44/76. C4b deposition was mostly restored when deglycosylated NK3 IgG was substituted, as depicted by mean fluoroscent intensity and histogram

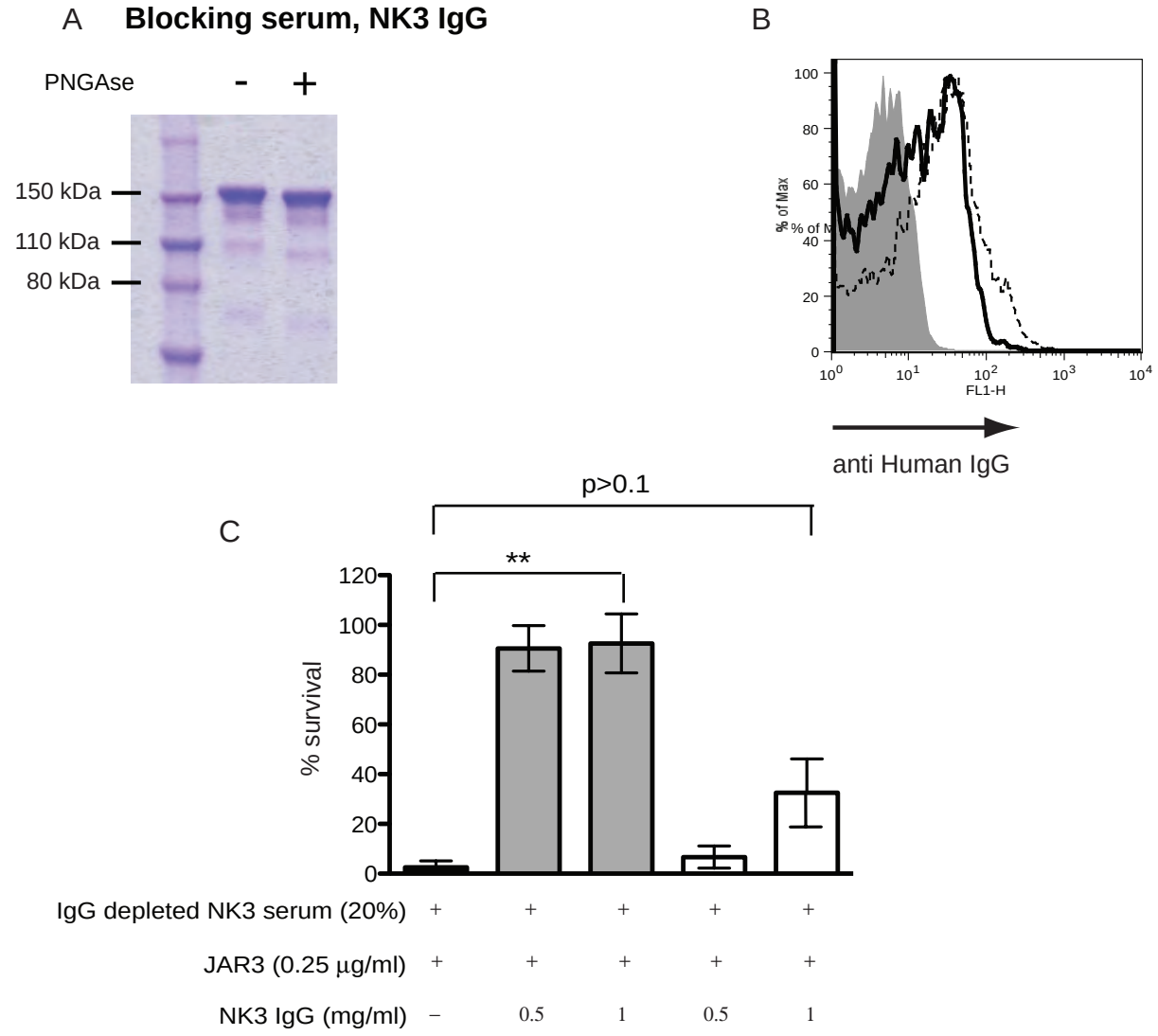


Figure 3.4. Blocking IgG requires Fc N-linked oligosaccharides to maintain blocking function. A) Deglycosylation of NK3 IgG by PNGase. Untreated and PNGase treated NK3 IgG were electrophoresed on a 12% Bis-Tris gel followed by either coomassie staining to monitor IgG deglycosylation. The deglycosylated NK3 IgG (3rd lane from left; PNGase +ve) migrates faster than the untreated IgG (2nd lane from left; PNGase –ve). B) Similar binding of untreated and PNGase-treated NK3 IgG to *N. meningitidis*. Serogroup B *N. meningitidis* strain H44/76 was incubated with 100 µg untreated (black plot) or deglycosylated (PNGase treated) NK3 IgG (dashed plot). IgG binding to bacteria was determined by flow cytometry using anti-human IgG-FITC. The secondary antibody control (grey shaded areea) represents bacterial incubation in the absence of blocking IgG. C) Loss of Fc glycan is associated with loss of blocking activity. Increasing (0.5 and 1 mg/ml) concentrations of deglycosylated (PNGase-treated) NK3 IgG (white bars) was added to a bactericidal reaction mixture containing strain H44/76, mAb JAR3 and IgG depleted serum NK3 (20% (v/v)) used as the complement source. The corresponding concentrations of untreated NK3 IgG (grey bars) served as a control. The y-axis indicates percent survival and each column represents the mean (±SEM) of 3 separate experiments.

plots (Figure 3.5 A, B). Controls included reaction mixtures that contained the complement source alone, complement plus intact blocking IgG and complement plus PNGase-treated blocking IgG; no increase in C4b deposition above levels seen with C' alone was seen in any of the other two controls, thereby showing that neither the intact or PNGase-treated blocking IgG activated the classical pathway by itself (Figure. 3.5 A). The level of classical pathway activation was independent of the amount of NK3 IgG or anti-fHbp mAb binding to the bacteria (Figure 3.5 D, E).

Blocking serum IgG does not effect classical pathway activation by anti-PorA monoclonal antibody.

In chapter II, I showed that blocking IgG did not block the bactericidal activity of a mAb against an abundant meningococcal outer membrane protein, PorA. In accordance with those observations, I show here that NK3 IgG did not diminish classical pathway activation by anti-PorA mAb, P1.7 (Figure 3.6 A, B). Binding of NK3 blocking IgG (Figure 3.6 C) and Por1.7 (anti-PorA mAb; Figure 3.6 D) were both ensured in these experiments.

Terminal sialylation of the N-linked Glycans of the blocking IgG is not responsible for its blocking activity.

Terminal α(2,6)-linked sialic acid residues of Fc N-linked oligosaccharides on IgG molecules downregulate inflammation by activating inhibitory Fcγ RIII receptors on immune cells (214). The level of sialyation of serum IgG is highly variable (215) and the role of terminal α(2,6)-linked sialic acid in complement activation is not completely understood. I hypothesized

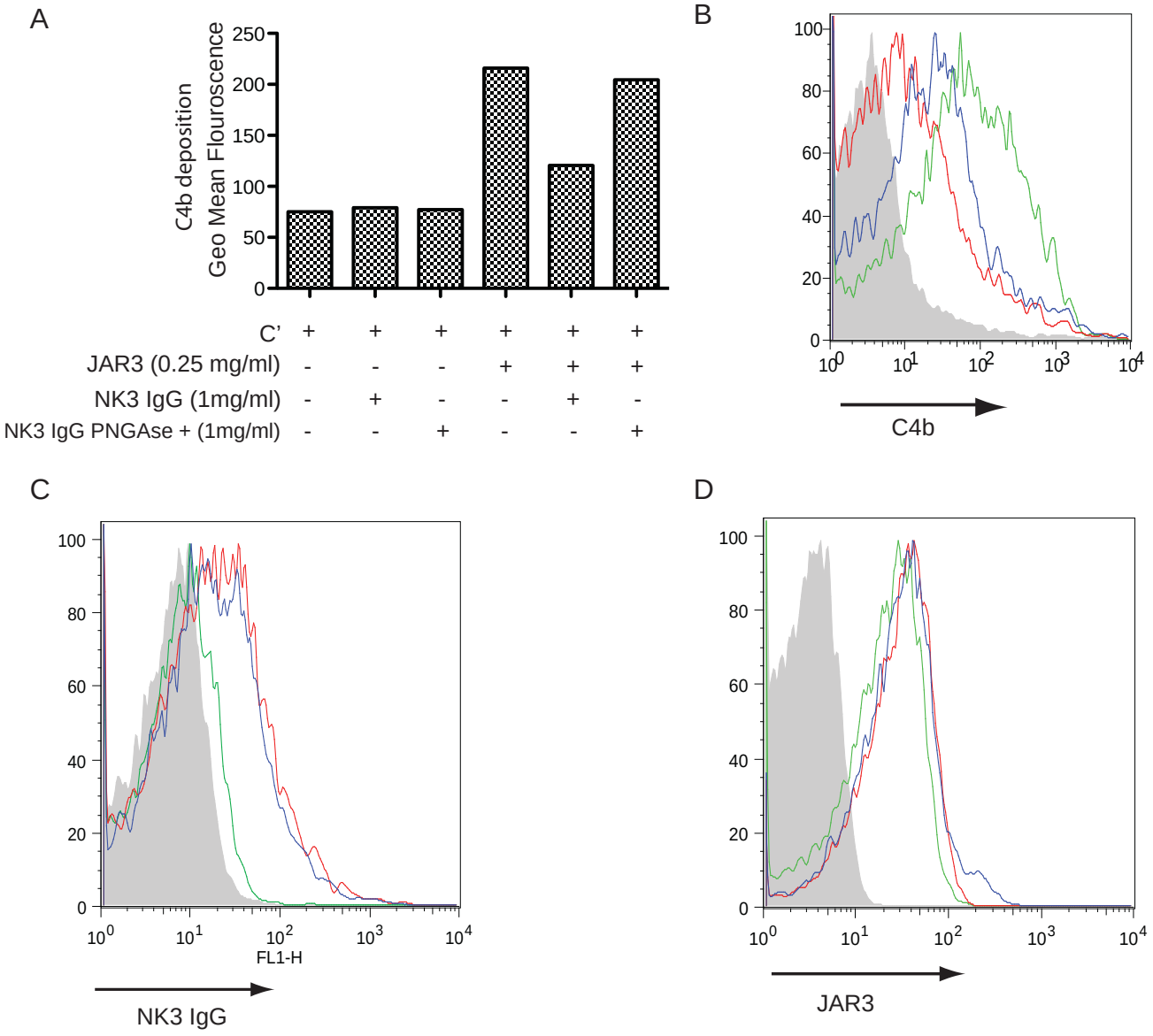


Figure 3.5. Blocking IgG inhibits the classical pathway activation of complement by JAR 3 mAb (anti-fHBP). N. meningitidis serogroup B strain H44/76 was incubated with mAb JAR3, IgG depleted NK3 serum used as a source of complement (C') and 100 µg of either untreated or PNGase-treated NK3 IgG. C4b deposition on bacteria was determined by flow cytometry and used as a measure of classical pathway activation. A) C4b deposition on bacteria (average of the geometric mean fluorescence of 2 experiments) mediated by JAR3 and IgG depleted serum NK3 (complement [C']) in the presence of NK3 IgG or PNGase-treated NK3 IgG. B) Histograms from one representative experiment in A. showing C4b deposition on bacteria X-axis; fluorescence on a log10 scale; y-axis, counts. C) Levels of NK3 IgG binding in NK3 containing reactions and D) levels of JAR 3 binding in JAR 3 containing reactions. All FACS plots representing bacteria incubated are color coded as follows: C' (solid grey plot); C'+JAR3 (Green plot); C'+JAR3+NK3 IgG (red plot) and C'+JAR3+ PNGase+ve NK3 IgG (blue plot).

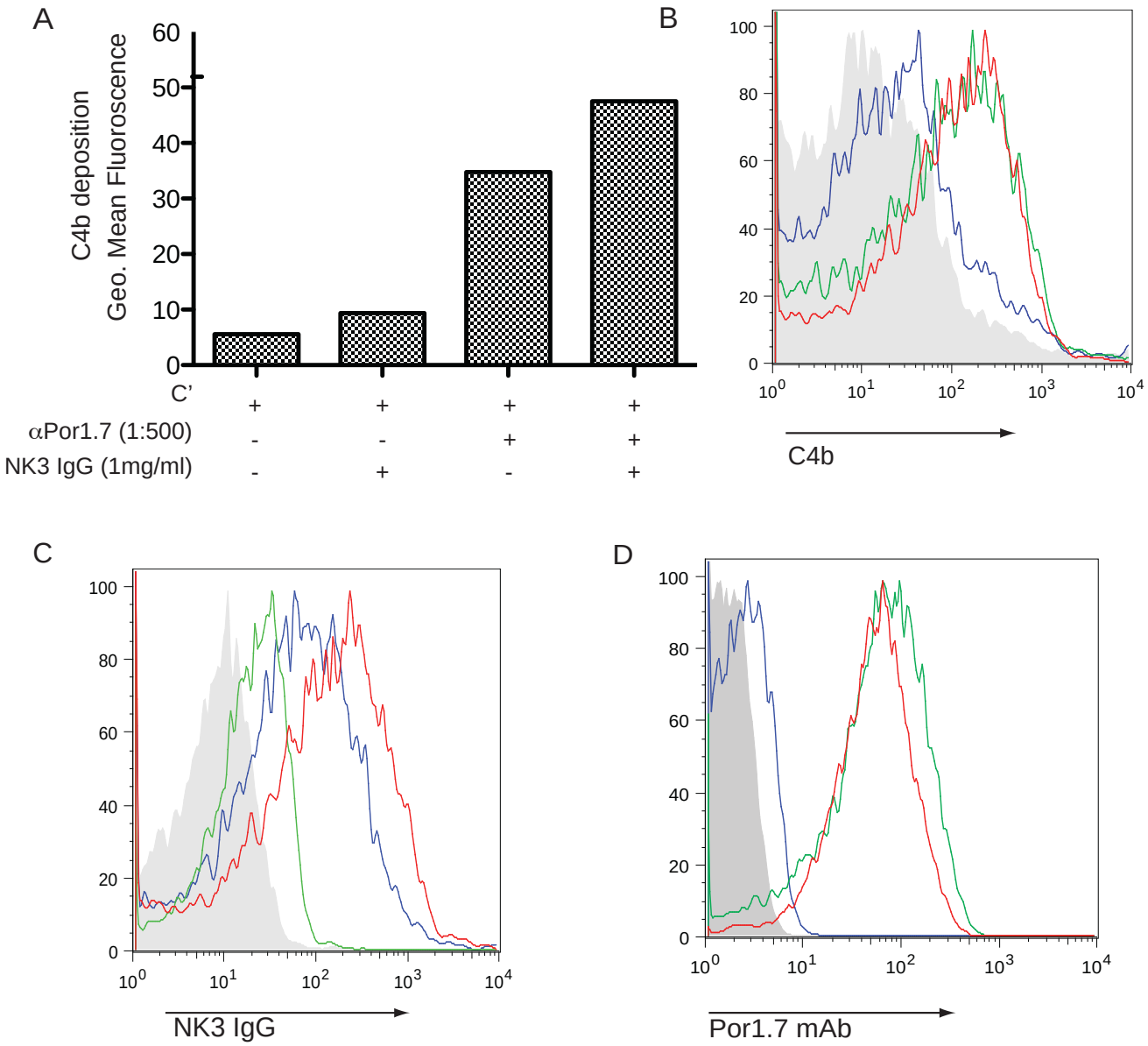


Figure 3.6. Blocking serum IgG does not inhibit classical pathway activation by Por1.7 mAb (anti-Por A). N. meningitidis serogroup B strain H44/76 was incubated with mAb Por1.7, IgG depleted NK3 serum used as a source of complement (C') and 100 µg of NK3 IgG. C4b deposition on bacteria was determined by flow cytometry and used as a measue of classical pathway activation. A) C4b deposition on bacteria (average of the geometric mean fluorescence of 2 experiments) mediated by P1.7 and IgG depleted serum NK3 (complement [C']) in presence or absence of NK3 IgG. B) Histograms from one representative experiment in A shoing C4b deposition on bacteria. X-axis; fluorescence on a log10 scale; y-axis, counts. C) levels of NK3 IgG binding in NK3 containing reactions and D) levels of Por1.7 binding in Por1.7 containing reactions. All FACS plots representing bacteria incubated are color coded as follows: C' (solid grey plot); C'+NK3 IgG (blue plot); C'+Por1.7 (green plot) and C'+Por1.7+ NK3 IgG (red plot).

that negatively charged terminal sialylation of blocking IgG N-linked glycans could be responsible for blocking activity, perhaps by interfering with C1q binding to proximate bactericidal antibodies. C1q-Fc interactions are ionic in nature and are influenced by charge (216). Alternately, terminally sialylated blocking IgG could recruit complement inhibitors such as C4bp and/or factor H that have the capacity to bind to polyanions and thereby inhibit complement activation. In order to determine the role of terminal sialic acid residues of N-linked oligosaccharides of blocking IgG upon its function, I successfully de-sialylated whole IgG purified from blocking serum using α(2,6) specific neuraminidase (Figure 3.7 A) and tested two doses of either untreated blocking IgG or desialylated blocking IgG for their ability to block the bactericidal activity of anti-fHBP mAb JAR (Figure 3.7 B). Loss of sialylation of blocking IgG did not inhibit its blocking activity. Interestingly, and contrary to my expectations at the time, I observed enhancement of blocking by desialylated blocking IgG compared to untreated IgG. This unpregulation of the blocking effect upon desialylation could be due to unmasking of particular sugar residues of the Fc N-linked glycans that possibly mediate or may be required for blocking.

‘Blocking’ serum IgG requires a heat stable serum factor to block

Several steps precede the deposition of C4b on a surface and interference with any of these steps could account for reduced C4b deposition that was seen in the presence of effective blocking by ‘blocking’ IgG. Engagement of the C1 complex by Fc, activation of C4 by C1s in the C1 complex and availability of targets for the activated metastable C4b molecule

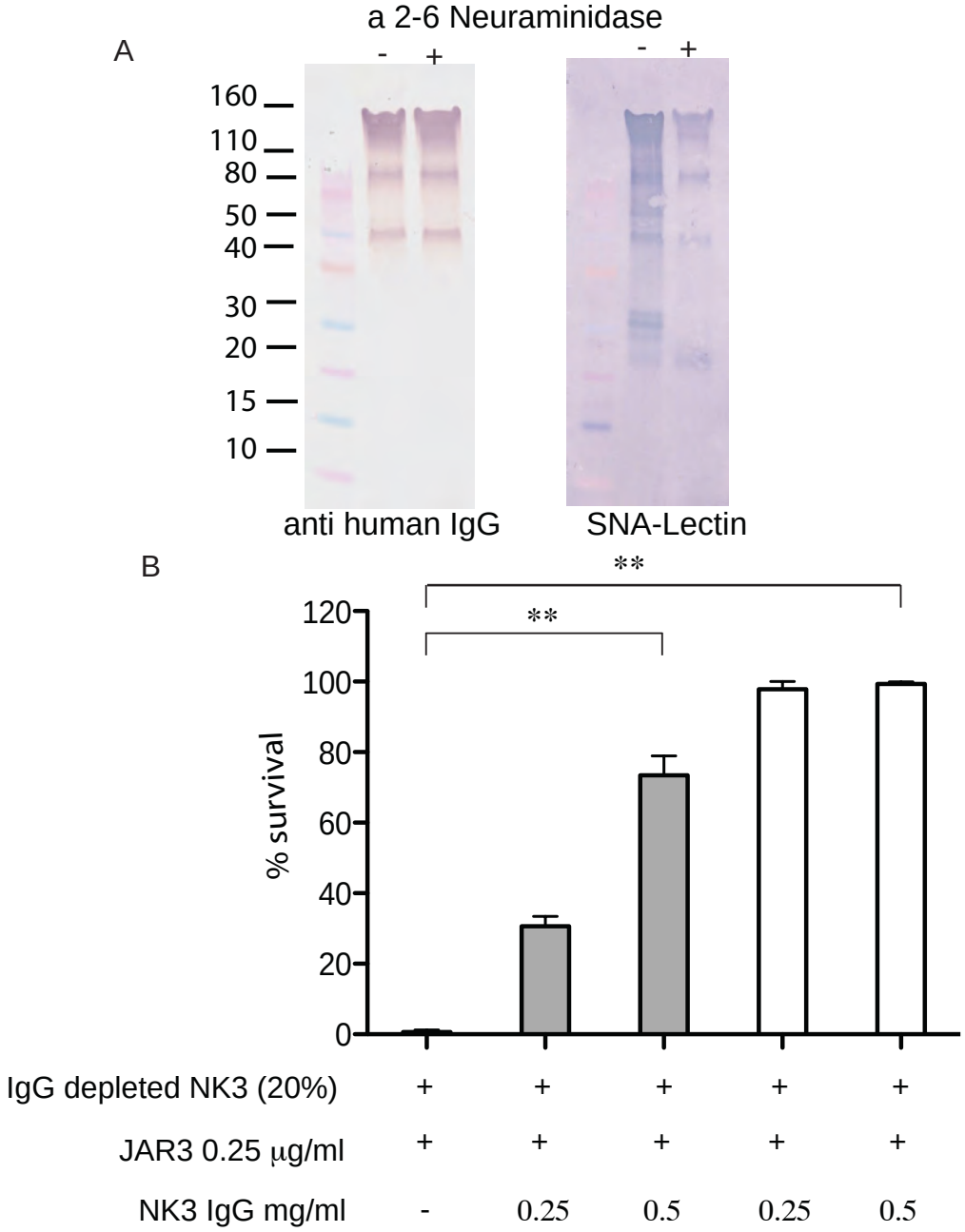


Figure 3.7. Desialylation of blocking serum IgG increases blocking activity. A) Confirmation of desialylation of IgG. Neuraminidase treated and untreated NK3 IgG were electrophoresed on a 12% Bis-Tris gel followed by western blotting and probing with anti-human IgG alkaline phosphate which served as a 'loading control' (left panel) or with biotinylated Sambrucus Niagra Lectin and alkaline phosphatase conjugated streptavidin to monitor desialylation (right panel). B) Either untreated (grey bars) or Neuraminidase treated (white bars) NK3 IgG was added incrementally (final concentration, 0.5 and 1 mg/ml) to a bactericidal reaction mixture containing strain H44/76, mAb JAR3 and IgG depleted serum NK3 (20%) used as a complement source. Corresponding doses of untreated NK3 IgG served as a control. The y-axis indicates percent survival and each column represents the mean values (±SEM) of 3 separate experiments.

must occur prior to C4b deposition on bacteria. As a first step in addressing this question, I attempted to 'recreate' the phenomenon of decreased C4b deposition by blocking IgG using purified complement components C1 and C4. C4b deposition was measured when strain H44/76 was incubated with purified C1 and C4 and JAR 3 either alone or in the presence of intact NK3 (blocking) IgG or PNGase-treated NK3 IgG (blocking ability lost). Reaction mixture containing only purified C1 and C4 without any antibody served as control for background (spontaneous) C4 activation and deposition. Reaction mixture containing sheep anti-C4b mAb and FITC conjugated goat-anti sheep IgG served as probing antibody control. Surprisingly, I observed that blocking IgG failed to affect a decrease in classical pathway activation by the JAR3 when purified C1 and C4 were used (Figure 3.8 A). However, downregulation of the classical pathway by the blocking IgG was restored upon addition of heat inactivated complement (IgG depleted blocking serum NK3) to the reaction mixture that contained C1 and C4 (Figure 3.8 B). The level of downregulation of classical pathway in this case was comparable to that caused by NK3 IgG on JAR3-mediated classical pathway activation when 'IgG depleted NK3 serum' was used as a complement source (Figure 3.8 C). The heat inactivated 'IgG depleted blocking serum NK3' showed minimal classical pathway activation by JAR3 (Figure 3.8 D). Thus, the blocking IgG requires an additional heat stable serum factor(s) in order to mediate its blocking activity against bactericidal mAb JAR3.

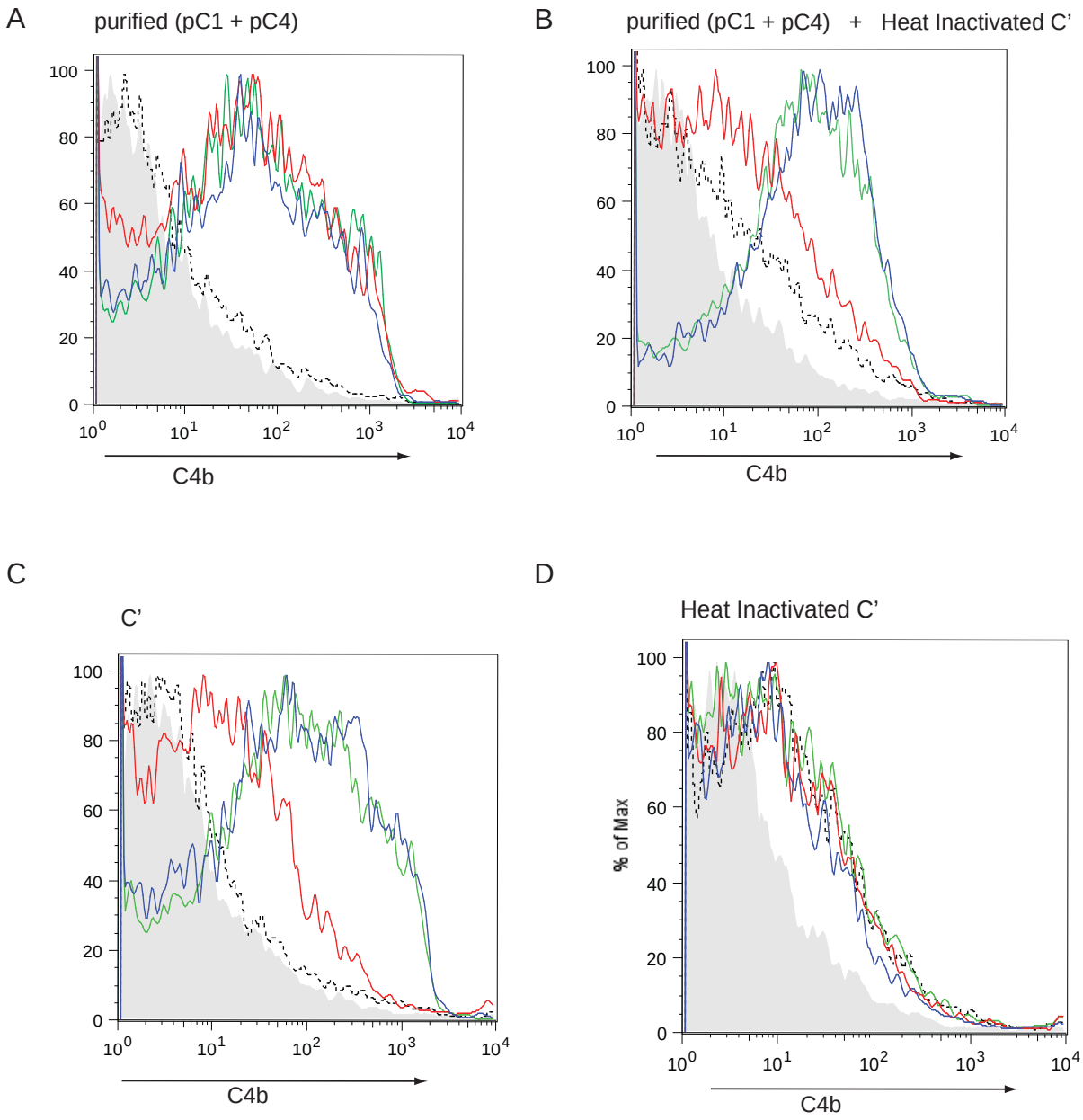


Figure 3.8. Blocking activity of NK3 IgG requires participation of a heat stable serum factor. N. meningitidis serogroup B strain H44/76 was incubated with mAb JAR3 alone (green plot) or along with NK3 IgG (1 mg/ml, red plot) or PNGase-treated NK3 IgG (1 mg/ml, blue plot) in presence of one of the following used as a complement (C') source indicated above each FACs plot: A) purified C1 (pC1) and C4 (pC4), B) pC1 and pC4 mixed with heat inactivated (IgG depleted NK3 serum, C') IgG, C) IgG depleted NK3 serum, C' and D) heat inactivated IgG depleted NK3 serum. Baseline level of C4 activation by C' alone (in the absence of any antibody) is shown by a dashed black line. Background binding of the detector antibody (anti-C4 IgG + FITC-sec Ab) binding is shown by solid grey shading. Classical pathway activation in presence of each complement source is depicted as level of C4b binding. X-axis; fluorescence on a log10 scale; y-axis, counts.

Mannan binding lectin (MBL) may be a possible heat stable factor required by the blocking IgG to block the bactericidal activity of JAR3.

MBL is a collectin family member found in the serum of most mammals that specifically binds to glucans, lipophosphoglycans and glycoinositol phospholipids that contain mannose, glucose, fucose or N-acetylglucosamine (GlcNAc) as their terminal hexose (217-219) (113). MBL is associated with three MBL associated serine proteases (MASPs), MASP-1 (220, 221), MASP-2 (222) and MASP-3 (223). Of these MASP-1 activates C3 directly (222, 224, 225) and MASP-2 cleaves C4 (226). MBL and C1q have been shown to compete for binding to human endothelial cells (227). IgG N-linked glycans terminating in N-acetyl glucosmanine or mannose bind MBL (228) and show reduced classical pathway activation due to lack of binding to C1q (229). I hypothesized that MBL could be the heat stable serum factor that acted together with blocking IgG to block the bactericidal activity of antibodies such as JAR3. MBL deficiency is relatively common, although complete lack of MBL in serum is rare (132). I utilized serum from an MBL-deficient individual described in an earlier publication from our laboratory (230) (Figure 3.9 A). IgG and IgM were depleted from this MBL-deficient serum to remove background antibody-dependent bactericidal activity for use as a complement source. I compared the ability of NK3 IgG to block the bactericidal activity of JAR3 in presence of one of two complement sources: i) either IgG and IgM depleted MBL-deficient serum or ii) IgG depleted NK3 serum (MBL-sufficient) (Figure 3.9 B). I showed that while the NK3 IgG blocks the bactericidal activity of JAR3 in presence of MBL-sufficient complement source, it was an ineffective blocker in MBL-deficient complement (Figure 3.9 B). These results

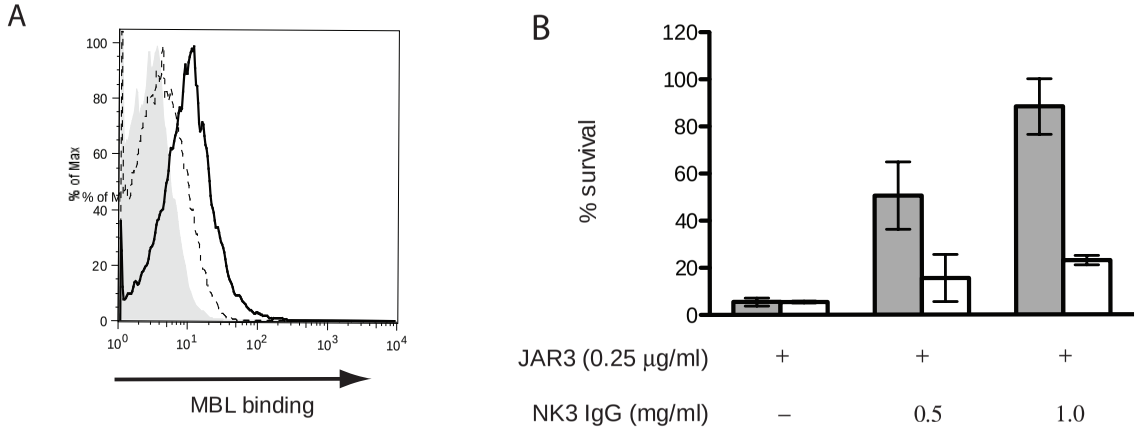


Figure 3.9. Mannan binding lectin (MBL) is the serum factor required for the blocking activity of anti-meningococcal blocking IgG. A) Confirmation of low MBL in MBL-'deficient' serum. *N. gonorrhoeae* strain 1291a (LOS terminates in a GlcNAc residue and binds to MBL) (225) was incubated with either 20% NK3 or 20% of MBL-deficient normal human serum. MBL binding to bacteria was determined by flow cytometry using anti-human MBL mAb (3E7). The background binding of 3E7 antibody is determined by bacterial incubation with 3E7 in the absence of serum. B) *N. meningitidis* serogroup B strain H44/76 was incubated with 0.25 µg/ml of anti-fHbp mAb JAR 3 together with increasing doses of NK3 IgG (0.5 and 1 mg/ml) and one of the following two complement sources: IgG-depleted NK3 (MBL sufficient) or IgG/IgM-depleted MBL-deficent serum are shown by the grey shaded and open bars respectively. Each bar shows the mean percent survival (±range) of 2 separate experiments. IgG binding to bacteria was determined by flow cytometry using anti-human IgG-FITC.. Percent survival of bacteria was determined in a serum bactericidal assay. Data with IgG-depleted NK3 (MBL sufficient) and IgG/IgM-depleted MBL-deficent serum, are shown by the grey shaded and open bars respectively. Each bar shows the mean percent survival (±range) of 2 separate experiments. IgG binding to bacteria was deter-

indicate that MBL could possibly be the heat stable serum factor required for the activity of blocking activity of IgG in NK3. However, further experiments are required to confirm and validate this finding.

3.3 Discussion

N. meningitidis is a major cause of bacterial meningitis epidemics worldwide. Only a small percentage of the general population is susceptible to meningococcal infections although a relatively large percentage is asymptomatic nasopharyngeal carriers. A major reason for variation in susceptibility to menigococcal infection is the difference in serum bactericidal activity against meningococci among individuals. In chapter II, I have shown that diminished SBA in some individuals occurs because of the presence of complement blocking IgG antibodies directed against pentameric 'AAEAP' motif contained in meningococcal outer membrane proteins Lip and Laz. In this chapter I showed that blocking IgG against *N. meningitidis* inhibits the SBA by downregulating the classical pathway of complement. Blocking requires glycosylation of the Fc portion of blocking IgG (Figure 3.4). This is in contrast with the mechanism of blocking against *N. gonorrhoeae* which does not require Fc (159). My results highlight important differences in the mechanism of blocking IgG in human serum directed against *N. meningitidis* and *N. gonorhoeae*.

In human IgG, Asn 297 in the CH2 domain of the constant region is substituted with N-linked oligosaccharides (Figure 3.1 A) (208, 209). Fc linked oligosaccharide is necessary for a number of antibody effector functions, including complement activation and antibody directed cell

cytotoxicity (208)208, (231, 232). Heterogeneity of the N-linked oligosaccharides can be manifest by differing terminal sugars including sialic acid, galactose or N-acetyl gluosamine; these sugar substitutions influence antibody effector functions (212). A possible role for MBL in blocking is consistent with the requirement of Fc glycan in mediating blocking. I have not yet characterized the terminal sugars present in Fcs of blocking antibody but identification of sugar-specific terminal substitutions on Asn 297-linked oligosccharides of blocking antibodies may strengthen the hypothesis of MBL being a part of the blocking antibody mechanism. I speculate that the Fc glycan of blocking IgG possesses either a terminal GlcNAc or mannose residue, which may enable it to engage MBL. Efforts to characterize the terminal glycan residue of the IgG are currently underway. Kaneko et al. have shown that terminal sialylation of IgG glycans inhibits inflammatory response mediated by human intravenous immunoglobulin (IVIG) preparations (214). However, I showed that sialylation has no role in facilitating blocking (Figure 3.7). On the contrary, desialylation of the blocking IgG enhanced its blocking activity. The inverse correlation between IgG sialylation and blocking also indirectly suggests the involvement of serum factor/s e.g. MBL in blocking because ‘capping’ of glycans by sialic acid decreases binding of MBL and possibly other serum factors (233).

It seems unlikely that MBL, which is an activator of the complement system, would directly contribute to blocking. I speculate that MBL may serve to recruit molecules that inhibit complement. Two candidates include α_2-macroglobulin and C1 inhibitor. Previous work in our laboraory showed that MBL in the context of serum did not activate complement on *N. gonorrhoeae*

because of the inhibitory effects of α_2-macroglobulin and C1 inhibitor on MBL (230). However, the exact mechanism used by these two molecules to render MBL ineffective in enhancing complement activation on gonococci was not defined. Several studies have shown that MBL is associated with α_2-macroglobulin (234-236) in serum, making my speculation plausible.

MBL deficiency predisposes to a variety of pathogenic infections (132) and its role in immune defenses may be particularly important in individuals who lack a mature repertoire of antibodies, particularly infants older that 3-6 months and young children. In these situations, MBL may serve as a substitute antibody functioning as an opsonin. MBL deficiency has been cited as a risk factor for meningococcal disease, but how MBL protects against this infection is not well understood (90). The level of binding of MBL to both *N. meningitidis* and *N. gonorrhoeae* (237, 238) is inversely proportional to the level of sialylation of lipooligosaccharide (LOS). MBL has been shown to initiate lysis and death of *N. gonorhoeae* only when pre-incubated before addition of potentially bactericidal serum (230).

I am currently purifying anti-H.8 specific antibody from serum by affinity chromatography over immobilized H.8/Lip. Once obtained, the N-linked glycans of the anti-H.8 blocking IgG will analyzed by mass spectroscopy. Although quite preliminary and speculative, this study privides a lead towards a possible negative role of MBL in complement activation and imparts an important role for Fc glycans in regulating complement activation on Neisseria. These findings have important implications for antibody-mediated immunity against pathogens.

3.4 Materials and Methods

Bacterial strains

N. meningitidis strains H44/76 (B:15:P1.7,16: ST-32; invasive isolate from Norway (1976)) (202) and MC58 (B:15:P1.7,16: ST-74; invasive isolate (United Kingdom (1985)) (6) have been described previously. Gonococcal strain 1291A has been used to analyze MBL binding (230) .

Monoclonal antibodies

mAb JAR 3 (IgG3) that recognizes variant 1 meningococcal factor H-binding protein (fHbp) that is expressed by strains H44/76 and MC58) was provided by Dr. Dan M. Granoff (Children's Hospital Oakland Research Institute). Anti-PorA mAb P1.7 (ascites fluid) was purchased from the National Institute of Biological Standards and Control (Potters Bar, Hertfordshire, U.K).

Serum bactericidal assays

Bacteria taken from an overnight culture on chocolate agar plates were re-passaged onto fresh chocolate agar and allowed to grow for 6 h at 37°C in an atmosphere containing 5% CO_2. Approximately, 2000 CFU of meningococci were incubated with IgG depleted serum (concentrations specified for each experiment), antibodies; the final reaction volume was maintained at 150 µl. Aliquots of 25 µl of the reaction mixtures were plated onto chocolate agar in duplicate at the beginning of the assay (t_0) and again after incubation at 37°C for 30 min (t_{30}). Survival was calculated as the number of viable colonies at t_{30} relative to t_0. The ability of purified NK3 IgG to

reduce the killing activity of a bactericidal reaction mixture by ≥50% compared to the baseline level of killing was defined as ‘blocking’.

Preparation of IgG depleted serum

All manipulations were performed at 4 °C. To deplete IgG, 2 ml of serum containing 10 mM EDTA to eliminate complement activation, was passed over a 1 ml protein G sepharose column (Sigma Catalog No. P3296; binding capacity >20 mg human IgG/ml) equilibriated with Tris-buffered saline (TBS); pH **7**. The fall through material (absorbed serum) was spin-concentrated and dialyzed against TBS using an Amicon Ultra-15 centrifugal filter device (Millipore). The absorbed serum was concentrated to near its original volume and stored as single-use aliquots at -80º C. Depletion of IgG was confirmed by flow cytometry, where complete loss of IgG binding to H44/76 was observed (Figure 2.12). The hemolytic activity of IgG depleted serum was quantified following reconstitution with 2 mM Ca^{++} and 2 mM Mg (resultant $C_{50}H$ ~50%). * We used 2X concentration of depleted serum vs. the intact serum counterpart in functional bactericidal assays to directly compare depleted with intact sera,

IgG purification

IgG that had bound to protein G sepharose following passage of serum through the column (as described above) was eluted with 0.1 M glycine-HCl, pH 3.0, and neutralized immediately with 0.2 M Tris in 0.5 M NaCl, pH 8.0. Protein containing fractions were spin-concentrated and dialyzed against Tris Buffered saline, pH. 7, using Amicon-15 Ultra device (30 kDa cutoff).

Generation of Blocking serum IgG F(ab)2 fragments.

Purified NK3 IgG was digested using immobilized pepsin bound to agarose beads (Pierce# 9001-75-6) in Tris-Cl buffer, pH 6 for 4hrs at 37^{o}C while shaking. The beads were spun down at 2000rpm for 5min. The F(ab)2 fragments in the supe was purified using sepharose G beads and spin-concentrated and dialyzed against Tris Buffered saline, pH. 7, using Amicon-15 Ultra device (30 kDa cutoff).

IgG De-glycosylation

Purified NK3 IgG was deglycosylated by incubation with 500U of PNGAse (NEB# P0705S) in sodium phosphate buffer, pH. 7.5, overnight at 37^{o}C. Deglycosylation of IgG was detected by running on 4-12% bis-Tris gel followed by staining with Imperial blue stain to see a distinct shift in migration of 150kDa IgG band. Deglycosylated IgG was then purified using a sepharose bound protein G beads and spin-concentrated/dialyzed against Tris Buffered saline, pH. 7 using an Amicon-15 Ultra device (30 kDa cutoff).

IgG De-sialylation.

Purified NK3 IgG was desialylation by incubation with 100 U of a 2-6/a 2-3 neuraminidase (NEB#P0720S) in sodium phosphate buffer, pH. 7.5 overnight at 37^{o}C. Desialylated IgG was then purified using a sepharose bound protein G beads and spin-concentrated/dialyzed against Tris Buffered saline, pH. 7 using an Amicon-15 Ultra device (30 kDa cutoff).

Flow cytometry analysis

Binding of human IgG and classical complement pathway components to *N. meningitidis* was determined by flow cytometry as described previously (147). Briefly, 40 µl of IgG depleted blocking serum along with anti-fHBP mAb was added to 10^8 bacteria suspended in 90 µl Hank's Balanced Salt Solution (HBSS) in presence or absence of purified blocking IgG for 30 min at 37 °C. Bacteria were washed and bacteria-bound IgG and classical complement pathway components were detected using FITC conjugated anti-human IgG (from Sigma) and anti-human C1q, C3 and C4 (from molecular probes). Binding of MBL in serum to *N. meningitidis* was incubating the bacteria incubating with the specific serum followed by primary probing with anti-MBL mAb 3E7 and secondary probing with FITC conjugated goat anti-mouse IgG. Data were generated using BD FACSCalibur (Becton Dickinson) flow-cytometer and analyzed using FlowJo software (Tree Star, Inc.).

Western blotting

Western blotting was used to determine binding of human IgG to meningococcal antigens. Bacterial lysates were separated on a 4–12% Bis-Tris gel (Invitrogen Life Technologies) using 2-(*N*-morpholino) ethanesulfonic acid (MES) running buffer, pH~7. Proteins were transferred to PVDF membranes (Millipore) and blocked with PBS-1% dry milk for 30 min at 24° C. Membranes were then incubated with heat-inactivated NHS (diluted 1:100 in PBS-0.05% Tween 20) for 15 h at 4°C. Membrane-bound IgG was detected using alkaline phosphatase conjugated anti-human IgG (Sigma) at a dilution of 1:1000 dilution in PBS-0.05% Tween 20 and 5-bromo-4-chloro-3-indolyl

phosphate/nitro blue tetrazolium (BCIP/NPT) purple liquid substrate (Sigma; Cat No. B3679). To determine level of sialylation of IgG binding to meningococcal antigens, blots were blocked with PBS-1% BSA for 30 min at 24° C. Sialylation of membrane-bound IgG was detected using biotinylated *Sambrucus niagra* Lectin and alkaline phosphatase conjugated streptavidin (Sigma) at a dilution of 1:1000 dilution in PBS-0.05% Tween 20 and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NPT) purple liquid substrate.

Generation of F(ab)2 fragments of purified IgG was detected by running untreated and immobilized pepsin treated IgG on 4-12% bis-Tris gel followed by staining with either 1) Imperial blue stain to see distinct 150kDa and 100kDa bands for whole IgG and F(ab)2 fragments or 2) transfer to a PVDF membrane followed by probing with alkaline phosphatase conjugated goat anti-human IgG Fab specific antibody. Deglycosylation of IgG was detected by running on 4-12% bis-Tris gel followed by staining with Imperial blue stain to see a distinct shift in migration of 150kDa IgG band.

Desialylation of IgG was detected by running neuraminidase treated IgG on 4-12% bis-Tris gel followed by transfer to a PVDF membrane. This was followed by staining with biotynilated sambrucus niagra lectin and alkaline phosphatase conjugated streptavidin and detection using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NPT) purple liquid substrate.

Chapter IV

Discussion

N. meningitidis is gram-negative diplococcus that is often found colonizing the human upper respiratory tract of about 10% of humans in non-epidemic settings. Invasive disease, fortunately, is a rare occurrence relative to rates of colonization. While most individuals are able to clear invading bacteria either through serum bactericidal activity or opsonophagocytosis, some persons remain susceptible to infection. Two major reasons cited for susceptibility to meningococcal disease are 1) lack of natural bactericidal antibodies in serum against the bacteria and 2) complement deficiency. In this thesis we have characterized an additional variable involved in modulation of natural immunity against *N. meningitidis* called 'blocking antibodies'. Blocking antibodies against meningoccci is not a new concept (163, 165, 166). The novelty of the antibodies we have described is their existence in normal sera, in contrast to prior reports that describe blocking antibodies in convalescent or post-vaccine sera. In this work we show that antibodies directed against specific outer membrane proteins Lip/H.8 and Laz of *N. meningitidis* can block the killing of the bacteria by otherwise bactericidal normal human serum or specific bactericidal antibodies directed against certain group B protein vaccine candidates.

The phenomena of blocking antibodies has been described several decades ago and can influence immunity against a wide range of pathogenic bacteria such as *Brucella abortus* (153), *B. melitensis* (154) and *Pseudomonas aeruginosa* (155) (156). More recently, blocking antibodies have been demonstrated against non-typhoidal strains of *Salmonella* in HIV patients in Africa (199). The first written account of blockings serum activity against arrange of gram negative bacteria including Neisseriae was provided

by Neisser and Wechsberg (196). Another important study, demonstrating the of role blocking antibodies in inhibiting killing of the bacteria by otherwise bactericidal serum was provided by Lewis Thomas in 1943 (162). This seminal study Thomas *et al*. is described in detail in the discussion of chapter II.

Previous studies have shown that IgA and IgG against serogoup C (164, 165, 239, 240) and W-135 (166) can block complement mediated effector functions in post vaccinee serum. However, in the studies mentioned above, the blocking antibodies identified were serogroup specific (i.e, directed against capsular polysaccharide following infection or immunization). Unlike previous studies, the blocking IgG identified by us in normal human serum is not serogroup (capsule) specific and can block SBA against a serogroup A meningococcal strain. At this point, we do not know whether the presence of these anti-H.8/Laz blocking antibodies predispose individuals to invasive meningococcal disease. A study comparing the presence of anti-H.8 antibodies in 'acute' sera (i.e., prior to the development of specific Ab against the invading strain) from persons with invasive disease with carefully matched controls during an epidemic of meningococcal disease may be one way to address this question.

Low immunogenicity of the serogroup B polysialic acid capsule has prompted extensive research to identify outer membrane proteins as potential vaccine candidates. Several genes encoding potential surface exposed protein antigens were identified (72, 73). An extensively studied and promising vaccine candidate is called fHbp (previously referred to as GNA1870 or LP2086) (75, 150). The ability of the blocking IgG to inhibit the

bactericidal activity of a monoclonal antibody against fHbp (Figure 2.5 A) is of considerable significance because it could identify individuals in whom the effectiveness of an fHbp-based vaccine may be blunted. Another interesting observation was that the blocking IgG did not inhibit the bactericidal activity of a mAb against PorA (Figure 2.5 B), an abundantly expressed antigen. PorA is the most immunogenic component both as a feature of natural immunity (106) and elicited by outer membrane vesicle based vaccines (191). Outer membrane vesicle vaccines 'tailor-made' for epidemics, such as those seen in Cuba, Norway and New Zealand, have proven to be efficacious (14, 16, 241, 242); the lack of an observed effect of anti-H.8 blocking IgG against anti-PorA Abs may be an advantage of these PorA-based vaccines.

A limitation of outer membrane vesicle vaccines is their relatively narrow stain coverage that is dictated by the type of PorA molecule(s) in the vaccine preparation. In order to circumvent this problem, one approach that has been tried is to delete PorA and over-express other more conserved proteins in the strain from which the vesicles are prepared. In light of our findings, it is important to consider the attenuating effects of anti-H.8 Abs that may be elicited by H.8/Laz in the vesicle preparations (69-71).

In the case of *N. gonorhoeae*, the specificity of blocking IgG is against the protein III (the homologue of Class 4 protein in *N. meningitidis*) (158). The role of Rmp as a blocking target in *N. meningitidis* is still unresolved and at times conflicting. One report suggests that mAbs directed against Rmp/Class 4 proteins do not appear to have significant blocking activity against *N. meningitidis* (161). In the same report, IgG purified from a recipient of the New Zealand phase II-9 vaccine trial a vaccinee whose serum reacted with

Rmp on an immunoblot was shown to posses blocking activity against serogroup B *N. meningitidis* strain H44/76 (161). Interestingly, the IgG from this vaccinee was shown to be directed against an epitope ‘’**EPEPEPEP**VVVV’ of Rmp, which, akin to the **‘AAEAP’** pentameric repeats of the H.8 antigen is Glu and Pro rich. Munkley *et al*, showed that the same anti-Rmp mAb used by Rosenqvist et al directed against an epitope ‘WKNAYFDK’ of the protein Rmp in meningococci could block the bactericidal activity of both mAbs and (otherwise bactericidal) serum (160). However the blocking activity of the serum (against meningococci) identified in our study, is not directed against Rmp. We show that the blocking antibody against meningococci in normal human serum is directed against the pentapeptide ‘AAEAP’ repeat containing H.8 domain of outer membrane proteins Lip (also called H.8) and Laz. These data suggest that subtle differences in accessibility and/or relative spatial orientation of membrane antigens in the two closely related Neisserial species may account for differences in the specificity of blocking antibody.

Surface components such as LOS, capsular polysaccharide and outer membrane proteins are important determinants of virulence of Neisseria spp (243-251). These surface molecules are highly variable in terms of antigenicity and expression levels between different serogroups and strains of meningococci. Efforts have focused on identifying a common antigenic determinant of pathogenicity among the different disease causing Neisseriae sp. Conserved antigens that are shared among diverse strains are also attractive from the point of vaccine development. The H.8 antigen was one of the first such identified antigens that was common to pathogenic Neisseriae

(167). However, the anti-H.8 antibody purified from humans or from mouse ascites was not bactericidal against Neiserriae, and further efforts to develop a H.8-based vaccine were abandoned (182).

Pathogenic Neisseriae express two genes encoding the “H.8 antigen” (252). The first identified gene was *lip* which is almost entirely comprised of the H.8 domain (186, 253) and the second gene that coded for the H.8-like pentatpeptide repeats is the lipid modified azurin (Laz) (186) which comprises of a N-terminal non conserved H.8 like domain and a 127 amino acid azurin-like domain. The sequences of both Lip and Laz are highly conserved across all pathogenic strains of *N. meningtidis and N. gonorrhoeae*. The fact that both proteins are found mostly in pathogenic neisseria (181) suggests an important function in virulence. Here we show generation of complement blocking antibodies against H.8 antigens of both proteins in normal human serum. It is also interesting that the ‘AAEAP’ pentameric repeats of the H.8 domain of Lip is duplicated as Laz in only Neisseria spp; other pathogenic microbes express azurin but without the “H.8-like” domain. The presence of these repeats must serve an important function and our work has shown that it contributes to immune evasion.

As discussed above, the presence and role of blocking antibodies against *N. meningitidis* has been previously reported. However, not much is known about the mechanism of action of these blocking antibodies. Earlier work done with blocking antibodies against Rmp in *N. gonorrhoeae* has shed some light in this aspect. Joiner *et al*. have shown that this blocking IgG against gonococcal Rmp has complement fixing ability and inhibits killing of the bacteria by diverting the activated C3 to alternate sites on the bacterial

surface (159). The blocking activity required only the F(ab)2 fragment of the anti-Rmp blocking IgG. Earlier work done by the same group showed that the C5b-9 (MAC) forms higher molecular weight complexes in resistant gonococcal strains as compared to susceptible strains (193). Collectively these data indicated that the blocking IgG against the Rmp protein of gonococci can itself fix complement itself but in an effete manner.

In contrast to blocking anti-Rmp antibodies against *N. gonorrhoeae* that do not appear limit the amount of complement activation on the bacterial surface, but rather alter the site of C3 deposition and perhaps the 'qualitative' aspects of MAC insertion, we show that anti-H.8 blocking IgG against *N. meningitidis* acts by down-regulating classical pathway activation by otherwise bactericidal antibodies (Figure 3.5). Also, this down-regulation of classical pathway activation and subsequent blocking activity required the N-linked oligosaccharide chains of the Fc portion of the blocking IgG (Figure 3.5 and 3.4). This highlights an important difference between the two pathogenic members of the Neisseria genus not only in terms of the target of blocking antibody target, but also the mechanism of blocking.

We further showed that the down-regulation of classical pathway by the anti-meningococcal blocking antibody that was evident when IgG-depleted serum was used as a complement source was not seen with purified complement components (C1 and C4) (Figure 3.8). This indicated a role for a distinct serum factor to mediate the blocking effect. Because of the requirement of Fc glycans in blocking, we hypothesized that a lectin in serum was the heat-stable molecule that participated in blocking. A soluble lectin, MBL could be the serum factor required for blocking based on the observation

that the use of antibody-depleted MBL deficient serum as a complement source did not facilitate blocking of JAR 3 mediated killing by NK3 IgG. We \need more experiments to validate this possibility. We speculate that MBL alone is unlikely to effect blocking because it has traditionally been regarded as an activator of complement. However, in serum, MBL can be associated with a complement inhibitor called α_2-macroglobulin (234) and we believe that it may be recruitment of α_2-macroglobulin that results in blocking. Prior work in our lab has shown that α_2-macroglobulin and another complement inhibitor, C1 inhibitor, can also block the complement activating properties of MBL on the gonococal surface (230). Experiments to address the role of α_2-macroglobulin and C1 inhibitor in the blocking initiated by human anti-H.8 antibodies are underway. Therefore we propose a hypothetical model in which the blocking IgG in normal human serum binds to the pentapeptide motifs of Lip and Laz in *N. meningitidis* and then recruits a MBL and α_2-macroglobulin (and also possibly C1 inhibitor) that down-regulates the activation of the classical pathway by a proximate, and otherwise bactericidal, antibody (Figure 4).

In order to bind to MBL, the N-linked glycans of the blocking IgG should terminate in either N-acetyl glucosamine (G0S0) (Figure 3.1 B) (207, 208, 254) or in high mannose residues (215, 255, 256). It is interesting that the murine mAb 2C3 directed against H.8 does not have blocking activity even though it is directed against similar or overlapping epitopes as the human anti-H.8. We believe that the glycan of the Fc of mAb 2C3 likely does not terminate in GlcNAc or Man and therefore may not bind to MBL. Mass spectrometric analysis of the glycan of 2C3 is currently being perfomed by our

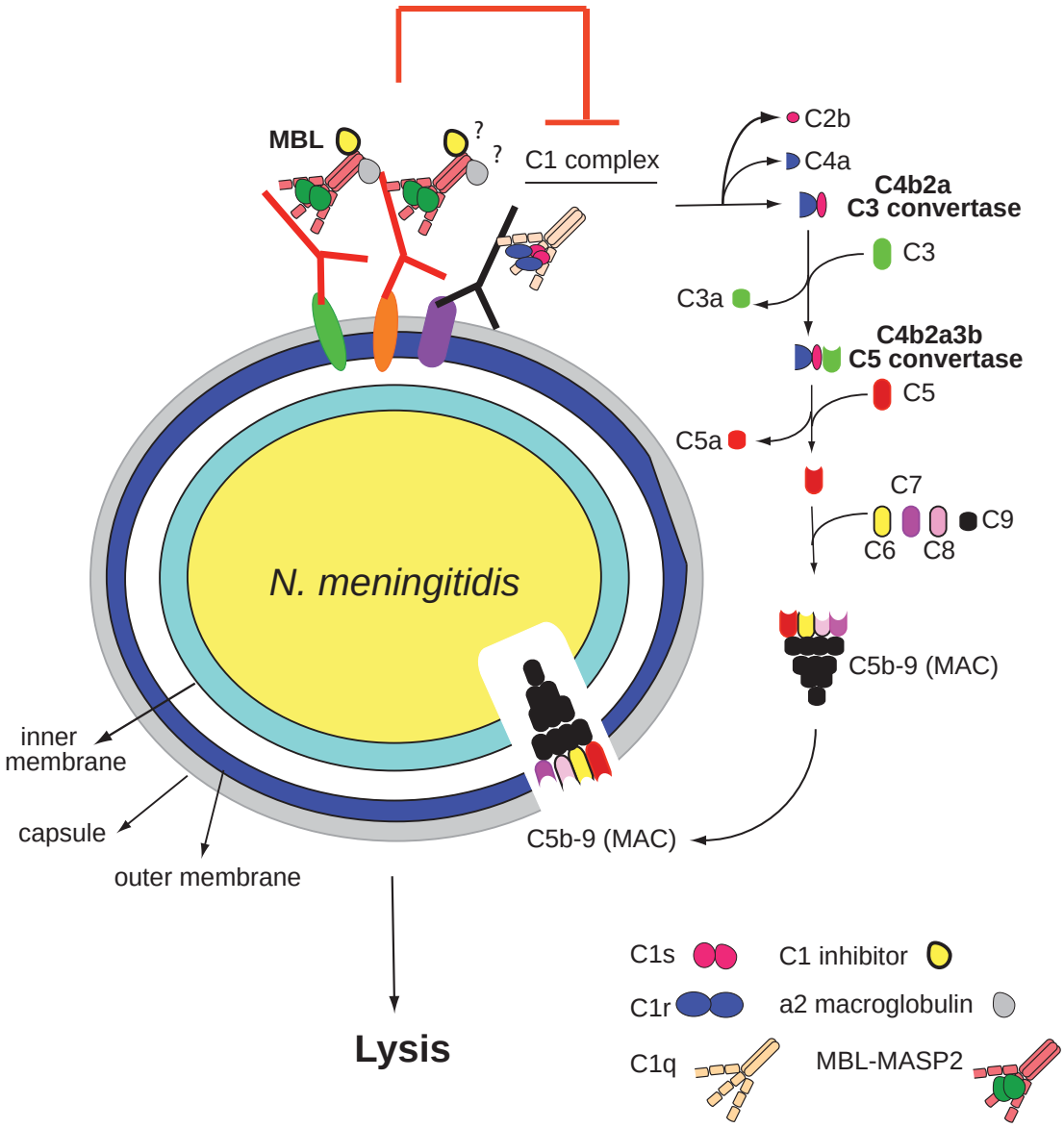


Figure 4. Proposed model for mechanism of action of blocking IgG. Based on ourdata I propose the following model to explain blocking by human anti-H.8 antibodies. Blocking IgG (red antibodies) binds to H.8 antigen -containing outer membrane proteins, Lip (green) and Laz (orange) on the surface of meningococci. These blocking IgGs bind in close proximity to antibodies (black) that are otherwise bactericidal, which are directed against relatively sparsely distributed antigens (purple) such as factor H binding protein. We hypothesize that the blocking IgG probably then recruits MBL (possibly in complex with C1 inhibitor , α2-macroglobulin) and prevents recruitment and activation of C1q by the bactericidal antibody, thereby blocking classical pathway activation and meningococcal killing.

collaborators at the National Research Council (Ottawa, Canada). As a part of our future directions we will purify the anti-H.8 human blocking IgG from the blocking serum using immobilized purified recombinant Lip/H.8 for analysis of its Fc glycans.

Another interesting point merits discussion. On one hand the findings in Chapter II show that specific outer membrane proteins such as, Lip and Laz are targets for complement blocking IgGs against *N. meningitidis*. On the other hand, the findings in Chapter III show that Fc N-linked glycosylation (we speculate that they teminate in either GlcNAc or Man residues) of the blocking IgG may facilitate recruitment of MBL, which results in blocking activity. A key question is which of the two factors a) the target antigen or b) IgG glycosylation is determines whether an anti-meningococcal IgG acts as a blocking antibody. Stated differently, would a bactericidal antibody directed against bactericidal targets such as fHbp or Por1.7 function as a blocking antibody if their Fc N-linked glycans were to bind to MBL? Studies to address this question will require characterization of the Fc glycans of these antibodies and modifying them (perhaps enzymatically) to resemble the glycans of anti-H.8 antibodies. Alternatively, the same chimeric human-mouse (Fc-Fab) antibodies containing terminal Man residues can be generated by expressing them in Lec-1 CHO cells that lack the enzyme N-acetyl glucosaminyl transferase I (257) (ref. Figure 3.1 A, B). These experiments will enable us to better understand how blocking occurs on the Neisseria but are beyond the scope of this thesis.

Another possible explaination for why the blocking IgG is directed specifically against H.8 could be because the H.8-specific B cell clones in

these individuals are deficient in the enzyme galactosyl tranferase that adds the terminal Gal residues on to the N-linked glycans of IgG or the enzyme mannosidase that cleaves the excess mannose residues in the N-linked glycan prescursor chain during post translational modification in the golgi apparatus. As a result the H.8 specific IgG terminates in GlcNac or high mannose residues, which might result in binding serum factors involved in blocking possibly MBL as our preliminary data indicates.

Anti-H.8 blocking IgG in normal human serum may contribute to variations observed in natural immunity and susceptibility to *N. meningitidis*. This study also provides insight into the mechanism of this blocking activity and identifies an important biological role for antibody glycosylation and function. Overall, the study provides important insights into natural immunity against *N. meningitidis* and has implications for vaccine develpment against meningococcal disease.

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