UNIVERSITY OF SOUTHAMPTON

INTERACTION OF *Neisseria meningitidis* WITH CELLS OF THE HUMAN MENINGES

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Submitted for the degree of Doctor of Philosophy

DEPARTMENT OF MICROBIOLOGY AND PATHOLOGY

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

SEPTEMBER 2000

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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Neisseria meningitidis is the leading cause of meningitis in the Western world. Recent studies have begun to elucidate the molecular mechanisms underlying the interactions of pathogenic meningococci with human epithelial and endothelial cell barriers in the early stages of meningococcal disease. However, there is little information on the later stages of infection concerning the interactions of meningococci with the cells of the leptomeninges. The human meninges comprise a series of three membranes- the dura mater, arachnoid and pia mater- which surround and protect the brain and the spinal cord. During meningitis, meningococci are thought to bind to and possibly invade the leptomeninges (arachnoid and pia mater), and initiate a compartmentalised inflammatory response. Normal leptomeningeal cells do not grow in culture whilst cells can be cultured from meningiomas which are tumours of the meninges. Meningioma cells share many of the same basic characteristics as normal meninges and can therefore be used as a model for investigating meningococcal - meningeal cell interactions.

Meningiomas of the meningothelial, transitional and anaplastic subtypes were taken at biopsy and meningioma cell lines were cultured from them. These cell lines were characterised for the presence of desmosomal desmoplakin which is indicative of normal leptomeninges and meningiomas, and also for the presence of other cytological markers including epithelial membrane antigen (EMA), cytokeratin and vimentin. A model was established to study the interactions of *Neisseria meningitidis* with meningioma cells. A panel of meningococcal phenotypic variants was selected from the pathogenic strains MC58 and MC59 which differed in their pilus, Opa and Opc expression. All bacteria were encapsulated and of the same LPS immunotype, although an acapsulate mutant was included as a comparison. Meningioma cells were cultured and infected with these meningococci. Total bacterial association was determined by viable counting, and internalisation was determined by pre-incubation with gentamicin. Meningococcal association with meningioma cells was also visualised by scanning and transmission electron microscopy and by confocal microscopy.

The adherence of encapsulated meningococci to human meningioma cells was predominantly pilus-mediated and subject to antigenic variation of the pilin subunit. In organisms with less adherent pili, the presence of Opa protein did increase adherence slightly, but Opc had no effect. Experimental evidence suggests that meningococci do not invade meningioma cells.

The work described in this thesis has established an *in vitro* model for studying the interactions of *Neisseria meningitidis* with cells of the human meninges which will can be used for further studies including receptor-mediated adhesion of meningococci to the meninges, and the production of pro-inflammatory cytokines and chemokines in the inflammatory response of meningitis. A greater understanding of these mechanisms may highlight possible avenues for therapeutic strategies to alleviate the sequelae associated with meningococcal meningitis.

PUBLICATION

Parts of the work presented in this thesis have formed the basis of the following publication:

Hardy, S.J., Christodoulides, M., Weller, R.O. and Heckels, J.E. (2000) Interactions of *Neisseria meningitidis* with cells of the human meninges. *Mol Microbiol* **36:** 817-829.

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ACKNOWLEDGEMENTS

I am eternally grateful to the many people who have afforded me considerable help in various forms throughout my PhD and during the construction of this thesis.

In particular, I would like to thank my family for their unconditional support throughout the whole process, and to Mark for putting up with the colonisation of his study during the write-up. To Myron for putting up with my years in the lab and endless emails afterwards, and Professor Watt for his pearls of wisdom throughout. I would also like to thank members of LC83 and Molecular Microbiology, past and present, for the fun times, and the people of the Imaging Unit and Neuropathology Department for the excellent help and advice that they willingly gave me.

However, perhaps the most thanks should go to John Heckels without whom I literally would not have been around to write this thesis. Thank you John.

DEDICATION

This thesis is dedicated to the memory of my Mother for the unconditional love, guidance, support and encouragement that she gave to me.

ABBREVIATIONS

ANOVA	analysis of variance
ASGP-R	asialoglycoprotein receptor
ATP	adenosine triphosphate
BBB	blood-brain barrier
B-CSFB	blood-cerebrospinal fluid barrier
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BMEC	brain microvascular endothelial cells
BSA	bovine serum albumin
CD	cytochalasin D
cDNA	complementary DNA
CEA	carcinoembryonic antigen
cfu	colony forming unit
CGM	CEA gene family member
CHO	chinese hamster ovary
CMP-NANA	cytidine monophosphate-N-acetyl neuraminic acid
CMVEC	cerebral microvascular endothelial cells
CNS	central nervous system
CSF	cerebrospinal fluid
dec'	decomplemented
DIC	disseminated intravascular coagulation

DMEM	Dulbecco's modification of eagles medium
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EMA	epithelial membrane antigen
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FNR	fibronectin receptor
GFP	glial fibrillary acidic protein
GM-CSF	granulocyte macrophage colony stimulating factor
GPI	glycosylphosphatidylinositol
HBEC	human brain endothelial cells
HBSS	hanks balanced salt solution
HMEC-1	human microvascular endothelial cells
HMFG	human milk fat globule-2
HSPG	heparan sulphate proteoglycan
HUVECS	human umbilical vein endothelial cells
ICP	intracranial pressure
IF	intermediate filament

Ig	immunoglobulin
Il	interleukin
IGF-I	insulin-like growth factor-I
IL	interleukin
KDa	kilodalton
LAMP	lysosome-associated membrane protein
LAP	lysosomal acid phosphatase
LBP	lipopolysaccharide binding protein
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LSCM	laser scanning confocal microscopy
mAb	monoclonal antibody
mCD14	membrane CD14
MCP-1	monocyte chemoattractant protein 1
MIP-1/2	macrophage inflammatory protein 1/2
moesin	membrane-organising extension spike protein
m_r	relative molecular mass
mRNA	messenger ribonucleic acid
NBT	nitro blue tetrazolium
OMP	outer membrane protein
PBS	Dulbecco's phosphate buffered saline
PBSB	Dulbecco's complete phosphate buffered saline

PCR	polymerase chain reaction
PI	propidium iodide
PK	pyruvate kinase
PMN	polymorphonuclear cell
PMSF	phenylmethylsulphonylfluoride
PP	proteose peptone
PVS	perivascular space
RANTES	regulated on activation, normal T cell expressed and secreted
RBC	red blood cell
RT-PCR	reverse transcriptase polymerase chain reaction
SAH	sub-arachnoid haemmorrhage
SAS	subarachnoid space
sCD14	soluble serum CD14
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SIgA	secretory immunoglobulin A
S-pilin	soluble pilin
TEM	transmission electron microscopy
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-1β	transforming growth factor beta
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	tumour necrosis factor alpha

Tris	tris(hydroxymethyl)methylamine
TBS	tris buffered saline
TTBS	tween-tris buffered saline
UHQ	ultra high quality water
VN	vitronectin
VNR	vitronectin receptor

CHAPTER 1 INTRODUCTION

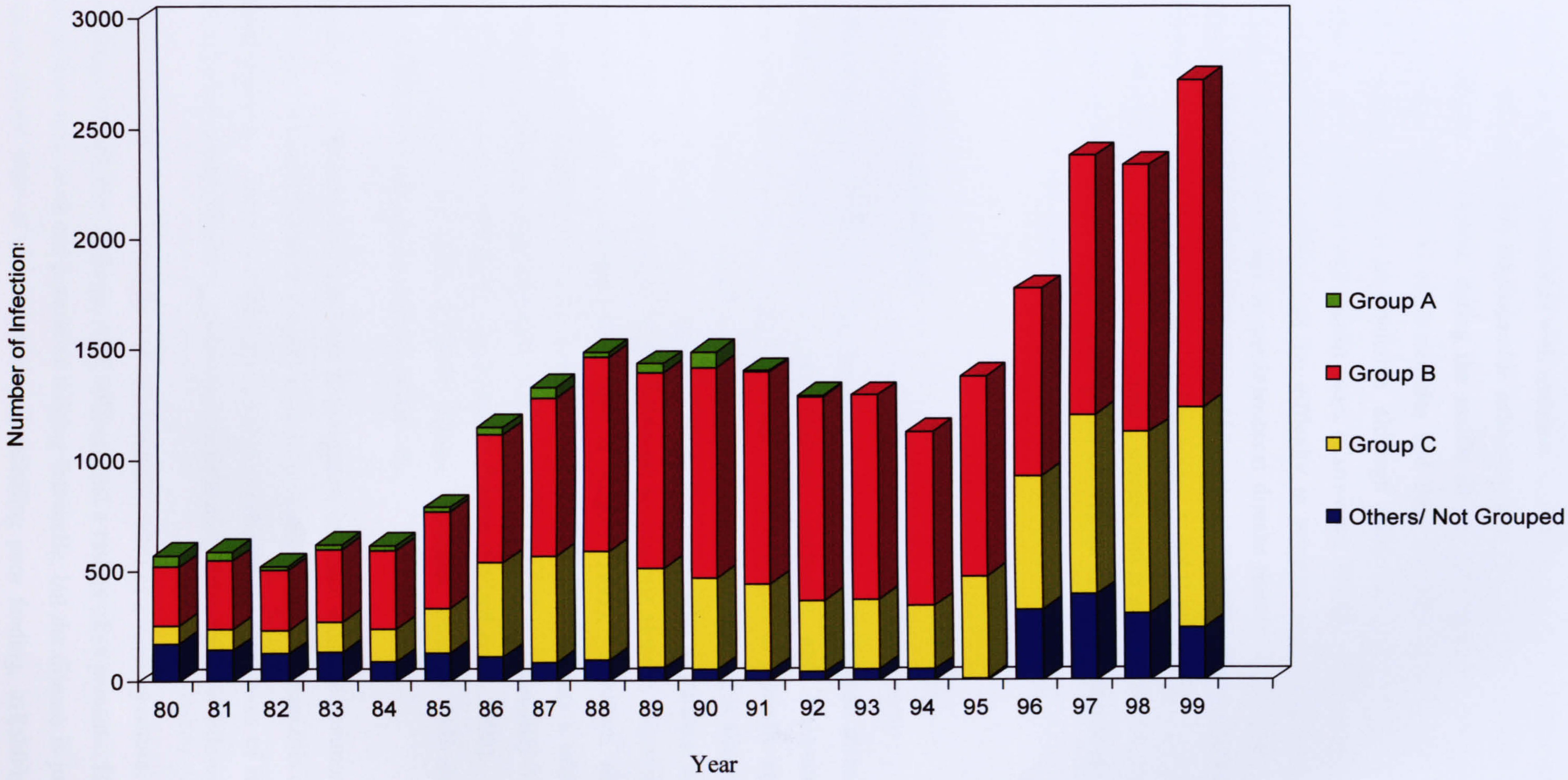
1.1 Neisseria meningitidis

Neisseria meningitidis is responsible for worldwide epidemic and endemic human disease, ranging from sub-clinical nasopharyngeal infection to fulminant, life threatening disease, and is the leading cause of meningitis in the Western world. In the UK, after the introduction of successful antibiotic therapy and despite increased public awareness of the symptoms associated with infection, in recent years the mortality rate of meningococcal disease has only decreased slightly, whereas the incidence of disease has increased (Figure 1.1) (Tunkel *et al.*, 1990; Cartwright, 1995a).

Although Hippocrates described symptoms now associated with meningitis in the 5th century BC, in modern times, the first clear description of meningococcal disease was not until 1806 when Vieusseaux identified it as a clinical entity. Subsequent identification of the aetiological agent was not achieved until 1887 when Anton Weichselbaum isolated an organism from the meningeal exudate of meningitis patients. The bacteria were initially called *Diplococcus intracellularis meningitidis* and were not assigned to the genus *Neisseria* until the turn of the twentieth century (Fredlund, 1993; Cartwright, 1995a).

The genus *Neisseria* includes the pathogenic *Neisseria meningitidis* (meningococci) and *Neisseria gonorrhoeae* (gonococci), but also includes organisms that generally do not cause disease, such as *Neisseria lactamica* and *Neisseria sicca*. Meningococci are aerobic, gram negative diplococci that are closely related to gonococci. Both of these species are obligate human pathogens, and both have an affinity for mucosal surfaces. The only known natural reservoir of meningococci is the human nasopharynx, and at any given time, approximately 10% of the British population are carriers. Transmission between individuals is thought to occur via aerosols of nasopharyngeal secretions between close contacts (Hart & Rogers, 1993; Cartwright, 1995a). Meningococcal disease is most common amongst young children (less than one year old), and there are smaller peaks of infection amongst teenagers and those entering closed communities, such as fresh military recruits (Cartwright, 1995a). In the UK, *Neisseria meningitidis* of serogroup B are the most prevalent cause of meningococcal meningitis, and in 1999 accounted for about 53% of cases, followed by 36% due to group C, and the rest from other serogoups or non-groupable strains (from PHLS communicable disease reports, 2000). Group A disease is more prevalent in third world countries, where it is associated with epidemics, especially in the "meningitis belt" of sub-Saharan Africa. Other serogroups rarely cause disease although group Y meningococci seem to have a predilection for causing pneumonia (Cartwright, 1995b).

Figure 1.1: Incidence of meningococcal disease in the UK over recent years.
Data compiled from PHLS communicable disease reports.



A death rate of up to 90% is associated with untreated cases and prior to the antibiotic era (Wolf & Birbira, 1968). The use of the sulphonamide antibiotics in the late 1930's resulted in a dramatic decrease in mortality. However, during the middle of the 20th century meningococcal strains became increasingly resistant to sulphonamides and their use was discontinued. Fortunately meningococci remain sensitive to penicillin although there has been a gradual decline in susceptibility to this antibiotic over recent years (Cartwright, 1995a). The shortage of medical expertise in third world countries, and the difficulty in accurate early diagnosis in developed countries means that mortality due to meningococcal disease remains unacceptably high. This coupled with the prospect of antibiotic resistance raises the desirability of prophylactic measures to prevent disease. A polysaccharide vaccine against groups A and C gives limited protection against disease in adults and a new vaccine against group C disease that is supposed to be immunogenic in infants has just been released. Yet, despite global efforts, an effective vaccine against group B meningococcal infection has remained elusive.

1.1.1 Meningococcal disease

In 1948, Banks (Banks, 1948) described meningococcal disease as a 'protean disease' due to its variable nature; he defined several meningococcal syndromes and designated the term 'meningococcosis' to encompass them all. Today, *N. meningitidis* is typically associated with causing illness of an acute form, usually meningitis and/or septicaemia, but it is also responsible for benign and chronic syndromes. Most commonly, acute disease presents as purulent meningitis, and this accounts for up to 50% of cases, whilst fulminant septicaemia alone is responsible for about 10% of disease (Steven & Wood, 1995; Hart & Rogers, 1993). However, meningitis and septicaemia are not mutually exclusive and the remainder of cases presents a mixed picture of disease. Isolated meningococcal meningitis has a mortality rate of approximately 5%, but this is increased if there is some septicaemic component to disease (Pollard *et al.*, 1998). It is not clear why or how meningococci cause different disease processes amongst individuals, but the immunological status of the subject may play a key role.

Infected patients can present with a number of symptoms, of which most are common to other less serious illnesses. As meningococcal disease tends to progress rapidly, misdiagnosis can contribute to increased mortality. There is often considerable overlap of the symptoms of meningococcal meningitis and septicaemia, but these syndromes can be broadly categorised as follows:

Meningitis is typically characterised by a severe headache, often of a bursting nature. There is also fever, vomiting, photophobia, lethargy, neck stiffness and a rash is often present. The very young will often present with fever and a tense or bulging fontanelle, but the disease is just as likely to present as an altered state of alertness or mood including poor feeding, irritability, and a high

pitched cry. With disease progression, signs of cerebral dysfunction (confusion, lethargy, decrease in consciousness) and raised intracranial pressure (ICP) may develop (including coma, bradycardia, cranial nerve palsies) (Tunkel & Scheld, 1995; Pollard *et al.*, 1998). Laboratory findings regarding the CSF in meningococcal meningitis, typically show increased protein concentration and raised leukocyte count (predominantly neutrophils), with a decrease in glucose concentration as well as in the overall blood:CSF glucose ratio (Tunkel & Scheld, 1995; Brandtzaeg, 1995).

Septicaemia is typified by fever, rash, vomiting, myalgia, abdominal pain sometimes with diarrhoea, tachycardia, hyptotension and cool peripheries, and there may be headache even in the absence of meningeal irritation. The early symptoms of disease are identical to other viral illnesses and when myalgia is prominent, meningococcal septicaemia is often confused with influenza. Fulminant septic shock can develop at a frightening speed, with disseminated intravascular coagulation (DIC) and pulmonary oedema, and in fatal cases death is usually a result of irreversible circulatory collapse and multiple organ failure (Pollard *et al.*, 1998; Steven & Wood, 1995; Devoe, 1982).

Of particular importance in meningococcal disease is the presence of a petechial or purpuric rash, haemorrhages in the skin caused by microbial damage to the endothelium with activation and depletion of the human coagulation system (Brandtzaeg, 1995), and found in the majority of cases of meningococcal disease. The presence of a rash is more likely to be present in illness of shorter duration prior to hospitalisation.

Approximately 14% of the survivors of acute meningococcal disease are left with long term sequelae (Steven & Wood, 1995). In meningitis survivors, these usually take the form of neurological complications due to cerebral herniation, resulting from raised ICP and thrombotic occlusion of blood vessels within the subarachnoid space (SAS) resulting in cerebral vasculitis. Hearing impairment is the most common neurological complication and probably occurs in about 9% of paediatric cases (Steven & Wood, 1995). The cranial nerves pass through the SAS and cranial nerve palsies also occur in meningococcal meningitis (Weller, 1990). The sequelae of meningococcal sepsis are generally a result of impaired circulation, most commonly renal failure and skin infarction, with some survivors even requiring skin grafts or the amputation of digits or whole limbs (Hudson *et al.*, 1993).

Other rarer forms of meningococcal disease include benign and chronic meningococcaemia, with *N. meningitidis* being present in blood cultures of both. The benign (or occult) form is an acute illness with fever and often a rash but no symptoms of meningitis or septicaemia; more serious disease may progress but the disease often resolves spontaneously. The symptoms of chronic disease last for longer than a week and include fever, a rash and painful joints (Steven & Wood, 1995).Other manifestations of meningococcal disease include arthritis, pericarditis and vasculitic skin lesions. In addition, *N. meningitdis* can cause primary arthritis, urethritis and pneumonia without features of acute meningococcal disease (Andersson & Krook, 1987).

1.1.2 Meningococcal carriage and immunity

Meningococci are normally carried in the human nasopaharynx as commensal organisms. In temperate climates the peak carriage rates of 25-40% occur in teenagers and young adults, whereas the peak incidence of disease is in children under one year of age. Transmission between individuals occurs during prolonged close contact, and probably by airborne spread of aerosol droplets of nasopharyngeal fluids, as occurs during sneezing and coughing. In susceptible individuals the development of disease is quite rapid, a virulent strain of meningococci may cause invasive disease within 2-10 days of acquisition (Hart & Rogers, 1993). There is general acceptance that the presence of bactericidal antibodies against meningococci in serum correlates with natural immunity to this infection (Goldschneider *et al.*, 1969). It has been suggested that in children, immunity against meningococci may be induced primarily by the carriage of the closely related but non-pathogenic organism *Neisseria lactamica*, resulting in the production of cross-reacting antibodies, as well as by the asymptomatic carriage of meningococci themselves (Cartwright, 1995b). The peak incidence of disease in children between the ages of six months and two years is probably due to the decline of maternal immunity coupled with the fact that such infants have not yet produced their own antibody repertoire (Goldschneider *et al.*, 1969; Cartwright, 1995b).

Bactericidal antibodies are those antibodies capable of activating the complement cascade leading to the formation of a membrane attack complex (MAC) and ultimately bacterial lysis (Griffiss, 1995). Complement-mediated lysis of meningococci by the classical and alternative pathways is believed to be the most important defence against invasive disease, and people who are deficient in terminal complement components (C5b-C9) are known to be at increased risk of infection, especially if they also lack antibodies against meningococci (Jarvis & Vedros, 1987; Densen, 1989).

It is possible that IgA antibodies for meningococci can have deleterious effects on the immunity to meningococcal infection. IgA molecules are less efficient complement activators than IgG or IgM, and IgA bound to meningococcal capsules are not bactericidal. From patients convalescing from meningococcal disease, IgA molecules have been identified that can block the binding of IgG and IgM binding and thus prevent their bactericidal activities. In addition, it has been proposed that exposure to certain enteric organisms can result in the production of secretory IgA antibodies that cross-react with meningococci in the nasopharynx, and which subsequently block the actions of lytic IgG and IgM molecules upon meningococcal invasion (Griffiss, 1995).

In summary, immunity to meningococcal infection is still not fully understood, but the presence of meningococcal antibodies and an intact complement system is recognised as being important, if not essential, in successful host immunity. The carriage of commensal *Neisseria* species and non-pathogenic meningococci throughout childhood is also likely to have a protective role.

1.1.3 Molecular structure of the meningococcus

Existing as diplococci with the flat surfaces of each organism opposed to one another, meningococci are approximately 0.6 x 0.8μm in size. As with other gram negative bacteria the cell envelope of the meningococcus consists of two cell membranes, one each side of a rigid peptidoglycan layer, and is frequently surrounded by an acidic polysaccharide capsule. The outer membrane (Figure 1.2) houses the bacterial structures that come into closest proximity with the host cells, and some of these structures are known to be important in meningococcal colonisation, invasion and disease outcome. Many of the exposed epitopes of meningococcal surface structures are known to vary (antigenic variation), whilst the expression of some surface structures can be turned on or off (phase variation). Antigenic variation may be an important evasion mechanism of the host immune response. Structures present in the outer membrane include lipopolysaccharide, pili, and the outer membrane proteins (OMPs), Opa, Opc and porins. During the course of normal growth, meningococci are known to release a substantial amount of the outer cell wall layer into the surrounding medium in the form of blebs (Devoe & Gilchrist, 1973). Such blebs contain a large proportion of LPS and also outer membrane proteins.

1.1.3.1 Capsule

Meningococci express a polysaccharide capsule on the surface of its outer membrane, which is not present in the related *Neisseria gonorrhoeae*. Meningococci are divided into the serogroups A, B, C, D, 29-E, H, I, K, L, W-135, X, Y, Z according to structural and antigenic differences in their capsular polysaccharide (Poolman *et al.*, 1995). The biosynthesis pathway of the group B meningococcal polysaccharide is controlled by the *cps* gene complex, which consists of five regions. Different regions are involved in the synthesis of capsular polysaccharide, its transport from the cytoplasm to the periplasm and from periplasm to the cell surface, and for the regulation of capsule production (Frosch *et al.*, 1990).

The capsule of the disease-associated serogroup B consists of repeating units of N-acetylneuraminic acid (sialic acid) and serogroups C, Y and W-135 also contain sialic acid in their polysaccharide. The presence of sialic acid in the capsule confers resistance to human complement-mediated attack. Attempts to produce a group B anti-capsule vaccine have failed due to the poor immunogenicity of this polysaccharide. This is thought to be due to structural similarities between the bacterial capsule and polysaccharides present on neural cell adhesion molecules (NCAMs) in the host brain (Finne *et al.*, 1983).

1.1.3.2 Lipopolysaccharide (lipooligosaccharide, endotoxin)

The outer membrane lipopolysaccharides (LPS) of the related organisms, *N. meningitidis* and *N. gonorrhoeae,* are structurally and antigenically similar. LPS is a bacterial glycolipid consisting of a

hydrophobic lipid backbone (lipid A) and a variable hydrophilic core of oligosaccharide moieties, but lacking O-side chains (van Putten, 1993). It is possible to use neisserial LPS as a typing antigen, resulting in a possible 12 immunotypes, designated L1 through to L12 (Poolman *et al.*, 1995). Immunotypes result from differences in the oligosaccharide component of LPS and most strains often express more than one immunotype. In group A meningococci, immunotypes L8, L9, L10 and L11 are found, with L10 and L11 being most prevalent and uniquely associated with this group. The immunotypes L1 through L9 are found in group B and C meningococci with L3,7,9 being more prevalent than the L2 and L1,8 immunotypes (Verheul *et al.*, 1993). The majority of meningococci isolated from the nasopharynx of carriers are of a short LPS species, whilst isolates from the blood and CSF are of a long LPS species. The longer LPS molecules contain additional carbohydrate residues, lacto-neotetraose groups (Galβ1-4GlcNAcβ1-3Galβ1-4Glc), that are endogenously sialylated (Meyer *et al.*, 1994). The addition of sialic acid to the terminal sugar residues is mediated by a membrane-associated sialyltransferase using cytidine 5'-monophosphate-*N*-acetylneuraminic acid (CMP-NANA) as the sialyl donor. Both meningococci and gonococci possess a sialyltransferase but only the former can synthesise CMP-NANA (Meyer *et al.*, 1994).

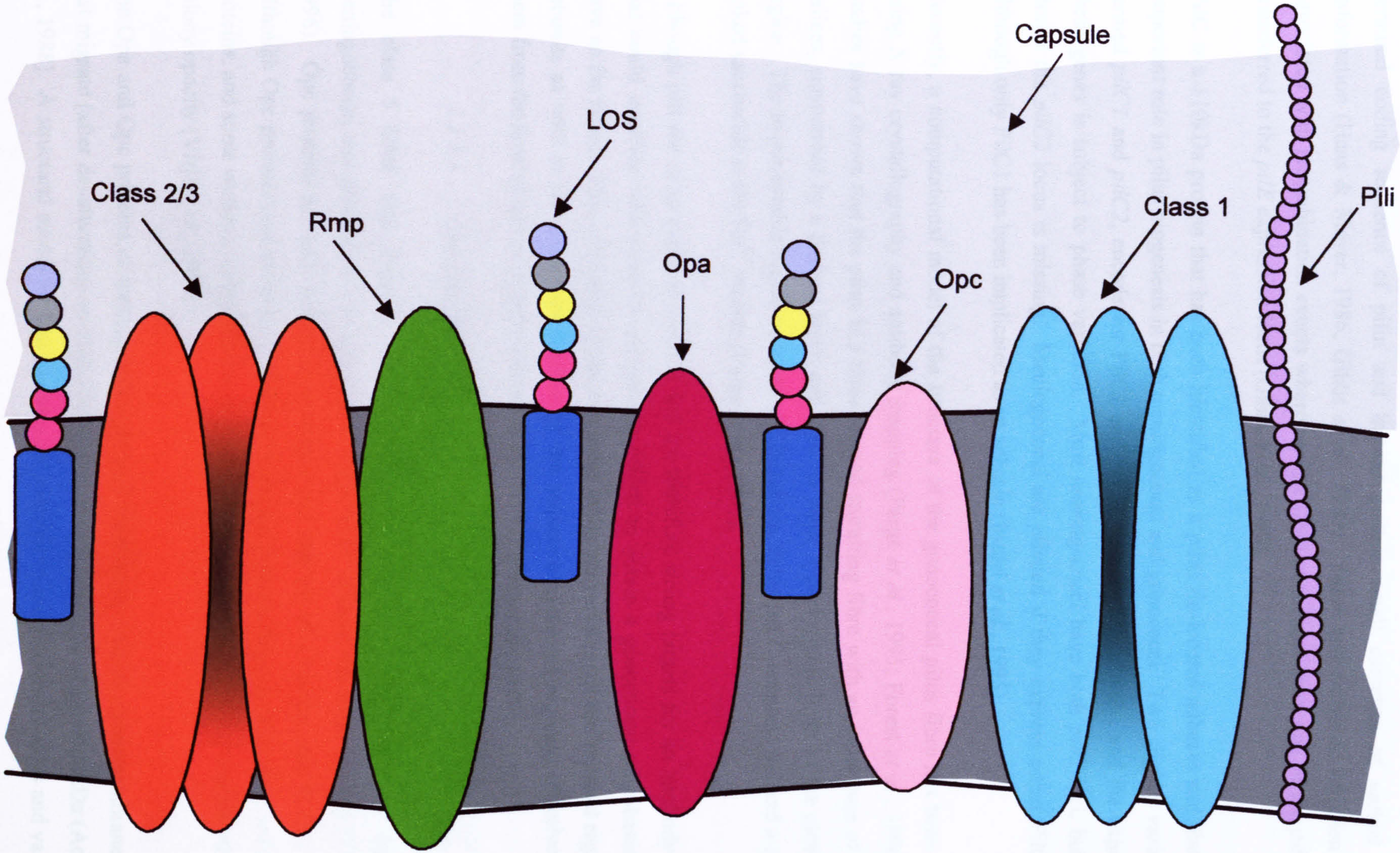
LPS is an important virulence factor in the pathogenesis of meningococcal infection and, as an endotoxin, has a key role in the initiation of the human inflammatory response resulting in meningitis.

1.1.3.3 Pili

Pili are filament-like structures that extend several micrometres from the meningococcal and gonococcal surface, with a diameter of approximately 6nm, and they are visible under TEM. Pili have several functions; they are required for the initial binding of meningococci to human cells, are implicated in interbacterial adherence and may play a role in transformation and in twitching motility (Meyer *et al.*, 1994). Neisserial pili undergo phase and antigenic variation, and the latter is considered a means by which bacteria evade the host immune system. Pilus fibres are formed by the ordered association of thousands of identical pilin (PilE) subunits, plus a few copies of pilus-associated proteins. Two types of pili, class I and II, have been identified in the meningococcus, whilst only the former have been found in gonococci. The classes of pili are antigenically and structurally distinct and are distinguished by the specific reaction of class I pili with the mAb SM1 which recognises the EYYLN epitope in the N-terminal domain of pilin (Diaz *et al.*, 1984; Virji *et al.*, 1989).

Neisserial pilin molecules consist of approximately 160 amino acids, and belong to the family of surface appendages termed type IV pili, whose other members include *Pseudomonas aeruginosa, Vibrio cholerae* and *Escherechia coli.* The N-terminal sequences of pilins of these family members share a high degree of homology and are highly conserved (Tonjum & Koomey, 1997). In contrast, the C-terminal domain is the predominant site of antigenic variation. Meningococcal class I pilin

Figure 1.2: Structure of the meningococcal outer membrane
Kindly provided by Professor JE Heckels



range in size from 17 – 22kDa, whilst the class II subunits are slightly smaller ranging from 15 – 17kDa (Diaz *et al.*, 1984). The neisserial genome harbours a complete pilin expression locus (*pilE*) that carries the pilus structural genes, and incomplete silent gene loci (*pilS*) that lack the common N-terminal coding sequence of pilin and instead contain small cassettes of variant sequence information (Haas & Meyer, 1986; Gibbs *et al.*, 1989). Phase and antigenic variation of pili is associated with recombination events where variant DNA sequences from one of the *pilS* loci are transferred to the *pilE* expression locus (Gibbs *et al.*, 1989).

PilC is a 110kDa protein that has been identified as a pilus tip-located adhesin and that plays an important role in pilus biogenesis in both meningococci and gonococci. Two slightly variant genes, termed *pilC1* and *pilC2*, encode for PilC1 and PilC2 proteins respectively, and the expression of these genes is subject to phase variation. Most meningococci have both *pilC* loci, but in some strains the *pilC2* locus is missing. Meningococci are piliated if they express either PilC protein, although only PilC1 has been implicated as an adhesin (Rudel *et al.*, 1995).

Recently, a computational model of the structure of the gonococcal pilus fibre has been proposed using X-ray crystallography and antibody mapping (Parge *et al.*, 1995; Forest *et al.*, 1996). These studies have shown that the pilus is a three-layered spiralling fibre with an inner core of coiled α-helices, surrounded by a β-sheet layer, and with an outer hypervariable layer as the most exposed region. The hypervariable region consists of a β-hairpin, extended C-terminal tail and a covalently linked saccharide at the Ser^{63} residue (Parge *et al.*, 1995).

Although pili are in an exposed position and are therefore strong targets for an antibody response that could interfere with receptor recognition, efforts to obtain a gonococcal pilus-based vaccine have so far failed. This is because of the enormous antigenic variation of the exposed regions pilin subunits as well as the concealed location of the sequence-conserved regions, effectively hiding them from the host's immune system (Meyer *et al.*, 1994; Forest *et al.*, 1996).

1.1.3.4 Opa and Opc proteins

The class 5 OMP Opa proteins are responsible for the opaque phenotype of agar-grown meningococcal and gonococcal colonies when viewed under oblique sub-stage lighting (Virji *et al.*, 1996). Opc proteins are only present in meningococci and are of a similar size to Opa proteins. Although Opc protreins are structurally distinct from Opa proteins they are still classified as class 5 proteins, and some workers justify this because they have observed that Opc expression still confers colony opacity (Virji *et al.*, 1992b).

The Opa and Opc proteins of meningococci are heat-modifiable, trimeric, transmembrane proteins that migrate (after denaturation) on SDS-PAGE with an apparent size of about 28kDa (Achtman *et al.*, 1988). A structural model for Opa proteins predicts four surface-exposed loops and variation in

these domains characterises distinct Opa proteins; loop 4 is conserved, loop 1 is semivariable with some structural similarity between different Opa proteins, while loops 2 and 3 are hypervariable (Bhat *et al.*, 1991; Kupsch *et al.*, 1993). It is predicted that Opc contains ten transmembrane strands and five surface exposed loops (Merker *et al.*, 1997). Opa proteins are subject to a high frequency of phase and antigenic variation, whereas Opc undergoes phase variation but is also subject to different levels of expression, which may vary from zero to the intermediate or high level. Whilst a single gonococcal strain can possess up to 11 or 12 *opa* genes encoding for nine or more Opa proteins (Bhat *et al.*, 1991), meningococci only encode up to four such proteins (Virji *et al.*, 1993a). In contrast, only one copy of the *opc* allele is present per genome for some meningococci, with other meningococci having none (Merker *et al.*, 1997).

Opa and Opc proteins are strongly implicated in the interactions of non-capsulated meningococci with human cells and their expression may be required for the internalisation of meningococci into these cells.

1.1.3.5 Other OMPs

The class 1 (PorA) and class 2/3 (PorB) OMPs of meningococci are porins that permit the passage of ions across the cell membrane. Gonococci also express a PorB OMP ($PorB_{IA}$ or $PorB_{IB}$), but they do not express a PorA protein, although they do possess a *porA* pseudogene. The class 4 OMP is called Rmp (reduction modifiable protein), and has homology with *E. coli* OmpA, which has been shown to have some pore-forming properties. The mucosal surfaces of human airways are severely iron depleted, and during such conditions of iron starvation the meningococcus expresses a number of OMPs that are indirectly responsible for the capture of iron.

1.1.3.6 IgA proteases

In humans, IgA is secreted onto mucosal surfaces where it is the principal mediator of mucosal immunity. There are two IgA subclasses, IgA1 and IgA2, but IgA1 predominates in nasopharyngeal secretions, the site of meningococcal colonisation (Kilian *et al.*, 1988). This is significant, as pathogenic neisseriae constitutively produce either one of two closely related proteases specific for human IgA1 (Plaut & Mulks, 1978). IgA proteases are ~106kDa endopeptidases encoded by single copies of *iga* genes, and are exported (secreted) into the extracellular environment in a two-step process across both of the bacterial membranes (Pohlner *et al.*, 1987). These enzymes cleave within the hinge region of IgA1, and the type 2 IgA1 protease has recently been ascribed a second function in which it can promote intracellular survival of meningococci and gonococci by altering the levels of a major human lysosomal protein (Lin *et al.*, 1997). In addition, Vitovski and co-workers (Vitovski *et al.*, 1999) have recently reported that although the majority of both invasive and colonising isolates of meningococci had IgA protease activity, the pathogenic strains possessed

elevated levels of the enzyme, supporting the view that IgA protease contributes significantly to virulence.

1.1.4 Serological classification

The classification of meningococci utilises the variations, between strains, of structures present on the surface of the outer membrane. Serogrouping makes use of differences in the polysaccharide capsule, resulting in A, B, C etc. Serotyping uses differences in the conformational epitopes of class 2/3 OMPs (PorB) to give 1, 2a, 2b etc. Serosubtyping resulting from variations in the linear epitopes of class 1 OMPs (PorA) gives rise to P1.1, P1.2, P1.3 etc. Class 1 OMPs have two variable regions (VR1 and VR2) resulting in serosubtypes such as P1.7,16b (Frasch *et al.*, 1985). Immunotyping results from differences in the oligosaccharide components of lipopolysaccharide molecules, giving L1, L2, L3 etc.

1.2 Human meninges

The term meninx, meaning a membrane, was first introduced by Erasistratus in the Hippocratic collection circa 400BC to describe the layers covering the central nervous system (CNS). The meninges represent a series of three membranes that surround and protect the brain within the skull, and the spinal cord within the spinal column (Figure 1.3). These are the dura mater, the arachnoid and the pia mater. The arachnoid is not considered as one of the original maters as it was not identified as a separate entity in its own right until the 17th century (O'Rahilly & Muller, 1986).

1.2.1 Origins of normal human meninges

During embryonic development the process known as gastrulation (gut formation) gives rise to the three primary germ layers common to higher animals:

ENDODERM - the innermost layer that forms the gut tube (and other organs),

ECTODERM - the outermost layer of epithelium (epidermis) that also gives rise to the entire nervous system,

MESODERM - between the two other layers, this is a looser layer of tissue composed of mesenchyme cells that form connective tissue and muscle.

The vertebrate nervous system is principally derived from two sets of ectodermal cells after gastrulation (Lu, 1998). The neural tube is created in the human embryo (O'Rahilly & Muller, 1986) during neurulation, whereby a thickened region of the ectoderm rolls up (neural fold) and pinches off to form a tube. This neural tube initially consists of a single cell layer of epithelium and will eventually form the neurons and supporting glial cells of the brain and the spinal cord (CNS). Neural crest cells are ectodermal cells that break loose from the epithelium along the line where the neural tube pinches off, and migrate out through the mesoderm to form most of the peripheral nervous system (Lu, 1998). In most of the body, cartilage, bone and other connective tissues arise from the mesoderm, whereas in the developing human head such tissues are derived from the differentiation of many of the neural crest cells, due to their pluripotent nature (Napolitano *et al.*, 1963; Lu, 1998). The meninges originate from cells of the neural crest and from mesodermal cells that migrate into the area of the developing neural tube in the form of undifferentiated mesenchyme (O'Rahilly & Muller, 1986; Al-Rodhan & Laws, 1991).

Despite the CNS being the most anatomically complex system in the body, there are principally just five groups of constituent cells: neurones, glial cells, microglial cells, connective tissue (that gives rise to the meninges) and endothelial cells (blood vessel components) (Underwood, 1992).

1.2.2 Structure and function of the normal human meninges

Three closely associated yet distinct membranous layers constitute the meninges; these are the dura mater (pachymeninx), arachnoid and the pia mater. Together, the arachnoid and pia mater comprise the leptomeninges, and there are many structural similarities between the cells of these membranes (Weller, 1995). Located between the arachnoid and pia is a well-organised, fluid containing space, the sub-arachnoid space, and this space is traversed by leptomeningeal trabeculae which join the arachnoid and pial layers (Figure 1.4) (Al-Rodhan & Laws, 1991; Weller, 1995). Overall, the meninges are composed primarily of meningothelial cells and varying amounts of extracellular connective tissue (Al-Rodhan & Laws, 1991).

1.2.2.1 The dura mater

The dura mater, hard mother, is intimately associated with the skull and comprises layers of fibroblasts intervened by dense extracellular collagen. The dura is a relatively thick membrane of two indistinctly separated parts. The periosteal (endosteal) region is outermost acting as the skull periosteum (Al-Rodhan & Laws, 1991), and osteoblasts are also found in this region (Weller, 1995). The meningeal region is the inner dural layer adjacent to the arachnoid. This innermost portion of

the dura is composed of about five layers of flattened cells (modified fibroblasts) that form an electron dense dural border cell layer (Lopes & Mair, 1974; Alcolado *et al.*, 1988; Nabeshima *et al.*, 1975; Schachenmayr & Friede, 1978). Weed (1938) stated that these inner dural cells formed a mesothelium. This layer is attached to the arachnoid and there is no sub-dural space (Al-Rodhan & Laws, 1991). The dural border layer was previously thought to have formed part of the arachnoid membrane (Lopes & Mair, 1974). However, this was probably due to a tear occurring between the dural layers during the removal of the arachnoid from the dura, so that some dura was still attached to the arachnoid before processing for microscopy. This would also explain the artifactual appearance of a sub-dural space. Previous studies of the meninges have concentrated on mammals other than man (Waggener & Beggs, 1967; Nabeshima *et al.*, 1975; Alksne & Lovings, 1972). Such studies confirmed the existence of direct contacts between the innermost dural border cells and the outermost arachnoid cells. Schachenmayr and Friede (1978) demonstrated that the same is true for humans and that the cells of these two membranes are attached much more tightly together than they are between the cells of the layers they belong to. The cells of these two layers lack any connective tissue fibres and can be designated as a dura-arachnoid interface layer. In comparison to small animals, this collagen-free zone is substantial in man. Morphologically distinct cell junctions between the fibroblastic cells of the dural periosteal and meningeal regions are rare and there are no tight junctions. The great strength of the dura comes from the interlacing of collagen fibrils. Occasionally cell junctions (gap junctions, desmosomes) are seen between the cells of the dural border layer and between these cells and the underlying arachnoid layer. They are not found between cells of the meningeal dura and the dural border layer, and are not as numerous in the dural border layer as they are in the arachnoid layer (Al-Rodhan & Laws, 1991; Weller, 1995; Waggener & Beggs, 1967).

1.2.2.2 The arachnoid and its relationship to the sub-arachnoid space

The arachnoid layer of the meninges is a delicate membrane consisting of two regions. The arachnoid barrier cell layer consists of several layers of translucent, tightly packed cells that follow with the dura. The arachnoid trabeculae are loosely organised, spindly cells that traverse the sub-arachnoid space (SAS) and attach to the pia mater (Al-Rodhan & Laws, 1991). The SAS contains cerebro-spinal fluid (CSF) and the larger blood vessels. The human arachnoid is similar to that of other mammals but is considerably thicker (Alcolado *et al.*, 1988). The arachnoid follows the surface of the underlying brain but does not enter the sulci. Characteristic of the meningothelial cells within the arachnoid barrier layer is the presence of morphologically distinct cell junctions (Al-Rodhan & Laws, 1991; Nabeshima *et al.*, 1975). The arachnoid barrier cells at the dural aspect of the arachnoid are linked by numerous desmosomes and an extensive system of tight junctions (Alcolado *et al.*, 1988; Nabeshima *et al.*, 1975). The presence of tight junctions is unique to these

Figure 1.3: Simplified schematic of the structural relationships between the meninges, brain, choroid plexuses and route of CSF.

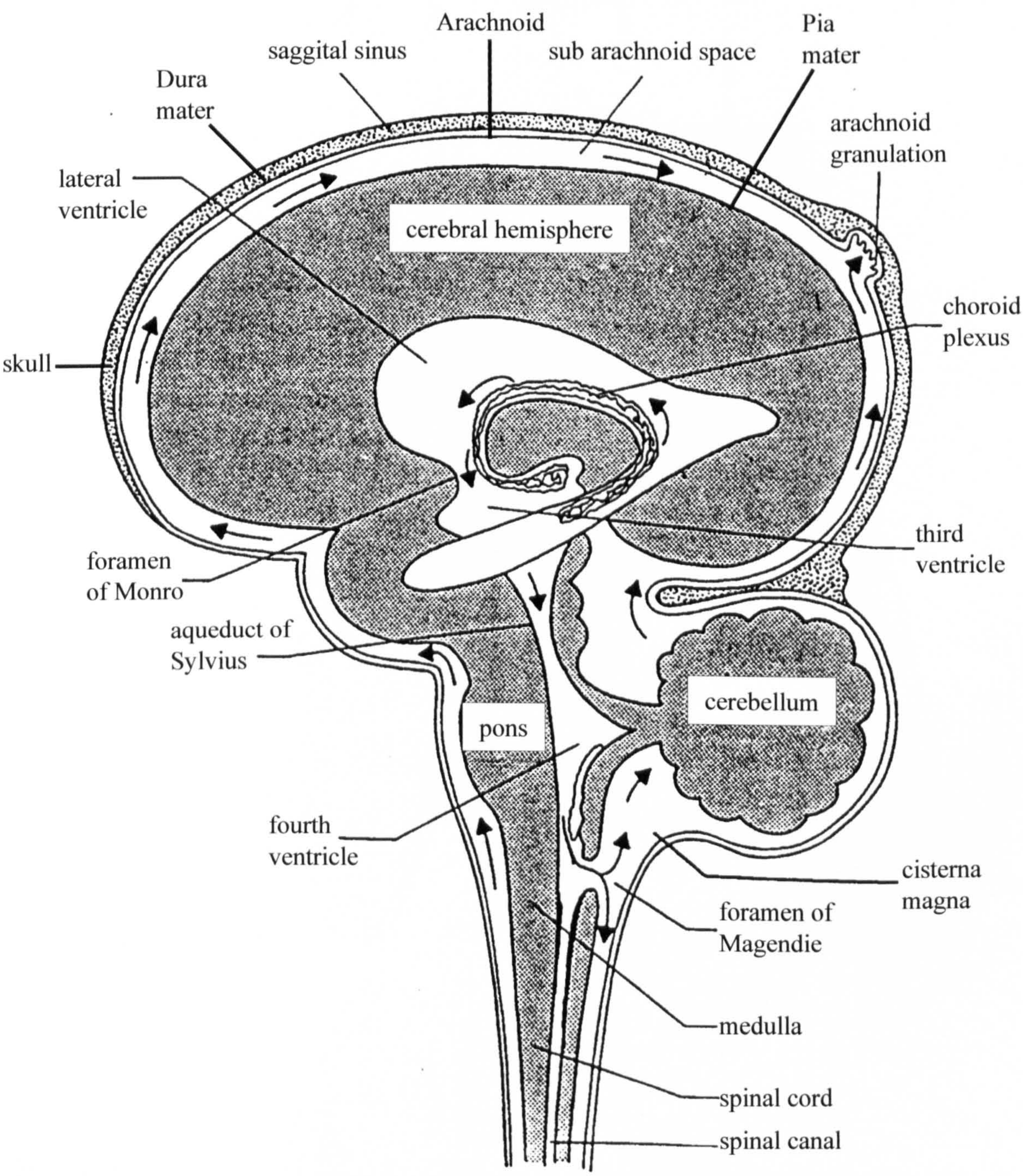
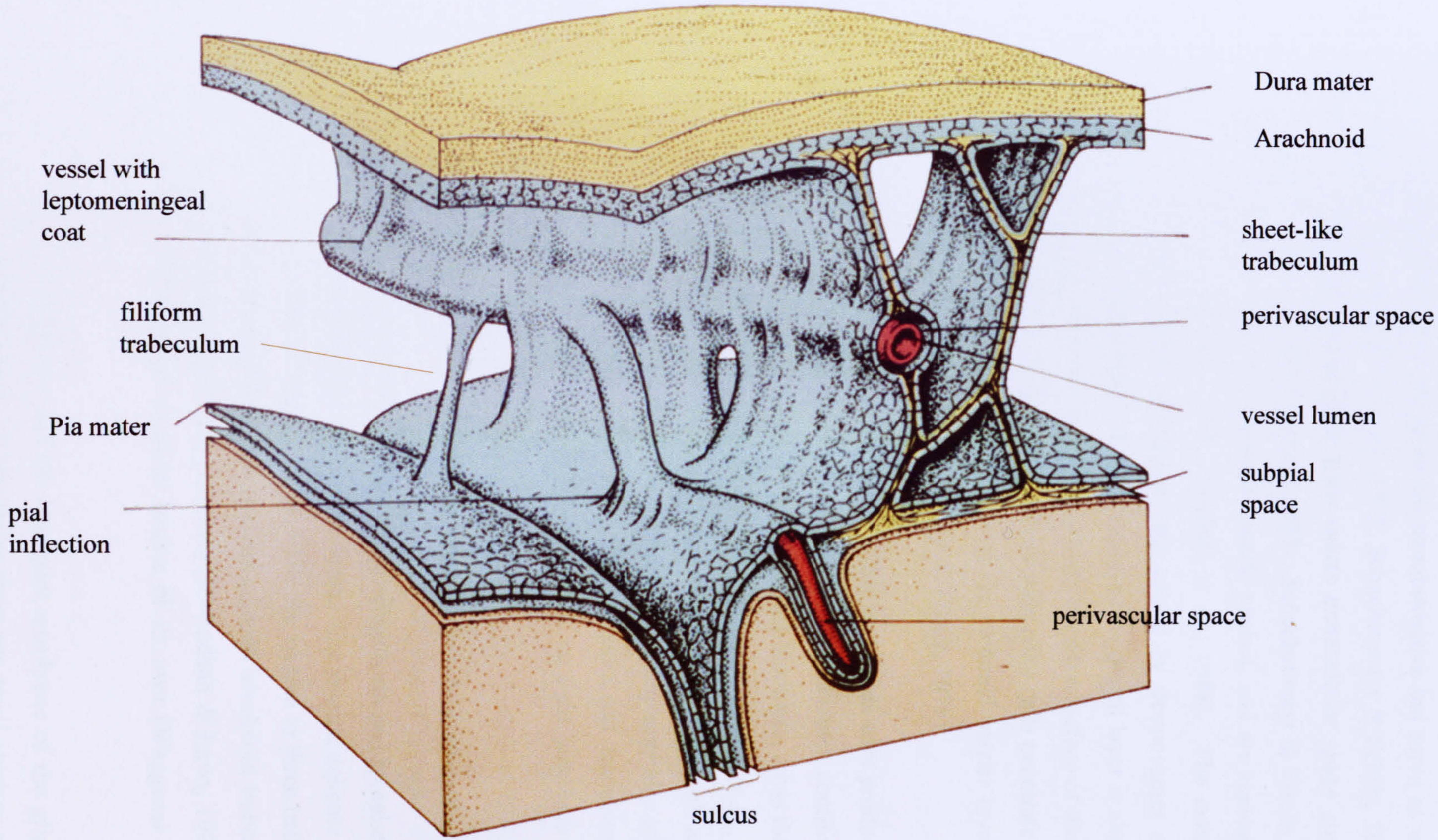


Figure 1.4: Structure of the human meninges and their inter-relationships.
(Adapted from Weller, 1995).



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cells in the meninges and confers the role of physiological barrier onto the arachnoid membrane. Such junctions are impermeable to CSF, protein macromolecules, water, small molecules, ions, and other soluble materials, whilst other junctions are nonobstructive but serve as attachment devices (Waggener & Beggs, 1967; Nabeshima *et al.*, 1975; Schachenmayr & Friede, 1978). Cells of the outer aspect of the arachnoid barrier layer have sparse extracellular space and no extracellular connective tissue (collagen) (Al-Rodhan & Laws, 1991; Schachenmayr & Friede, 1978). The cells of the inner aspect border on the SAS, are more loosely packed, and are separated by bundles of collagen fibres (Al-Rodhan & Laws, 1991; Alcolado *et al.*, 1988). The central region of the arachnoid consists of closely packed polygonal cells joined by desmosomes and gap junctions (Alcolado *et al.*, 1988). The inner aspect of the arachnoid barrier cell layer is characterised by two features. Firstly, a continuous basement membrane covers the inner surface of the arachnoid barrier layer and thus borders on the SAS. Secondly, arachnoid trabecular cells penetrate the surface of the basement membrane and attach to the inner surface of the archnoid barrier layer by desmosomes and gap junctions (Lopes & Mair, 1974; Schachenmayr & Friede, 1978).

Arachnoid trabeculae enclose the SAS blood vessels within a collagen core produced by and coated in leptomeningeal cells. The cellular coating of the trabeculae becomes continuous with the pia mater at the interface of these cells. Trabeculae are of varying thickness, cross the SAS at irregular intervals, and probably exist as sheets rather than narrow cords (Alcolado *et al.*, 1988). The cells appear to be specialised fibroblasts with long cytoplasmic processes. As well as attaching to the arachnoid barrier layer and pial cells on the surface of the brain, they may also attach to each other and to pial cells covering blood vessels in the SAS. Gap junctions and desmosomes connect these leptomeningeal cells, and within this layer of the arachnoid, no tight junctions appear (Lopes & Mair, 1974; Schachenmayr & Friede, 1978).

1.2.2.3 The pia mater and its relationship to the sub-arachnoid space

The pia mater, soft mother, completely covers the surface of the brain and spinal cord, following all of their contours in detail and entering the sulci of the brain. The pia is a delicate membrane that is mostly one cell thick, but in different regions of the CNS can be two to three cells thick. Cells of the pia mater share a number of morphological similarities with arachnoid trabecular cells, being flattened and joined by desmosomes and gap junctions (Al-Rodhan & Laws, 1991; Weller, 1995; Alcolado *et al.*, 1988). The pia has no basal lamina of its own (Waggener & Beggs, 1967; Nabeshima *et al.*, 1975).

Between the pial cells of the meninges and the basement membrane of the glia limitans on the surface of the brain, is the subpial space. In this space there are small clusters of collagen fibrils and small arteries and veins entering and leaving the brain (Hutchings & Weller, 1986). The

interface between pial cells and the brain surface is therefore very similar to that found between leptomeningeal cells and the smooth muscle cells of vessel walls found in the SAS.

Perivascular spaces (PVS) (Virchow-Robin spaces) are the spaces between blood vessel and neural tissue as vessels leave or enter the brain. Previously it was thought that the PVS of the brain were in direct contact with the SAS, allowing free passage of CSF between the SAS and PVS. However, more recent studies suggest that the pia mater separates these spaces from the SAS. The pia mater is reflected off of the surface of the brain and is continuous with the meningeal coating of the arteries and veins in the SAS. The perivascular and subpial spaces are therefore separated from the SAS (Alcolado *et al.*, 1988; Hutchings & Weller, 1986; Zhang *et al.*, 1990). The perivascular spaces of arteries are not continuous with the subpial space. As arteries extend downwards from the SAS, through the subpial space, and into the cerebrum developing into arterioles, they are externally bounded by a continuous layer of pial cells. As the arterioles get smaller, their pial coating becomes incomplete. The same is not true with veins, which lose their pial coating as they enter the subpial space. Arterioles within the subpial space have also been shown to have a coating of cells which resemble pial cells, but which are not continuous with it. Such pial cells did not possess desmosomes but instead were attached by nexus junctions (Zhang *et al.*, 1990).

Just as cells within the arachnoid layer have a barrier role, so cells of the pia mater form a regulatory interface between the surface of the brain and the CSF, and between brain arterioles and surrounding neural tissue. This could have a role in the protection of the brain from metabolites in the CSF. Hutchings and Weller (1986), studied the permeability of the pia to India ink in postmortem samples and to inflammatory cells and red blood cells in patients who died from leptomeningitis and sub-arachnoid haemorrhage (SAH) respectively. The pia was found to be impermeable to India ink injected into the SAS and no ink particles were found in the cerebral perivascular spaces. In the patients who died of SAH, the SAS was filled and distended by red blood cells, but no erythrocytes were found in the cortical perivascular spaces. These observations suggest that the pia is a barrier to particulate matter. In samples taken from patients who died of leptomeningitis, polymorphonuclear leukocytes and macrophages were found in the SAS and the perivascular and subpial spaces. The distribution of the inflammatory cells indicates that they must have had to penetrate the pia mater as they pass from blood vessels and into the CSF (Hutchings & Weller, 1986). The pia is also regarded as having enzymatic barrier functions, and glutathione S-transferase (Carder *et al.*, 1990) and catechol-*O*-methyltransferase are both present in pial cells (Kaplan *et al.*, 1981). Both of these enzymes have a role in the degradation of neurotransmitters and thus may inhibit or regulate their entry through the pia and into the brain. Feurer and Weller (1991) investigated the possible enzymatic and endocytotic barrier functions of the pia in normal frozen leptomeningeal tissue, and of cultures of meningeal cells derived from meningiomas. They found that cells derived from meningioma cells had similar barrier properties to those of the normal leptomeninges.

1.2.2.4 The arachnoid villi and granulations

Arachnoid villi and granulations are essentially specialised segments of the meninges whose apical surface is opposed to the venous sinus. In humans, they provide the main pathway for CSF back into the blood (Al-Rodhan & Laws, 1991; Weller, 1995). The difference between arachnoid villi and granulations is due to the former being smaller and not visible to the naked eye. With increasing age, granulations become more complex and lobulated, and when they are especially pronounced or elaborate they are called Pacchionian bodies (Wolpow & Schaumburg, 1972). They are most prominent in the superior saggital sinus but can be present in any of the major dural venous sinuses. Granulations are an extension of the arachnoid and the SAS which extends from the surface of the brain into the central portions of the arachnoid granulation. At the base of a granulation, a thin layer of cells from the arachnoid barrier layer penetrates the outer dural layers of the venous sinus and expands to form a central region of collagenous trabeculae and many interwoven channels. Around most of the granulation is a thick fibrous dural cupola (Upton & Weller, 1985; Yamashima *et al.*, 1988). In some sites, the endothelium lining the dural venous sinuses may cover the entire arachnoid granulation whilst in other sites, only the apex of the granulation is covered. In the latter case, a specialised clump of arachnoid cells form a cap of about 150μm thick located on top of the collagenous core. Such arachnoidal cap cells form an external seal on the outer surface of the arachnoid membrane and are exposed to the venous blood of the sinus (Kepes, 1986; Upton & Weller, 1985). The cap region of the granulation is only attached to the venous endothelium by an area about 300μm in diameter. The rest of the granulation is separated from the endothelium by the dural cupola and a subdural space. The channels within the granulations are coated with arachnoid cells and lined with collagen, and may contain macrophages. Erythrocytes may be found in such channels following sub-arachnoid haemorrhage, suggesting that these channels are continuous with the SAS, and that they are CSF drainage pathways (Upton & Weller, 1985; Weller, 1995). The bulk flow of CSF from granulation to venous sinus probably occurs via micropinocytotic vesicles and intracytoplasmic vacuoles in the venous endothelium (Yamashima *et al.*, 1988). Arachnoidal cap cells (or arachnoid cell clusters) are closely packed and polygonal in shape with interdigitating processes. They have oval nuclei and translucent cytoplasm, and are attached to each other by desmosomes. Based on their morphological characteristics, cap cells are clearly a specialised segment of the arachnoid barrier layer (Al-Rodhan & Laws, 1991).

1.3 The blood-brain and blood-CSF barriers

The internal milieu of the brain is maintained by the presence of cellular and enzymatic barriers, which restrict the passage of circulating cells, macromolecules and ions into the CNS. The CNS is

essentially isolated from the intravascular space by the blood-brain barrier (BBB) and from the CSF by the blood-CSF barrier (B-CSFB), and homeostasis is maintained. The BBB exists at the cerebral capillaries of the brain parenchyma, whilst the B-CSFB exists at the choroid plexus epithelium, and the blood vessels present within arachnoid trabeculae in the SAS (Figures 1.3 and 1.4). Confusingly, many authors consider the BBB to encompass all three of the barriers mentioned above, but for the purposes of this study, the barrier present within the leptomeninges is considered as part of the B-CSFB.

1.3.1 Cerebral microvasculature

The endothelial cells of blood vessels within the brain differ from those of peripheral tissues in several ways. Cerebral endothelial cells exhibit complex tight junctions with a high electrical resistance, although the transcellular resistance of the endothelial cells within the vessels of the pia (and presumably of the meninges) is much lower than that of the brain parenchymal vasculature (Perry *et al.*, 1997). These vessels are rarely fenestrated and possess few pinocytic vesicles. In addition, they specifically express several proteins which may be required for protective, metabolic or transport activities at the BBB, and presumably also at the B-CSFB (Dehouck *et al.*, 1992; Townsend & Scheld, 1995). It was previously assumed that the endothelial tight junctions prevented the entry of leukocytes into the CNS and that such cells could only gain entry when the BBB or B-CSFB was damaged in some way (e.g., from the inflammatory response associated with meningitis). It is now known that leukocytes cross the BBB and B-CSFB as part of normal physiology, although it is not known whether the route through the endothelium is trans- or paracellular (Perry *et al.*, 1997).

1.3.2 Choroid plexus epithelium

The choroid plexuses are microscopic fingerlike projections of the vascular system located in the lateral, third and fourth cerebral ventricles (Weller, 1995). They consist of a central core of blood vessels surrounded by a single layer of epithelial cells. Together with the leptomeninges, the choroid plexus acts as a barrier between the blood and the CSF. Whilst the barrier properties of the leptomeninges are largely passive, the choroid plexus is actively involved in regulating the concentrations of molecules in the CSF. The endothelial cells within the choroid plexus are highly fenestrated allowing the entry of CSF ingredients from the blood. Up to 90% of the CSF is manufactured by the choroidal epithelial cells. The anatomical foundations of the choroidal B-CSFB are the tight junctions between the cells of the epithelial layer. These tight junctions prevent

the free passage of substances, and micronutrients are transported through the epithelial layer by active pumplike carriers (Davson, 1976; Spector & Johanson, 1989; Pron *et al.*, 1997).

1.4 Meningiomas

A meningioma is a benign tumour of the meninges of the CNS (Al-Rodhan & Laws, 1991). It is one of the most common neoplasms of the CNS, accounting for between 13-19% of all primary intracranial tumours (Cruikshank *et al.*, 1975). The term 'meningioma' was given by Cushing to encompass all of the tumours of the meninges. Previously, such tumours had been named to reflect several of their different properties, such as their gross appearance and the histogenic characteristics of the main tumour cell (Kepes, 1982).

1.4.1 Localisation and aetiology

Although meningiomas can occur anywhere where there are meninges, or cells of meningeal derivation, they appear to have a predilection for certain locations. The most frequent anatomical sites for meningiomas are the parasagittal region, sphenoidal wing, olfactory groove and foramen magnum. Spinal meningiomas are less common than intracranial ones. Generally, cranial meningiomas have an attachment to the dura, but pial forms without any such attachment also exist. Whilst it is not uncommon for meningiomas to infiltrate the adjacent dura and overlying bone, actual brain invasion is rare (Kepes, 1982).

Meningiomas are derived from cells of the arachnoid membrane. The mechanism by which arachnoid cells are transformed into meningiomas may involve the loss of part of, or the whole of chromosome 22. It has been suggested that the aetiological agents of meningiomas include trauma, high- or low-dose irradiation, DNA tumour viruses or neurofibromatosis (an autosomal dominant hereditary disorder) (Al-Rodhan & Laws, 1991; Kepes, 1982).

The incidence of meningiomas occurs more frequently in females than males, at the ratio of 2:1. This could be a result of the effects of female sex hormones on meningiomas. The female hormone oestrogen is known to increase the rate of growth of some tumours and pre-existing meningiomas in some women become larger during pregnancy when progesterone levels are at an increased level. Meningiomas, in both men and women, have been shown to express oestrogen and progesterone receptors, often at a high level (Kepes, 1982). Among other receptors that they express are insulin-

like growth factor-I (IGF-I) (Lichtor *et al.*, 1993), integrins, and transmembrane glycoproteins such as CD44 (Figarella-Branger *et al.*, 1997).

1.4.2 Origins

Cushing and Eisenhardt believed that within a meningioma there is a basic cell that sometimes exists in pure form but that otherwise undergoes morphological changes to create the different meningioma variants. This pure component cell of a meningioma is called the meningothelial cell (Kepes, 1982; Napolitano *et al.*, 1963). In 1902, Schmidt noted histological similarities between normal arachnoid villi and meningiomas and proposed that these tumours originated from the cap cells of the arachnoid villi. The current theory is that meningiomas are derived from any part of the arachnoid membrane.

Some knowledge into the nature of the non-neoplastic arachnoidal cells is necessary to understand the nature of meningiomas. The arachnoid cap cells form the external covering cell layers of the arachnoid membrane, and have no real histological parallels in the rest of the human body. Normally epithelial cells assume the role of covering a surface or lining a cavity. However, in some areas of the human body, there are non-epithelial cells assuming an epithelial role. These include mesothelial cells that line serous cavities and synovial cells that line joints and tendon sheaths. Such cells are still in contact with the underlying mesenchyme and tumours derived from these cells often show a mixture of mesenchymal and epithelial characteristics. However, mesothelial and synovial cells form a lining of cavities whilst arachnoid cap cells form an external seal of the SAS (Kepes, 1982; Kepes, 1986). A further difference between arachnoid cells and mesothelial or synovial cells is that the arachnoid villi also serve to funnel the CSF into the venous circulation of the dural sinuses.

Meningiomas are well known for their diverse histological appearance (Meis *et al.*, 1986) and can exhibit properties of both mesenchymal and epithelial differentiation. The multipotential character of the arachnoidal cell is thought to give rise to such histologic diversity. Alternatively, these cells may constitute most of a meningioma but with other component cells of the meninges such as fibroblasts or endothelial cells also taking part in the formation (Al-Rodhan & Laws, 1991; Kepes, 1986). There is some confusion in the literature as to whether meningiomas should be classified as either epithelial or mesenchymal - it would seem that they can be a mixture of both. Kepes suggested that arachnoidal cells represent a "mixed cell population derived from both neural crest and mesenchymal elements, blended together in a homogeneous cell mass, which may give evidence of either its mesenchymal or neuroectodermal derivation or both" (Kepes, 1986). It may be that meningiomas (derived from leptomeningeal cells) are composed of mesenchymal cells with an epithelial function. It is most likely that the mixed mesenchymal and epithelial properties of

meningiomas reflect their derivation from the meninges, which are known to have dual embryological origins.

1.4.3 Histological classification and structural characteristics

Meningioma cells share cellular characteristics with normal meningeal cells, from which they are derived. Meningothelial cells have a tendency to form cellular whorls that is unparalleled by any other cell type known to histologists, except in the normal meninges from whence these tumours originate. These whorls also form when meningiomas are grown in tissue culture and when provided with suitable scaffolding they can even become three-dimensional. Whorls occur due to the tendency of meningothelial cells to wrap themselves around structures (such as blood vessels) that they encounter during growth. Such whorls frequently exist in the normal meninges where, as in meningiomas, they can undergo hyalinization and calcification. Hyalinization involves the internal structure of whorls becoming less discernible, whilst calcification involves the deposition of calcium apatite crystals between the cells. Both meningiomas and arachnoid villi often possess psammoma bodies ('sand' bodies) which occur when calcium and phosphorous are deposited in collagen whorls (Al-Rodhan & Laws, 1991; Kepes, 1986; Kepes, 1982). Psammoma bodies and hyaline inclusions are considered as potential markers for epithelial potential in meningiomas (Kepes, 1982). Some meningiomas are syncytial whereby the cellular components cannot be distinguished from one another and exhibit a blurring of the cell membranes. Normal meninges and meningiomas also consistently express intermediate cytoskeletal filaments and desmosomes (Al-Rodhan & Laws, 1991; Kepes, 1982).

Other features shared by normal leptomeninges and meningiomas are the presence of certain enzymes. These include alkaline phosphatase, lactic dehydrogenase (Kepes, 1982), glutamine synthetase and catechol-*O*-methyltransferase (Feurer & Weller, 1991). Meningiomas also exhibit endocytic activities as in leptomeningeal cells (Feurer & Weller, 1991).

Meningiomas are classified according to their histological characteristics, Table 1.1.

Meningioma cells of the subtype *meningothelial* are considered the most similar to their non-neoplastic counterparts, and are most likely to have a more epithelial nature. The cell membranes are not well defined and nuclear pleomorphism is quite common, often resulting in giant cells with single or multiple nuclei (Lantos *et al.*, 1997). In *fibroblastic meningiomas*, the cells have a tendency to become increasingly spindly whereby the cells are elongated, bipolar, with spindly nuclei, and the cells resemble fibroblasts. This term is not universally applied to exactly the same

Table 1.1: 1997 WHO histopathological classification of tumours of meningothelial cells

Tumour	Subtype
Meningioma	Meningothelial
	Fibrous (fibroblastic)
	Transitional (mixed)
	Psammomatous
	Angiomatous
	Microcystic
	Secretory
	Clear cell
	Chordoid
	Lymphoplasmacyte-rich
	Metaplastic
Atypical meningioma	-
Papillary meningioma	-
Anaplastic (malignant) meningioma	-

cell types. Some pathologists use it to describe meningiomas comprising of spindly cells, with no collagen formation. Others reserve the term for cells that are visibly laying down collagen fibres. In some meningiomas, there is a coexistance of meningothelial and spindly cell types. Such tumours are referred to as *transitional meningiomas* as they do not truly contain a dual cell population but instead contain cells capable of expressing dual characteristics. Studies with the electron microscope have shown that meningothelial, fibroblastic and transitional meningiomas have no basic differences in their ultrastructural cell morphology. *Psammomatous meningiomas* are variants of meningothelial meningiomas but which contain many psammoma bodies. They are most common in the spinal canal. *Angiomatous meningiomas* have the same general features as meningothelial meningiomas but are highly vascularised. Blood vessels do not dominate the whole tumour. *Atypical* meningiomas are characterised by deviation from the normal benign histopathological pattern of the previous meningiomas, including increased mitotic activity. These tumours do not invade the dura or adjacent bones. The *papillary* and *anaplastic* (malignant) forms of meningioma are more consistent with epithelial differentiation. The term 'malignancy' when applied to meningiomas, implies the tumour is invasive and has spread to other sites in the body. Whilst papillary forms are aggressive they are not usually invasive and so are not classified as malignant (Al-Rodhan & Laws, 1991; Kepes, 1982; Kepes, 1986; Whalen *et al.*, 1995).

1.4.4 Characterisation of meningiomas using cellular markers

Previously, many studies have been concerned with the immunohistochemical investigation of large numbers of meningiomas using a wide range of epithelial, mesenchymal and extracellular matrix protein markers. These include: collagen (Kartenbeck *et al.*, 1984), fibronectin (Rutka *et al.*, 1986), laminin (Rutka *et al.*, 1986), vimentin (Kartenbeck *et al.*, 1984; Schwechheimer *et al.*, 1984), cytokeratin (Theaker *et al.*, 1986; Radley *et al.*, 1989), carcinoembryonic antigen (CEA) (Ng & Wong, 1993), epithelial membrane antigen (EMA) (Theaker *et al.*, 1986; Radley *et al.*, 1989), human milk fat globule-2 (HMFG) (Ng & Wong, 1993), desmoplakins (Parrish *et al.*, 1987; Feurer & Weller, 1991), glial fibrillary acidic protein (GFAP) (Rutka *et al.*, 1986; Parrish *et al.*, 1987), S-100 protein (Theaker *et al.*, 1986), factor VIII related antigen (von Willebrand factor) (Rutka *et al.*, 1986) and desmin (Kartenbeck *et al.*, 1984). For a given marker, not all meningiomas will stain positively. Normal arachnoid cells have been shown to be strongly immunoreactive with antibodies raised against desmoplakin (Kepes, 1986; Parrish *et al.*, 1986), EMA, vimentin, S-100 protein (Meis *et al.*, 1986; Theaker *et al.*, 1986) in frozen or paraffin sections, and cytokeratin (Meis *et al.*, 1986; Frank *et al.*, 1983) in cultured cells.

Cytokeratin, vimentin, desmin and GFAP are intermediate filaments (IFs). These protein filaments form part of the cell cytoskeleton and, together with actin filaments and microtubules, are responsible for movement within the cell. Intermediate filaments are ropelike structures of about 10nm diameter and seem to provide mechanical strength to the cell. They form an extensive network surrounding the nucleus and extending out to the periphery of most animal cells. Different cell types usually express different IFs. Generally, normal cells contain only 1 type of IF. Staining cultured cells with antibodies to a cytoplasmic IF results in the appearance of delicate, threadlike filaments surrounding the nucleus and reaching out to the plasma membrane. In epithelial cells such filaments are keratin filaments and these attach to specialised cell junctions; desmosomes (maculae adhaerentes) which attach neighbouring cells, and hemidesmosomes, which attach cells to the underlying basal lamina.

Desmosomes are cytoskeletal-associated structures present in epithelia, myocardium, dendritic follicle cells of lymph nodes and the arachnoidal cells of the meninges. They are also present in meningiomas. Desmoplakins I and II (intracellular attachment proteins) are the most prominent protein components of a dense plaque on the cytoplasmic side of the desmosome to which intermediate filaments attach (van der Voort *et al.*, 1998). An anti-desmosomal monoclonal antibody (11-5F) that was immunoreactive with desmoplakins I and II, was developed by Parrish and co-workers (Parrish *et al.*, 1987). In frozen sections of meningiomas, all were found to stain positively with 11-5F. The staining pattern for meningotheliomatous, transitional, fibroblastic and psammomatous meningioma subtypes was punctate but, in the two cases of angioblastic tumours, the staining pattern was fibrillar. This antibody was also shown to distinguish between meningioma

Table 1.2: Types of intermediate filament.

Cell Type	IF Expressed	Mw
Epithelial	Keratins:	
	type I (acidic)	40 - 70KDa
	type II (neutral/basic)	40 - 70KDa
Mesenchymal – including: fibroblasts, endothelial cells, white blood cells	Vimentin	58KDa
Muscle	Desmin	53KDa
Glial – astrocytes, some Schwann cells	Glial Fibrillary Acidic Protein (GFAP)	51KDa
Neuronal	Neurofilaments: NF-L	68 - 70KDa
	NF-M	160KDa
	NF-H	200KDa
Nuclear lamina of eukaryotic cells	Nuclear Lamins A,B,C	65 - 70KDa

Compiled from (Lu, 1998; Fawcett, 1986).

and glioma as none of the gliomas stained positively with 11-5F. Although this antibody did not discriminate between meningiomas and metastatic carcinomas, which were all immunoreactive, histological differentiation between these types of tumour is not usually a problem. Other studies (Ng & Wong, 1993) have also found meningotheliomatous, transitional and fibroblastic meningiomas to be immunoreactive for desmoplakins.

Cytokeratin is characteristic of epithelial cells and is not found in cells of mesenchymal origin. It forms bundles of tonofilaments that terminate in desmosomes at sites of cell-to-cell adhesion. Vimentin is found in cell types derived from embryonic mesenchyme. It is most consistently found in intimate association with the nuclear envelope and may provide the nucleus with mechanical support or stabilise its position in the cell. In cell types that contain more than one kind of IF, vimentin is always one of them (Fawcett, 1986). Kartenbeck (Kartenbeck *et al.*, 1984) and Schwechheimer (Schwechheimer *et al.*, 1984) have shown that vimentin IFs are also capable of attaching to desmosomal plaques in both normal arachnoid cells and meningiomas. This has led to the definition of a new class of cells; the desmofibrocytes (Kartenbeck *et al.*, 1984). In meningiomas where there is co-expression of cytokeratin and vimentin IFs, it seems that the cytokeratin filaments are constitutively attached to desmosomes whilst vimentin filaments are free within the cell (Kartenbeck *et al.*, 1984). Desmin IFs are not present in arachnoid or meningioma cells, and only very rarely has a meningioma shown positivity for GFAP IFs (Wanschitz *et al.*, 1995).

The literature is ambiguous regarding the expression of cytokeratin in meningiomas. In a study of transitional, fibroblastic and syncytial subtypes, Halliday and colleagues (Halliday *et al.*, 1985)

found that none of them showed immunoreactivity for cytokeratin. Instead, in all cases, vimentin was found to be the only IF expressed. In contrast, Theaker and co-workers (Theaker *et al.*, 1986) found cytokeratin expression in meningiomas and observed that this was especially related to the presence of hyaline bodies, and was generally related to meningothelial tumours. It is now generally regarded that cytokeratin is frequently expressed in meningiomas and that it is frequently co-expressed with vimentin. However, it seems that the proportion of meningiomas expressing vimentin IFs is greater than the meningiomas expressing cytokeratin IFs.

The expression of EMA is a feature indicative of epithelial differentiation and is widely expressed in epithelial tissues (Theaker *et al.*, 1986; Ng & Wong, 1993). EMA is closely related to, but apparently distinct from human milk fat globule-2. They are both glycoproteins within the membranes of epithelial cells, although EMA is a more sensitive marker for detecting epithelial differentiation (Ng & Wong, 1993). Investigations into EMA expression in paraffin and frozen sections of meningiomas of different histological subtypes have shown that between 50-100% were positive (Theaker *et al.*, 1986; Meis *et al.*, 1986; Radley *et al.*, 1989). Histological subtype did not seem to have any bearing on whether a result is expected to be positive or not. EMA staining in cultured meningioma cells has been investigated (Ng & Wong, 1993), and in meningothelial, transitional and hemangiopericytic meningiomas, all were positive for EMA.

S-100 protein was previously considered to specifically stain cells of neuroectodermal origins, but some studies have shown otherwise (Theaker *et al.*, 1986). Nevertheless, normal meninges are immunoreactive for S-100 and many meningiomas show the same pattern of reactivity (Theaker *et al.*, 1986; Meis *et al.*, 1986). Collagen, fibronectin and laminin are glycoproteins of the extracellular matrix that forms the leptomeningeal basement membranes. Studies from the laboratories of Rutka (Rutka *et al.*, 1986) and Ng & Wong (Ng & Wong, 1993) have shown that meningioma and leptomeningeal *in vitro* cultures stained positively for these markers, and that frozen sections of meningiomas stained positively for fibronectin and at least one of the collagens. These results indicate that leptomeningeal and meningioma cells can behave like stromal cells (by the production of collagen, a characteristic of mesenchymal cells) and epithelial cells (in their production of desmosomes). Immunoreactivity for carcinoembryonic antigen (CEA) has been found in meningiomas of a secretory subtype (Ng & Wong, 1993). Factor VIII related antigen is a marker for endothelial cells and has been shown to be negative in all meningiomas studied (Rutka *et al.*, 1986).

In summary, it appears that with the exception of desmoplakin, no other cellular marker can be used with confidence in determining meningioma. Indeed, meningiomas may be positive or negative for EMA, cytokeratin, vimentin or S-100 protein and still be considered as a tumour of the meninges. However, using a panel of several of these antibodies, information is obtained on the immunohistochemical nature of meningiomas, and the possibility of glioma is also ruled out.

1.4.5 Tissue culture

Despite occasional reports in the literature (Frank *et al.*, 1983; Rutka *et al.*, 1986; Murphy *et al.*, 1991; Motohashi *et al.*, 1994), primary human leptomeningeal cells cannot be reliably cultured *in vitro* (Feurer & Weller, 1991). In contrast, meningioma cells grow readily in tissue culture, and display many of the features associated with normal leptomeningeal cells. It is noteworthy that when grown in tissue culture, meningothelial, transitional and fibroblastic meningiomas are found to be morphologically identical (Schwechheimer *et al.*, 1984; Rutka *et al.*, 1986). Intersetingly, Murphy and colleagues (Murphy *et al.*, 1991) transfected normal leptomeningeal cells with an SV40 large T antigen gene construct to yield an immortalised cell line designated LTAg2B. These cells were found to be positive for the expression of desmoplakin, cytokeratin and vimentin in a similar staining pattern to primary cultures of leptomeningeal cells.

1.5 Meningococcal - host cell interactions

Meningococci and gonococci are obligate human pathogens. The study of the molecular mechanisms of the pathogenesis of meningococcal infection has therefore been hampered by the lack of a suitable animal model. Obviously, the direct use of humans in the study of these potentially pathogenic organisms is very limited. The only studies possible with human subjects, so far, have been experimental gonococcal urethritis in men. The lack of a suitable animal model necessitates the use of *in vitro* human models to study the pathogenesis of these bacteria. Tissue sections, cells, cell lines and organ cultures obtained from humans can provide detailed information about the adhesive, invasive and cytotoxic nature of these bacteria. From the judicious use of cells obtained from different anatomical sites, it may be possible to follow the path of meningococci through the human body.

1.5.1 Models for studying Neisserial-host cell interactions

Although meningococci and gonococci infect different anatomical sites, there is uniformity in their mechanism of infection and the infection experiments designed to investigate their pathogenesis are similar. Most studies have concentrated on the epithelial sites of bacterial colonisation, and the invasion of endothelium. There are very few reports regarding the subsequent entry into the bloodstream and the interaction of meningococci with components of the BBB, B-CSFB and brain.

1.5.1.1 Organ culture

Organ culture studies have enabled experimental investigations into the initial interactions between pathogenic *Neisseria* and the intact human mucosa, and the subsequent colonisation of these surfaces. The tissues that are predominantly used are specific for the sites of natural infection by these bacteria. The fallopian tube organ culture (FTOC) model has typically been used for gonococcal infection studies and is particularly relevant as salpingitis is a major complication of gonorrhoea (Johnson *et al.*, 1977; McGee *et al.*, 1983). The nasopharynx is the only natural habitat of meningococci and the initial colonisation of humans by these bacteria has been studied using the nasopharyngeal organ culture (NPOC) model (Stephens *et al.*, 1983; Stephens *et al.*, 1986; Stephens & Farley, 1991). This model utilised tissue taken mainly from child adenoids, the lymphoid glands of the nasopharynx, but also from the actual nasopharyngeal mucosa. The tissues comprised ciliated and non-ciliated columnar epithelium, and underlying lymphoid tissue. The presence of ciliated epithelium is important because the nasopharynx of young children is composed mostly of such cells and this age group is a particular target group for meningococcal infection (Stephens *et al.*, 1986).

Organ culture studies have provided some information on the types of human mucosal cells that are colonised, the cytotoxic effects of meningococci and gonococci and the roles that different bacterial components have in the colonisation and invasion process. However, there are disadvantages to these systems. Fresh human tissue is often difficult to obtain, has limited viability and there is usually variability between tissue from different individuals. Human tissue also lacks humoral factors and other important components of the inflammatory response (e.g., leukocytes and complement). The use of human tissue also necessitates the use of antibiotics for sterilisation.

1.5.1.2 Cells and cell lines

Some of the problems associated with using human organ culture have been overcome with the use of human cells and cell lines. Different human cells and cultured cell lines have been used in the study of neisserial pathogenesis. Many of the *in vitro* assays used to study meningococcal and gonococcal infections use the same cells and cell lines. The types of cell used broadly fall into four categories; epithelial, endothelial, and immune cells, and components of the BBB and B-CSFB, although the latter really represents both epithelial and endothelial cells.

The following epithelial human cells and cell lines have been used in studies of meningococcal pathogenesis: isolated buccal and nasal mucosal cells; primary cultures of uroepithelial and nasopharyngeal cells; Chang (conjunctival), Hep-2 (larynx carcinoma), A549 (lung carcinoma), Hec-1B (endometrial adenocarcinoma), ME-180 (cervical carcinoma), T_{84} (lung metastasis of colonic carcinoma), HT29 (colonic) and A431 (epidermoid carcinoma) cell lines. The most commonly used endothelial cells are primary cultures of HUVECs (human umbilical vein

endothelial cells), whilst HMEC-1 (human microvascular endothelial cells) and brain microvascular endothelial cells (BMEC) have also been used. Cells of the immune system, including isolated monocytes, macrophages and polymorphonuclear cells (PMN), and immune cell lines have also been used.

Buccal epithelial cells are squamous epithelial cells that are easily obtained from scrapings of the buccal mucosa. Both gonococci and meningococci adhere to these cells, but they do not invade them (Pearce & Buchanan, 1978; Stephens *et al.*, 1981; Trust *et al.*, 1983; Stephens & Whitney, 1985). The early studies by Stephens and co-workers (Stephens *et al.*, 1981), suggested that meningococci only bound to certain cells in the body due to expression of specific receptors by these cells. Although buccal cells have been extensively used, there are several disadvantages of which the most important is that these cells are not normally colonised by pathogenic *Neisseria.* Although isolated epithelial squamous cells from the nasal mucosa provide a model that more closely describes the natural site of bacterial colonisation similar problems apply here as to buccal cells. Problems associated with isolated tissues and cells have been largely overcome by the use of cell lines.

Human cell lines have provided more anatomically relevant models of infection. Primary cell lines (e.g., conjunctival, endometrial, endothelial) can be difficult to establish and maintain and can only be passaged a limited number of times. Immortalised cell lines, often derived from tumours, are less fragile and can be cultured to high passage numbers without significant deviation from the original tumour cells. Cell lines have been used in attachment and invasion assays, and in cytotoxicity studies. Investigations into meningococcal interactions with the BBB have so far utilised HBEC (human brain endothelial cells) (Pron *et al.*, 1997), CMVECS (cerebral microvascular endothelial cells) (Townsend & Scheld, 1995) and T_{84} cells (Pujol *et al.*, 1997) as models of the endothelial components of the BBB. The relevance of T_{84} cells is that they form a polarised monolayer with tight junctions, as present in the endothelium of the BBB and B-CSFB. However, caution must be observed using these cells as they are epithelial cells derived from a human colonic carcinoma, and so they are not endothelial cells and they are derived from an anatomically irrelevant site. More recently, cell lines have been used to investigate the host cell receptors that meningococci and gonococci may utilise in the establishment of infection.

Cultured animal cells of epithelial and endothelial origins have also been used in attachment assays, including COS (african green monkey kidney) and CHO-K1 (chinese hamster ovary) cells. Cell lines derived from animals are typically transfected with human cell surface molecules that act as receptors for bacterial outer membrane structures. These studies make use of the tropism of meningococci for human cells.

1.5.2 Pathogenesis of meningococcal meningitis

To be a successful pathogen, meningococcal interactions with host cells are multifactorial, involving the invasion and survival in several different cellular environments within the human body. Firstly, the meningococcus must colonise and invade the host mucosal surfaces. Next, it must invade the underlying blood vessel endothelium and survive in the intravascular space. After its journey through the bloodstream it must cross the B-CSFB and survive in the CSF. From *in vitro* studies of meningococcal-host cell interactions and with the analysis of clinical isolates of meningococci from different anatomical sites, information has been collected on the ways in which meningococci survive their journey to the meninges. The phase and antigenic variation of several of their outer membrane structures is believed to enable their survival at different sites (Stephens, 1989; Nassif & So, 1995).

1.5.2.1 Mucosal colonisation and invasion

Meningococci are harboured, usually, in the nasopharynx of asymptomatic carriers. The transmission of meningococci between individuals occurs via contact with nasopharyngeal secretions, either from airborne droplets or by direct contact. To colonise and survive in the nasopharynx, meningococci must overcome host defence mechanisms including the mucus barrier, secretory IgA and ciliary clearance. Before they can associate with mucosal cells, meningococci must first cross the mucus barrier, a viscoelastic gel 2-5μm thick (Stephens & Farley, 1991). There are two subclasses of sIgA with sIgA1 predominating in nasopharyngeal secretions. Pathogenic *Neisseria* produce extracellular IgA1 proteases, which cleave, and hence inactivate, sIgA1 whilst non-pathogenic strains, such as *N. lactamica*, do not produce these proteases (Kilian *et al.*, 1988).

Organ culture models have established some of the events occurring during the initial colonisation of the host, including mucosal cytotoxicity, attachment and mucosal invasion. The attachment of gonococci to FTOC is species specific as shown by Johnson and colleagues (Johnson *et al.*, 1977). These workers found that whilst gonococci adhered rapidly to the mucosa of human fallopian tubes, attachment to rabbit, porcine or bovine tubes was negligible. Using light microscopy and scanning and transmission electron microscopy, both meningococci and gonococci have been shown to bind selectively to the microvilli of only some non-ciliated mucosal cells (Stephens *et al.*, 1983; McGee *et al.*, 1983). These pathogens rarely associate with mucus (Rayner *et al.*, 1995). Large numbers of meningococci have been seen to bind to the microvilli between 4-12 h after infection (McGee *et al.*, 1983; Rayner *et al.*, 1995). During colonisation, the cilia of neighbouring cells stopped beating and are sloughed from the tissue (McGee *et al.*, 1983). This is beneficial to the bacteria, as to colonise these surfaces, they must contend with the host ciliary clearance mechanisms. The loss of ciliary activity did not require attachment of bacteria to the ciliated cells, implying that some soluble bacterial factor induced ciliostasis either directly or indirectly, by inducing the release of host cytokines. Viable bacteria were required for ciliary damage and attachment. During adherence

assays, meningococci have been seen to produce blebs (Rayner *et al.*, 1995) which are known to contain LPS, OMP and capsular polysaccharide. Meningococcal LPS did not contribute to ciliary damage in the nasopharyngeal model, but did in the FTOC model (Stephens *et al.*, 1986). Upon binding to the tips of the microvilli, the bacteria are trapped by these structures against the host cell surface where they are then phagocytosed (Stephens *et al.*, 1983). In the NPOC model, meningococci have been seen within membrane-bound vacuoles in the apical region of the non-ciliated cells. The bacteria seem to remain in this apical position and there is no evidence of bacteria passing through the basement membrane into the subepithelial tissues adjacent to the lymphoid tissue. Enteric organisms such as *Salmonella typhimurium* are known initiate murine infection by invasion of the M cells of the Peyer's patches, so it is possible that meningococci could cross the mucosal barrier by transcytosis through the M cells of the tonsils (Jones *et al.*, 1994). In any event, meningococci are observed in subepithelial tissues after 18-24 hours of infection, suggesting that they have penetrated the epithelial layer (Stephens *et al.*, 1983). It is not clear whether meningococci enter the subepithelial tissues by parasite-directed endocytosis (McGee *et al.*, 1988; Stephens & Farley, 1991) or via an intercellular route through disrupted tight junctions (Rayner *et al.*, 1995). The presence or absence of capsule appears to have no effect on the ability of meningococci to invade the non-ciliated cells (Stephens *et al.*, 1993).

Cell culture assays have supported organ culture studies in that meningococci and gonococci adhere to epithelial cells (Virji & Everson, 1981) and have established that they also invade these cells (Shaw & Falkow, 1988; Stephens, 1989). The work of Virji and colleagues (Virji *et al.*, 1991; 1995b) with HUVECs, has established that both meningococci and gonococci associate with, and invade, human endothelial cells. The use of cells grown on a microporous membrane confirms that meningococci and gonococci do cross both epithelial and endothelial layers (Birkness *et al.*, 1995; Ilver *et al.*, 1998). Merz *et al* (1996) have shown that meningococci traverse T_{84} cells in a time course identical to that established in organ culture studies. It is possible that the route of passage across epithelial and endothelial cell layers is either transcellular or paracellular, or a combination of both. However, during the meningococcal infection of T_{84} cells, tight junctions remained intact suggesting a transcellular route (Merz *et al.*, 1996; Pujol *et al.*, 1997). More recently the transcellular theory has been supported by the work of Wang and co-workers (Wang *et al.*, 1998), who have shown that during the infection of T_{84} cells with gonococci, the transepithelial electrical resistance of the cell monolayers was sustained.

1.5.2.2 Bacteraemia

Little is known about the events that occur after meningococci enter the circulation and the establishment of bacteraemia. Certainly, meningococci must survive in the intravascular space and evade host defence mechanisms in the blood, especially circulating components of the alternative complement system and immune effector cells (Densen, 1989). The resistance of group B

meningococci to phagocytosis and the alternative complement pathway is due to the inhibitory effects of its capsular and LOS sialic acid and, typically, meningococci isolated from the blood are encapsulated. In the circulation system, the presence of meningococcal LPS initiates the coordinated release of the proinflammatory cytokines and chemokines, including tumour necrosis factor α (TNF-α), and interleukins 1β, 6 and 8 (Il-1β, Il-6 and Il-8) (Brandtzaeg *et al.*, 1992). LPS is also responsible for activation of the coagulation system and suppression of fibrinolytic pathways which, in concert with its inflammatory role, contribute to DIC and multiple organ failure in septic shock (Karima *et al.*, 1999). LPS is thus a potent inducer of inflammation and is responsible for triggering the extensive and detrimental host response seen in meningococcal meningitis and sepsis.

1.5.2.3 Pathophysiology of meningitis

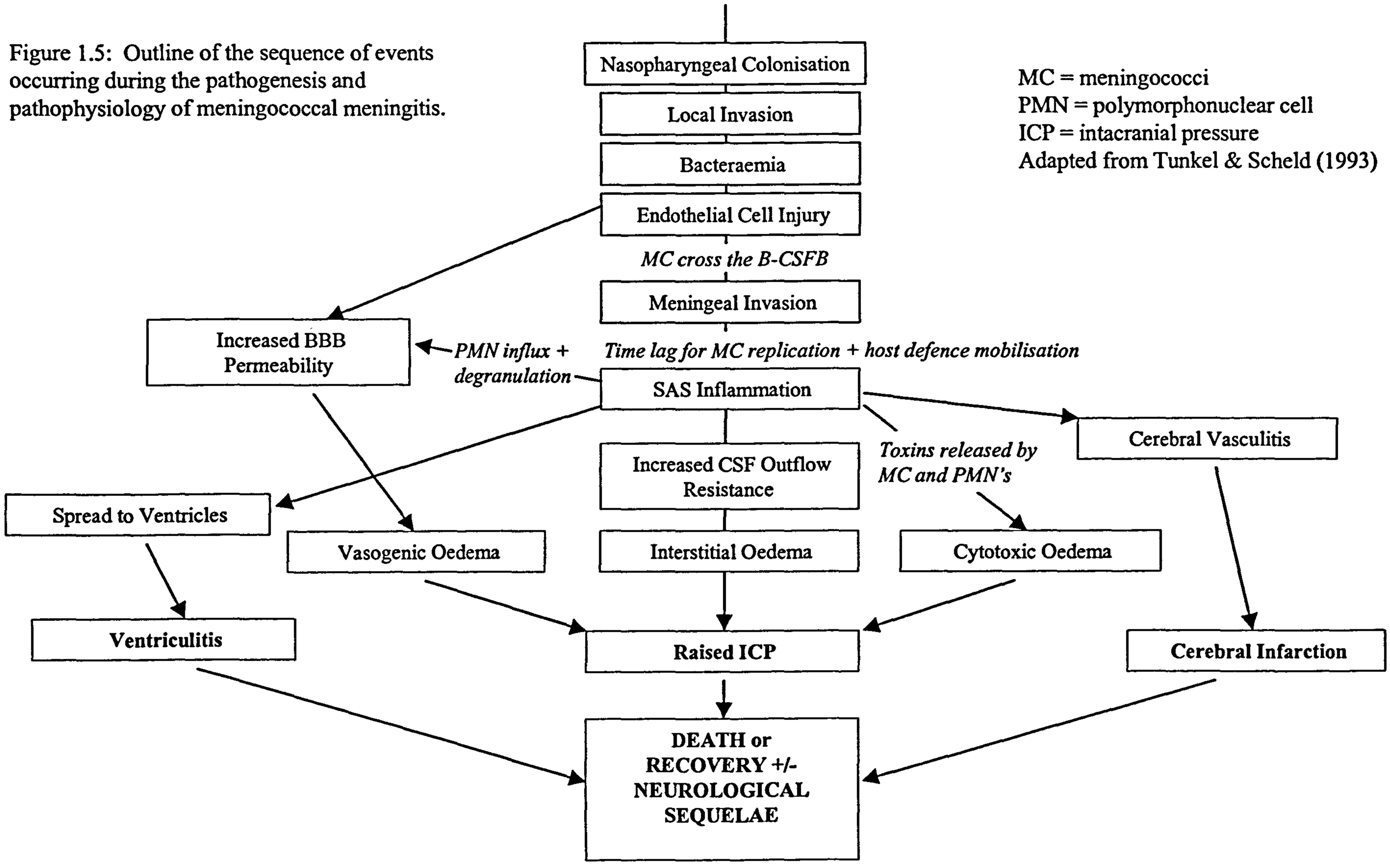
Although it is possible that meningococci travel directly from the nasopharynx, through other tissues to reach and enter the SAS, it is more likely that they invade via the systemic circulation. This is supported by the recovery of meningococci from the CSF of rhesus monkeys after intravenous injection (Nassif & So, 1995) and also that in some individuals infected with meningococci, bacteria were cultured from the blood before being cultured from the CSF (Devoe, 1982). From the blood, meningococci must breach the B-CSFB to reach the SAS and meninges, and it is not known whether this occurs at the B-CSFB located at the meningeal capillaries or at the choroid plexus. From a fatal untreated case of fulminant meningococcaemia, meningococci have been seen to adhere to the endothelial cells of the choroid plexus in greater numbers than to those of the meninges (Pron *et al.*, 1997). However, the choroid plexus is served by an increased blood supply, which may result in the delivery of more meningococci to this site. In addition, TEM analysis showed that meningococci were not internalised by the underlying choroidal epithelial cells or located between them, suggesting that invasion of the B-CSFB may be via a different route. An alternative hypothesis is that meningococci gain access to the SAS via invasion of the thin-walled veins and venules in the meninges where, unlike in arteries, there is minimal smooth muscle surrounding the vessel. As described in Section 1.3.1, the endothelial cells of the cerebral microvasculature act as a highly selective barrier and it is not known how meningococci invade these cells. During this process though, endothelial cells are damaged, and it is likely that they are thus activated to release cytokines and chemokines, and to up-regulate their expression of leukocyte adhesion molecules. Once meningococci enter the SAS the virtual absence of host humoral defences in the CSF (especially immunoglobulin and complement activity) probably contributes to meningococcal survival (Epstein *et al.*, 1992). After meningococci have entered the SAS and proliferated in the CSF, the presence of increasing levels of LPS contributes to the host inflammatory response initiated by endothelial cell damage. The role of leptomeningeal cells in the inflammatory response and recruitment of neutrophils is not known. Although meningococci are present in the SAS of meningitis victims they do not actually invade the underlying cerebral tissue, even though the brain may become oedematous and haemorrhagic.

From analysis of cytokine profiles in patients with meningitis, it has been suggested that the local release of proinflammatory cytokines into the SAS, including TNF-α, IL-1β, Il-6, and Il-8, is a coordinated host response to meningococcal infection (Brandtzaeg, 1995). Both TNF-α and IL-1β are implicated as mediators of damage to the B-CSFB and either molecule is capable of activating the cerebral endothelium to express surface adhesion molecules required for the recruitment of leukocytes, predominantly neutrophils. To facilitate neutrophil diapedesis, the endothelial tight cell junctions are then widened. Upon entry into the SAS, the locally produced TNF-α and IL-1β stimulate the neutrophils to degranulate and release their toxic contents in the vicinity of the cerebral microvasculature, thus worsening the inflammation. Following the inflammatory response, blood vessels that run through the SAS can become inflamed (cerebral vasculitis), resulting in thrombotic occlusions that can cause small infarctions on the surface of the brain, or more extensive infarction deep within the cerebral hemispheres if larger vessels are involved (Weller, 1990). The B-CSFB becomes increasingly permeable due to the opened endothelial intercellular junctions allowing the influx of protein, particularly albumin, and vasogenic brain oedema ensues (Brandtzaeg, 1995; Perry *et al.*, 1997; Tunkel & Scheld, 1993). Cerebral oedema hampers the efficiency of venous drainage resulting in increased CSF outflow resistance (interstitial oedema). Ultimately, cerebral oedema leads to raised intracranial pressure and subsequent herniation of brain tissue (Devoe, 1982; Tunkel *et al.*, 1990). In rare cases, the infection and inflammation of meningitis may spread from the meninges to the cerebral ventricles to cause ventriculitis, which is usually fatal (Weller, 1990).

1.5.2.4 Cytokines and chemokines in meningococcal disease

Cytokines play an important role in the host inflammatory response to meningococcal challenge. TNF-α, Il-1 and Il-6 are classical proinflammatory cytokines detected in the CSF during meningococcal meningitis. The former two are produced by haematopoietic cells and various tissue cells in response to mediators such as LPS, whereas Il-6 is produced by monocytes, macrophages, endothelial cells and other tissue cells in response to Il-1 and TNF-α stimulation. To regulate the effects of the proinflammatory cytokines during meningitis, anti-inflammatory cytokines are also produced, particularly Il-10 and TGF-β. Il-10 is produced by monocytes, macrophages, and B and T lymphocytes in response to immunological challenge, and is present in high concentrations in the CSF of meningococcal meningitis sufferers. This cytokine has been shown to inhibit the synthesis of TNF-α, Il-1α/β, Il-6, Il-8 and colony stimulating factors such as GM-CSF from human monocytes and neutrophils (Tauber & Moser, 1999; Lehmann *et al.*, 1995). After bacterial LOS was injected into the SAS of rabbits, Il-10 was shown to significantly decrease the concentration of TNF-α at this site and subsequently decrease CSF inflammation (Paris *et al.*, 1997), and presumably, this cytokine has similar effects in humans. TGF-β is mainly produced by monocytes, macrophages and T lymphocytes, and it appears to have a similar anti-inflammatory role to Il-10

Figure 1.5: Outline of the sequence of events occurring during the pathogenesis and pathophysiology of meningococcal meningitis.



MC = meningococci
PMN = polymorphonuclear cell
ICP = intacranial pressure
Adapted from Tunkel & Scheld (1993)

(Tauber & Moser, 1999). Chemokines are a family of cytokines produced by leukocytes and tissue cells, which induce chemotactic migration in leukocytes. They contain a highly conserved four-

cysteine motif in their primary amino acid structure, and can be further divided into two principal subfamilies, depending on the arrangement of the two amino-terminal cysteines. If these are adjacent then the chemokine belongs to the CC subfamily, and if there is an intervening amino acid residue then it belongs to the CXC subfamily. In general, the CC chemokines (eg MCP-1, MIP-1α/β, RANTES) act on monocytes and other leukocytes, but not neutrophils or B lymphocytes, whereas the CXC (eg Il-8) chemokines are more selective for neutrophils and T or B lymphocytes. Il-8, MCP-1 and MIP-1 are all detected in the CSF during bacterial meningitis, and it is likely that these chemokines contribute to the ordered influx of neutrophils, and then monocytes (Tauber & Moser, 1999; Diab *et al.*, 1999).

1.5.3 *Neisserial* virulence factors and their receptors

Some of the structures that are responsible for the adhesive and invasive characteristics of meningococci and gonococci have become apparent. Among others, capsule, pili, Opa and Opc proteins, and LPS play important roles in the meningococcal attachment and invasion of human cells. The human receptors for these bacterial virulence factors are beginning to be elucidated.

1.5.3.1 Pilus

It is now well established that pili are required for the attachment of meningococci and gonococci to eukaryotic cells. Pili are currently the only bacterial structure implicated in the adhesion of encapsulated meningococci, or bacteria with sialylated LPS immunotypes, to such cells. In organ culture studies, meningococcal pili were shown to act as important adhesins to the non-ciliated cells (McGee *et al.*, 1983), in encapsulated and non-encapsulated strains (Stephens *et al.*, 1986). The presence of capsule reduced the binding of piliated bacteria when compared to acapsulate piliated bacteria (Stephens *et al.*, 1981; Stephens *et al.*, 1993). The reason for increased adherence to organ culture cells upon loss of capsule is not known (Stephens *et al.*, 1993). It may be that, as with epithelial and endothelial cells grown in culture, the loss of capsule enables other outer membrane virulence factors to act as adhesins. It has been shown that capsule expression does exert a partial inhibitory effect on pilus-mediated interactions with endothelial cells (Virji *et al.*, 1995b). This could be due to a charge-mediated phenomenon as both capsule and host cell surface carry a net negative charge. Alternatively, capsule may mask some adhesive ligands along the length of the pilus. The important role of pili is supported by the fact that meningococci isolated from the nasopharynx of carriers, and from the CSF of patients with meningococcal disease, were shown to be heavily piliated (Devoe & Gilchrist, 1975). However, several studies have shown that both

meningococcal and gonococcal pili are downregulated during the invasion of epithelial cells (Makino *et al.*, 1991; deVries *et al.*, 1996; Ilver *et al.*, 1998). These findings suggest that after attachment, the non-piliated phenotype may be a prerequisite for invasion of epithelial cells.

Pilus expression is regulated by both phase variation, and by antigenic variation of the pilin subunit (PilE), and PilC proteins. Intrastrain antigenic variation of pilin, and differences in expression of class I and II pili, have been shown to affect the pilus-mediated adhesion of *Neisseria meningitidis* to epithelial cells. In contrast, such variations have little effect on the interactions with endothelial cells (Virji *et al.*, 1992a; Nassif *et al.*, 1993). As pilin variants can confer different levels of meningococcal adhesiveness onto epithelial cells, the bacteria may be provided with a way of modulating their virulence. Pilin antigenic variation may also show tropism to different host cell types (Pinner *et al.*, 1991). It has been suggested that the high adhesiveness of some pilin variants is due to the formation of pili bundles which may induce inter-bacterial interactions (Marceau *et al.*, 1995). The bundling of pili could lead to enhanced binding to host cells because several pilus tip adhesins would be together at the extremity of the bundle. Although both meningococcal and gonococcal pili are glycocsylated (Parge *et al.*, 1995; Stimson *et al*, 1995), recent studies (Marceau *et al.*, 1998) have shown that this has little effect on adherence properties. The PilC proteins of pili have also been implicated in the adherence of meningococci (both class I and II pili) and gonococci to epithelial cells. PilC proteins have been identified as pilus tip adhesins (Rudel *et al.*, 1995) and are also associated with the bacterial cell surface (Rahman *et al.*, 1997). In meningococcal strains it is the PilC1 protein that is implicated in adherence, and expression of this protein is transiently up-regulated during the initial contact of bacteria with human or other eukaryotic cultured cells. The up-regulation of PilC1 is essential to pilus-mediated adhesion but does not increase the actual level of piliation; it is possible that a basal level of PilC1 is required for pilus assembly but that higher production is required to localise this protein to the pilus tip to fulfil its role as an adhesin (Nassif *et al.*, 1994; Taha *et al.*, 1998).

Piliated *Neisseria* have been shown to bind to the epithelial cell surface transmembrane glycoprotein, CD46 (membrane cofactor protein), probably via the PilC1 protein (Kallstrom *et al.*, 1997). The CD46 protein is involved in the downregulation of the complement cascade by binding of the C3b/C4b components, and is also known to be a receptor for the measles virus. Although there is no evidence for a direct interaction of PilC with the CD46 receptor, CD46 blocking antibodies as well as purified CD46 can prevent gonococcal adherence to target cells (Kallstrom *et al.*, 1997). CD46 is present on virtually every human cell and tissue type except on RBCs. However, as *Neisseria* are known to bind erythrocytes in a pilus-dependent manner, another pilus receptor must exist on these cells (Kallstrom *et al.*, 1997). The binding of piliated *Neisseria* to human epithelial cells has been shown to elicit actin rearrangements and clustering of tyrosine-phosphorylated proteins in the host cells, and suggests that microbe-human cell interactions may activate host signal transduction cascades (Merz & So, 1997). It is likely that such host cell

responses are involved in the triggering of the inflammatory response, which is a characteristic of meningococcal meningitis.

1.5.3.2 Opa and Opc proteins

It is well established that in non-capsulate meningococci, the outer membrane proteins Opa and Opc have a modulatory role in bacterial interactions with host cells; this is also seen with Opa proteins in gonococci. Whilst meningococcal Opa proteins are expressed equally in nasopharyngeal and systemic isolates, Opc expression is often confined to the former suggesting that these proteins may have a role in the colonisation of different host niches (Achtman *et al.*, 1991). The outer membrane Opa proteins have been shown to mediate neisserial interactions with human neutrophils and epithelial cells, and endothelial cells to a lesser extent, and have a role in the invasion of these cell types. The Opc protein is unique to meningococci, and has been shown to mediate the cellular invasion of cultured epithelial and endothelial cells (Virji *et al* 1992b).

In capsulate meningococci, Opa and Opc proteins are generally regarded to be ineffective as adhesins to cultured human epithelial, endothelial and phagocytic cells due to a masking effect by the capsule (Virji *et al.*, 1991; Virji *et al.*, 1993a). Although meningococci isolated from the blood are predominantly capsulate and piliated, different environmental conditions may affect capsulation, and nasopharyngeal isolates are often non-groupable and presumably capsule-deficient. However, conditions of low level capsule expression do enable the Opa and Opc proteins of invasive strains to function, and recent studies have shown that if receptors for Opa proteins are present at a high density, then Opa-mediated adhesion is enhanced (Virji *et al.*, 1996). Sialylated LPS has also been shown to have an inhibitory role on Opa and Opc function in certain bacterial interactions with epithelial, endothelial and phagocytic cells (Virji *et al.*, 1996).

In non-capsulate meningococci, expression of the Opc protein confers the ability for these bacteria to adhere to and invade human epithelial and endothelial cells. Interestingly, the Opc mediated interaction is dependent on rearrangements in the host cell cytoskeleton and, in the absence of Opa, the use of the microfilament inhibitor Cytochalasin D (CD) results in the inhibition of both invasion and total cell association. This is interesting because it is in contrast to studies which showed that CD increased Opa mediated association, although invasion was inhibited (Virji *et al.*, 1992b; 1994). Opc-expressing *Neisseria meningitidis* are known to target integrin host-cell receptors on endothelial cells to subsequently invade these cells. Integrins are a superfamily of heterodimeric transmembrane molecules that consist of two chains; one of 16 α chains and one of eight β chains. They specialise in mediating cell adhesion and communication by binding to a wide array of ligands, including extracellular matrix (ECM) proteins, plasma proteins and transmembrane glycoproteins (Binnerts *et al.*, 1996). Many integrin ligands utilise the RGD (Arg-Gly-Asp) ligand recognition motif which is recognised by $\beta3/\beta5$ and some $\beta1$ and $\beta2$ integrins. Using polarised Huvecs, studies have shown that, in the presence of serum, Opc-expressing meningococci associate

with the apical surface of endothelial cells. Opc is able to interact with multiple ECM components and serum proteins. Meningococcal adhesion to integrins occurs via a bridging mechanism whereby the bacteria bind serum proteins that are natural ligands of integrins and which contain the RGD motif. Meningococci bind to the serum glycoprotein vitronectin and use it to bind to the integrin vitronectin receptor (VNR αvβ3); the fibronectin receptor (FNR α5β1) may also be involved (Virji *et al.*, 1994; 1995b). For efficient Opc-dependent host-cell interactions, meningococci must express Opc at a high density on their outer membrane surface (Virji *et al.*, 1995b). The Opc protein is also known to mediate the meningococcal adherence to and invasion of epithelial cells (Virji *et al.*, 1992b). Recently, (deVries *et al.*, 1998) it has been shown that Opc also binds to cultured epithelial cell heparan sulphate proteoglycan receptors in a similar manner to the Opa_{50} protein of the gonococcal strain MS11 (see subsequent Opa section). The presence of capsular and/or LPS-associated sialic acids has an inhibitory effect on Opc-mediated interactions with epithelial cells. It is likely that LPS-associated sialic acids prevent these interactions by interfering with the Opc-proteoglycan receptor interaction. In contrast, capsule may prevent these interactions by steric hindrance rather than by direct interference with Opc-receptor binding (deVries *et al.*, 1998).

Many studies have focused on the role of gonococcal Opa proteins, and it is likely that meningococcal Opa proteins behave in a similar manner. The initial pilus mediated adhesion of *Neisseria* to host cells is believed to be enhanced by subsequent Opa binding to these cells. The strong adherence of gonococci to host cells appears to be essential for gonococcal internalisation, and the invasive gonococcal phenotype is typically non-piliated but Opa expressing (Makino *et al.*, 1991). Opa proteins may therefore have a role in the bacterial invasion of host cells after the loss of pili. In both meningococci and gonococci, different Opa proteins show tropism for different human cell types. Kupsch (Kupsch *et al.*, 1993) and Weldbeser (Waldbeser *et al.*, 1994) have shown that of the large repertoire of Opa proteins in gonococci, within a strain, some are implicated in epithelial cell interactions, whilst the majority confer interactions with neutrophils. In addition, Opa proteins play a role in endothelial cell interactions, and distinct Opa phenotypes mediate the interactions of gonococci with human monocytes (Knepper *et al.*, 1997). For instance, the gonococcal strain MS11 encodes for 11 variant Opa proteins, which can be divided into two groups according to the type of molecule they recognise on human cells; heparan sulphate proteoglycans or members of the CD66 family.

Opa proteins that mediate gonococcal-epithelial cell interactions, have been shown to bind to cell surface, syndecan-like, heparan sulphate proteoglycans (HSPGs) (van Putten & Paul, 1995). HSPGs are expressed ubiquitously on eukaryotic cells; the syndecan receptor is the most common member of the proteoglycan group, and is distributed on a variety of epithelia. The binding of certain Opa proteins, for instance Opa_{50} in strain MS11, to a syndecan HSPG molecule results in the internalisation of gonococci by some epithelial cell lines. However, the binding of gonococcal Opa proteins to proteoglycan receptors is not always sufficient to mediate the internalisation of these

bacteria into epithelial cells. A 78/68 kDa factor in serum, identified as vitronectin, has been implicated as a mediator of bacterial internalisation into normally nonphagocytic epithelial cells (Duensing & van Putten, 1997). Another serum factor, fibronectin, is also implicated in this process. Vitronectin and fibronectin are adhesive glycoproteins present in the blood and extracellular matrix of various tissues, including mucosal sites (Gomez-Duarte *et al.*, 1997). These serum factors bind to the surface of Opa proteins, and then to integrins on the epithelial cell surface via the RGD bridging molecule. This interaction occurs in concert with the binding of Opa to HSPG receptors, and results in internalisation of the bacterium (Gomez-Duarte *et al.*, 1997; van Putten *et al.*, 1998). Opa mediated interactions with HSPGs have also been shown to promote strong binding of Opa_{50} expressing gonococci to endothelial cells, although internalisation was integrin dependent and required the presence of serum factors (Dehio *et al.*, 1998).

Although a small proportion of Opas have been shown to mediate their actions on epithelial cells through interactions with HSPGs, the majority of Opas expressed by *N. meningitidis* and *N. gonorrhoeae* strains, have been shown to adhere and invade cells expressing CD66. However, recently, it has also been reported that some meningococcal Opa proteins have the ability to interact with both HSPG and CD66 receptors (Virji *et al.*, 1999). Members of the CD66 carcinoembryonic antigen family (CEA or CD66), are human cellular receptors that belong to the immunoglobulin superfamily (reviewed by Hammerström *et al.*, 1993). Within the CEA family are two subfamilies; the CEA subgroup (Table 1.3) and the pregnancy-specific glycoproteins. *In vivo*, CD66 proteins are probably involved in intercellular adhesion. The individual members of the CD66 family have different tissue distributions but, throughout the family, the N-terminal domain is highly conserved (reviewed by Hammerström *et al.*, 1993). Meningococcal Opa proteins that attach to epithelial cells and neutrophils via CD66, do so by binding to a common target site in the N-terminal domain (Virji *et al.*, 1996a,b). More than 95% of meningococci and gonococci, isolated from mucosal and disease sites, are able to bind the CD66a molecule whereas, so far, no strains have been found to bind to CD66b (Chen *et al.*, 1997; Gray-Owen *et al.*, 1997). In the case of CD66a, it is also important to note that encapsulated strains of meningococci with sialylated LPS are still capable of binding to this molecule, as previous studies have questioned the contribution that Opa has in the *in vivo* colonisation of host tissues (Virji *et al.*, 1996). When Opa proteins bind to CD66 molecules on PMNs, they trigger the non-opsonic phagocytic uptake of bacteria and subsequent generation of an enhanced respiratory burst, which is likely an effective bactericidal response (Belland *et al.*, 1992). It would therefore seem that in this setting, the expression of Opa proteins that bind to CD66 molecules is not a survival advantage. CD66e receptors are not expressed on PMNs, but on a variety of epithelial and endothelial tissues, and it is likely that the specificity of different Opa proteins for distinct CD66 molecules influences the tropism of certain bacteria for specific tissues. In addition, CD66 receptors are up-regulated on cytokine activated epithelia and endothelia, suggesting that Opa proteins could mediate the binding of pathogenic *Neisseria* to inflammatory tissues *in vivo* (Gray-Owen *et al.*, 1997a). Recently, evidence has been presented that the binding of

Opa proteins to cellular CD66 receptors mediates the transcellular traversal of gonococci across T_{84} epithelial cell monolayers (Wang *et al.*, 1998).

Table 1.3: The carcinoembryonic antigen cell adhesion molecule (CEACAM) branch of the CEA family

(Beauchemin *et al*,. 1999)

CD66 member	Name	Expressed on	Known Ng receptor functions	Known Nm receptor functions
CD66a (BGP)	Biliary glycoprotein	PMN; Many normal tissues, eg: epithelia of cervix, uterus, tonsils; endothelia of endometrium vessels lymphoid tissue	Opa receptor on PMN, where it triggers an enhanced respiratory burst	Opa receptor on PMN and other cells
CD66b (CGM6)	CEA gene family member 6	PMN	No evidence that this is a receptor for any Ng Opa	No evidence that this is a receptor for any Nm Opa
CD66c (NCA)	Non-specific crossreacting antigens	PMN	Opa receptor on PMN	Possible Opa receptor on PMN
CD66d (CGM1)	CEA gene family member 1	PMN	Opa receptor on PMN	Possible Opa receptor on PMN
CD66e (CEA)	Carcinoembryonic antigen	Various epithelial and endothelial tissues, eg Squamous epithelium of uterus; Endothelium of lymph nodes, inflammatory tissue Up-regulated in epithelial carcinomas, some secretory meningiomas Not on granulocytes	Opa receptor on epithelial and endothelial cells	Opa receptor on epithelial and endothelial cells

Compiled from: Gray-Owen *et al.*, 1997b; Chen & Gotschlich, 1996; Virji *et al.*, 1996, 1999.

The high frequency phase transitions of Opa proteins allow non-invasive gonococci and meningococci to acquire an invasive phenotype and *vice versa* (Waldbeser *et al.*, 1994; Virji *et al.*, 1996). Such transitions may also protect meningococci from phagocytosis by neutrophils. When human PMNs were infected with Opa expressing meningococci, surviving bacteria were found to be

Opa- revertants, suggesting that the lack of Opa expression may enable bacteria to escape recognition by CD66 on these cells (Virji & Heckels, 1986; Virji *et al.*, 1996).

Recent studies have shown that as well as roles in adhesion and invasion, Opa protein may promote the intracellular survival of gonococci. Once internalised, if gonococci (and meningococci) are to survive and grow, it is likely that they will require a host carbon source of which the most likely candidates are pyruvate and lactate. Recently, using cultured human epithelial cells, it has been shown that intracellular gonococci co-localise with host pyruvate kinase (PK) via their Opa proteins to acquire pyruvate produced from phosphoenolpyruvate (Williams *et al.*, 1998). The ATP produced from this reaction may also be utilised by gonococci. It is likely that internalised, Opa expressing meningococci also interact with PK for intracellular survival.

1.5.3.3 LPS

The majority of meningococcal isolates from the nasopharynx of carriers are non-capsulate and express a non-sialylated LPS species. In the disease state, bacteria isolated from the blood or CSF are almost exclusively encapsulated, with sialylated LPS (Meyer *et al.*, 1994). The unsialylated phenotype permits meningococcal invasion of epithelial cells but renders the bacteria sensitive to host bactericidal activity (Meyer *et al.*, 1994). The terminal sialic acid confers resistance to antibody- and complement-mediated killing, presumably by masking target epitopes. Sialylation also interferes with meningococcal adherence to PMNs thus preventing phagocytosis. Different LPS immunotypes have negligible effects on the adherent properties of encapsulated meningococci to human cells (Virji *et al.*, 1995b). Switching between an unsialylated and a sialylated phenotype may be the basis for the phase variation of LPS, allowing the meningococcus to survive in different host anatomical sites (van Putten, 1993).

The sialylated terminal lacto-N-neotetraose structures of LPS are similar to glycosphingolipids present on the membranes of many human cells. In gonococci (and presumably meningococci) the presence of this LPS structure may be important for either of two equally valid reasons. Firstly as an example of molecular mimicry, the bacteria are more 'host-like' and are able to avoid immune recognition (Smith *et al.*, 1992). Secondly, the synthesis of these asialoparagloboside-like structures may be to act as ligands for human cell receptors. Recent reports (Porat *et al.*, 1995a; Porat *et al.*, 1995b) have shown that on the hepatic HepG2 cell line, there are two receptors for the lacto-N-neotetraose structure of gonococci. One of these receptors is the human asialoglycoprotein receptor (ASGP-R), an integral membrane protein that specifically recognises ligands with a terminal galactose residue. Its normal physiological role is to remove serum glycoproteins that have lost their terminal sialic acid residues. Amongst others, these receptors are present on macrophages, endothelial cells, sperm cells, hepatocytes and male urogenital cells. Gonococci have been shown to bind to ASGP-R on HepG2 cells and to upregulate receptor expression (Porat *et al.*, 1995a). It is likely that the ASGP-R has a role in *in vivo* gonococcal infections as the presence of this receptor

has been reported on infected primary human urethral epithelial cells, a model for gonococcal infection (Harvey *et al.*, 1997). The oligosaccharide region of gonococcal LPS has also been shown to bind to a 70kDa protein (p70) of HepG2 cells. This receptor has a higher affinity for gonococcal LPS than the ASGP-R, and is antigenically similar to the gonococcal Opa proteins which are known to bind to LPS. However, the relevance of this receptor under *in vivo* gonococcal infection is not known (Porat *et al.*, 1995b).

It appears that the activity of LPS is mediated by specific cellular receptors, and it has been suggested that on human cells there are multiple LPS binding sites and perhaps multiple receptors (Tohme *et al.*, 1999). However, the signalling pathways that transduce the LPS signal to result in the release of proinflammatory mediators is not clear. CD14 is a 55kDa glycosylphosphatidylinositol (GPI) – anchored protein found on the surface of monocytes, macrophages and polymorphonuclear leukocytes (mCD14), and as a soluble protein in the blood (sCD14). In conjunction with the serum protein LPS binding protein (LBP), mCD14 acts as a receptor for LPS. In epithelial and endothelial cells which do not have mCD14, LPS exerts its effects via sCD14 (Hailman *et al.*, 1994). However, CD14 does not possess a cytoplasmic domain or any apparent signal transduction motif, suggesting that another cell surface domain must be present to transduce signals across the cell membrane. Recently, the 78kDa protein moesin (membrane-organising extension spike protein) has been identified as an independent LPS receptor on human monocytes, and it is suggested that this protein may function as a signal transducing coreceptor for CD14 (Tohme *et al.*, 1999). In addition, members of the integrin family may also interact with LPS (Virji, 1996).

1.5.3.4 IgA proteases

In addition to the specific protease activity of these bacterial enzymes for human IgA1, they have also been shown *in vitro* to promote the intracellular survival of meningococci and gonococci. Using epithelial cell lines, the neisserial type 2 IgA1 protease was found to target the human lysosome-associated membrane protein 1 (LAMP-1) which contains an IgA1-like hinge region (Lin *et al.*, 1997; Hauck & Meyer, 1997), resulting in its degradation. LAMP-1 is a major integral membrane protein of lysosomes and late endosomes; it is thought to be important in maintaining the stability of these compartments by protecting the membranes from digestion by the hydrolytic enzymes within them. The intracellular growth rate of bacteria that had been genetically modified to produce no IgA1 protease was greatly reduced compared to the wild-type parent strain, and this mutant could not affect the turnover rate of LAMP-1 in infected cells. However, a reduction in LAMP-1 levels was seen in cells infected with meningococci and gonococci that expressed IgA protease, and this may result in an overall decrease in the number of functional lysosomes, suggesting that IgA1 protease may directly promote the intracellular survival of these bacteria. It is however, still unclear how this enzyme gains access to LAMP-1. Although internalised bacteria are

often seen within a phagosomal membrane, if such a phagosome fused with a late endosome or a lysosome then IgA protease would gain contact with LAMP-1 (Lin *et al.*, 1997). In addition, neisserial infection of epithelial cells results in reduced levels of three other lysosomal markers, LAMP-2, lysosomal acid phosphatase (LAP) and CD36, although this is not due to direct cleavage by the protease and is likely an indirect effect of the cleavage of LAMP-1 (Ayala *et al.*, 1998). Experimental evidence therefore suggests that IgA proteases may emerge as important virulence determinants, by lessening the effects of humoral immunity and by promoting the intracellular survival of invasive *Neisseria.*

1.5.3.5 Porins

Meningococci produce two porins designated PorA and PorB, whereas gonococci only express the latter. *In vitro* investigations have shown that porins of meningococci and gonococci can spontaneously translocate into artificial lipid membranes, as well as into eukaryotic plasma membranes where they assume functional voltage-gated ion channel activity similar to that seen in native eukaryotic porins. In contrast, porins of the commensal, *Neisseria sicca*, did not transfer into lipid bilayers, suggesting that pore-forming ability may rest only with pathogenic organisms (Mosleh *et al.*, 1998). Purified porins of meningococci and gonococci have been shown to interfere with neutrophil signalling mechanisms, inhibiting their phagocytic capacity (Bjerknes *et al.*, 1995). In addition, purified gonococcal porin has been shown to interfere with phagosome maturation in macrophages (Mosleh *et al.*, 1998) suggesting that porins have an important pro-survival role for intracellular bacteria. Support for this hypothesis has come from investigations utilising gonococci that have either had their *porB* gene mutated or replaced with one from a commensal *Neisseria* species. Both mutants induced an enhanced respiratory burst from, and uptake by, HL60 cells (differentiated to simulate neutrophil functions), and were significantly decreased in their ability to invade Chang epithelial cells in comparison to the wild type parent strains (Bauer *et al.*, 1999). The results from this study also suggest that it is possible for PorB to be a co-stimulatory factor in the invasion of epithelial cells mediated by the Opa_{50} protein of the gonococcal MS11 strain. It is also possible that neisserial porins influence the dynamics of host cell actin rearrangements during infection, as both PorA and PorB proteins were shown to nucleate and alter actin filament structure (Giardina *et al.*, 1998).

1.5.4 Signalling events occurring during infection with pathogenic Neisseria

Much progress has been made concerning the cellular interactions and host-cell signalling events that occur during infection with gonococci, although less is known about the events occurring during meningococcal infection. The inflammatory response that occurs during gonococcal infection is characterised by a massive influx of neutrophils and monocytes into the infected tissues,

Table 1.4: Functions of Neisserial virulence factors and their host-cell receptors

Virulence factor	Nm or Ng	Cellular association	Host-cell receptor used	Virulence function
Capsule	Nm	-	-	Inhibits phagocytosis by PMN Masks Opa/Opc
Pilus/PilC	Both	Epithelial Endothelial	CD46 on all cells except RBCs	Binding of capsulate organisms
Opa	Both	Epithelial Endothelial PMN	CD66 on PMN, epithelial, endothelial Heparan sulphate proteoglycans on epithelial	Adhesion Invasion Intracellular survival
Opc	Nm	Epithelial Endothelial Monocytes	Integrins on endothelial Proteoglycans on epithelial	Adhesion Invasion
LPS	Both	Epithelial	ASGP-R, p70, CD14/LBP, Moesin, Possibly integrins	Adhesion Invasion Sialylated form inhibits Opa/Opc
IgA proteases	Both	-	-	Protease cleaves human IgA1 Promote intracellular survival
Porins	PorA and PorB in Nm PorB in Ng	All?	-	Unclear, may dampen the phagocytic capacity of neutrophils and macrophages

Adapted from: Jerse & Rest, 1997; Dehio *et al.*, 1998; Nassif *et al.*, 2000.

and consequently the epithelial layer is destroyed allowing the bacteria access to deeper tissues, and the opportunity for disseminated disease. Naumann and colleagues (Naumann *et al.*, 1999) have summarised the co-ordinated sequence of events that can happen upon gonococcal infection as follows: 1) cytoskeletal reorganisation, 2) invasion or phagocytic uptake, 3) intraphagosomal accommodation, 4) nuclear signalling, 5) cytokine/chemokine release, and finally, 6) apoptosis (Naumann *et al.*, 1999). Direct contact of piliated or Opa-expressing gonococci with epithelial cells

results in the activation of the early transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), Rho-GTPases and stress response kinases, and subsequent production of proinflammatory cytokines (Naumann *et al.*, 1997; 1998). In addition, translocation of the gonococcal porin may also be involved in the initiation of cell signalling, and could play a key role in the induction of apoptosis by the activation of cysteine proteases (Muller *et al.*, 1999). It is likely that further studies will reveal that pili, Opa proteins and porins mediate a similar sequence of events during meningococcal infection. In addition, signalling pathways may be activated directly by the targeting of integrins by meningococcal Opc proteins, with subsequent cytoskeletal arrangements which facilitates endocytosis of these bacteria by human epithelial and endothelial cells (Virji, 1996).

1.6 Aims of the project

Little information is known about the later stages of meningococcal disease, after meningococci have entered the SAS, concerning the interactions of these bacteria with the cells of the human leptomeninges. A hypothesis was formed based on the major observation that during meningitis, *Neisseria meningitidis* targets the leptomeningeal cells of the subarachnoid space.

Hypothesis: Meningococci associate with leptomeningeal cells, and that this interaction is mediated by specific ligands.

As normal human leptomeninges do not reliably grow in culture, this thesis reports on the use of meningioma cells grown *in vitro*, as a model for studies on the interaction between meningococci and the human leptomeninges.

Aims: The aims of the work were to:

A] Establish the growth of meningioma cells *in vitro*, and to characterise the cells with respect to normal leptomeningeal cells;

B] Investigate the nature of the interactions between meningococci and human meningeal cells with respect to association and invasion;

C] Identify meningococcal surface antigens which participate in such interactions; and to investigate the potential use of the model to study mechanisms which might be involved in the generation of the acute inflammatory response characteristic of meningococcal meningitis.

CHAPTER 2 MATERIALS AND METHODS

2.1 Growth of bacteria

All bacteria were grown on proteose peptone (PP) agar. This was prepared according to the method of Zak (Zak *et al.*, 1984) from the following components.

All components except supplements A and B were sterilised by autoclaving at 15lb in^{-2} for 15min. Supplements A and B were added to the autoclaved solution after cooling to 50°C.

Materials:	Amount per litre (distilled water)
Proteose peptone (Difco)	10g
Bacto-agar (no.1, Oxoid)	10g
Starch (Merck-BDH)	1g
$K_2HPO_4.3H_2O$ (Merck-BDH)	5.24g
$K_2H_2PO_4$ (Merck-BDH)	1g
Sodium chloride (Sigma)	5g
Supplement A	8ml
Supplement B	2ml

Supplement A:	Amount per 800ml
Glucose (Merck-BDH)	100g
L-glutamine (Merck-BDH)	10g
para-amino-benzoic acid (Sigma)	13mg
β-nicotinamide adenine dinucleotide (Sigma)	250mg
Thiamine hydrochloride (Sigma)	3mg
Co-carboxylase (Sigma)	100mg
Cyanocobalamin (Sigma)	10mg
Ferric nitrate (Merck-BDH)	20mg

These components were dissolved in distilled water, filter sterilised, and stored at -20°C.

Supplement B:	Amount per 200ml
L-cysteine hydrochloride (Sigma)	26g
Adenine (Sigma)	1g
Guanidine hydrochloride (Sigma)	30mg
Uracil (Sigma)	800mg
Hypoxanthine (Sigma)	320mg

All of the above, except cysteine, were dissolved in 100ml boiling 0.1M hydrochloric acid. The cysteine was added after cooling to room temperature and the final volume made up to 200ml with water. The solution was filter sterilised and stored at -20°C.

2.2 Bacterial strains and growth conditions

The strains of *Neisseria meningitidis* used in this study are listed in Table 2.1. The MC58 and MC59 variants were case isolates from an outbreak in Stroud, Gloucestershire in the mid-1980's. Strains C114 and C311 were obtained from Professor JR Saunders, University of Liverpool. The original *Neisseria gonorrhoeae* strain P9 was a case isolate (Lambden & Heckels, 1979a). The commensal Neisseria strains were obtained from the National Type Culture Collection (NTCC); *Neisseria lactamica* (NTCC 10617) and *Neisseria sicca* (NTCC 4591). Original strains and phenotypic variants picked from them were stored at –196°C as thick suspensions in proteose peptone solution containing 10% (v/v) glycerol. For experiments requiring bacterial cultures, these frozen stocks were used to inoculate PP agar plates which were then incubated in a moist atmosphere at 37°C with 5% CO_2 for 16 to 18 h.

Table 2.1: *N. meningitidis* strains

Strain	Origin	Serogroup	Serotype	Subtype	Reference
MC58	CSF	B	15	P1.7,16b	(McGuinness *et al.*, 1991)
MC59	CSF	B	15	P1.7,16b	(McGuinness et al., 1991)
C114	CSF	C	2b	Nd[b]	(Perry *et al.*, 1987)
C311	CSF	B	NT[a]	Nd[b]	(Perry *et al.*, 1987)

a: Non-typable

Nd[b]: Not determined

2.2.1 Selection of phenotypic variants

Non-piliated variants from strains MC58 and MC59 expressing class 1 pili were obtained by selecting single large colonies from bacteria grown overnight at 30°C. Size variation was used as the differentiating property (Blake *et al.*, 1989). Variants lacking Opa proteins were selected as single colonies using their decreased opacity when viewed under a stereo microscope equipped with a substage flat polished mirror (Carl Zeiss Jena).

2.3 Testing of *Neisseria meningitidis* for oxidase activity

(Cruikshank *et al.*, 1975)

The organisms to be tested were grown for 16-18 h on PP agar. A few drops of N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (Sigma) solution (1% w/v) were placed on the colonies. The presence of oxidase activity was signified by an immediate colour change from pale brown to a strong purple.

2.4 Gram staining of bacteria

(Cruikshank *et al.*, 1975)

Materials:

Gram crystal violet (Difco)
Gram iodine (Difco)
Acetone (Merck-BDH)
Gram safranin (Difco)

One or two colonies were spread onto a clean glass microscope slide with a drop of distilled water to create an emulsion. Bacteria were fixed to the slide by passing it through a bunsen flame. The slide was flooded with crystal violet for 1 min and then rinsed with running tap water. Iodine was used as a mordant and was added for 1 min and then rinsed off with water. The bacteria were destained with acetone for a few seconds, rinsed with water and the counterstain safranin was added for 30 seconds then rinsed off with water. After blotting dry, the sample was viewed under a Leitz Dialux light microscope under oil with a 100x objective.

2.5 Detection of pilin, Opa, Opc and PilC proteins

Proteins were detected by electrophoresis and immunoblotting using antibodies raised to the relevant protein. Gradient gels were used for pilin, Opa and Opc, and linear gels were used for PilC. In addition, the presence or absence of pili was confirmed by electron microscopy.

2.5.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.5.1.1 Gradient gels

All SDS-PAGE for the detection of pilin, Opa and Opc proteins was performed using a 10 - 25% w/v acrylamide gradient as described by Heckels (Heckels, 1981), with the discontinuous buffer system of Laemmli (Laemli, 1970).

Materials:

Acrylamide monomer solution 50% w/v acrylamide (Merck-BDH), and 1.3% w/v N,N'-methylene bisacrylamide (Merck-BDH) in uHQ.

SDS solution 2% w/v SDS (Merck-BDH) in uHQ water.

Separating gel buffer 1.2M Tris-HCl (IBI), pH 8.8.

Stacking Buffer 0.25M Tris-HCl, ph6.8, 0.08% v/v TEMED (IBI).

Glycerol (Merck-BDH).

Ammonium persulphate (IBI) 1% w/v, freshly prepared.

TEMED.

Running buffer 25mM Tris-HCl, 192mM glycine (Merck-BDH), 0.1% w/v SDS, pH 8.3, freshly prepared.

Dissociation buffer 125mM Tris-HCl, pH6.8. 4% w/v SDS, 20% v/v glycerol, 10% v/v mercaptoethanol (Merck-BDH), 0.002% w/v bromophenol blue (Merck-BDH).

2.5.1.2 Construction of gradient gels

The glass gel casting plates (Life Technologies) were thoroughly cleaned with methanol (100%). The plates were separated by plastic spacers (1mm thick) and were held together with bulldog clips. The edges were sealed with 1% w/v molten agarose. Solutions of 10 and 25% acrylamide were prepared according to Table 2.2 and deaerated to remove dissolved oxygen. The separating gel was cast using a triple channel peristaltic pump (Pharmacia, P-3) to dimensions of 1x110x170mm. Water-saturated butanol was carefully layered onto the surface of the gel, to exclude air, and the gel was left to polymerise at room temperature for 1 h. The butanol was removed and the top of the gel was rinsed with distilled water. The stacking gel was prepared according to Table 2.3 and cast onto

the separating gel to dimensions of 1x35x170mm. The desired comb was inserted and the gel was left to polymerise at room temperature for 30 min. After polymerisation, the comb was removed and the wells were washed with distilled water.

Table 2.2: Composition of separating gel

Acrylamide concentration	25%	10%
Acrylamide monomer solution	6.25ml	2.5ml
Separating gel buffer	3.1ml	3.1ml
SDS solution	0.62ml	0.62ml
Glycerol	1.25ml	-
Water	1.1ml	0.25ml
*Ammonium persulphate	0.125ml	0.25ml
*TEMED	6.2µl	6.2µl

*these were added after the solutions were deaerated and immediately before gel casting.

Table 2.3: Composition of stacking gel

Acrylamide concentration	4%
Acrylamide monomer solution	1ml
Stacking gel buffer	6.25ml
SDS solution	0.62ml
Water	3.37ml
*Ammonium persulphate	1.25ml

*this was added after the solution was deaerated and immediately before gel casting.

2.5.1.3 Sample preparation

Whole cell lysates were prepared from bacterial strains grown overnight and which were scraped into 1ml of water containing 0.05% w/v sodium azide and 1mM PMSF. Protein concentration was measured indirectly using the absorbance of DNA at 260nm. Previous studies have shown that an A_{260} of 12 is equivalent to 1mg/ml protein. Using this standard, samples were prepared to contain 5µg of protein and adjusted to a volume of 10µl with water. This was mixed 1:1 with dissociation buffer to a final volume of 20µl, for loading into a well formed by a 20 tooth comb.

2.5.1.4 Gel running conditions

The loaded wells were carefully overlaid with running buffer prior to filling the gel tank reservoirs with buffer (Life Technologies V15.17 gel tanks). Gradient gels were run at 200 volts for 18-20 h at 4°C.

2.5.1.5 Linear Gels

All SDS-PAGE for the detection of PilC proteins was performed using a 10% w/v acrylamide linear gel using the discontinuous buffer system of Laemli (Laemli, 1970).

Materials:

As described in section 2.5.5.1 Gradient gels.

2.5.1.6 Construction of linear gels

The glass gel casting plates (Biometra) were cleaned as in section 2.5.1.2 and were assembled as per the manufacturers instructions. The 10% acrylamide solution was prepared according to Table 2.4, and was deaerated before the separating gel was cast using a pasteur pipette to dimensions of 1x140x170mm. After polymerisation the stacking gel was prepared according to Table 2.4 and cast onto the separating gel to dimensions of 1x45x170mm.

Table 2.4: Composition of linear gel solutions

	Separating gel (10% acrylamide)	Stacking gel (4% acrylamide)
Acrylamide monomer	7.8ml	1ml
Separating gel buffer	9.6ml	6.25ml
SDS solution	2.67ml	0.62ml
Water	18.72ml	3.37ml
*Ammonium persulphate	0.76ml	1.25ml
*TEMED	19.2µl	-

*added after the solutions were deaerated and immediately before gel casting.

2.5.1.7 Sample preparation

Samples were prepared as described in section 2.5.1.3 except that the protein concentration was adjusted to 100µg with water and was mixed 1:1 with dissociation buffer to a final volume of 40µl, for loading into a well formed by an 18 tooth comb. A biotinylated molecular weight standard

mixture (Sigma) was made up according to the manufacturers instructions and 10µl was loaded into the relevant wells.

2.5.1.8 Gel running conditions

The loaded wells were carefully overlaid with running buffer prior to filling the gel tank reservoirs with buffer (Biometra tanks) and the gels were run at 60 volts for 18 h at room temperature.

2.5.2 Staining of polyacrylamide gels

Staining was carried out by the method of Fairbanks *et al.* (1971) (Fairbanks *et al.*, 1971). After electrophoresis the stacking gel was removed and the proteins were fixed and stained in a stain solution containing 10% v/v glacial acetic acid (Merck-BDH), 20% v/v isopropanol (Merck-BDH) and 0.5mg/ml PAGE Blue 83 (Merck-BDH) for 1h. The gels were destained with several washes of 10% v/v glacial acetic acid and 10% v/v isopropanol until the background staining was minimal.

2.5.3 Storage of polyacrylamide gels

For long term storage the gels were either sealed in plastic bags containing destain solution or dried onto filter paper using the Biorad gel drier. For a long term record, the gels were photographed using Kodak technical pan film (TP 135-36).

2.6 Western blotting of polyacrylamide gels and detection of transferred proteins

2.6.1 Electrophoretic transfer of proteins to nitrocellulose sheets

The method was adapted from that of Towbin (Towbin *et al.*, 1979).

Materials:

Blotting buffer 20% v/v methanol in running buffer
Nitrocellulose Protran nitrocellulose, 0.45µm (Schleicher & Schuell)
Chromatography paper 17 Chr (Whatman)

The gel was placed in blotting buffer for 30 min to equilibrate. Four pieces of chromatography paper and 1 sheet of nitrocellulose were cut to the same size as the gel and soaked in blotting buffer.

The surface of the anode of a Bio-Rad Trans blot semi-dry transfer was wetted with blotting buffer and onto it the following were placed in this order: 2 pieces chromatography paper, 1 sheet nitrocellulose, equilibrated gel, 2 pieces chromatography paper. Air bubbles were removed using a glass pasteur pipette as each layer was placed. A constant current of 0.8mA/cm^2 of gel was applied for 1 h with gradient gels and for 2 h with linear gels.

2.6.2 Immune detection of proteins bound to nitrocellulose

Materials:

Tris buffered saline (TBS) 20mM Tris-HCl, 500mM sodium chloride (Sigma), pH 7.5

Tween-Tris buffered saline (TTBS) TBS plus 0.05% v/v Tween 20 (Merck-BDH)

Block buffer 5% w/v non-fat milk powder (Marvel^{TM}) in TTBS

Antibody diluent 1% w/v gelatine (Merck-BDH) in TTBS

Substrate components

Substrate buffer 100mM Tris-HCl, 100mM sodium chloride, 2mM magnesium chloride (Merck-BDH), pH 9.5

Nitro blue tetrazolium (NBT) 30mg/ml in 70% dimethylformamide (DMF) (Sigma/Romil)

5-Bromo-4-chloro-3-indolyl phosphate (BCIP) 15mg/ml in 100% DMF (Sigma)

Just before use, 1ml each of NBT and BCIP solutions were added to 100ml of substrate buffer.

Antibodies

Table 2.5: Antibodies used for the detection of pilin, class II pilus, Opa, Opc and PilC

Antibody	Reactive with	Source	Working dilution	Species raised in
SM1	Pilin (Class I pilus)	In-house (Virji & Heckels, 1983)	1:25000	Mouse
U101	Class IIa pilus	Gift from M Achtman	1:10000	Mouse
AG123	Class IIb pilus	Gift from M Achtman	1:10000	Mouse
4B12	All Opa proteins	M Blake (Achtman *et al.*, 1988)	1:5000	Mouse
B306	Opc	M Achtman (Achtman *et al.*, 1992)	1:5000	Mouse
ABJ	PilC1	AB Jonsson (Jonsson *et al.*, 1991)	1:2000	Rabbit
602724 T_3	PilC1	X Nassif (Taha *et al.*, 1998)	1:50	Rabbit

After protein transfer, the nitrocellulose sheets were washed in TTBS (3x5min) to remove traces of SDS. The section of nitrocellulose containing the biotinylated standards was cut off and then all sheets were incubated with block buffer for 1 h to block non-specific antibody binding. All incubations were done at room temperature. Block buffer was removed from the sample sheets and the nitrocellulose was washed in TTBS (3x5min). The primary antibodies were made up in antibody diluent according to the dilutions in Table 2.5 and were added for 1h. The molecular weight standards remained in the block buffer for the duration of the primary antibody stage.

Unbound antibody was removed by washing in TTBS (3x5min), and at this stage an extra amplification step was incorporated into the staining for PilC by adding a biotinylated anti-rabbit IgG (KPL) at 1:1000 in block buffer for 1h. After washing in TTBS (3x5min) the next step for PilC detection, and the secondary stage for the biotinylated standards, was a streptavidin alkaline phosphatase conjugate (Zymed) used at 1:1000 in block buffer for 1h. The secondary antibody for all of the other samples was an alkaline phosphatase conjugated goat anti-mouse antibody (BioRad), which was used at 1:1000 in block buffer. The nitrocellulose sheets were then washed in TTBS (3x5min) and in TBS (3x5min). Antibody bound to the nitrocellulose was detected by addition of the substrate. The colour reaction was monitored carefully and was stopped in cold water washes once the bands had reached the desired intensity and before background staining occurred. The blots were stored dry in the dark and then photographed using Kodak technical pan film (TP 135-36).

2.7 Transmission electron microscopy to confirm piliation

The presence or absence of pili in all strains was confirmed by electron microscopy following negative staining (Diaz *et al.*, 1984).

Bacterial strains were grown overnight and scraped into PBS to give a concentration of between $1x10^8$-$1x10^9$ cfu per ml; a 4µl sample was then put onto a Formvar coated copper grid and left for two min. The grids were then washed three times with PBS, and 4µl of 1% (w/v) uranyl acetate was added for 2 min. The uranyl acetate was removed and the grids were washed four times with uHQ water, and allowed to dry for 30 min at RT. The samples were viewed under a Philips 201 transmission electron microscope at 60kV.

2.8 Detection of lipopolysaccharide

Lipopolysaccharide was detected by both visualisation on a polyacrylamide gel with silver staining and by whole cell ELISA using antibodies raised to specific LPS immunotypes.

2.8.1 LPS on low molecular weight polyacrylamide gels

Samples containing LPS were run out on low molecular weight gels. When compared to gradient SDS-PAGE (section 2.5.1), this system enhanced the resolution of low molecular weight proteins. All polyacrylamide gels were constructed according to the method of Schagger and Von Jagow (Schagger & von Jagow, 1987), using a discontinuous buffer system and tricine as the trailing ion.

Materials:

Acrylamide monomer solution 48% w/v acrylamide, and 1.5% w/v N,N'-methylene bisacrylamide. Solubilised at 27°C in a water bath and filtered through a scintered glass funnel.

SDS solution 20% w/v.

Gel buffer 3M Tris-HCl, pH 8.5, and 0.3% w/v SDS.

Ammonium persulphate 10% w/v, freshly prepared.

TEMED.

Anode buffer 0.2M Tris-HCl, pH8.9.

Cathode buffer 0.1M tricine (Merck-BDH), 0.1% w/v SDS, and 0.1M Tris-HCl, ph8.2.

Sample buffer 4% w/v SDS, 12% v/v glycerol, 2% v/v 2-mercaptoethanol, 0.02% w/v Brilliant blue G (Merck-BDH), and 0.05M Tris-HCl, pH6.8.

2.8.1.1 Construction of linear gel

The glass gel casting plates were prepared as in section 2.5.1.2, and the separating and spacer gel solutions were prepared according to Table 2.6 and then deaerated. The separating gel was poured into the casting mould to a height of 10cm and then the spacer gel was carefully overlaid to a height

of 3cm. Water was carefully layered onto the spacer gel and, after polymerisation, the water was removed and the stacking gel was cast on top of the spacer gel.

Table 2.6: Composition of low molecular weight gel solutions

	Separating gel	Spacer gel	Stacking gel
Acrylamide solution	6.7ml	2.0ml	1.0ml
Gel buffer	6.7ml	3.33ml	3.1ml
Glycerol	2.1ml	-	-
Water	4.5ml	4.63ml	8.4ml
*Ammonium persulphate	67µl	33µl	100µl
*TEMED	6.7µl	3.3µl	10µl

*these were added after deaerating and immediately before gel casting.

2.8.1.2 Sample preparation

Samples of whole cell lysates containing 10µg protein were prepared to 20µl final volume with sample buffer, heated at 40°C for 30 min and loaded into a single well on a 20 well gel.

2.8.1.3 Gel running conditions

The gels were loaded into Life Technologies V15.17 gel tanks and the tank reservoirs were filled with the appropriate anode and cathode buffers. The components of the samples were allowed to stack up by running the gel at 30 volts for 1 h and separated by increasing the voltage to 90 volts and running for a further 16 h.

2.8.1.4 Silver staining of LPS

This LPS silver staining method is that described by Hitchcock and Brown (Hitchcock & Brown, 1983). All stages were carried out at 37°C using pre-warmed solutions and a clean glass dish.

Materials:

Fixing solution 25% v/v isopropanol, 7% v/v glacial acetic acid.

Oxidising solution 0.7% w/v periodic acid, 2.67% v/v fixing solution.

Silver staining solutions 35% w/v ammonia solution (Merck-BDH),
0.1M sodium hydroxide (Merck-BDH)
20% w/v silver nitrate solution (Merck-BDH).

Developing solution 0.05% w/v citric acid (Merck-BDH) in 0.05% v/v formalin.

Stop solution 0.1% v/v acetic acid.

After electrophoresis the gel was placed in fixing solution for 30 min and then shaken in oxidising solution for 5 min. The silver stain solution was made up as follows; 0.56ml of ammonia solution (35% w/v) was added to 18.8ml of sodium hydroxide solution (0.1M). With constant stirring on a white background, silver nitrate solution (20% w/v) was added dropwise until the saturation point was reached (about 3.4ml). Finally, this solution was diluted to 100ml with water. After washing in water (8x4min), the gel was incubated with the silver staining solution for 10 min. The gel was washed with water (4x10min) and then incubated with developing solution until the LPS bands had reached the desired intensity. At this point the reaction was stopped by the addition of stop solution for 1 h. For a long term record, the gel was photographed as in section 2.5.3.

2.8.2 Whole cell ELISA for determination of LPS immunotype

This method was carried out as described by Scholten (Scholten *et al.*, 1994).

Materials:

Wash buffer 8.5% w/v sodium chloride, 0.5% v/v Tween 20.

Substrate buffer components

3,3',5,5'-tetramethylbenzidine (TMB)

DMSO

0.1M sodium acetate (Merck-BDH) (adjusted to pH6.0 with 2M citric acid - Merck-BDH)

30% w/v hydrogen peroxide.

Just before use, 3.6mg TMB was added to 1ml DMSO and added dropwise to 100ml 0.1M sodium acetate containing 10µl hydrogen peroxide.

Antibody diluent 0.85% w/v sodium chloride, 0.06% w/v Tris-HCl, 1% w/v bovine serum albumin (BSA) (Sigma), 0.05% v/v Tween 20, pH 7.4 with acetic acid.

Meningococci to be tested were grown for 16-18 h and the growth from 1 plate was scraped into 1ml water containing 0.05% (w/v) sodium azide and 1mM PMSF. The samples were inactivated by heat at 56°C for 30 min. The concentration of the samples was adjusted to an optical density (OD) of 0.10, in PBS, measured in a standard 1cm cuvette at 620nm with a Cecil CE559 automatic scanning spectrophotometer. Flat bottomed 96 well polystyrene microtitre plates (Sterilin) were coated with the relevant antigen (100µl per well) using 1 well per antibody to be tested. The plates were incubated overnight at 37°C in a humid environment. The coating buffer was removed and the wells were washed 3 times with wash buffer before the addition of diluent (100µl per well) to block

non-specific binding sites. After 1 h at 37°C, the diluent was removed and the primary antibodies, made up in diluent at dilutions according to Table 2.7, were added (100µl per well) and incubated

Antibodies

Table 2.7: mAbs used for LOS immunotyping of *N. meningitidis* strains

mAb	Immunotypes reactive with	Working dilution	Source
1) Mn14F21-11	L1	1:300	JT Poolman (Scholten *et al.*, 1994)
2) 1-9C4	L3,7,9	1:300	...
3) Mn15A11	L7,9	1:300	...
4) Mn11A11G	L10,11,12	1:300	...
5) Mn14F20-11	L1,8,10	1:300	...
6) Mn4C1B	L6	1:300	...
7) Mn3A8C	L5	1:300	...
8) Mn4A8-B2	L3,7,9	1:1000	...
9) 9-2-L379	L3,7,9	1:10000	Gift from W Zollinger
10) 2-1-L8	L8	1:10000	JT Poolman (Scholten *et al.*, 1994)
11) 14-1-L10	L10	1:10	...

for 1 h at 37°C. The antibodies were removed and the wells were washed 3 times. The secondary antibody was then added using goat anti-mouse horse radish peroxidase conjugated antibody (Zymed) diluted 1:2000 in diluent (100µl per well) and incubated for 1h. The secondary antibody was removed and the wells were washed 4 times. The substrate was added (100µl per well) for 10 min in the dark at room temperature. The reaction was stopped by the addition of 1M sulphuric acid (Merck-BDH) (50µl per well). The absorbance of each well at 450nm was measured using an Anthos htII microtitre plate reader. The OD cut off values for these antibodies was designated as 0.2, which was twice the background.

2.9 Detection of capsule

The presence of capsule was determined by both the use of an agglutination assay utilising latex particles sensitised with specific antisera, and by a dot blot using an antibody raised against meningococcal group B capsule (gift from W Zollinger).

2.9.1 The sensitised latex agglutination assay

The test was performed using the Slidex meningite-kit 5™ as described by the manufacturers instructions, and utilised the sensitised latex, black card and mixing sticks supplied.

Materials:

Anti-*N. meningitidis* group A serum from rabbit.
Anti-*N. meningitidis* group B monoclonal antibody from mouse.
Anti-*N. meningitidis* group C serum from rabbit.
Negative control 0.15M sodium chloride.

Meningococci to be tested were grown for 16-18 h and half of the growth of 1 plate was scraped into 1ml PBSB. Clumps of bacteria were removed by centrifugation at 450g and the supernatant was briefly vortexed. One drop of antisera for each sample and negative control to be tested was placed on a circle of a black mixing card. Next to this circle was placed 30µl of bacterial sample or negative control. The samples were mixed with the antisera and the card was gently rocked for 2 min. A positive reaction was characterised by white areas of agglutination in a clear background. The samples stayed clear if the test was negative.

2.9.2 Immunodot blot assay for capsule detection

Materials:

TBS
TTBS
Block buffer: 3% w/v gelatine in TTBS
Antibody diluent: 1% w/v gelatine in TTBS
Substrate components: as described in section 2.6.2

The protein concentration of the bacterial whole cell lysates was calculated as described in section 2.5.1.3 and then the samples were adjusted with water to contain 1mg/ml of protein. Strips of nitrocellulose paper were cut to a width of 0.3cm and sample (3µl) was added to the middle of a strip and left to air dry. Strips were washed in TTBS (3x5min) and then incubated in block buffer for 1 h. All incubations were done at room temperature. Block buffer was removed and the nitrocellulose was washed in TTBS (3x5min). The primary antibody was 2-2-B, an IgM monoclonal raised against group B capsule (gift from W Zollinger, WRIAR, Washington, DC). The samples were incubated for 1 h with primary antibody at a concentration of 1:100 in antibody

diluent. Unbound antibody was removed by washing in TTBS (3x5min) and the secondary antibody was added and incubated for 1 h. The secondary antibody was an alkaline phosphatase conjugated goat anti-mouse IgM (Sigma) used at 1:10000 in antibody diluent. The nitrocellulose strips were then washed in TTBS (3x5min) and TBS (3x5min). Antibody bound to the nitrocellulose was detected by addition of the substrate; the colour reaction was monitored carefully and was stopped in cold water washes when the desired colour intensity had been reached.

2.10 Human cell lines and tissue culture

2.10.1 Meningioma tissue

Meningioma tissue was obtained from patients undergoing surgical removal of meningeal tumours and was provided by the Department of Neuropathology, Southampton General Hospital.

Materials:

Collection medium 5% v/v foetal calf serum (FCS) (GibcoBRL) in PBS.

Growth medium 20% v/v FCS, 2mM L-glutamine (Sigma), 100IU/ml penicillin (Sigma), 100IU/ml streptomycin (Sigma), 10µg/ml gentamicin (Sigma) in Dulbecco's modification of eagles medium (DMEM) (GibcoBRL).

Trypsin-Ethylenediaminetetraacetic acid (EDTA) 0.125% v/v trypsin (Sigma), 0.02% w/v EDTA (IBI) in HBSS.

Trypsin-EDTA 0.05% v/v trypsin, 0.02% v/v EDTA (Sigma) in HBSS.

Hanks balanced salt solution (HBSS) (GibcoBRL).

Freezing medium 10% v/v Dimethyl sulfoxide (DMSO) in growth medium.

All tissue culture growth surfaces were coated with collagen type I from rat tail (Sigma) at 6µg per cm^2 according to the manufacturers instructions.

2.10.1.1 Primary culture of meningioma tissue

Within 30 min of tumour excision, tissue was placed into collection medium. Blood clots and necrotic tissue were removed with a scalpel and the remaining tissue was rinsed several times in HBSS. The tissue was cut into pieces smaller than $1mm^3$ and homogenised gently in a glass homogeniser. The tissue pieces were washed in HBSS and centrifuged for 10 min at 250g. Trypsin (0.125%)-EDTA (0.02%) was added and incubated for 30 min at 37°C with gentle shaking. The enzymatic activity was inhibited by dilution of the trypsin-EDTA with an equal volume of growth medium. The clumps of cells were allowed to settle for 3-5 min and the supernatant was removed and then processed separately to the clumps. The cells in the supernatant were centrifuged at 250g

for 10 min and were washed in HBSS before being centrifuged again. The cells were resuspended in growth medium and seeded into 80cm^2 tissue culture flasks (Nunc). The clumps of cells were washed in HBSS and centrifuged at 250g for 10 min. These cells were resuspended in growth medium and seeded into 80cm^2 tissue culture flasks. All cultures were maintained in a humid environment at 37°C with 5% CO_2. Growth medium was typically changed every 3 days.

2.10.1.2 Subculture of meningioma cells

Upon or just before reaching confluence, meningioma cells were passaged. Growth medium was removed and the monolayer was rinsed twice with PBS to remove debris and traces of FCS. Enough trypsin-EDTA (0.05%-0.02%) was added to just cover the flask surface for 5-10 min under normal growth conditions. Trypsin activity was deactivated by the addition of an equal volume of growth medium and the cells were centrifuged at 250g for 10 min. The cells were resuspended in growth medium and typically diluted 1:4 into 80cm^2 tissue culture flasks.

2.10.1.3 Frozen storage of meningioma cells

After the cells were removed from the substrate by trypsin and centrifuged to a pellet, the cells were resuspended in freezing medium and transferred into cryogenic storage tubes (Nunc). An 80cm^2 flask typically provided enough cells for 2 tubes containing 1ml cells each. The cryo-tubes were wrapped in tissue paper, placed in a small polystyrene box packed with tissue paper and frozen at -70°C overnight before they were then stored at -135°C.

2.10.2 Chang cells

Chang conjunctival epithelial cells were obtained from the European Type Culture Collection (ETCC) at Porton Down, UK.

Materials:

Growth medium 10% v/v FCS, 2mM glutamine in DMEM.
Trypsin-EDTA 0.05% v/v trypsin, 0.02% w/v EDTA in HBSS.
Freezing media 10% v/v DMSO in growth medium.

Chang cells were maintained in uncoated 80cm^2 tissue culture flasks at 37°C under 5% CO_2. Just before confluence they were passaged in the same way as meningioma cells but typically at a 1 in 10 dilution. The cells were frozen down in the same way as meningiomas but at a density of $3x10^6$ cells/ml freezing media.

2.10.3 MRC-5 cells

MRC-5 cells are human diploid embryonic lung fibroblasts (Jacobs *et al.*, 1970) and they were obtained from the ETCC.

These cells were cultured in the same way as Chang cells but were typically split at a 1 in 3 dilution.

2.11 Staining human cells for cellular markers

Meningioma cells were grown to approximately 60-80% confluence on 13mm diameter coverslips (Western) coated in 0.01% w/v collagen (Sigma). Chang and MRC-5 cells were grown on uncoated coverslips. Cells were stained for various cellular markers.

Materials:

Table 2.8: Fixing solutions and primary antibodies for staining

Fixative	Primary antibody	Source	Working dilution
Ice cold methanol	Desmoplakin I & II	Boehringer Mannheim	1:20
Ice cold methanol	Vimentin	Sigma	1:200
Acetone	Cytokeratin AE1/AE3	Zymed	1:20
3%w/v Paraformaldehyde	Epithelial membrane antigen (EMA)	Dako	1:20
Ice cold methanol	PG-M1 (CD68)	Dako	1:200
Ice cold methanol	CD46	Pharmingen	1:100

Block buffer 10% v/v rabbit serum (Life Technologies) in PBS.

Secondary antibody Fluorescein isothiocyanate conjugated rabbit anti-mouse antibody (Dako). Used at 1:100 in PBS. Kept in dark.

Propidium iodide (Sigma). Used at 10-25μg/ml in PBS. Kept in dark.

2.11.1 Staining procedure

The culture media was removed and the monolayers were washed 3 times with PBS. The monolayers were fixed with the relevant fixative for 10 min. At this time the fixative solutions were removed and, with the exception of the paraformaldehyde fixed coverslip, the monolayers were washed 3 times in PBS. The paraformaldehyde was removed and the free aldehyde groups were neutralised by the addition of 50mM ammonium carbonate for 5 min. The monolayer was then washed with PBS 3 times. Non-specific binding of the primary antibody was prevented by the addition of block buffer for 30 min at 37°C. The primary antibodies were made up in block buffer, according to the dilutions in Table 2.8. The block buffer was removed and the primary antibodies were added to the monolayers for 1 h at 37°C with an additional coverslip for EMA being incubated overnight at 4°C. The monolayers were washed 3 times in PBS before the addition of the secondary antibody for 1 h at 37°C. The monolayers were again washed 3 times in PBS before the addition of propidium iodide for 10 min at room temperature. The monolayers were then washed 6 times in PBS. The coverslips were mounted face down onto clean glass microscope slides using an anti-fade fluorescent mounting medium (DAKO). Staining was visualised under a Leica DMRB fluorescent microscope using an epifluorescence system of illumination.

2.12 Infection experiments for quantitation of bacterial association with human cells

For all infection experiments where bacterial association with host cells was quantified, the meningioma cells were grown to confluence on collagen coated 96 well tissue culture plates, in normal culture medium without antibiotics. Meningioma cells between passage 3-8 were used for all bacterial association infection experiments. Chang cells were grown to confluence on uncoated 96 well plates in normal culture medium.

Infection experiments in this section were based on the procedures of (Virji *et al.*, 1991; Virji *et al.*, 1992b).

Materials:

Wash buffer 2% v/v decomplemented FCS (heat treated at 56°C for 30 min to deactivate serum complement factors) in PBS.

Maintenance media 2% v/v dec' FCS, 2mM glutamine in DMEM.

Plating buffer 0.05% v/v B salts (Oxoid) in PBS.

Lysing buffer 1% w/v saponin (Sigma) in PBS, filter sterilised (0.22µm filter, Gelman), 2% v/v dec' FCS.

Gentamicin 200µg/ml gentamicin (Sigma) in maintenance media.

Cytochalasin D (CD) 2μg/ml CD (ICN) in maintenance media.

All solutions were pre-warmed to 37°C.

2.12.1 Standard method

Bacteria were grown for 18 h and the growth from 2 plates was scraped into 1ml plating buffer. The clumps of bacteria were removed by centrifugation at 300g. A sample was diluted 1:10 in 1% (w/v) SDS, 0.1% (w/v) sodium hydroxide (SDS/NaOH) and the absorbance at 260nm was read. Previous studies had shown that an A_{260} of 1 (at a 1:10 dilution) was equivalent to $3.6x10^9$ colony forming units (cfu) per ml of neat bacterial suspension. Bacterial numbers were adjusted to the desired number for the particular experiment in maintenance medium (typically $2.5x10^8$ cfu per ml). To obtain the actual infecting dose the remaining neat bacterial suspension was diluted with plating buffer and plated onto agar plates for viable counting. Cell monolayers were washed once with wash buffer and the bacteria were added to the wells. The volume of the bacterial suspension added, and the time they were allowed to associate with the cell monolayer for, depended on the experiment. The plates were incubated under normal culture conditions for the monolayers. For each time and sample point there were triplicate wells. At the designated time point for total bacteria-host cell association, the bacteria were removed from the wells and the monolayers were washed 4 times with wash buffer. The cells were lysed by the addition of lysing buffer (150μl per well) with incubation at 37°C for 15 min and shaking on a Titertek plate shaker for the first and last minute. The lysed samples were diluted with plating buffer and 15μl of each sample used to inoculate agar plates. For the determination of bacterial internalisation, the bacteria were removed and the monolayer was washed 3 times with wash buffer to remove excess non-adherent bacteria. Gentamicin solution (200μl per well) was added for 1.5 h, under normal culture conditions, to eliminate all extracellular bacteria. The gentamicin was removed and the monolayers were washed and lysed as for the associated wells.

2.12.2 Incubation with Cytochalasin D

To determine whether bacteria that survive after gentamicin treatment are truly internalised, the microfilament activity of the cell monolayer was inhibited by the addition of cytochalasin D (CD). Monolayers were incubated with 2μg/ml CD solution (125μl per well) or maintenance media (125μl per well) for 30 min prior to the addition of bacteria. After 30 min, $5x10^7$ bacteria in 125μl of maintenance media was added to each well, diluting the CD to 1μg/ml, and incubated for 6h or 24h. After these time points, the monolayers were either processed for total bacterial association or internalisation as in the standard method.

2.12.3 Statistical Analysis

All statistical analysis was done using the SPSS 9.0 for Windows package (SPSS Inc.). The relative association of different bacterial variants was compared using one-way ANOVA analysis to compare the level of significance between the means taking values with P=<0.05 as significant.

2.13 Infection experiments for visualisation of bacterial association with human cells

The association of meningococci with meningioma cells was visualised by staining infected monolayers with fluorescent markers and viewing under a confocal microscope or by processing the samples for scanning electron microscopy (SEM). To determine visually whether meningococci were internalised by meningioma cells, infected monolayers were processed for transmission electron microscopy (TEM).

2.13.1 Fluorescent staining of infected cell monolayers

Meningioma cells were grown until they were approaching confluence, on size O (13mm diameter) uncoated glass coverslips in 24 well tissue culture plates.

Materials:

Wash buffer 2% v/v dec' FCS in PBS.
Maintenance medium 2% v/v dec' FCS, 2mM glutamine in DMEM.
Human cell label - a sulfonated carbocyanine 5μM SP-DilC_{18}(3) (Molecular Probes) in PBS.
Bacterial label - primary antibody: M418-422 at 1:1000.
- secondary antibody: FITC conjugated rabbit anti-mouse at 1:100.

The bacterial suspensions for infection experiments were prepared as in section 2.11.1 and corrected to $2.5x10^8$ cfu/ml in maintenance medium. Cell monolayers were washed once with wash buffer and the bacterial suspension was added (1ml per well). The bacteria were removed and the monolayers were washed 3 times with PBS. The monolayers were incubated with SP-DilC_{18}(3) (in accordance with the manufacturers instructions) for 2 min at 37°C, 5% CO_2 and then for 10 min at 4°C. This solution was then removed and the monolayers were washed 3 times with PBS before

being fixed in 3.7% formaldehyde (in PBS) at 37°C for 10 min. The monolayers were washed 3 times with PBS and post-fixed in acetone for 10 min at room temperature. After washing 3 times in PBS the monolayers were incubated with M418-422, an in-house polyclonal mouse antisera raised against total meningococcal outer membrane proteins, overnight at 4°C. The wells were washed again with PBS 3 times and incubated with the secondary antibody for 1 h at 37°C. After washing in PBS, the coverslips were mounted face down onto clean glass microscope slides with anti-fade fluorescent mounting media.

2.13.2 Confocal microscope analysis

Samples were viewed under a Leica TCS 4D laser scanning confocal microscopy system using an epifluorescence system of illumination. The software program used was Microsoft SCANware 4.2. Images were obtained by simultaneous 2 channel scanning at 488 and 568nm to excite FITC and propidium iodide (PI) or SP-DiIC$_{18}$(3) respectively. The emission wavelengths of these fluorochromes were detected at 525nm for FITC, 617nm for PI and 573nm for SP-DiIC$_{18}$(3). Scanning was done in the xy plane at a standard scanning speed. Voltages and offsets for the photomultipliers were set for optimum image detection and resolution. The 2 channel series of optical slices obtained were combined and viewed as a 3-dimensional image via the software processing. The final image was viewed using a red/green combined look-up table. For a visual record, images were displayed on a high resolution flat screen monitor and were then photographed using a bench mounted camera, with Kodak Elite 100ASA daylight colour film.

2.13.3 Processing of infected cell monolayers for SEM

Meningioma cells were grown on collagen coated, size 2 (13mm diameter) glass coverslips in 24 well tissue culture plates until confluence.

Materials:

Wash buffer 2% v/v dec' FCS in PBS.

Maintenance medium 2% v/v dec' FCS, 2mM glutamine in DMEM.

Cacodylate buffer 0.1M sodium cacodylate (Merck-BDH), pH 7.4.

Fixative 3% v/v glutaraldehyde in cacodylate buffer, pH 7.4.

Cacodylate-sucrose buffer 0.1M sodium cacodylate, 0.23M sucrose (Merck-BDH), pH 7.4.

Post fixative 2% w/v osmium tetroxide in cacodylate buffer pH 7.4.

Bacterial suspensions for infection experiments were prepared as in section 2.11.1 and adjusted to $1x10^8$ cfu/ml. The monolayers were washed once with wash buffer and bacteria were added (500µl of the meningococcal suspension per well). Bacteria were removed and the monolayers were washed 4 times with DMEM before the addition of fixative for 2 h at 4°C. The coverslips were processed by the electron microscopy unit at Southampton General Hospital according to the following protocol. The coverslips were washed overnight in cacodylate-sucrose buffer and post-fixed in post fixative for 2 h. After rinsing in distilled water, the coverslips were dehydrated through graded ethanols at 70%, 90% and 100% (3x10 min each). The specimens were dried in a critical point dryer and mounted onto Cambridge 0.5 inch stubs before sputter coating with gold palladium.

2.13.4 SEM analysis

Samples were viewed under a Hitachi S-800 scanning electron microscope at 15kV with a tilt of 40°, and were then photographed using Ilford HP5 plus 400ASA film.

2.13.5 Processing of infected monolayers for TEM

Meningioma cells were grown on collagen coated, size 0 (13mm diameter) thermanox plastic coverslips in 24 well tissue culture plates until confluence.

Materials:

Wash buffer 2% v/v dec' FCS in PBS

Maintenance medium 2% v/v dec' FCS, 2mM glutamine in DMEM

PIPES buffer 0.1M piperazine-NN'-bis-2-ethanesulphonic acid, pH 7.2

Main fixative 3% v/v glutaraldehyde, 4% w/v formaldehyde in 0.1M PIPES buffer, pH 7.2, pre-warmed to RT

Rinse buffer 0.1M PIPES buffer, pH 7.2

Post fixative 1% w/v osmium tetroxide in 0.1M PIPES buffer, pH 7.2

Graded alcohols 30, 50, 70, 95% v/v ethanol in water, absolute ethanol

Propylene oxide

Epoxy resin Spurr's resin

Bacterial suspensions for infection experiments were prepared as in section 2.11.1 and were adjusted to $2.5x10^8$ cfu/ml. The monolayers were washed once with wash buffer and bacteria were added (1ml of the meningococcal suspension per well) at 37°C under 5% CO_2. Bacteria were removed and the monolayers were washed with wash buffer (4x) before the addition of the main fixative for 10 min at RT, and 50 min at 4°C. The coverslips were washed in rinse buffer (2x10

min) and post-fixed for 1 h at RT. The samples were washed in rinse buffer (2x10 min) and dehydrated through graded ethanol solutions at 30, 50, 70 and 95% (1x10 min each) and 100% ethanol (2x20 min each). The samples were then brought to resin by the addition of propylene oxide for 10 min which was then removed and replaced with a 50:50 propylene oxide:resin mix overnight on a rotator, and then with neat resin for 6 h. The coverslips were then embedded in fresh resin, which was polymerised at 60°C for 20-24 h.

Silver sections were cut on a Leica OMU3 ultramicrotome diamond knives, and mounted onto 200 mesh copper/palladium grids. The sections were then stained in saturated alcoholic uranyl acetate solution for 15 min in the dark, followed by Reynolds lead stain for 5 min in the presence of sodium hydroxide pellets to absorb excess carbon dioxide.

2.13.6 TEM analysis

The samples were viewed under a Philips 201 transmission electron microscope at 60kV.

2.14 Detection of cytokine mRNA produced by human cells challenged with meningococci

Measurement of cytokine expression in meningioma cells challenged with meningococci was investigated by reverse-transcriptase (RT)-PCR assays for a range of pro-inflammatory cytokines and chemokines.

Meningioma cells were grown on collagen coated 24 well tissue culture plates until confluence, and 48h prior to meningococcal challenge, they were growth arrested by reducing the concentration of FCS in the growth medium to 0.1% (v/v).

2.14.1 Details of challenge experiment

Bacterial suspensions were prepared as in section 2.12.1 and corrected to $2.5x10^8$ cfu/ml in maintenance medium. The monolayers were washed once with wash buffer and bacteria were added (1ml of the meningococcal suspension per well) for 3h.

Materials:

Wash buffer 0.1% (v/v) dec' FCS in PBS

Maintenance medium 0.1% (v/v) dec' FCS, 2mM glutamine in DMEM

2.14.2 Extraction of total RNA

The bacteria were removed and the monolayers were washed with wash buffer (4x) before homogenisation of the cells with Trizol reagent (0.5ml per well) (Life Technologies) for 5min at room temperature. Chloroform was added at a ratio chloroform:sample of 1:5, with vigorous shaking for 15sec followed by incubation at room temperature for 3min. Phase separation occurred when the samples were centrifuged at 12000g for 10min at 4°C, after which the RNA was present in the upper aqueous phase. The RNA was precipitated from the upper aqueous phase by the addition of isopropyl alcohol at a ratio of 1:1 isopropyl alcohol:sample, with brief mixing by hand followed by incubation at room temperature for 10min. The RNA was pelleted by centrifugation at 12000g for 10min at 4°C, washed in ice cold ethanol (75% v/v), and centrifuged at 12000g for 10min at 4°C. The pellet was placed under vacuum for 5-10min until almost dry then redissolved in 10µl uHQ water. To eliminate any contamination with genomic DNA, 1µl each of DNase (1U/µl) and DNase buffer (both Life Technologies) was added with brief vortex mixing and the sample was incubated for 15min at room temperature, after which the DNase was deactivated by heating at 65°C for 10min.

2.14.3 Synthesis of cDNA

The samples were split equally, 5µl in each tube, one half to serve as the –RT enzyme control. The samples were made up to 17.2µl with uHQ water, 100ng of Poly-T (in 2µl) was added, and the samples were mixed and centrifuged briefly. The tubes were placed on a heating block at 90-95°C for 5min, snap cooled on ice, and centrifuged briefly. Two 'mastermixes' were prepared, with and without the RT enzyme, using uHQ water to replace RT enzyme in the latter. The annealed RNA/primer samples were then made up to a final volume of 40µl with either one of the mastermixes:

	Final Concentration
Buffer, 5x superscript (Life Technologies)	20% (v/v)
DTT (Promega)	0.1M
dNTP (Promega)	10mM
Acetylated BSA (Promega)	1mg/ml
RNase inhibitor (Promega)	40 000 U/ml
Superscript, reverse transcriptase (RT) enzyme (Life Technologies)	200U/ml

The samples were incubated for 1h at 42°C to allow polymerisation, after which the RT enzyme was inactivated by heating at 90°C for 5min, snap cooled on ice and centrifuged briefly. The resulting cDNA was then stored at –20°C.

2.14.4 Cytokine PCR

Amplification of cytokine cDNA was carried out by PCR; 1µl of each sample was diluted to 10% (v/v) with uHQ water and this was added to a PCR mastermix to a final volume of 30µl per reaction.

PCR mastermix	Final concentration:
Buffer-$MgCl_2$ free (Promega)	10% (v/v)
dNTPs (Promega)	1.25mM
Primer 1-sense*	30µM see Table#
Primer 2-antisense*	30µM see Table#
25mM $MgCl_2$ (Promega)	1.5mM
Taq enzyme (5units/µl) (Promega)	0.2µl per reaction

* Primers were stored in lyophilised aliquots and resuspended in uHQ water before use. The primer concentration was determined by diluting 1:200 with uHQ water to a final volume of 1ml, and measuring the OD_{260} using uHQ as the blank. The final concentration was calculated using the equation: oligonucleotide (µg/ml) = dilution factor x A_{260} x 33. Primer stocks were then stored at –20°C.

Positive control samples were cDNAs from cell lines known to constitutively express the cytokine of interest (Table 2.9), and in the negative control, the cDNA was replaced with uHQ water. The sequences of the forward and reverse primers used are listed in Table 2.9, with β-actin primers used as an internal control in each reaction. Primer sequences were as published by other workers (Jung *et al.*, 1995; Yang *et al.*, 1997). The reaction was carried out for differing numbers of cycles depending on the transcript being studied, and using various annealing temperatures (Table 2.9). All PCR reactions were carried out using a Perkin Elmer Cetus GeneAmp PCR System 9600.

PCR products were separated using agarose gel electrophoresis and visualised by ethidium bromide staining.

Materials:

TBE 0.89M Tris, 0.89M boric acid (Merck-BDH), 0.02M EDTA, pH 8.3
Agarose gel 2% (w/v) agarose (SeaKem LE) in TBE.
Loading buffer (6x) 30% (w/v) glycerol, 60% (v/v) TBE, 0.25% (w/v) orange G dye (Merck-BDH)
Ladder Mix (MBI Fermentas)
Ethidium bromide 1µg/ml (w/v) (Merck-BDH)

A 10µl aliquot of PCR product was mixed with 2µl of 6x loading buffer and subjected to electrophoresis at 100V until the bands were well resolved (usually about 45 min) on a 2% (w/v) agarose gel (15x10x1cm horizontal slab) submerged in TBE buffer, using a BioRad 'wide-mini'

electrophoresis tank. The gel was then gently agitated in for 30 min in ethidium bromide solution, followed by rinsing in water twice for 30s each. The stained DNA bands were then visualised by illuminating the agarose gels with ultraviolet light using a transilluminator (Ultra-Violet products Inc), and photographed using Polaroid T667 film.

Table 2.9: Details of the forward (F) and reverse (R) primer sequences, cycle numbers and annealing temperatures (Tm) for PCR reactions of cytokines.

Gene	Sequence 5' 3'	Cycle number	Tm (°C) (F/R)	cDNA product size (base pairs)	Control cell line
β-actin F	TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA	35	52/65	661	CASKI
R	CTA GAA GCA TTG CGG TGG ACG ATG GAG GG				
Il-1α F	GTC TCT GAA TCA GAA ATC CTT CTA TC	40	46/51	420	CASKI
R	CAT GTC AAA TTT CAC TGC TTC ATC C				
Il-1β F	AAA CAG ATG AAG TGC TCC TTC CAG G	38	52	388	CASKI
R	TGG AGA ACA CCA CTT GTT GCT CCA				
Il-6 F	ATG AAC TCC TTC TCC ACA AGC GC	40	52/65	628	CASKI
R	GAA GAG CCC TCA GGC TGG ACT G				
Il-8 F	ATG ACT TCC AAG CTG GCC GTG GCT	42	52	289	CASKI
R	TCT CAG CCC TCT TCA AAA ACT TCT C				
GM-CSF F	ACA CTG CTG AGA TGA ATG AAA CAG TAG	38	45	286	CASKI
R	TGG ACT GGC TCC CAG CAG TCA AAG GGG				
MCP-1 F	TCT GTG CCT GCT GCT CAT AGC	40	59/57	510	CASKI
R	GGG TAG AAC TGT GGT TCA AGA GG				
MIP-1α F	CAG GTC TCC ACT GCT GCC	40	45/52	252	HUT 78
R	CAC TCA GCT CCA GGT CAC T				
TGF-1β F	GCC CTG GAC ACC AAC TAT TGC T	38	70/73.6	161	CASKI
R	AGG CTC CAA ATG TAG GGG CAG G				
TNF-α F	CGG GAC GTG GAG CTG GCC GAG GAG	40	86/72	355	CASKI
R	CAC CAG CTG GTT ATC TCT CAG CTC				
RANTES F	ATG AAG GTC TCC GCG GCA CGC CTC GCT GTC	40	45/52	252	CASKI
R	CTA GCT CAT CTC CAA AGA GTT GAT				

CHAPTER 3 CHARACTERISATION OF MENINGOCOCCI AND MENINGIOMA CELLS

Before investigations could take place into how isolates of *Neisseria meningitidis* interact with human meningeal cells, both components had to be established and characterised in an *in vitro* culture system. Firstly, a panel of meningococci that differed with respect to different surface antigens was established. Then cells were isolated from human meningioma tissue and characterised to confirm that they were of meningeal origin.

3.1 Selection of meningococcal phenotypic variants

The adhesive and invasive characteristics of meningococci to cultured epithelial and endothelial cells are known to be modulated by phase and/or antigenic variation of surface antigens as described in Chapter 1. To investigate the role of different meningococcal surface components on the bacterial association with and invasion of human meningeal cells, a panel of phenotypically variant bacteria was obtained and characterised with respect to the expression of these components.

Meningococcal disease isolates from the CSF are invariably found to be both encapsulated and piliated (Devoe & Gilchrist, 1975), and so it is likely that these components are of particular importance to the success of *N. meningitidis* as a meningeal pathogen.

3.1.1 Characterisation of meningococci

The meningococcal strains used in this study were clinical isolates from the CSF of patients with meningitis and are outlined in Table 2.1.

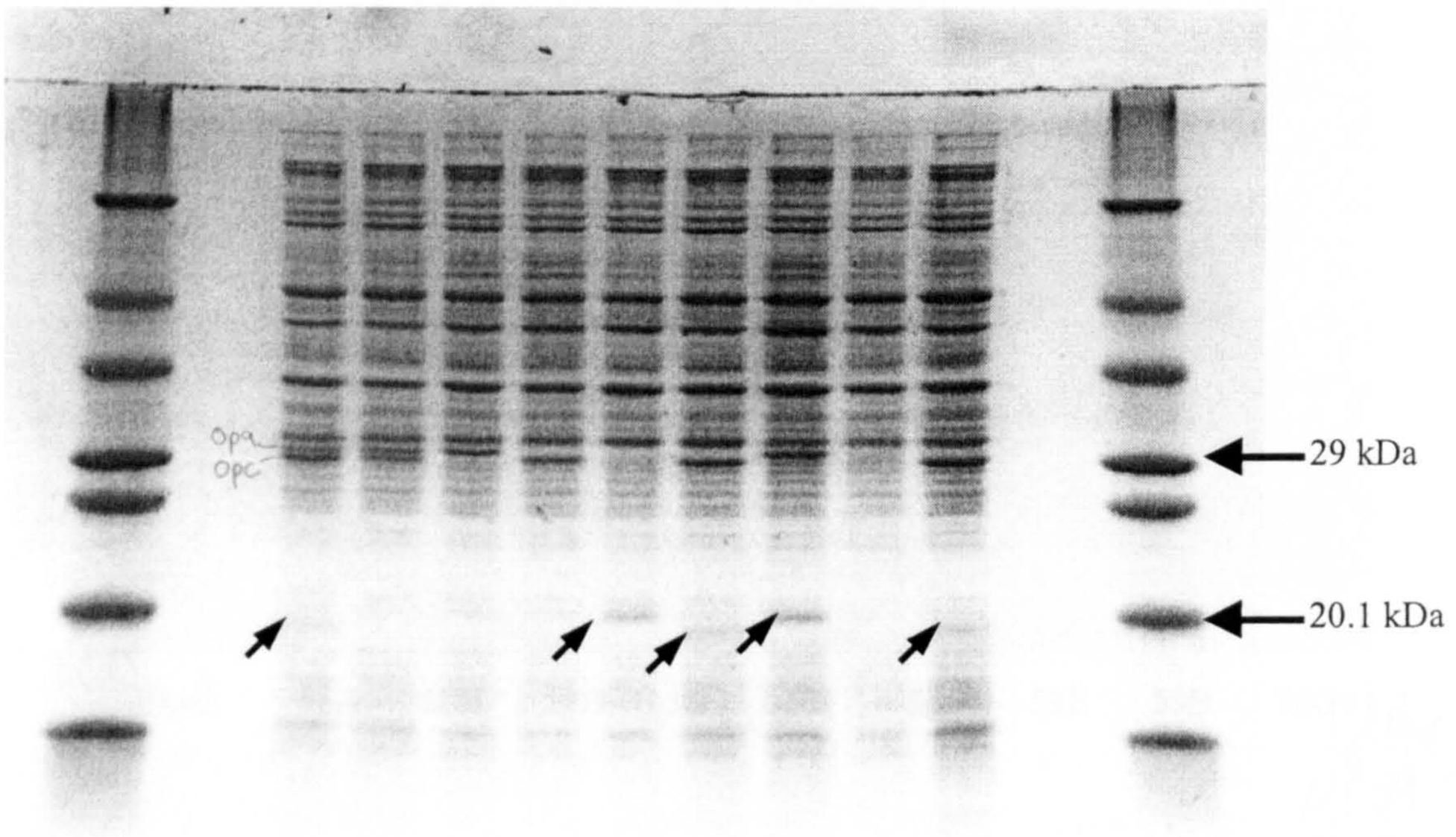
The original MC58 and MC59 isolates were initially characterised with respect to pilus (pilin subunit), Opa and Opc protein expression. Whole cell lysates of meningococci were subjected to SDS-PAGE on gradient gels. The expression of pilin, Opa and Opc proteins was then detected by immunoblotting and staining with the mAbs SM1 (anti-class I pilin), 4B12 (anti-Opa) and B306 (anti-Opc) (see Table 2.5 for details of mAbs used) as shown in Figures 3.1, 3.2 and 3.3

respectively. The MC58 isolate was found to be $Pil^+Opa^+Opc^+$ (58A) and the MC59 isolate was $Pil^+Opa^+Opc^-$ (59E). Although both isolates were piliated with class I pilin (piliation was defined by positive reactivity with SM1), the pilin bands were shown to differ in their apparent M_r; the MC58 pilin protein migrating further than, and hence being of smaller M_r, than that of MC59 (Figure 3.2). Bacterial cell lysates were subjected to SDS-PAGE using a gradient gel and stained for protein (as described in Section 2.5). The M_r of the pilin bands was then calculated by comparing the distance that these bands migrated to the distance migrated by protein standards of known M_r that were tested on the same gel (Figure 3.1). The pilin proteins of MC58 and MC59 were found to be 19KDa and 20KDa respectively. A variant that had been isolated previously in this laboratory, from $Pil^+Opa^+Opc^+$, was found to be $Pil^-Opa^+Opc^+$ (58H). Other non-piliated variants were obtained by selecting single large colonies from plates of piliated $Pil^+Opa^+Opc^+$ and $Pil^+Opa^+Opc^-$ grown overnight at 30°C (Figure 3.2), using the size variation between Pil^+ and Pil^- colonies as the differentiating factor. Bacteria lacking Opa proteins were selected as single colonies using their decreased opacity when viewed under a stereo microscope equipped with a substage flat polished mirror (Figure 3.3). All meningococci derived from MC58 expressed Opc, whilst all MC59 variants lacked this expression (Figure 3.4). By a combination of these selection techniques other bacteria were then selected; MC58 variants lacking in their Opa expression, $Pil^+Opa^-Opc^+$ (58F) and $Pil^-Opa^-Opc^+$ (58C), and MC59 isolates differing in their pilus and Opa expression, $Pil^-Opa^+Opc^-$ (59D), $Pil^+Opa^-Opc^-$ (59I) and $Pil^-Opa^-Opc^-$ (59B) as outlined in Table 3.1. All piliated bacteria showed the same reactivity with SM1 as the isolate that they originated from, with all Pil^+ MC58 variants expressing smaller M_r pilin subunits than the MC59 variants. This difference was subsequently represented by designating Pil^+ MC58 isolates as Pil_{Ia}^+, and all Pil^+ MC59 isolates as Pil_{Ib}^+. There were no such differences with the Opa proteins (Figure 3.3), and all Opa^+ isolates expressed Opa proteins that migrated to the same distance on SDS-PAGE. All selected bacteria were confirmed as meningococci by testing for their gram negative reaction in the Gram stain, and a positive oxidase test.

As meningococci can produce pilin proteins without assembling mature pili, the presence or absence of pili was confirmed by electron microscopy following negative staining with uranyl acetate (data not shown). These experiments confirmed the data from immunostaining with the anti-pilin SM1 Ab described above. In addition, bacteria with type Ib pilin appeared to be more piliated than isolates with type Ia pilin, and bundling of pilus fibres was not observed in either isolate under these conditions.

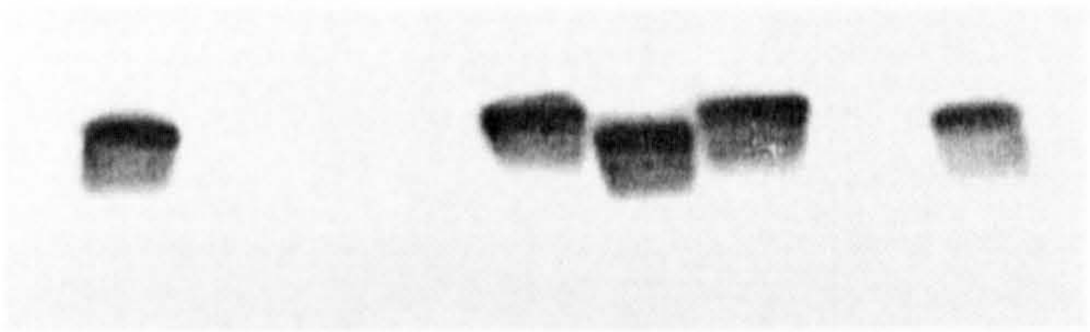
The expression of PilC in the MC58/59 variants was tested using immunoblotting with an anti-PilC polyclonal antibody (ABJ) raised against the C-terminal half of the PilC1 protein of *N. gonorrhoeae*. When reacted with this antibody, whole cell lysates of bacterial isolates possessing the PilC protein produced a band that corresponded to a Mr of 110KDa (Figure 3.5). The meningococcal group C and B strains C114 and C311 respectively, were included as controls as

Figure 3.1: Meningococcal variants subjected to SDS-PAGE and protein stain.
The pilin bands are roughly in line with the 20.1kDa protein standard band (see arrows).
The Opa and Opc bands are also apparent just below the 29kDa standard.



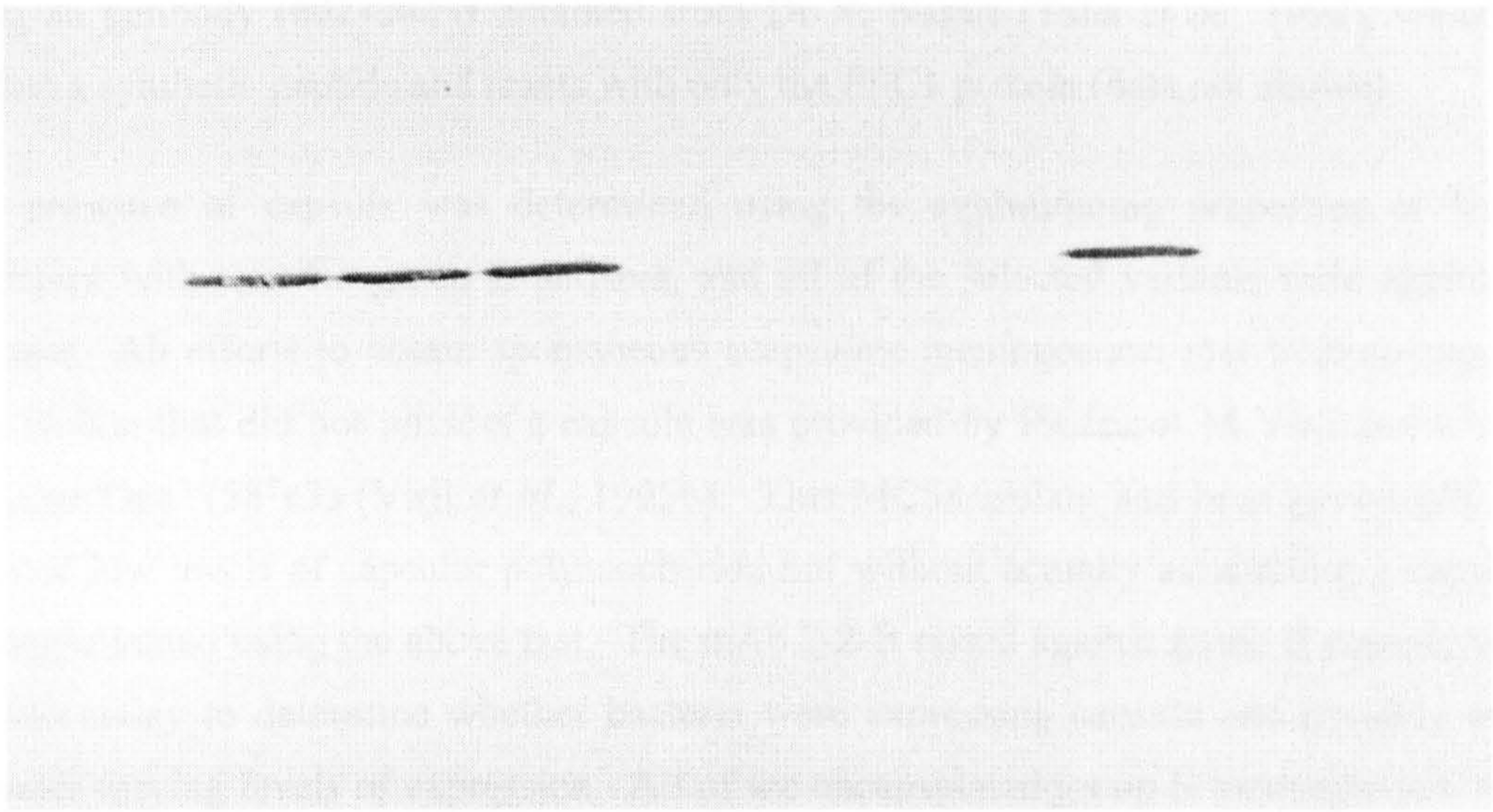
58A 58H 59D 58C 59I 58F 59E 59B 58G

Figure 3.2: Meningococcal variants reacted with SM1 to stain for pilin proteins.



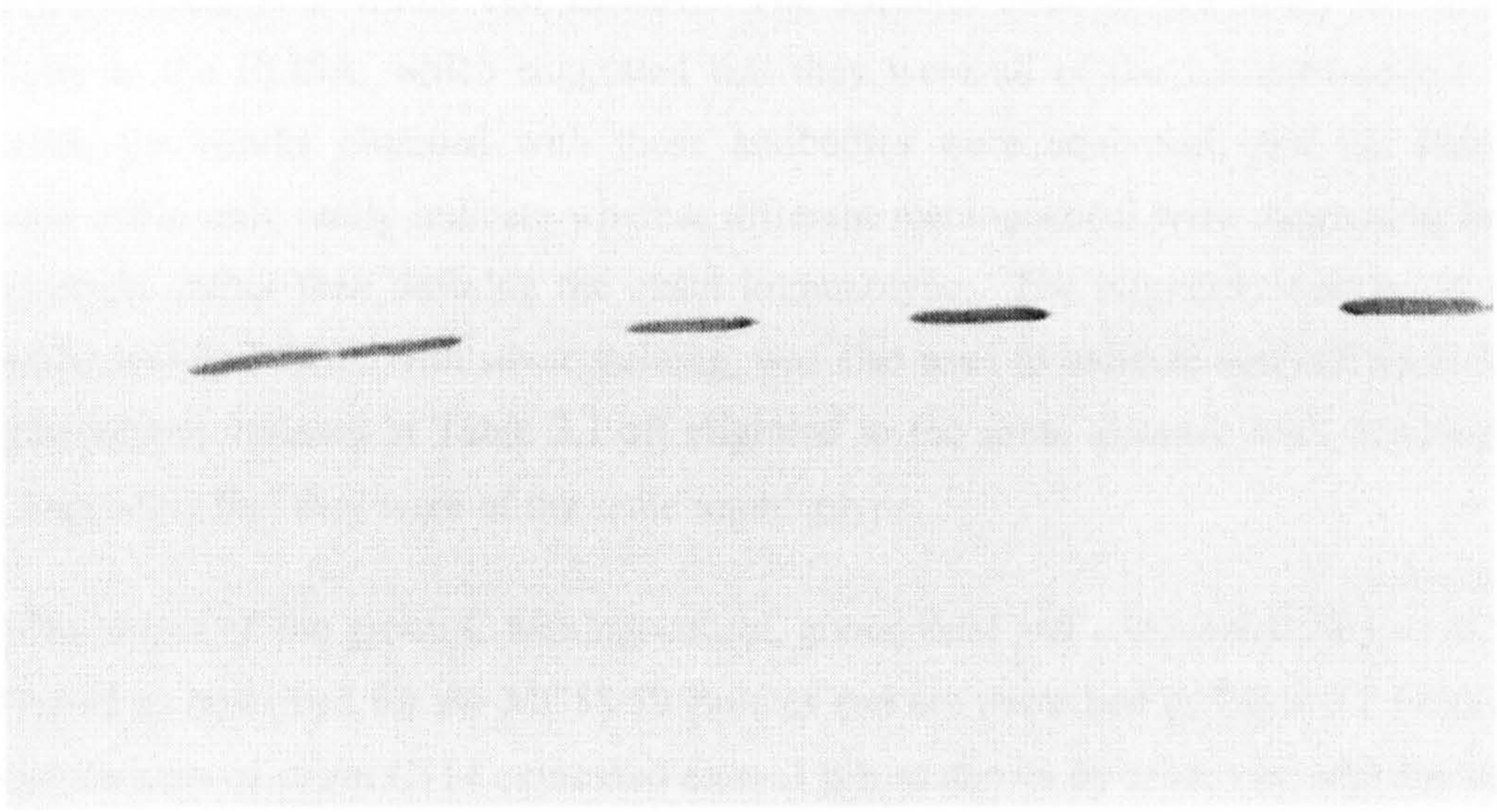
58A 58H 59D 58C 59I 58F 59E 59B 58G

Figure 3.3: Meningococcal variants reacted with mAb 4B12 to stain for Opa proteins



Blank 58A 58H 59D 58C 59I 58F 59E 59B 58G

Figure 3.4: Meningococcal variants reacted with mAb B306 to stain for Opc proteins



Blank 58A 58H 59D 58C 59I 58F 59E 59B 58G

they were found to have high PilC expression. The MC58/MC59 variants expressed equal amounts of the PilC protein, regardless of their piliation status, and these levels were quite low in comparison with the control strains. In addition to the PilC band, the antibody was also positive for many proteins of Mr less than 110KDa, and for one protein greater than 110KDa. Figure 3.5 shows only the top portion of the blot incorporating the PilC bands. Very similar results were obtained using an antibody ($602724T_3$) obtained from Dr X. Nassif (Taha *et al.*, 1998), which was raised against a synthetic peptide and reacts with only the PilC1 protein (data not shown).

The presence of capsule was determined using the agglutinating properties of latex particles sensitised with specific group B antisera, and all of the selected variants were agglutinated using this test. All efforts to obtain spontaneous acapsulate meningococci met with no success, and the only isolate that did not possess a capsule was provided by Professor M Virji and which was also $Pil^{+}_{Ia}Opa^{-}Opc^{+}$ ($58^{+}¢3$) (Virji *et al.*, 1995b). This MC58 isolate had been genetically modified to produce low levels of capsular polysaccharide but without actually assembling a capsule, and was not agglutinated using the above test. The mAb 2-2-B raised against group B capsule was used in a dot blot assay to determine whether bacteria were expressing capsule and possibly to distinguish between varying levels of expression. All of the encapsulated group B meningococci were positive using this test, with no apparent variation in level of expression, and a group C strain (C114) and gonococcal strain (P9-1), included as controls, were negative (Figure 3.6). However, the group B capsule deficient mutant also showed reactivity with the antibody, which was presumably due to the presence of capsule components rather than the fully assembled capsule structure.

LPS immunotyping was done using a panel of antibodies, which recognised different combinations of immunotypes, in a whole cell ELISA. The variants used in this study all showed similar reactivity in the ELISA, which suggested that they were all of the L3 immunotype (Table 3.1). However, the results obtained with these antibodies were equivocal, and the reaction pattern obtained could only really indicate whether different meningococci were expressing the same LPS immunotype, rather than defining the exact immunotype. The migratory pattern of LPS on low molecular weight PAGE, with silver staining, was also used to indicate similarities in immunotype. The phenotypic variants in Table 3.1 all migrated to the same distance with this method (Figure 3.7), suggesting that they were of the same immunotype.

The phenotypes of the group C meningococcal, gonococcal and commensal *Neisseria* strains were established as described for the MC58/59 isolates and are described in Table 3.2 (data not shown). Piliated variants of strain C114 expressed class II pili as shown by reactivity with the mAb AG123; in addition, the commensal *N. lactamica* was also found to express class II pili. Gonococcal pilin proteins were of a higher molecular weight than both the Pil_{Ia}^{+} and Pil_{Ib}^{+} meningococcal isolates, whereas gonococcal Opa proteins had a lower molecular weight than the meningococcal Opa proteins.

Figure 3.5: Meningococcal variants reacted with Ab ABJ to detect PilC expression. The PilC band is present just below the 116kDa protein standard band; the PilC band for each variant is labelled with an arrow.

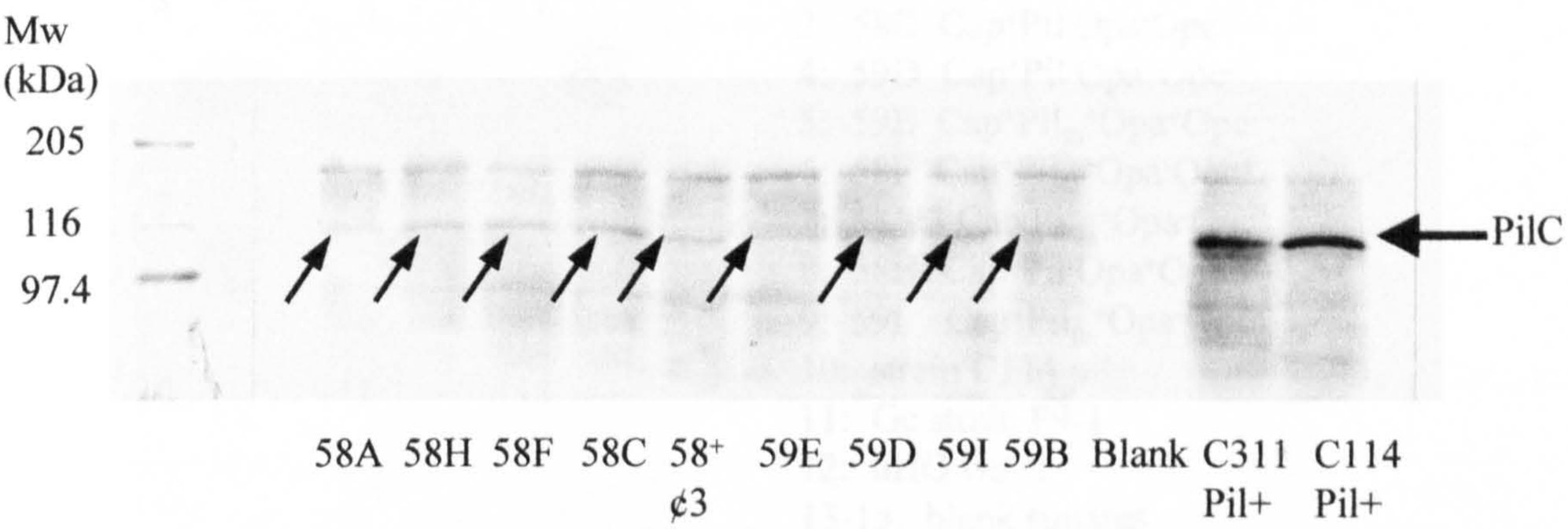
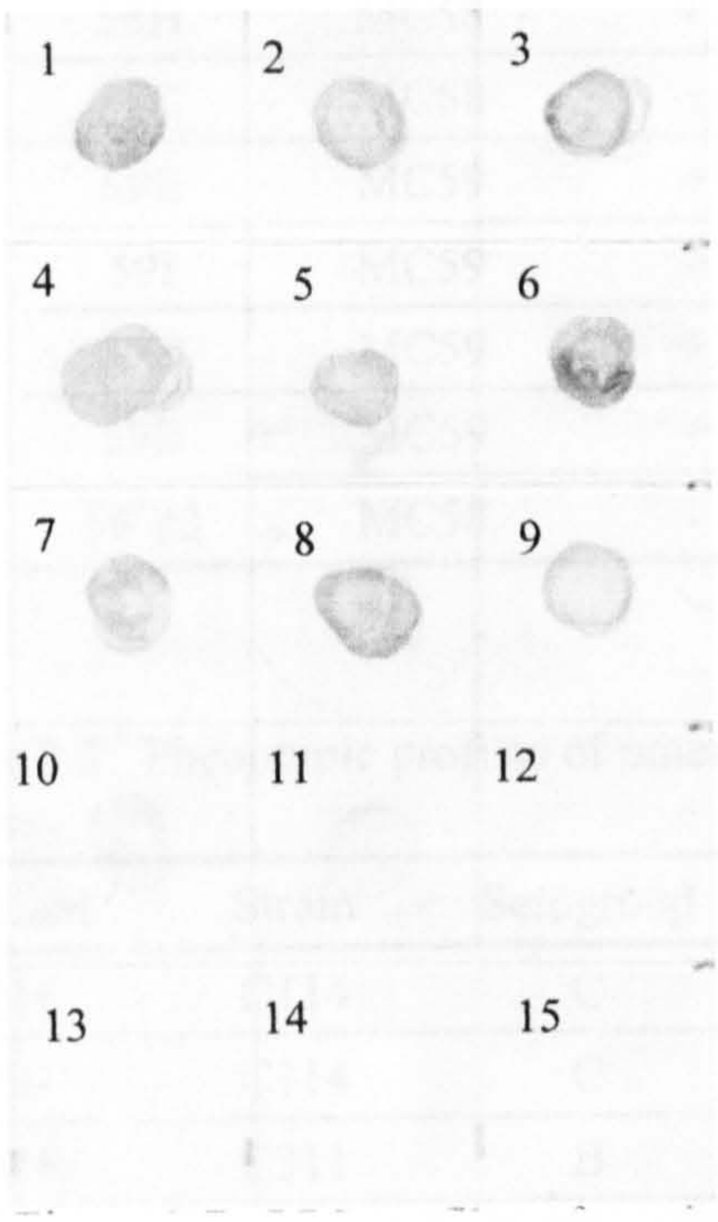


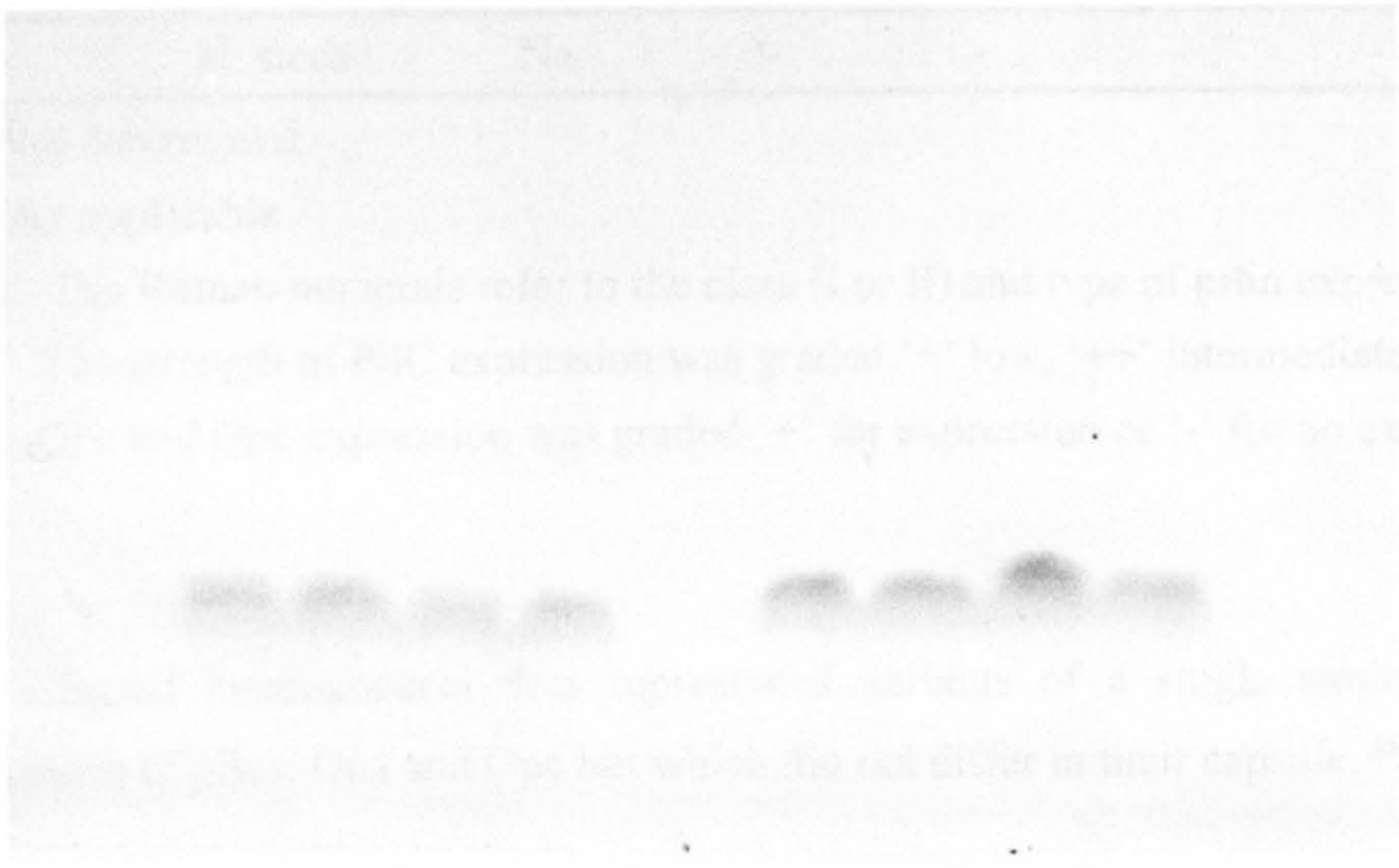
Figure 3.6: Meningococcal variants reacted with 2-2-B Ab to detect capsule expression



Key:

1: 58A $Cap^{+}Pil_{Ia}{}^{+}Opa^{+}Opc^{+}$
2: 59B $Cap^{+}Pil^{-}Opa^{-}Opc^{-}$
3: 58C $Cap^{+}Pil^{-}Opa^{-}Opc^{+}$
4: 59D $Cap^{+}Pil^{-}Opa^{+}Opc^{-}$
5: 59E $Cap^{+}Pil_{Ib}{}^{+}Opa^{+}Opc^{-}$
6: 58F $Cap^{+}Pil_{Ia}{}^{+}Opa^{-}Opc^{+}$
7: $58^{+}¢3$ $Cap^{-}Pil_{Ia}{}^{+}Opa^{-}Opc^{+}$
8: 58H $Cap^{+}Pil^{-}Opa^{+}Opc^{+}$
9: 59I $Cap^{+}Pil_{Ib}{}^{+}Opa^{-}Opc^{-}$
10: strain C114 pil+
11: Gc strain P9-1
12: uHQ water
13-15: blank squares

Figure 3.7: LPS profiles of meningococcal variants after low molecular weight PAGE and silver staining.



58A 58H 58F 58C Blank 59E 59D 59I 59B

Table 3.1: Phenotypic profiles of serogroup B MC58 and MC59 meningococci.

Variant	Strain	Capsule	Pilus[a]	Opa	Opc	PilC	LPS
58A	MC58	+	+ (Ia)	+	+	+	3
58F	MC58	+	+ (Ia)	-	+	+	3
58H	MC58	+	-	+	+	+	3
58C	MC58	+	-	-	+	+	3
59E	MC59	+	+ (Ib)	+	-	+	3
59I	MC59	+	+ (Ib)	-	-	+	3
59D	MC59	+	-	+	-	+	3
59B	MC59	+	-	-	-	+	3
58^{+}¢3	MC58	-	+ (Ia)	-	+	+	3

Table 3.2: Phenotypic profiles of other meningococcal, gonococcal and commensal Neisseria strains

Variant	Strain	Serogroup	Capsule	Pilus[a]	Opa	Opc	PilC[b]	LPS
Pil+	C114	C	+	+ (II)	+	Nd	+++	Nd
Pil-	C114	C	+	-	+	Nd	Nd	Nd
Pil+	C311	B	+	+ (I)	Nd	Nd	+++	Nd
P9-1	P9	Na	-	-	-	-	Nd	Nd
P9-2	P9	Na	-	+ (I)	-	-	Nd	Nd
P9-16	P9	Na	-	-	+	-	Nd	Nd
P9-17	P9	Na	-	+ (I)	+	-	Nd	Nd
-	N. lactamica	Na	-	+ (II)	-	-	Nd	Nd
-	N. sicca	Na	-	-	-	-	Nd	Nd

Nd Not determined

Na Not applicable

Pilus[a] The Roman numerals refer to the class (I or II) and type of pilin expressed (a or b).

PilC[b] The strength of PilC expression was graded '+' low, '++' intermediate, or '+++' high.

Pilus, Opa and Opc expression was graded '+' for expression or '-' for no expression.

The selected meningococci thus represented variants of a single strain that differed in their expression of pilus, Opa and Opc but which did not differ in their capsule, PilC or LPS profiles.

3.2 Characterisation of meningiomas

The *in vitro* growth of cells derived from human meningioma tissue was investigated as a prospective model for the normal human leptomeninges. To achieve this, meningioma cells were characterised with respect to known cellular markers for leptomeningeal cells.

3.2.1 Growth and morphology of cultured human cells

The pieces of meningioma tumour tissue obtained were processed for meningioma cell culture as described in the previous chapter. Meningioma cells were deemed to be cell lines after the first sub-culture, and were labelled numerically with the prefix 'M' (Table 3.3). Previously in this laboratory, meningioma cell lines M5 through to M14 had been established, and upon resuscitation, most of these cell lines were still viable and grew well in culture. The fresh samples processed in this study were cell lines M15 to M25 (Table 3.3). These tumours were confirmed as meningiomas by the Neuropathology Department using histological methods, and classified into the respective subtypes, which included meningothelial (5), transitional (3), anaplastic (1) and atypical (1) subtypes. Cell lines were established for ten out of the 11 new samples, and growth was more rapid when the tumour tissue was processed on the same day as the resection; primary culture meningioma cells were seeded into 80cm^2 flasks and these were sometimes confluent within just one day. The growth rate of the different meningioma cell lines varied between samples, irrespective of both the subtype and size of the tissue piece, which ranged from less than 1cm^3 to pieces as large as 3x2x0.5cm. The meningioma cell lines grew well in culture until at least passage nine, and the meningothelial line M18 was grown to passage 15 with no obvious signs of senescence. In healthy proliferating cultures, the passage number appeared to have no effect on the rate of growth. The growth rate of the cultured meningioma cells used was not measured in this study, but the proliferative activity of some meningioma tumours was tested by the Department of Neuropathology using the mAb Ki67, which detects a nuclear antigen that is only present in cells undergoing mitosis. Those tumours with a high Ki67 index were more likely to grow well in culture (Feurer & Weller, 1991) and the only meningioma from which cells failed to grow in this study (M22) showed no mitosis (Table 3.3).

The morphological appearance of cultured meningioma cells was monitored by light microscopy, and was found to vary for an individual cell line at different stages of confluence. Although there was some variation between different cell lines, it was not possible to distinguish between the meningioma subtypes meningothelial, transitional or anaplastic on the basis of their morphology in culture. Since cultured meningioma cells could resemble fibroblasts or epithelial cells depending on

the level of confluence, the cell lines MRC-5 and Chang were used as controls to compare meningioma cell morphology with that of fibroblasts and epithelial cells respectively. MRC-5 cells were typical fibroblasts; at subconfluence (Figure 3.8) they existed as multipolar or bipolar spindly cells, with a length more than twice their width and were well spread over the culture surface. At confluence (Figure 3.9) they assumed a bipolar appearance, were less well spread and grew in parallel arrays with frequent whorl formation. Chang cells displayed normal features of epithelial cells; polygonal cells with more regular dimensions that grow in discrete patches with other cells, although cells at the edges of these patches sometimes became fibroblastic in shape (Figures 3.10, 3.11).

In general, meningioma cells at subconfluence (exponential phase of growth) resembled fibroblasts and were often bipolar or stellate with frequent cell processes, prominent nuclei and nucleoli, and quite grainy cytoplasm. Also common were curvilinear edges, which sometimes joined up with other cells to form complete circles. At confluence, meningioma cells were more epithelial-like; there was more uniformity of cell shapes, with the reduction of cell processes, to adopt a flat polygonal appearance. The boundaries between cells were frequently unclear at this stage and thus the cells were sometimes difficult to see under the light microscope. Typically, meningioma cells grew in culture to cover the substrate and at confluence, there was usually contact inhibition between cells although not all of the monolayers were tightly packed and cell processes from a cell sometimes overlapped other cells.

The growth and morphology of four meningioma cell lines of different subtypes was recorded in more detail (meningothelial M18, M21; transitional M17; anaplastic M16). The meningothelial cell lines grew at a similar rate, which was faster than both the transitional and anaplastic cell lines.

On the basis of cellular morphology, the meningothelial cell lines M18 and M21 were virtually indistinguishable (Figures 3.12, 3.13). When seeded at sparse densities these cells were mostly tripolar with cellular processes and curvilinear edges; they generally resembled fibroblasts. At subconfluence, cultures contained a mix of cell sizes and shapes, mostly polygonal, with easily visible oval-shaped nuclei and nucleoli; the nucleus typically accounting for about 10% of the total cell area. The perinuclear cytoplasm was quite grainy and became more translucent towards the periphery of the cells. Large and small curvilinear edges were common, with some complete circles of cells, and processes were often seen extending out to other cells. When these cells had reached confluence, the cell borders were very difficult to distinguish and there were fewer visible cell processes.

The transitional and anaplastic cell lines, M17 and M16 respectively, were very similar to each other morphologically (Figures 3.14, 3.15). They shared features with the meningothelial cells described above, but differed in that the transitional and anaplastic cells had a more grainy cytoplasm, generally longer cell processes and a tendency to form more curvilinear edges. The

For photography using phase-contrast microscopy, control cell lines were grown in uncoated 25cm^2 tissue culture flasks, and meningioma cultures in collagen coated 25cm^2 flasks.

Figure 3.8: Subconfluent MRC-5 fibroblasts. Multipolar or bipolar cells, with a length more than twice their width. Magnification x47.

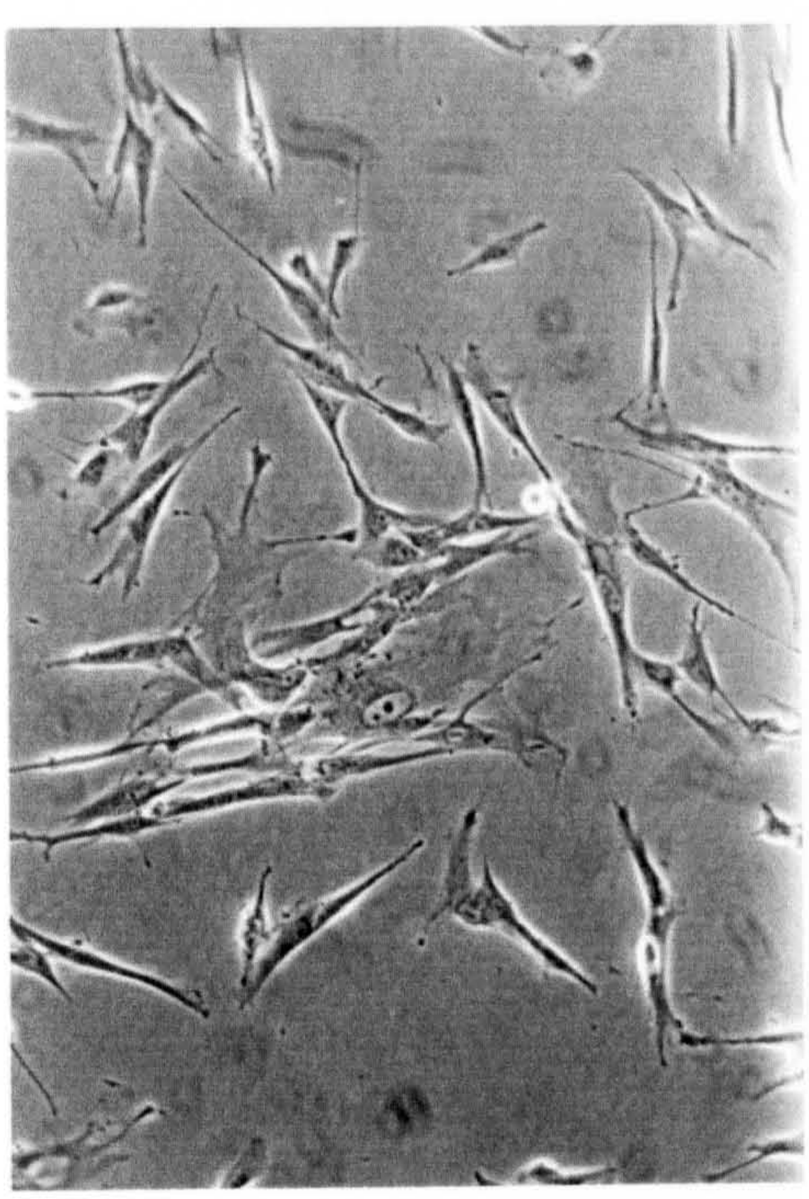


Figure 3.9: Confluent monolayer of MRC-5 cells. Bipolar cells growing in parallel arrays with whorl formation. Magnification x47.

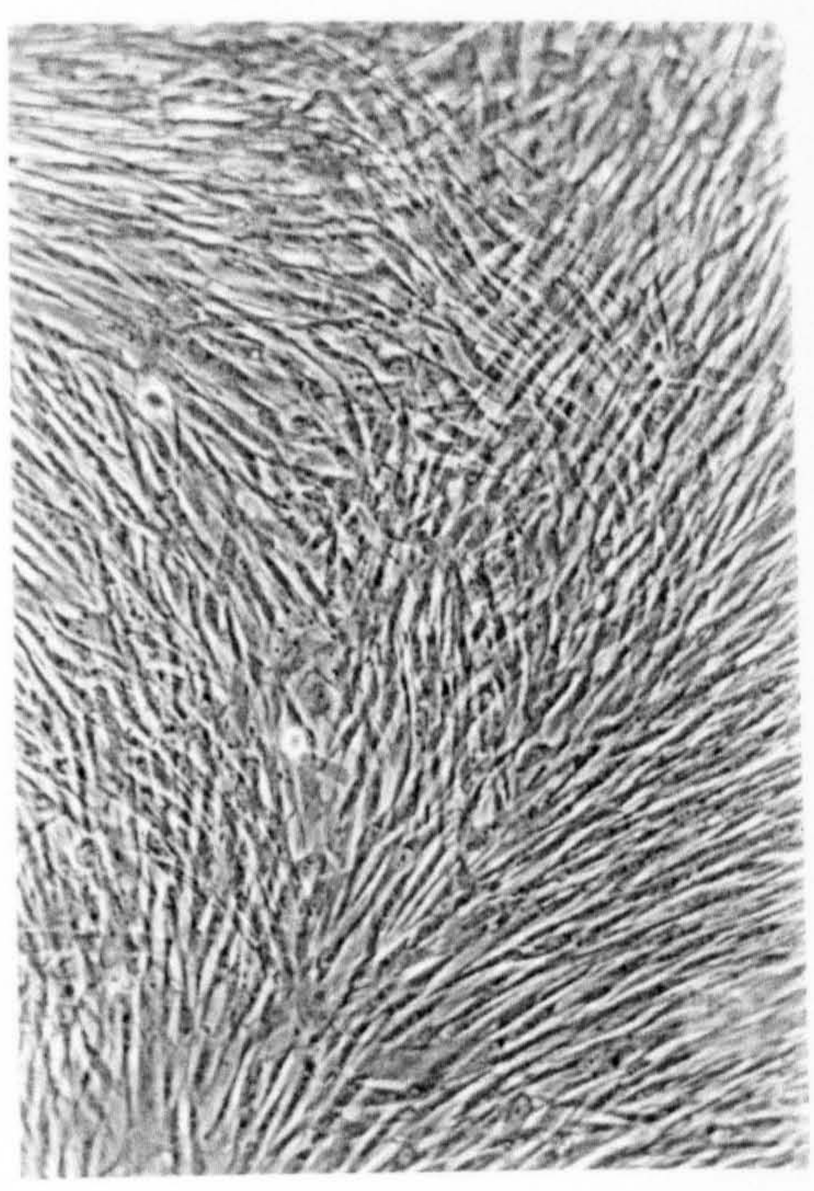


Figure 3.10: Subconfluent Chang epithelial cells. Magnification x47.

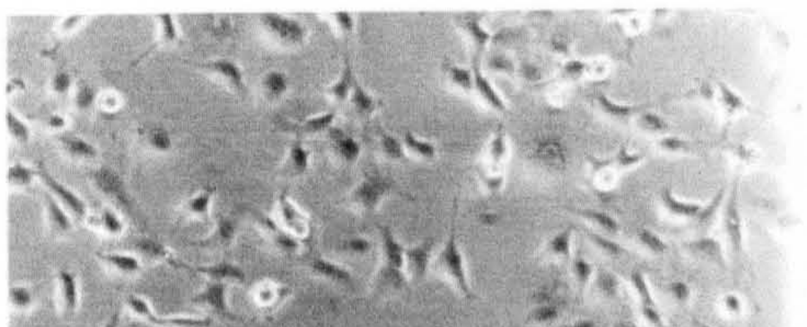


Figure 3.11: Confluent monolayer of Chang epithelial cells. Polygonal cells with more regular dimensions than fibroblasts. Magnification x47.

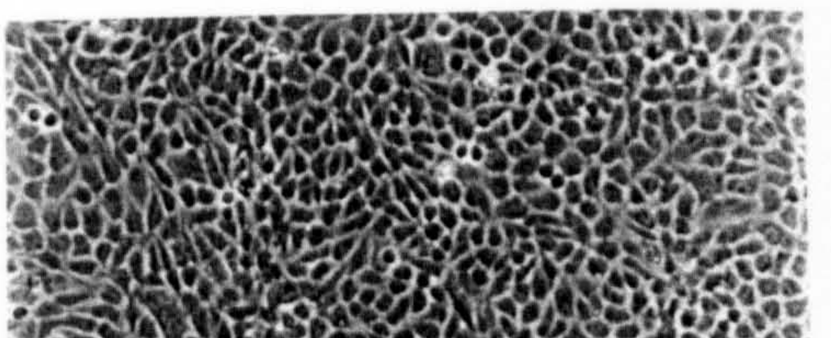


Figure 3.12a: Meningioma 18 (meningothelial) at passage 6 showing mostly polygonal cells with some curvilinear edges. Magnification x47.

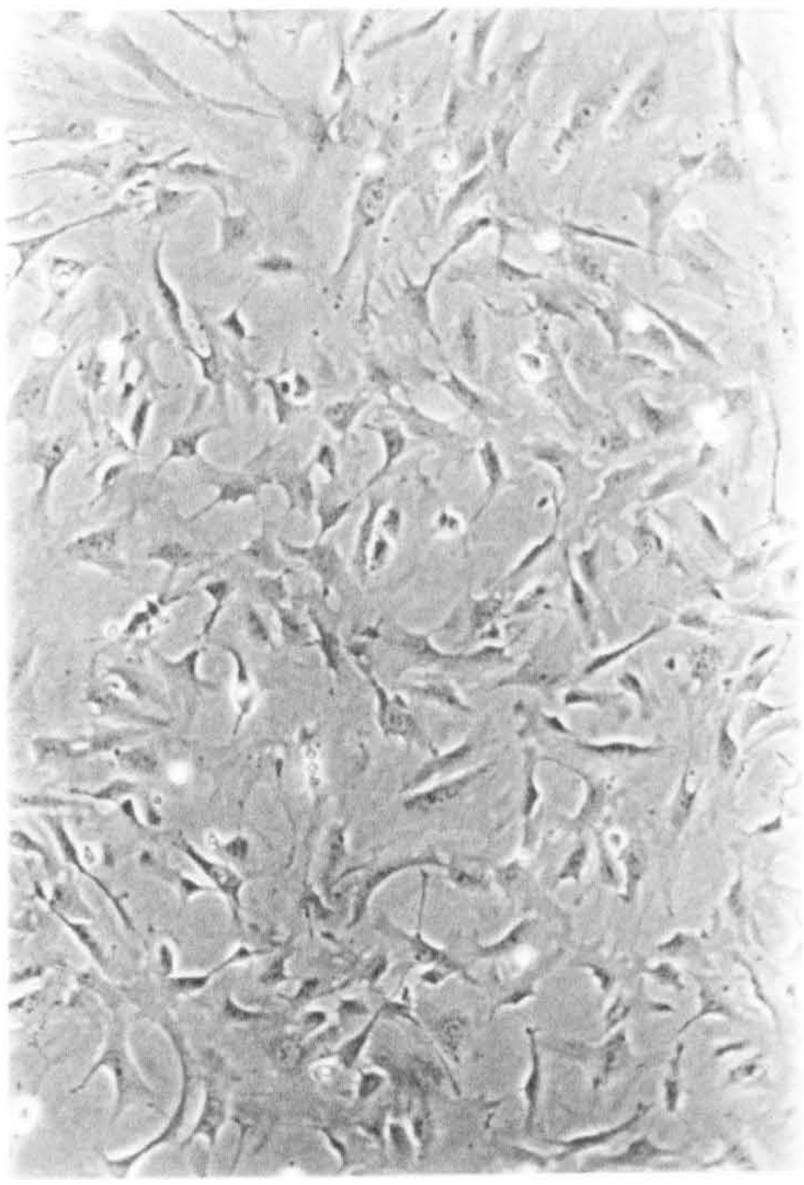


Figure 3.12b: Meningioma 18 at passage 9. Magnification x85.

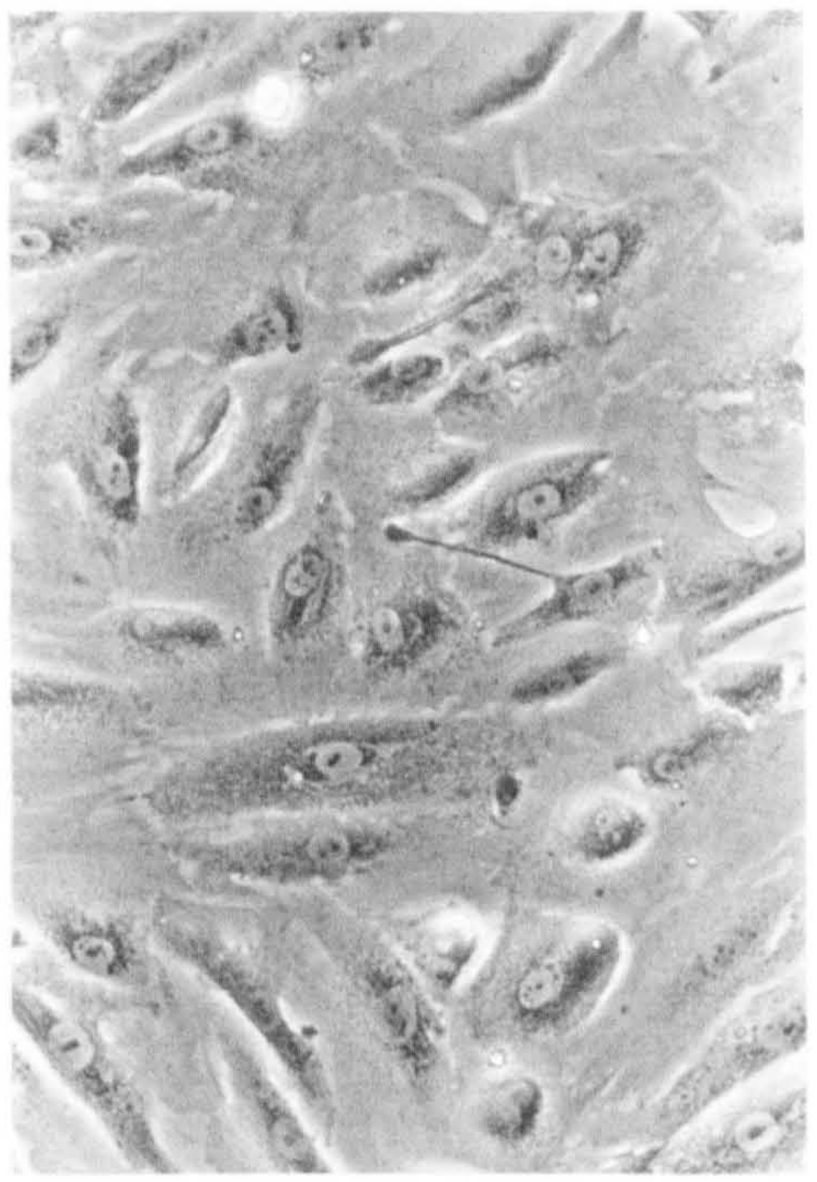


Figure 3.13a: Meningioma 21 (meningothelial) at passage 7 approaching confluence. Magnification x47.

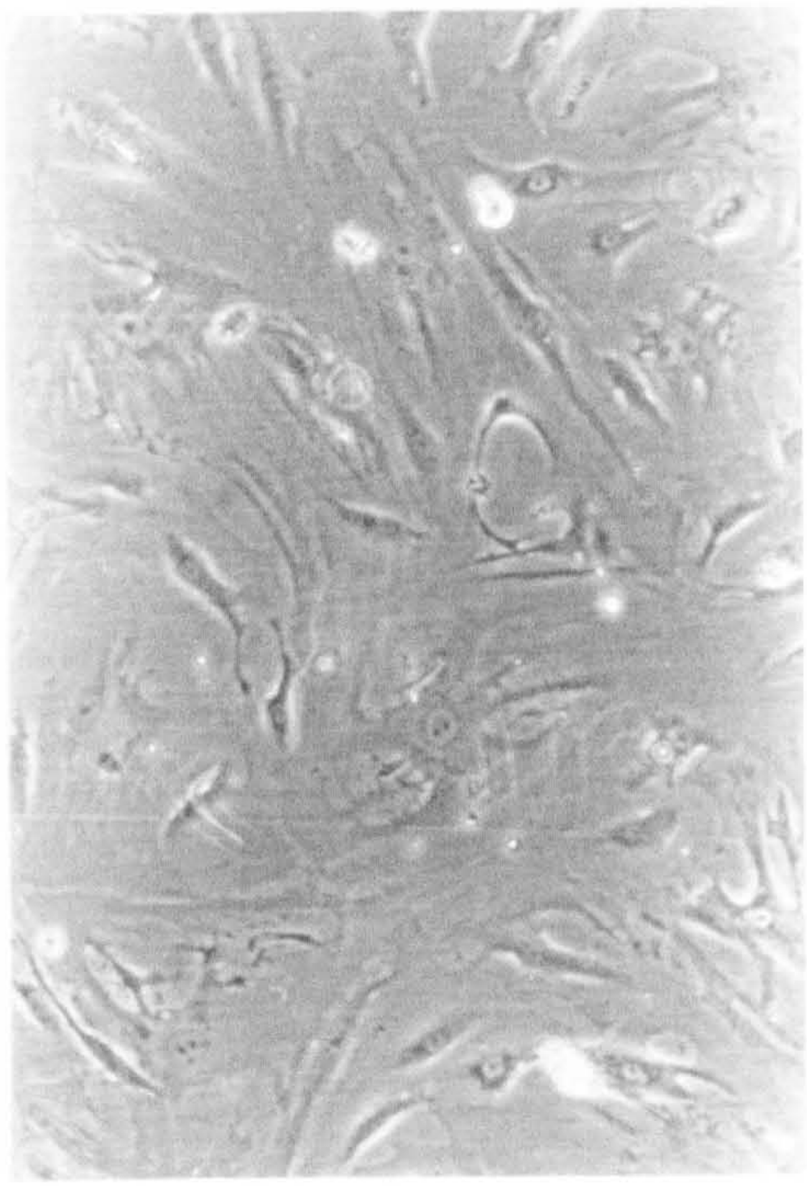


Figure 3.13b: Higher magnification of M21 in Figure 3.13a
Magnification x85.

Figure 3.14a: Meningioma 17 (transitional) at passage 6 showing quite large cells with curvilinear edges.
Magnification x47.

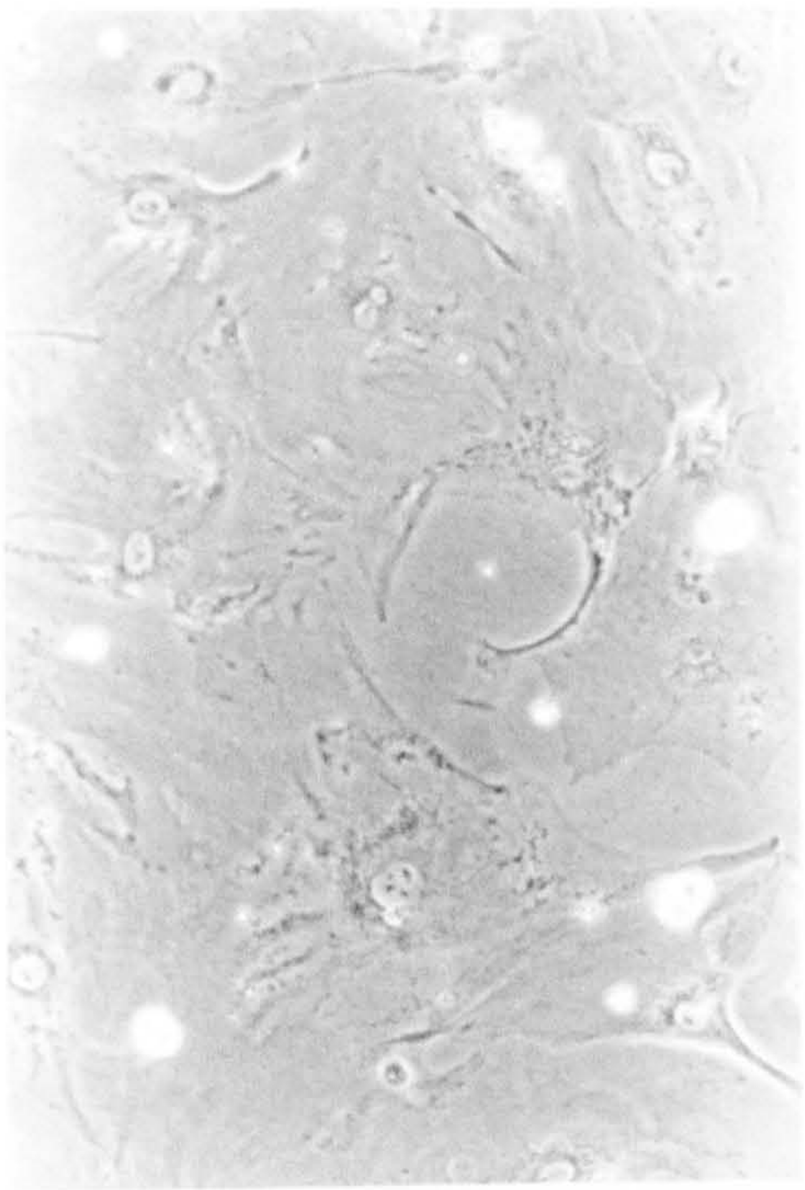


Figure 3.14b: Higher magnification of M17 in Figure 3.14a.
Magnification x85.

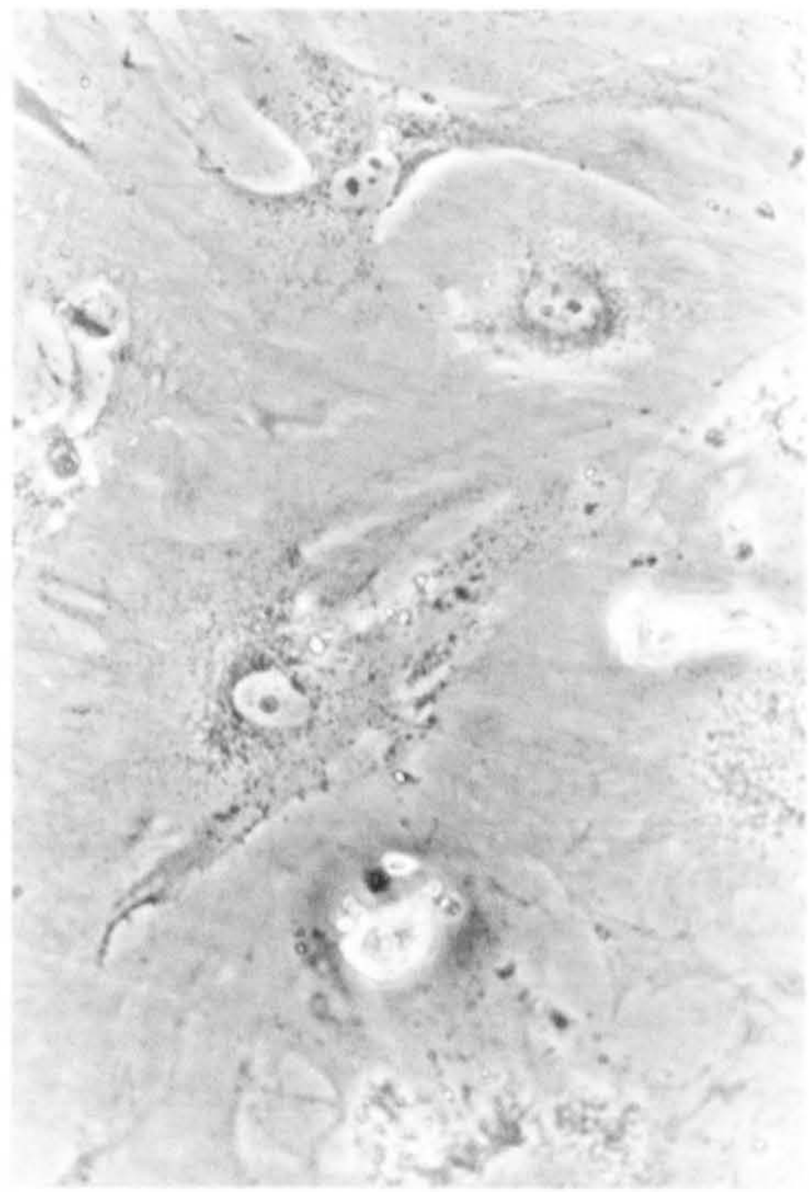


Figure 3.15a: Meningioma 16 (anaplastic) at passage 6 showing a mixture of cell shapes and sizes.
Magnification x47.

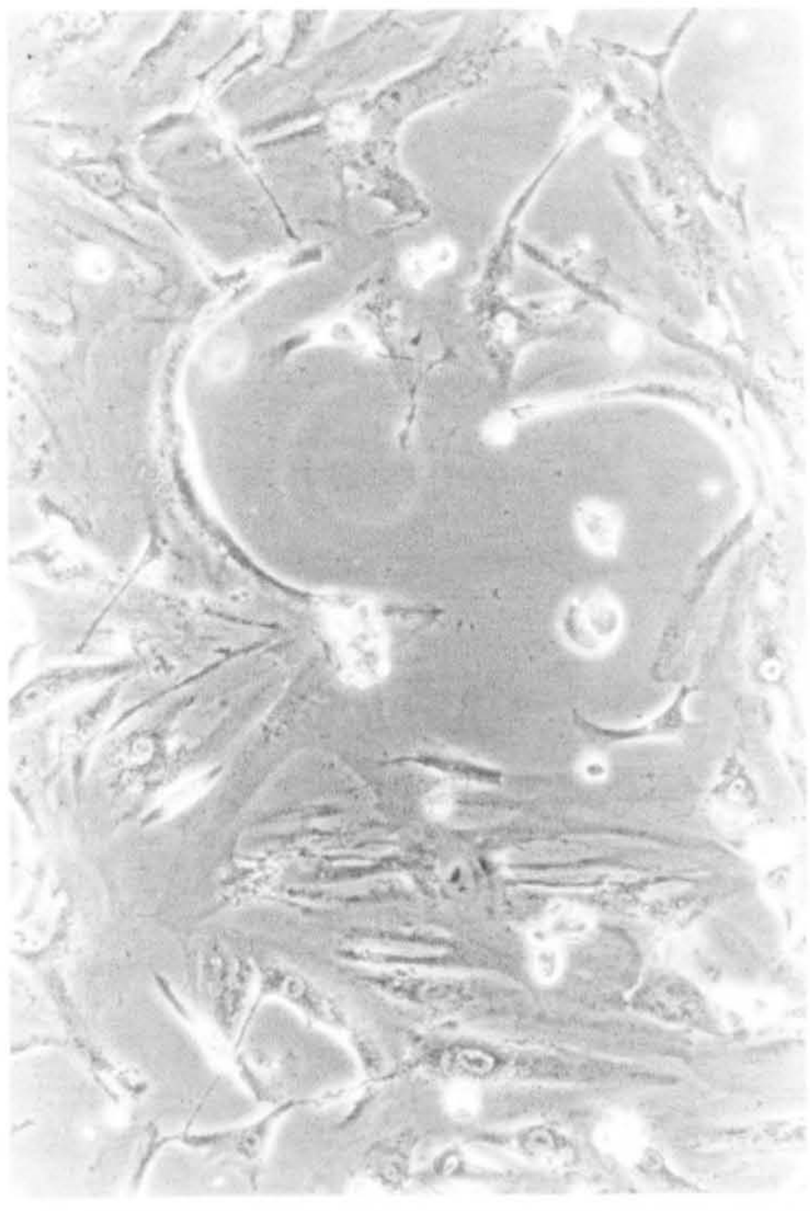
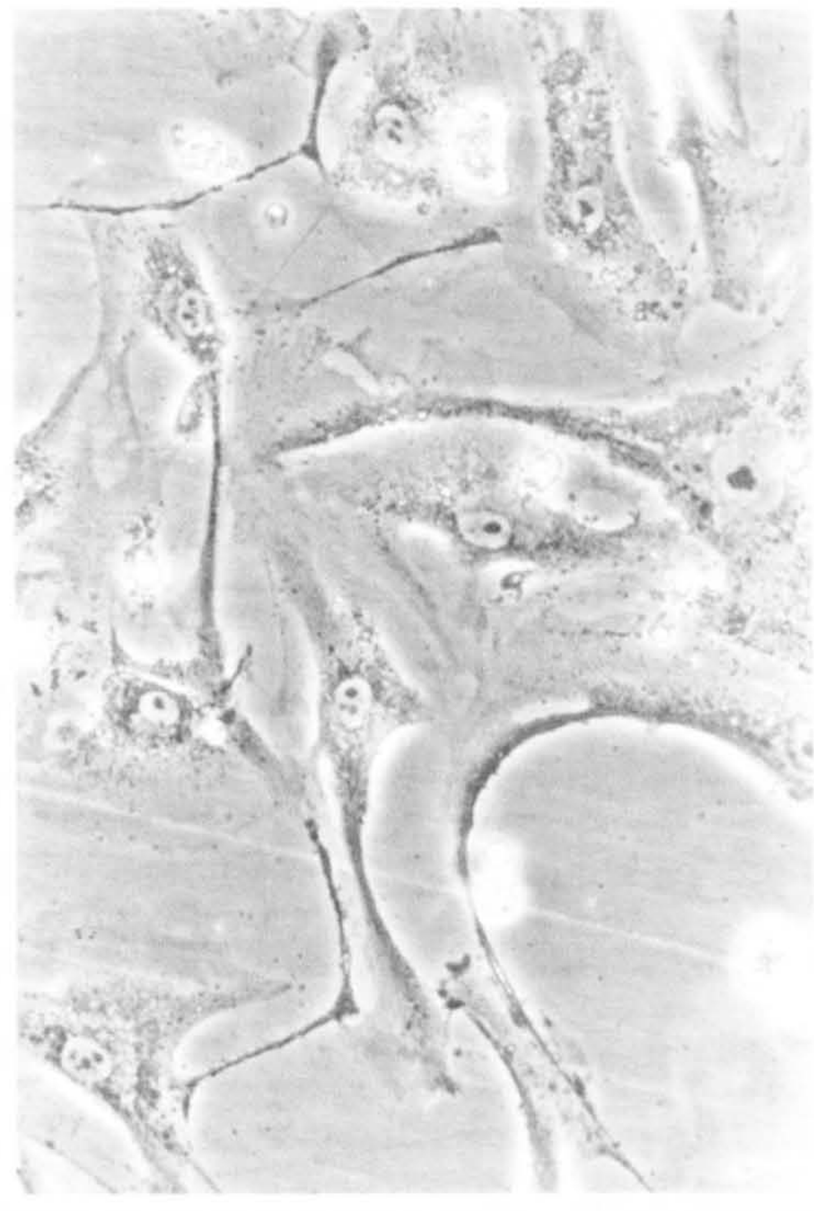


Figure 3.15b: Higher magnification of M16 in Figure 3.15a.
Magnification x85.



transitional cells appeared to be approximately twice the size of the meningothelial cells and slightly larger than the anaplastic cells. Anaplastic cells at subconfluence were often of mixed shapes and sizes, and sometimes grew in parallel whorl-like formation similar to that seen with fibroblasts, but as the cultures became more packed these structures were not so clear.

Overall, the growth rate and morphology of these meningioma cells did not appear to differ between passages.

3.2.2 Staining for cellular markers

It was important to establish that the cells cultured from meningioma tissue were actually meningioma cells, and therefore of meningeal origin. In addition, it was necessary to assess and compare the epithelial and mesenchymal characteristics of individual cell lines. To this end, meningioma cells were grown to sub-confluence on collagen coated glass coverslips and stained with a panel of cellular markers using an indirect immunofluorescent technique, with FITC as the flurochrome (see Table 2.8 for details of antibodies used). The methodology for the immunofluorescent characterisation of meningioma cells was established using the control cell lines (Chang, MRC-5) and meningioma cell lines previously obtained (M5 – M14). Initially, all samples were fixed in ice-cold methanol regardless of which antibody was being used. The Chang cell line was expected to stain positively for the epithelial markers desmoplakin, cytokeratin and EMA, so that when the samples showed inconclusive results for cytokeratin and EMA, different fixatives were tried. Optimum staining was found with acetone for cytokeratin and paraformaldehyde for EMA. In addition, EMA provided a clearer staining pattern when incubated with the sample overnight at 4°C. All samples were counterstained with propidium iodide, which stains nuclear material orange. Table 3.3 outlines the staining characteristics of the control cell lines and of all the meningioma cultures. Appropriate epithelial (Chang) and fibroblastic (MRC-5) cell lines were included as controls and these were grown to subconfluence on uncoated glass coverslips. Negative controls for each cell line consisted of the normal staining procedure minus the primary antibody but with PI, and hence the only staining in these samples was of the nuclear material, which appeared orange under fluorescence (Figure 3.16).

Desmosomes are found in leptomeningeal cells and their derivatives, and the presence of such structures within a culture was deemed indicative of true meningioma cells and confirmed that these cells were of leptomeningeal origin. The presence of desmosomes was determined by the use of a desmoplakin mAb that stained desmosome-associated protein plaques (desmoplakin I and II). Positive samples were characterised by punctate staining around the cellular borders, which represented the presence and site of the desmosomes. Within each positive sample, all of the cells showed staining for desmosomes indicating a pure culture of meningioma cells. With the exception

of M25 cells, all of the meningioma lines tested were positive for desmoplakin (Figure 3.17), whilst ependymoma cells were desmoplakin negative. Nine out of the eleven meningioma cell lines stained strongly for desmoplakin (Table 3.3), including the four cell lines whose morphology was previously recorded in detail (meningothelial M18, 21; transitional M17; anaplastic M16). Each meningioma was tested for the presence of desmosomes at more than one passage number, and no significant differences were observed between them. Overall, it was not possible to distinguish between meningioma subtypes using the strength of desmoplakin staining. Desmosomes are also found in epithelial cells but not in fibroblasts, so the Chang and MRC-5 cell lines were used as positive and negative controls respectively for the desmoplakin antibody (Figures 3.18, 3.19).

Staining for the cellular markers EMA, cytokeratin and vimentin in meningiomas and normal leptomeninges is well documented. Although not diagnostic for meningioma, as such tumours can be positive or negative for any of these markers, in this study they were used to assess how meningiomas compared with the leptomeninges, and to provide information on their epithelial and mesenchymal nature.

EMA is a membrane antigen often present in epithelial and meningeal cells, and the staining pattern of positive cells can be membranous and/or intracellular. Six out of 11 meningiomas gave a result of '+' or above, with the other five samples giving a mixture of results (Table 3.3). Meningothelial M18 cells were positive for EMA and the staining pattern was one of diffuse dots over the entire cell (Figure 3.27). Transitional M17 cells stained strongly for EMA but the staining pattern for these cells appeared as a disjointed outline of the cell membrane (Figure 3.28). Within a positive monolayer of meningioma cells there were always some cells that did not stain for EMA. Chang epithelial cells were the positive control for this marker and they stained quite uniformly in an intracellular manner similar to that of the M18 cells (Figure 3.29). The negative control cell line of fibroblasts showed no reactivity with EMA (Figure 3.30).

Cytokeratin, an epithelial intermediate filament, shows filamentous staining throughout the cell length much like vimentin. The staining for this marker showed considerable variation between different cell lines, and out of the 11 meningiomas tested, only four scored '+' or above (Figure 3.23). In addition, three samples showed very mixed staining (+/-) and three more gave a weak or negative result. Very strong cytokeratin staining was exhibited by the transitional M17 cells (Figure 3.24). In a population of cells scoring '+' or above for cytokeratin, there were some cells that did not stain. No significant differences were seen between the staining of cells of different passage numbers, although meningothelial M21 cells did show some variation (as seen previously with the vimentin staining). Chang cells were uniformly positive for cytokeratin (Figure 3.25) whereas MRC-5 cells failed to stain (Figure 3.26).

Vimentin, the intermediate filament usually found in cells of mesenchymal origin, typically shows characteristic filamentous staining throughout the length of the cell, especially in a perinuclear

position. For vimentin stained meningioma cells, the results were not as clearly defined as with the desmoplakin staining (Table 3.3). Of the 12 meningiomas tested nine gave a score of '+' or above and of these, most stained strongly as illustrated in Figure 3.20 with meningothelial M18 cells. With all of the positive samples, the majority of the cells were positive, but some did not appear to stain. There were no significant differences in staining between cells of different passage numbers, although meningothelial M21 was recorded as '+/++' because of some variation between samples. Interestingly, the transitional M17 cells showed only positive staining for vimentin in contrast to the very strong staining seen with the epithelial marker, cytokeratin. The positive control for vimentin was the MRC-5 fibroblast line, which represented a homogeneous population of strongly staining cells (Figure 3.22). In contrast, all Chang epithelial cells were negative (Figure 3.21). Although vimentin is a positive marker for fibroblasts, the presence of these cells as contaminants in meningioma cultures was excluded for desmoplakin positive cell lines, because desmoplakin staining was uniform throughout these cultures and desmosomes are not present in fibroblasts.

It was possible that macrophages from the original meningioma tumours could be present as contaminant cells in meningioma cell cultures, and this was a concern for the use of these cells in future infection studies. As macrophage are terminally differentiated cells, it was unlikely that they would survive in tissue culture for longer than the first passage and so it was decided to only use meningioma cells of P3 or greater for infection experiments. However, to ensure that macrophage were absent from meningioma cultures, meningothelial M18 cells were stained using CD68, a macrophage specific marker. Figure 3.31 shows that no staining was evident for CD68 indicating that no macrophage were present in this culture. Isolated macrophages were used as a positive control for CD68 to reveal amorphous perinuclear staining (data not shown).

During the course of this study, a very preliminary experiment to look at receptor expression on meningioma cells was carried out. The membrane cofactor protein CD46 is widely distributed on human cells, and is a putative receptor for the PilC protein of piliated Neisseria (Kallstrom *et al.*, 1997). Meningothelial M18 cells and Chang epithelial cells were stained for the presence of CD46. Chang cells were very strongly positive for this marker and the staining pattern appeared as a thick band marking the boundary of each cell (Figure 3.32). M18 cells stained positively with CD46 but not as strongly as the Chang cells and to a different pattern; CD46 appeared to be similar to the diffuse staining seen with EMA on M18 cells (Figure 3.33).

Figure 3.16: Negative control for M18. Just the nuclei are stained orange with propidium iodide, and the background is clean.
The scale bar represents 20μm.

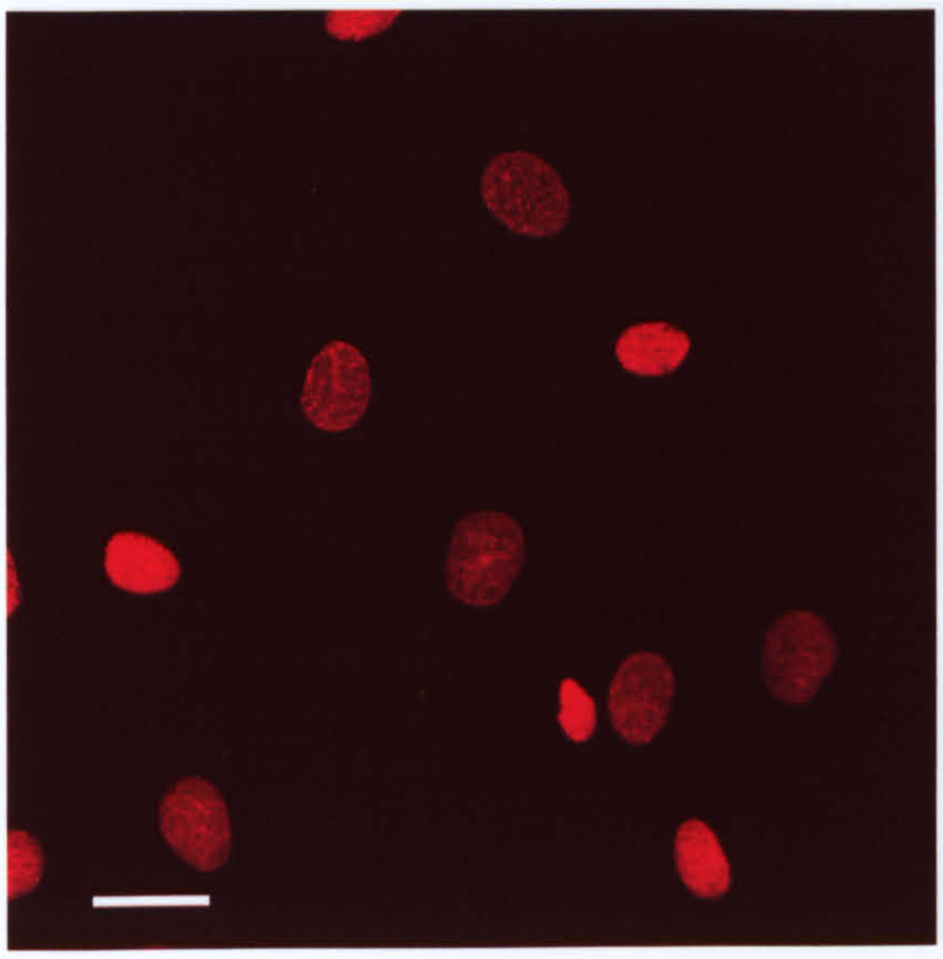


Figure 3.17: M18 with strong desmoplakin stain for desmosomes, which are present as punctate staining around the cells.
The scale bar represents 10μm.

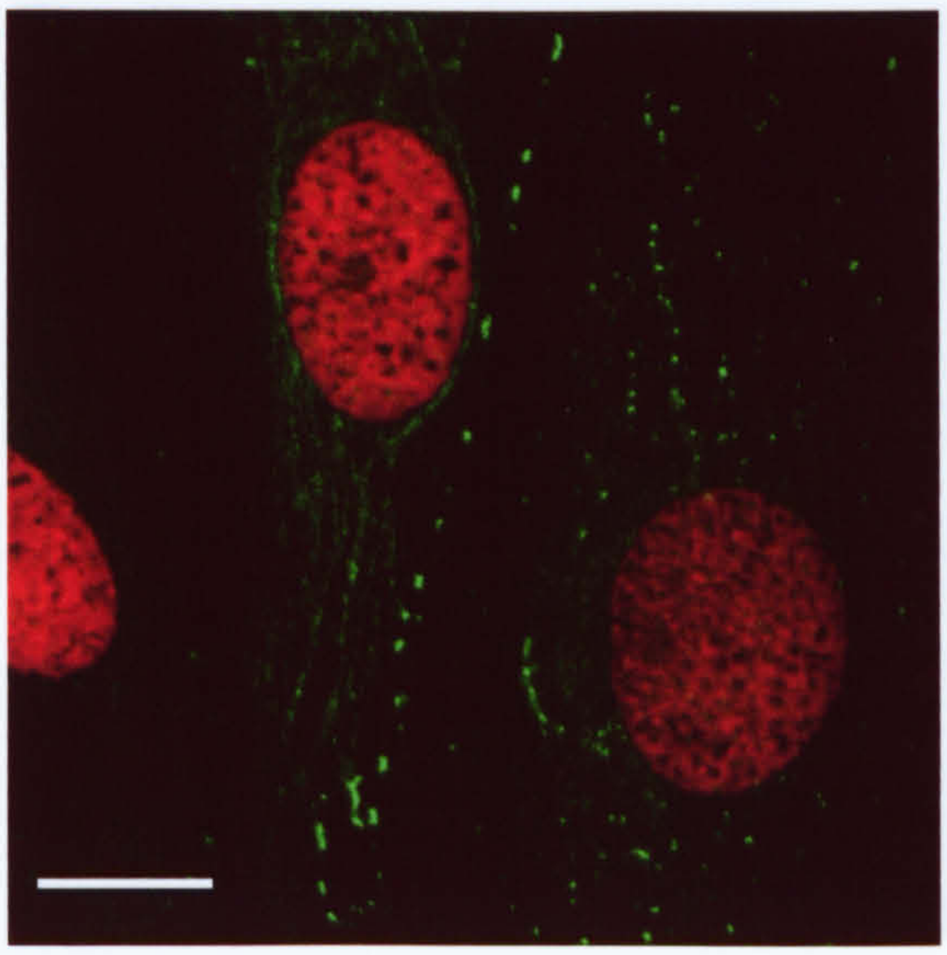
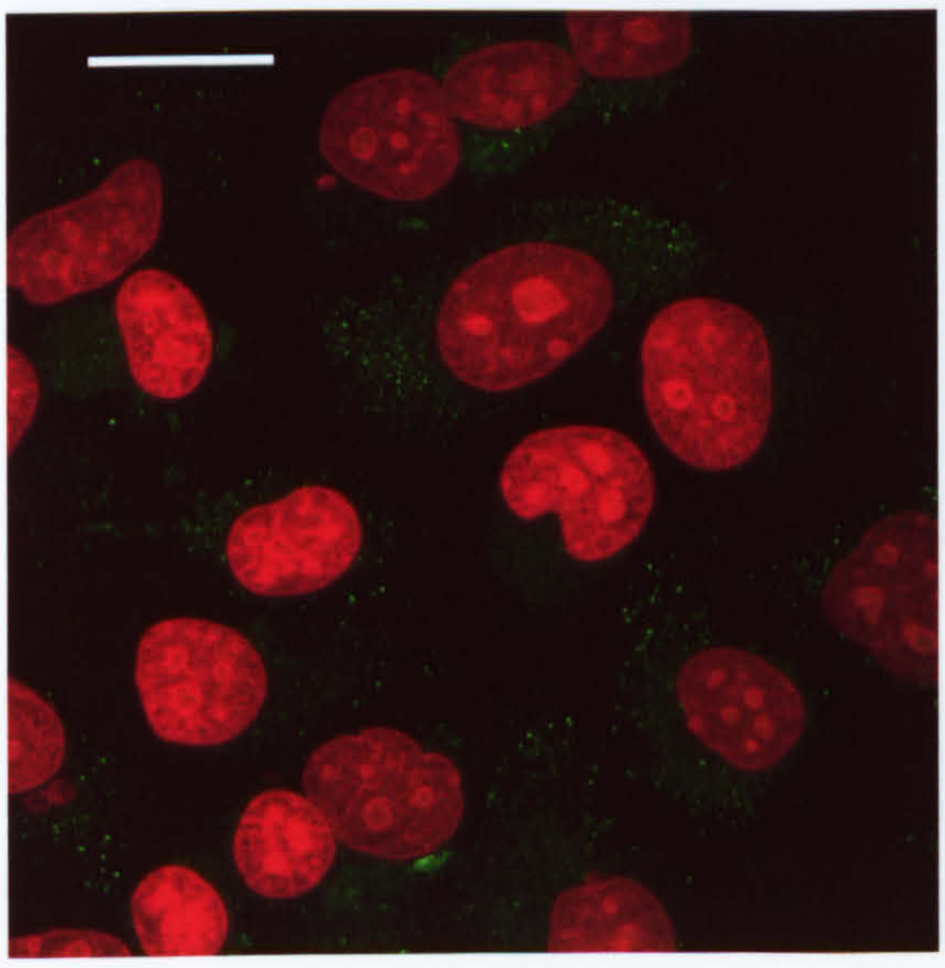


Figure 3.18: Chang cells and positive desmoplakin stain. Desmosomes can be seen (faintly) around the cells.
The scale bar represents 20μm.

Figure 3.19: MRC-5 cells and desmoplakin. The fibroblasts show no staining for desmosomes.
The scale bar represents 40μm.



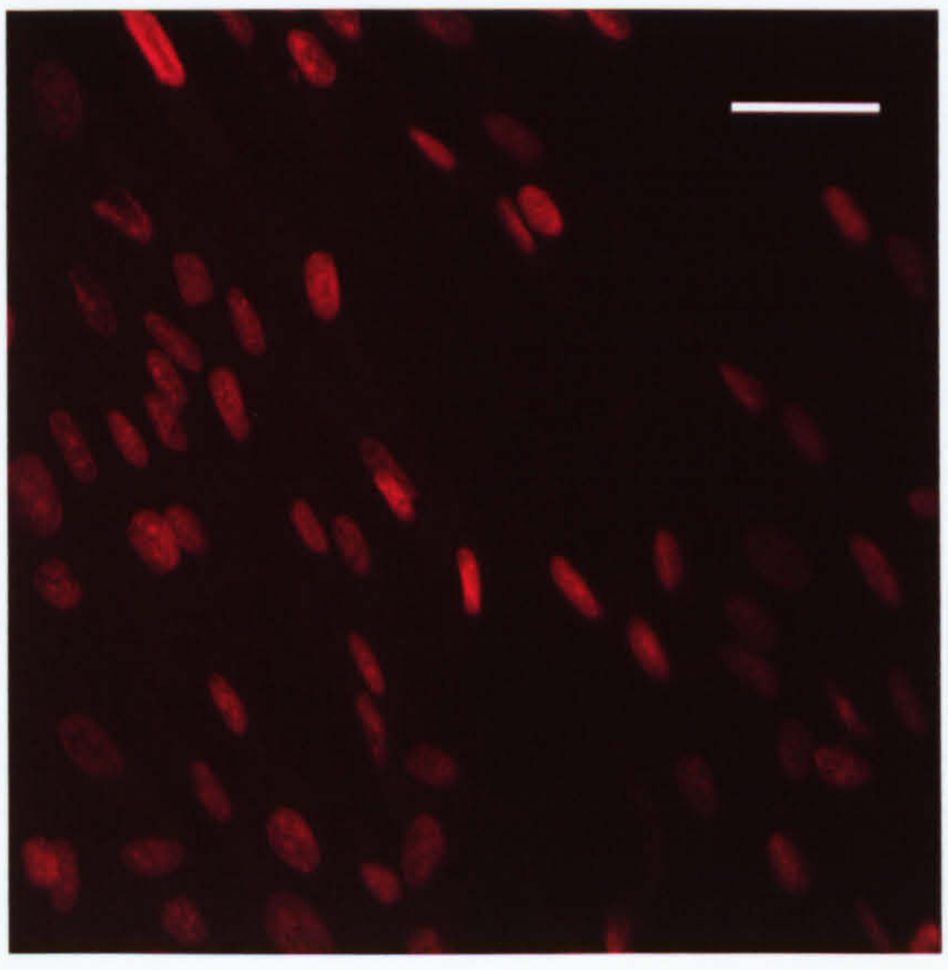


Figure 3.20: M18 showing strong staining for vimentin intermediate filaments.
The scale bar represents 20μm.

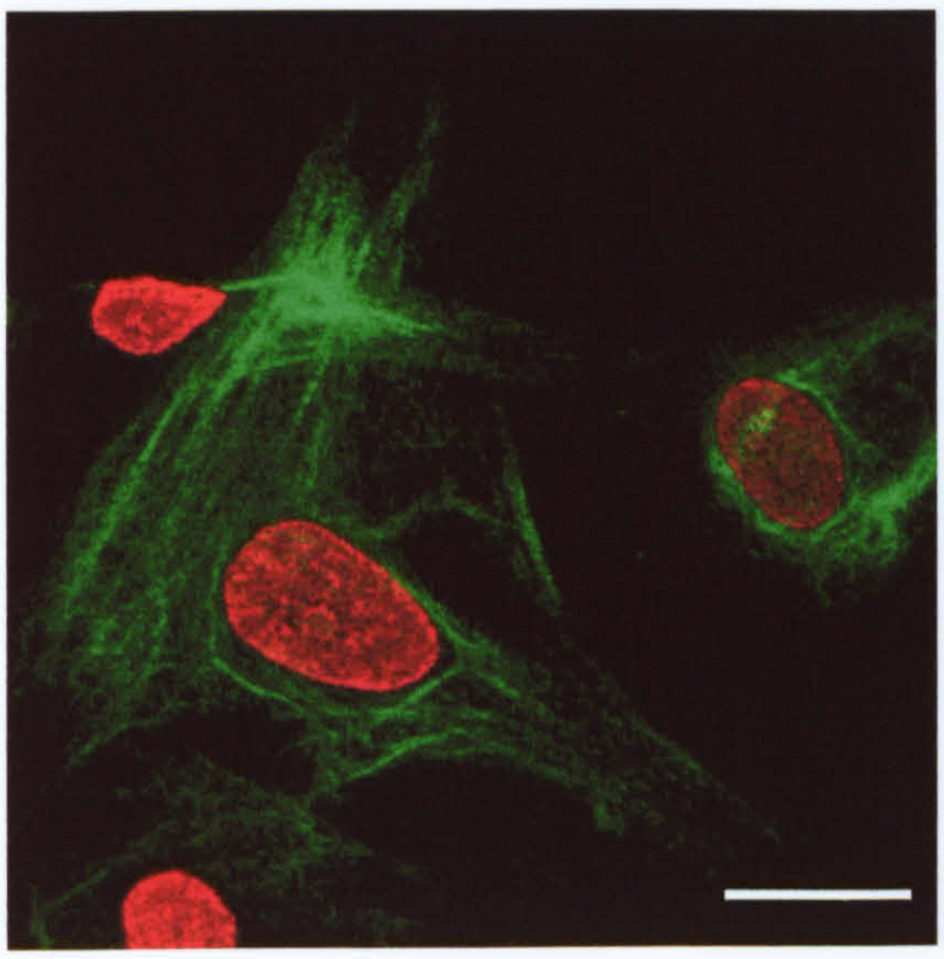


Figure 3.21: Chang cells showing no staining for vimentin.
The scale bar represents 20μm.

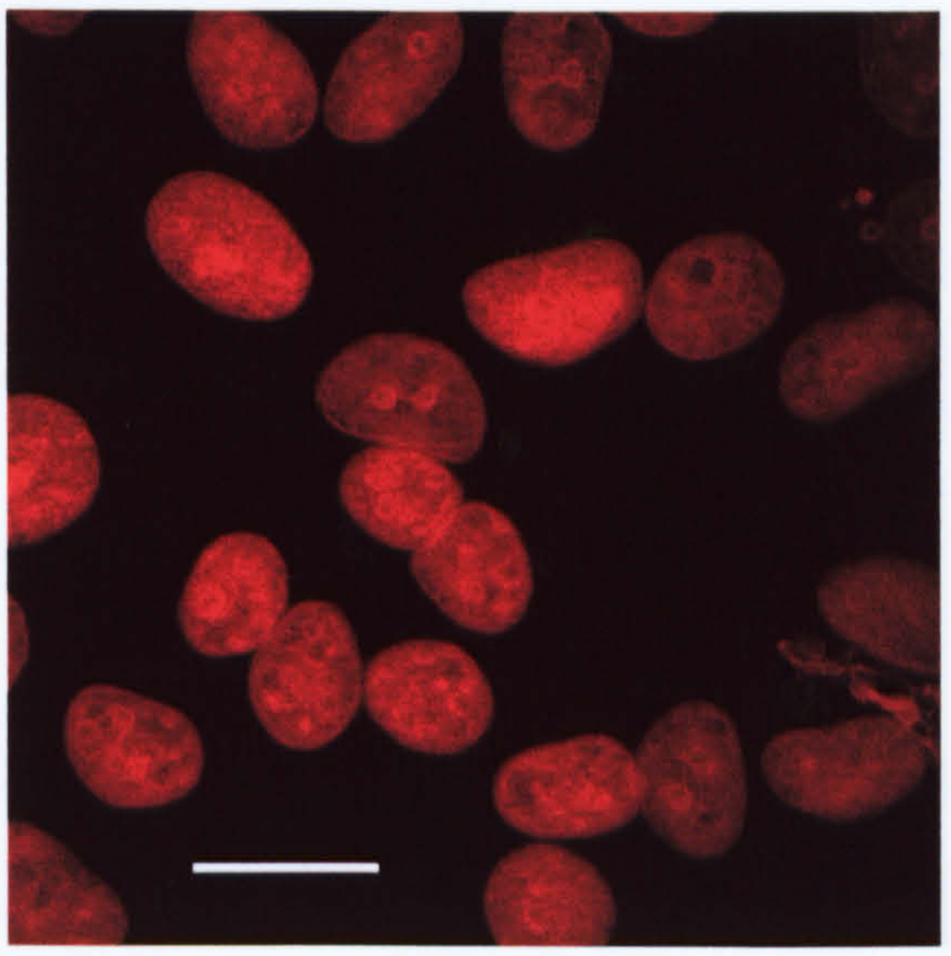


Figure 3.22: MRC-5 cells with strong staining for vimentin.
The scale bar represents 40μm.

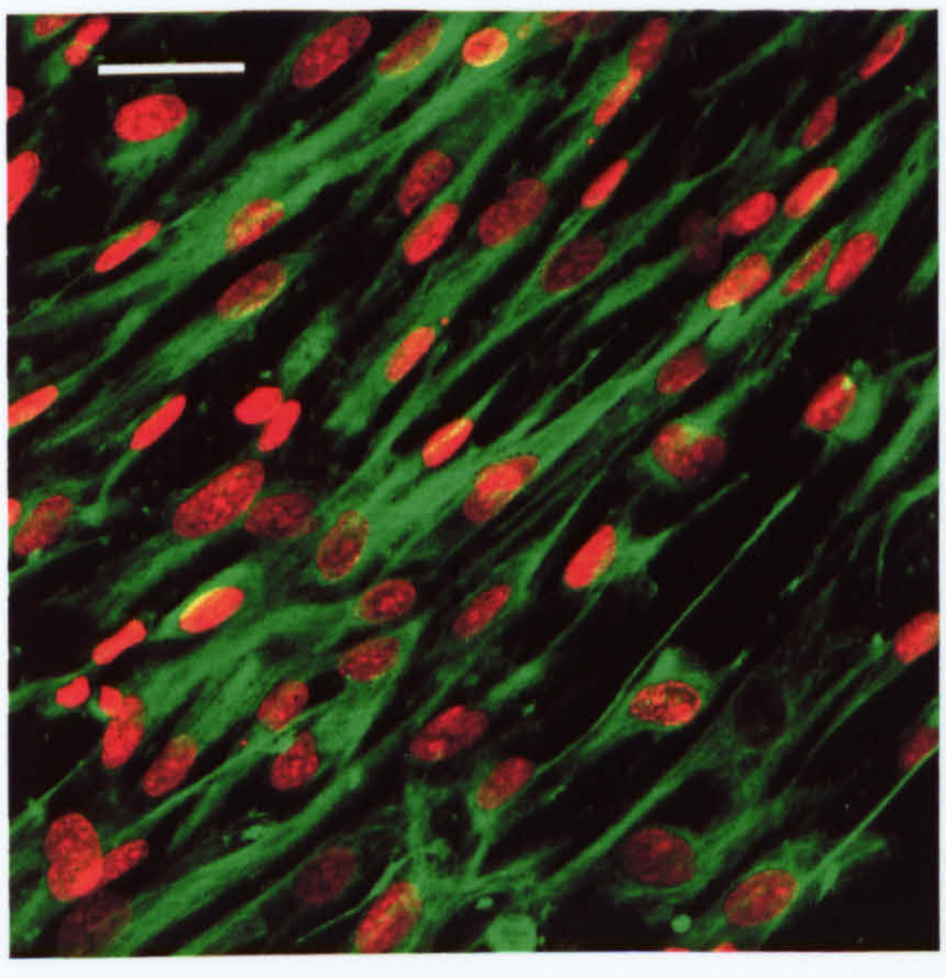
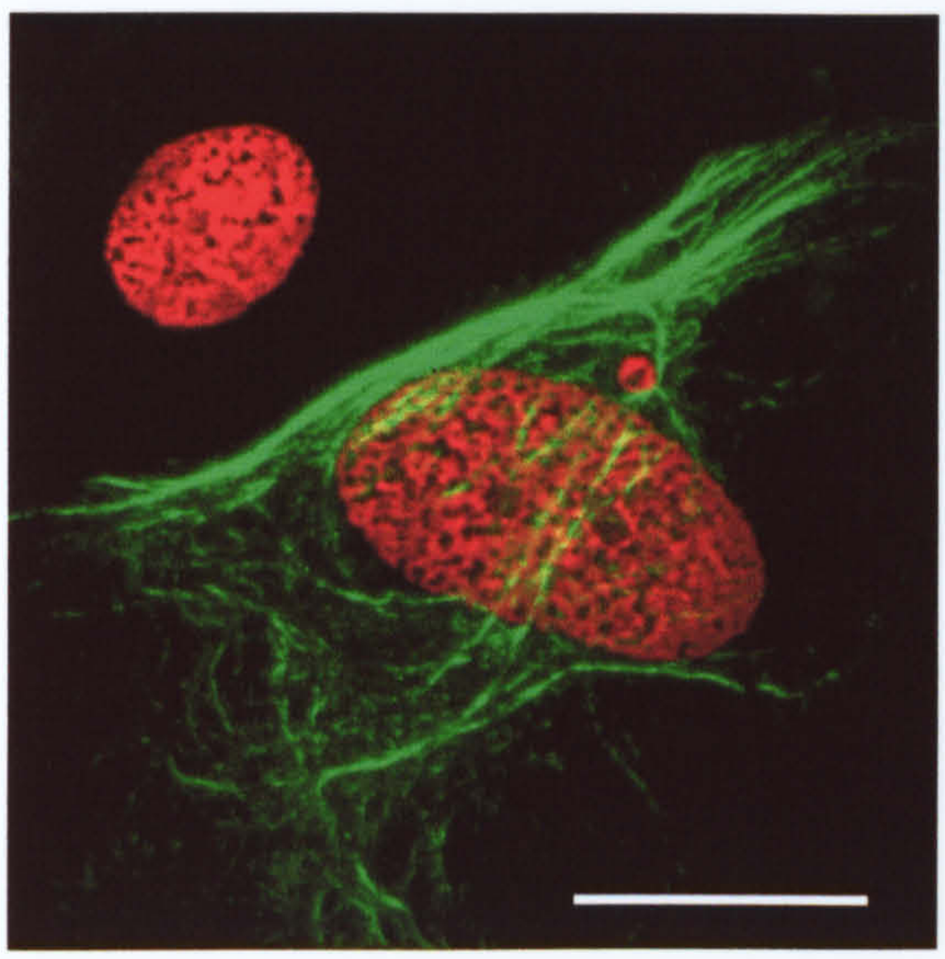


Figure 3.23: M18 showing strong staining for cytokeratin intermediate filaments.
The scale bar represents 20μm.

Figure 3.24: M17 showing very strong staining for cytokeratin intermediate filaments.
The scale bar represents 40μm.



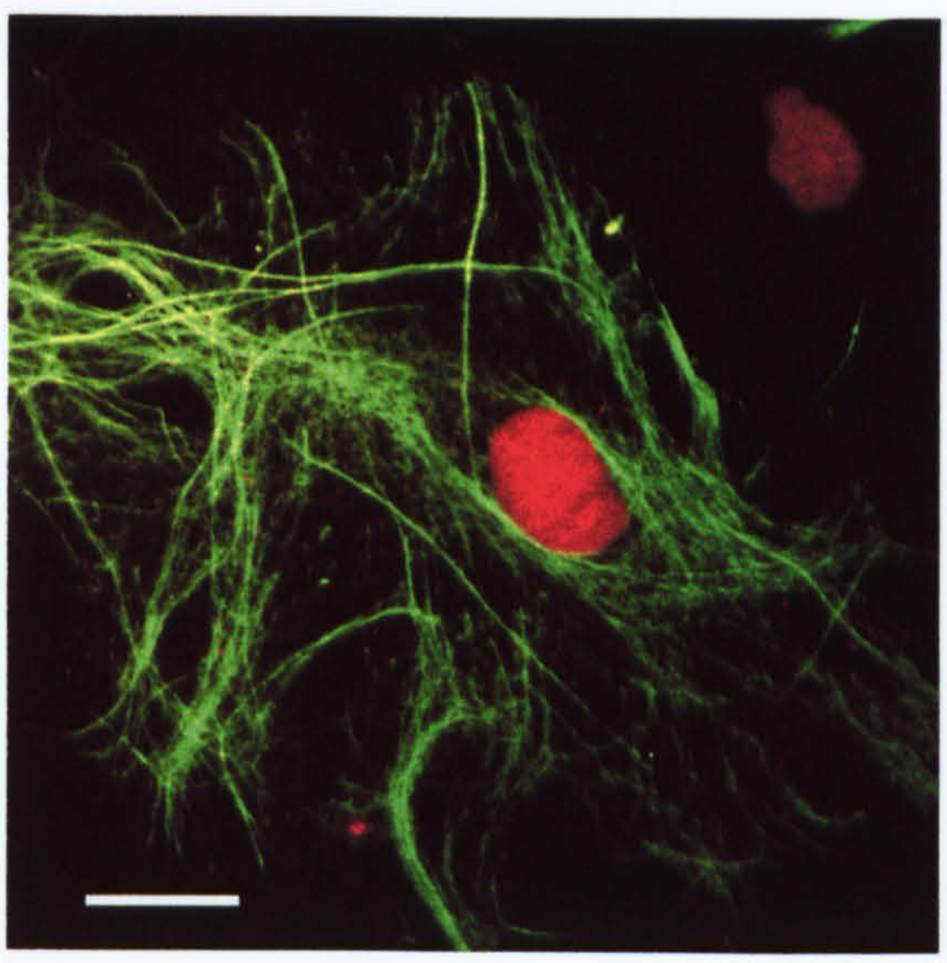
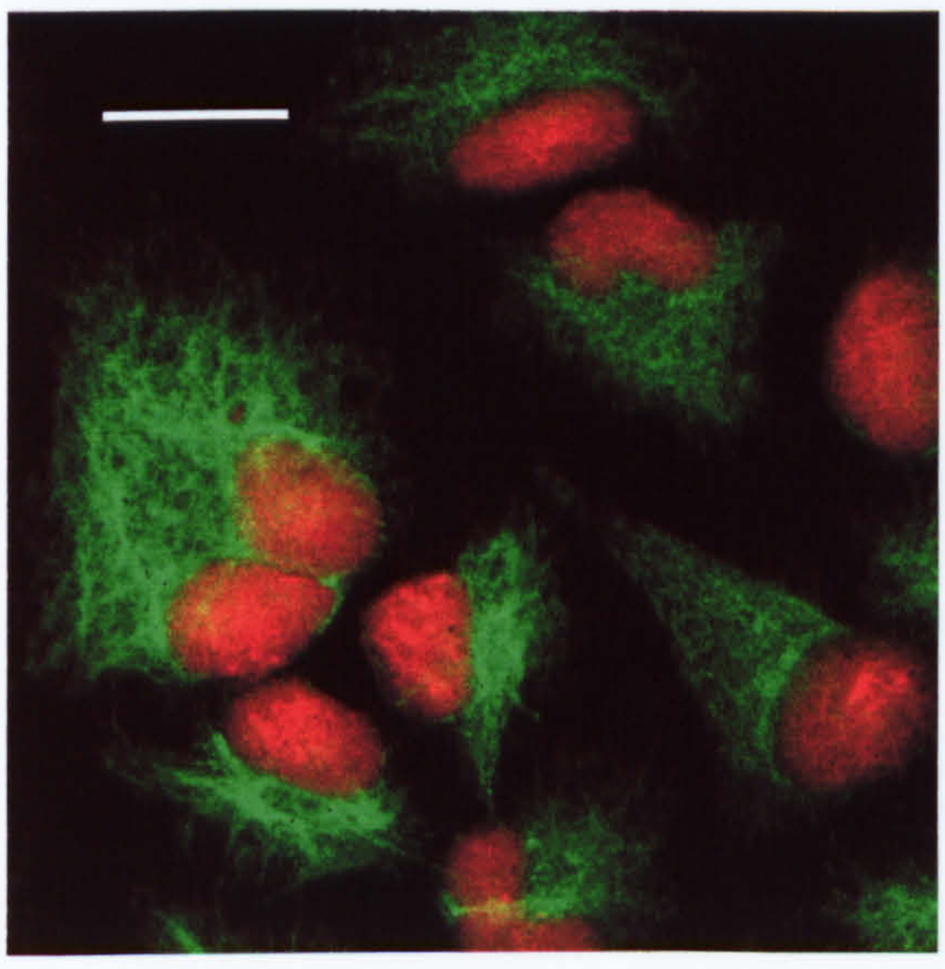


Figure 3.25: Chang cells with strong staining for cytokeratin.
The scale bar represents 20μm.

Figure 3.26: MRC-5 cells negative for cytokeratin.
The scale bar represents 40μm.



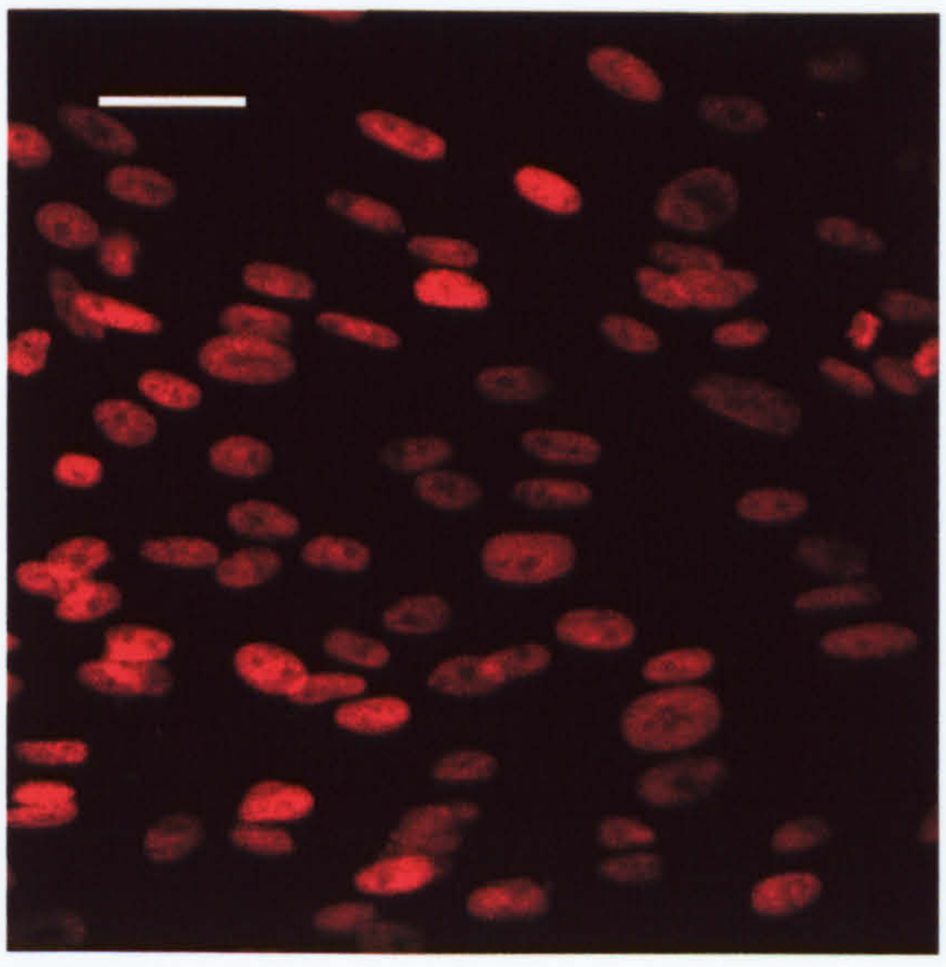


Figure 3.27: M18 cells showing positive intracellular staining for EMA.
The scale bar represents 20μm.

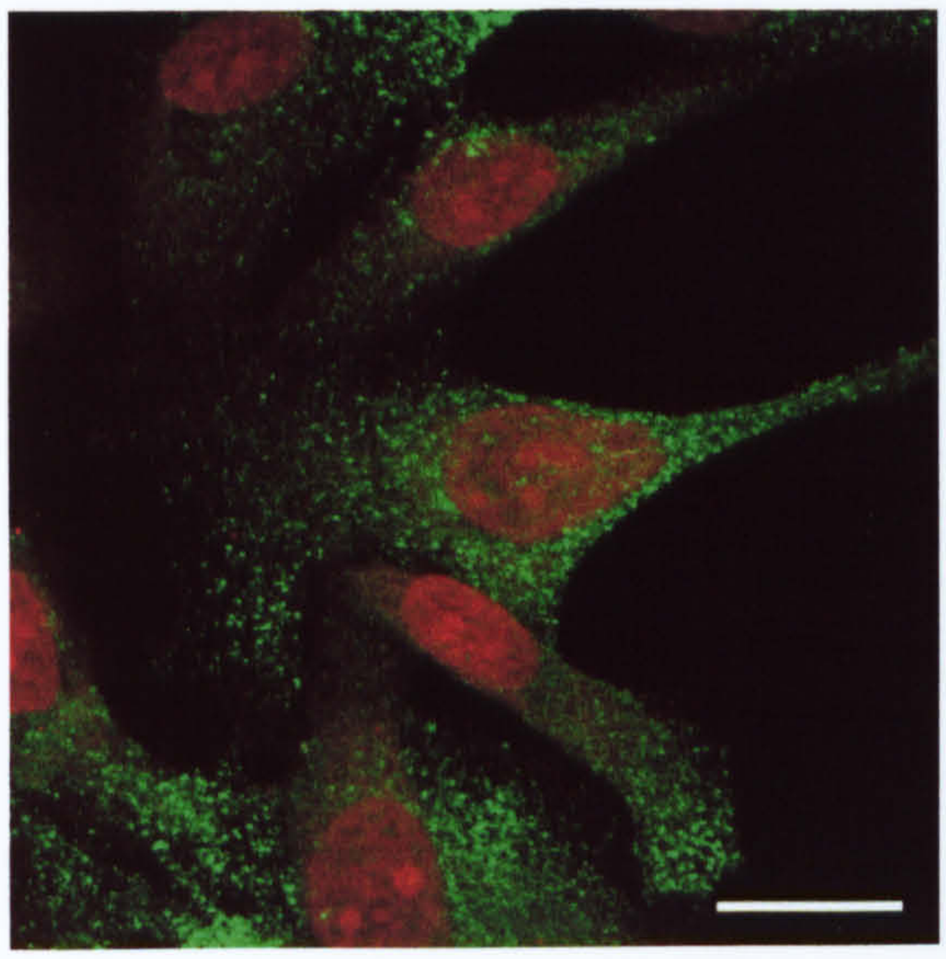


Figure 3.28: M17 cells showing strong membranous staining for EMA. Unfortunately, the PI staining was omitted from this sample and so the nucleus of this cell is not visible
The scale bar represents 40μm.

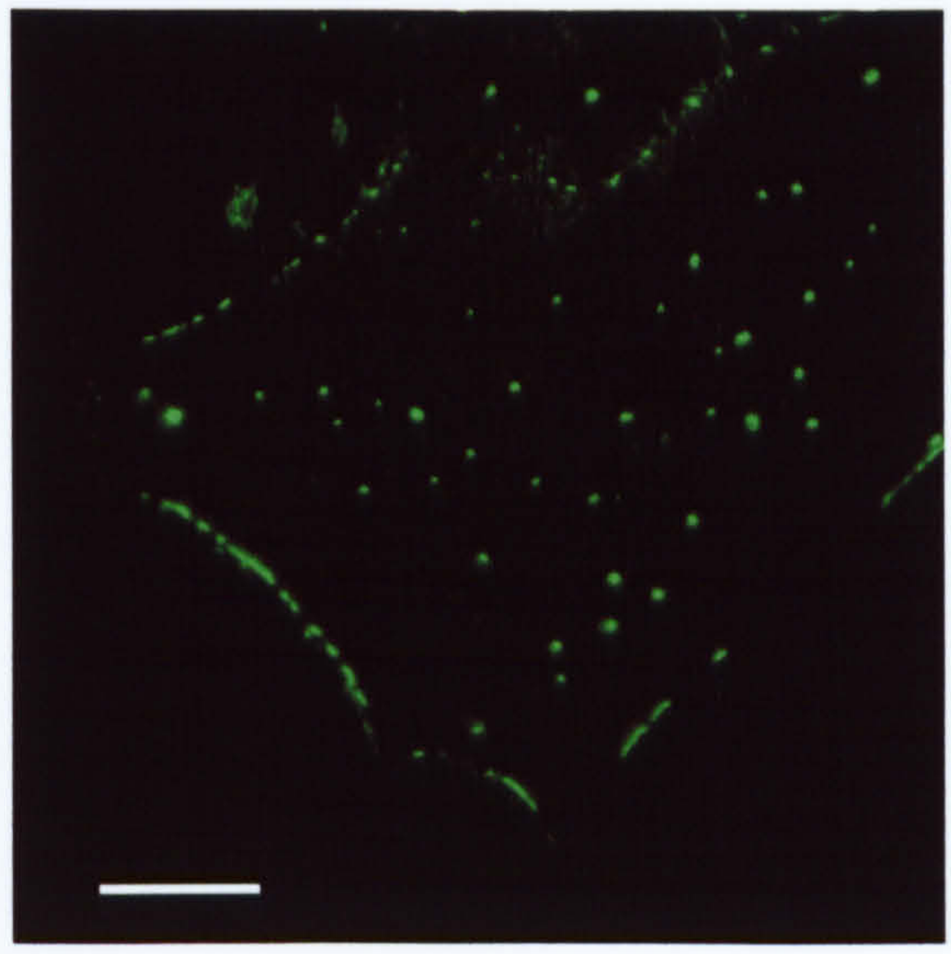


Figure 3.29: Chang cells showing positive staining for EMA.
The scale bar represents 20μm.

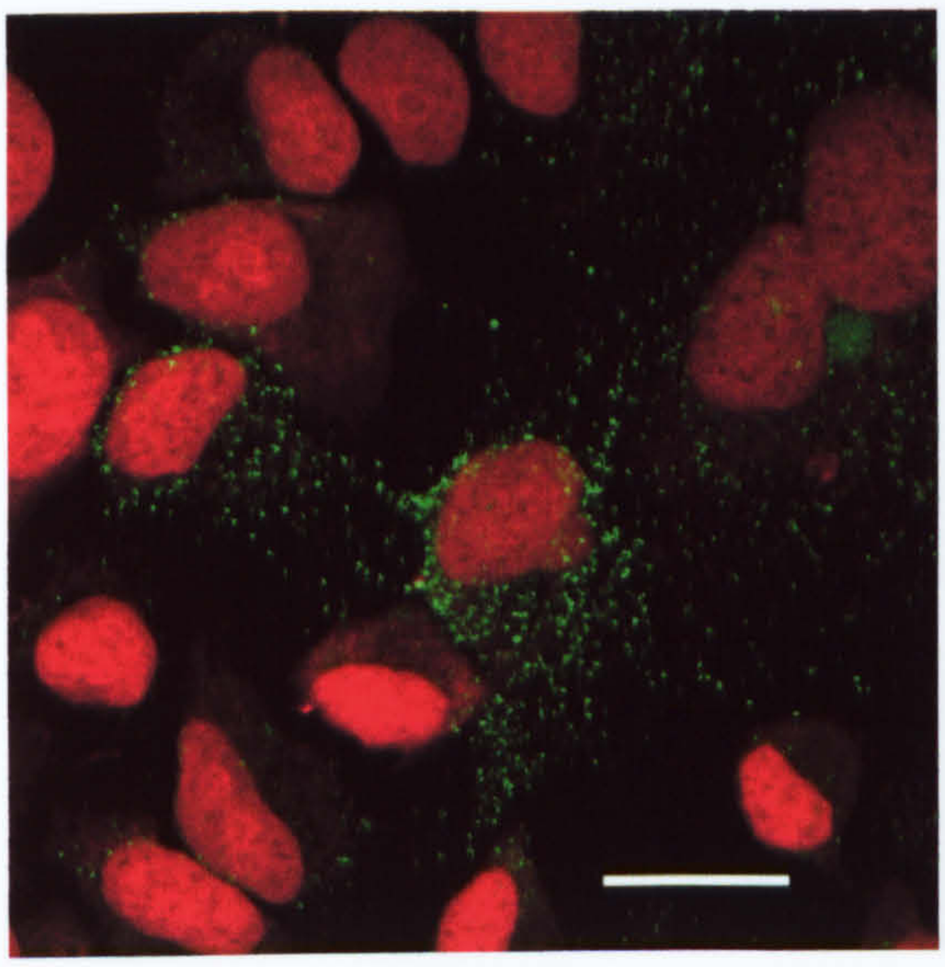


Figure 3.30: MRC-5 cells showing no staining for EMA.
The scale bar represents 40μm.

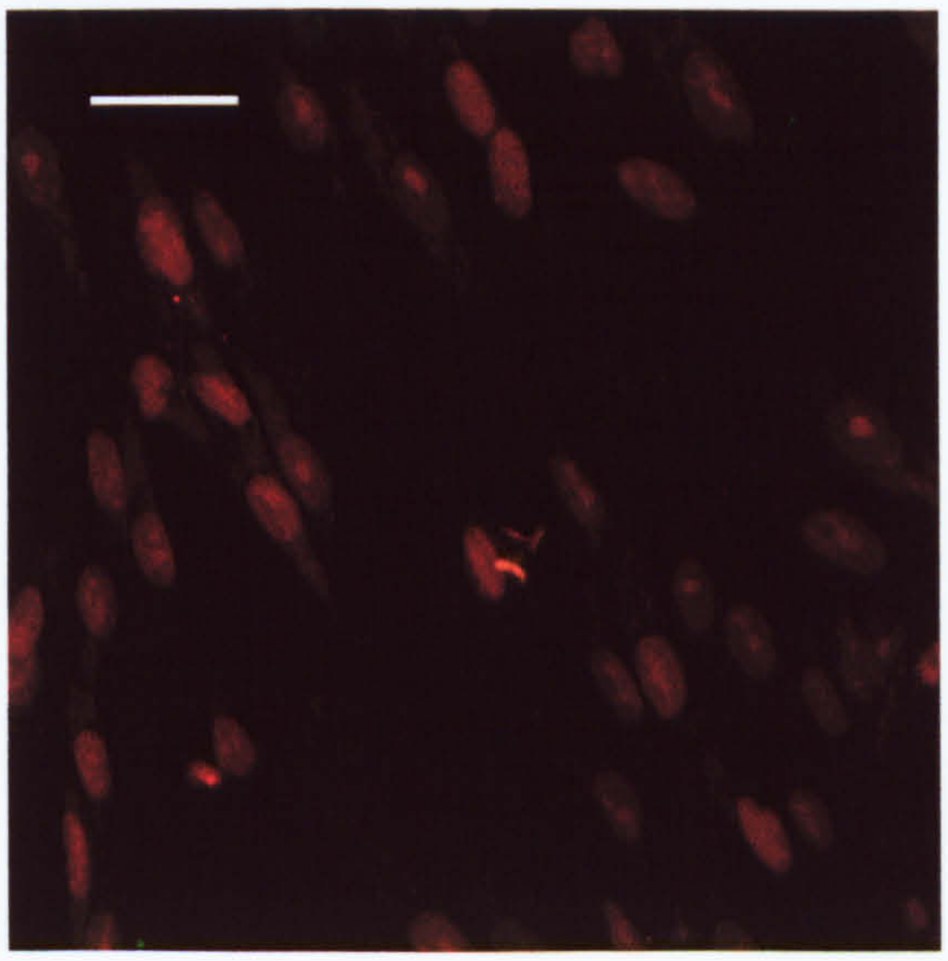


Figure 3.31: M18 cells showing no staining for CD68, indicating that macrophages are not present in the culture.
The scale bar represents 20μm.

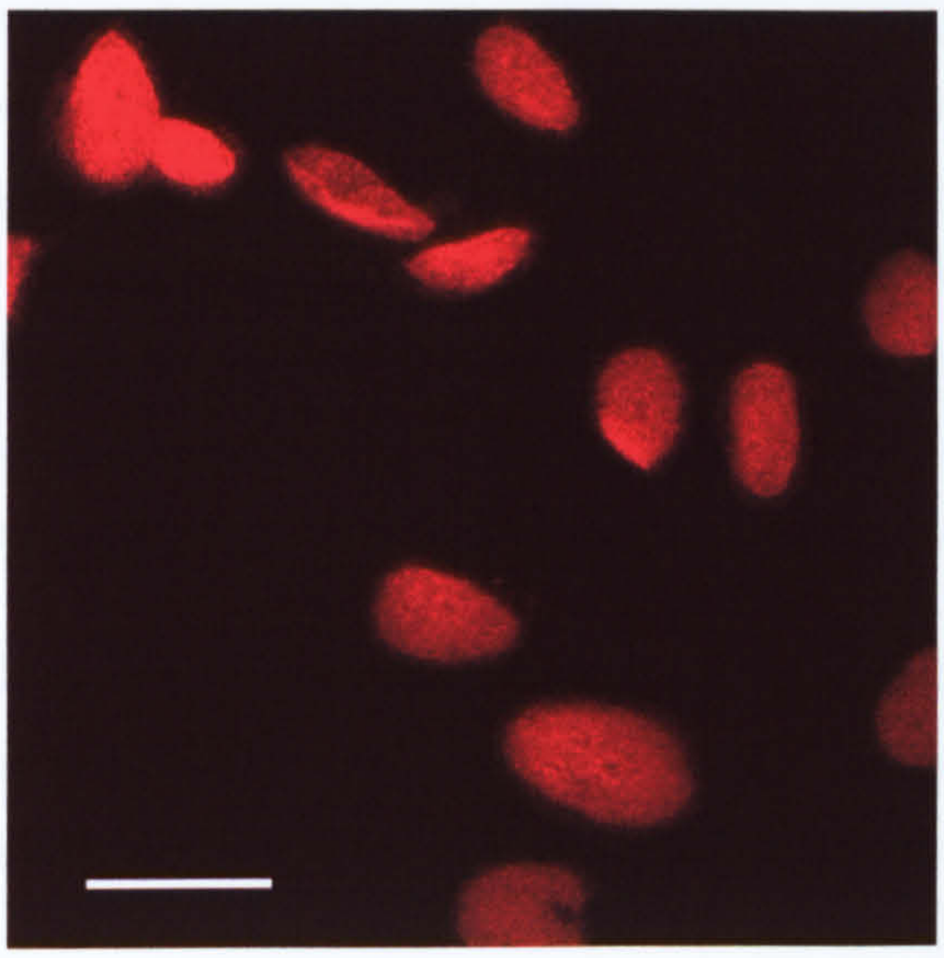


Figure 3.32: Chang cells showing strong staining for CD46.
The scale bar represents 20μm.

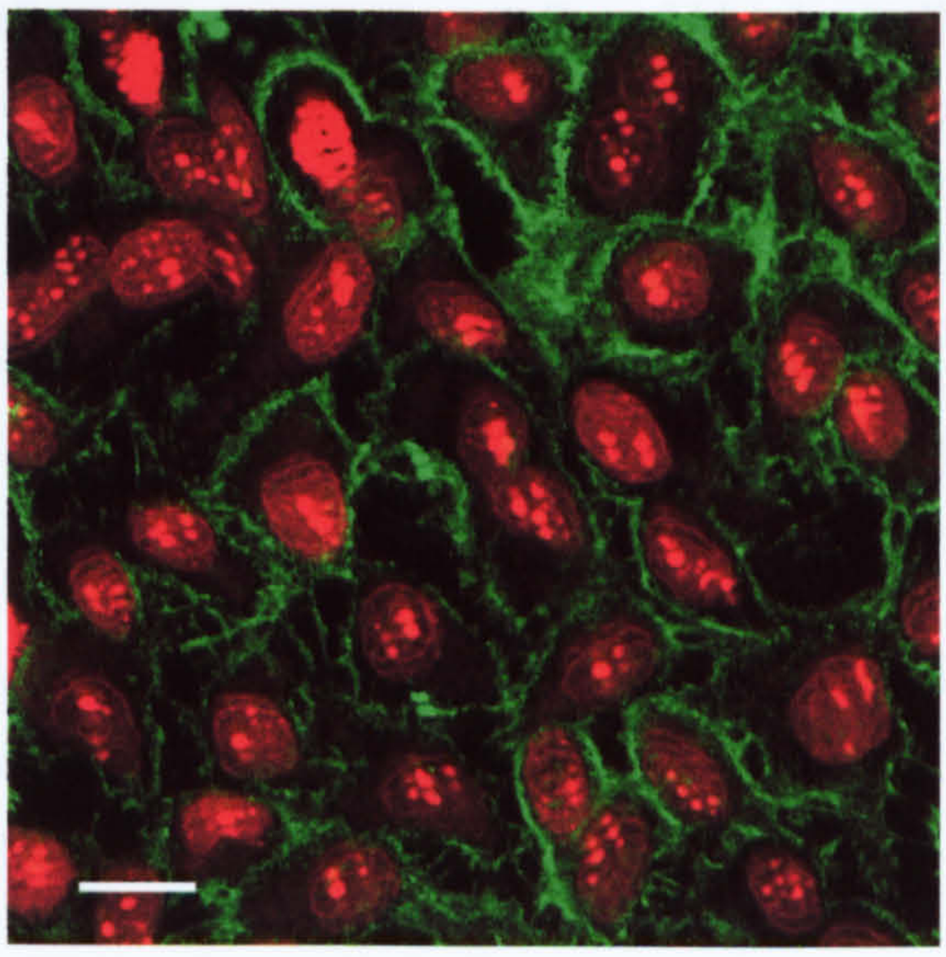
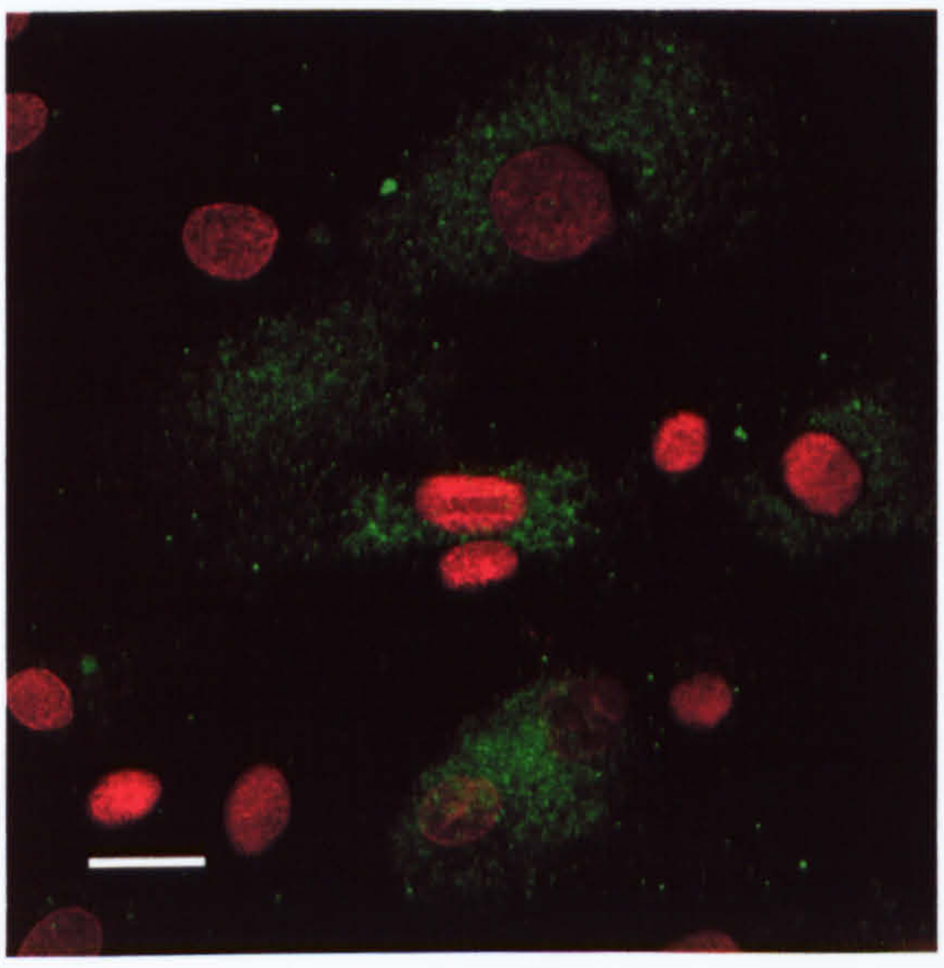


Figure 3.33: M18 cells showing positive staining for CD46.
The scale bar represents 20μm.



The growth and characterisation of meningioma cultures using cellular markers, and the type of meningioma that the cells were derived from, formed the basis of the selection criteria for cells to use in subsequent infection experiments, and is summarised in Table 3.3. Although meningothelial cells are thought to resemble most closely normal leptomeningeal cells, it was decided to also use other meningioma subtypes for additional experiments. Stocks of meningioma cell lines obtained in previous years were limited and so only fresh cell lines were used. Two meningothelial cell lines were chosen for infection studies, M18 and M21, which showed similar growth in culture and overall staining patterns. Although the meningothelial M25 tumour was confirmed histologically as being a meningioma, cells cultured from it did not stain conclusively for desmoplakin and it was not used in any further studies. Other meningothelial tumours either did not grow in culture (M22) or were from the spinal region (M20) and were not used further in this study. The M17 cell line was chosen to represent transitional meningioma cells; these cells showed stronger positivity for epithelial markers but were also positive for vimentin. The anaplastic cell line M16 showed similar staining patterns to the other selected meningiomas.

Overall, meningioma cells grew well in culture and represented a useful model for the study of the leptomeninges. In addition, meningioma cultures were not contaminated by macrophage, and preliminary experiments indicated that these cells would be of use in receptor studies.

Table 3.3: Details of control and meningioma cell lines used in this study.

Cell line	Age/sex	Record	Histological subtype	Growth in culture	Desmoplakin	EMA	Cytokeratin	Vimentin	CD68	CD46	Ki index
Chang	/	/	/	Yes	+	+	+	-	nd	+++	nd
MRC-5	/	/	/	Yes	-	-	-	+++	nd	nd	nd
M5	72/-	S843H90	Transitional	Yes	nd	nd	nd	nd	nd	nd	Low
M7	80/-	S938P90	Meningothelial	Yes	++	Weak +	+/-	+/-	nd	nd	High
M9	74/F	S1000K90	Transitional	Yes	++	+/-	Weak +	++	nd	nd	nd
M10	43/M	S1005S90	Anaplastic	Yes	+	+?	Weak +	++	nd	nd	nd
M11	40/F	S29B/91	Recurrent angioblastic	Yes	++	-	-	++	nd	nd	nd
M12	69/M	S35F/91	Meningothelial	Yes	++	+/-	+/-	++	nd	nd	nd
M13	42/F	S109U/91	Transitional	Yes	nd	nd	nd	nd	nd	nd	nd
M14	46/F	S135R/91	Meningothelial	Yes	++	+	+/-	+/-	nd	nd	Probably high

(cont.)

Table 3.3: Details of control and meningioma cell lines used in this study (cont.).

Cell line	Age/sex	Record	Histological subtype	Growth in culture	Desmoplakin	EMA	Cytokeratin	Vimentin	CD68	CD46	Ki index
M15	46/F	S166H	Ependymoma	Yes	-	nd	nd	+	nd	nd	nd
M16	69/M	S1460857	Anaplastic	Yes	++	+	++	++	nd	nd	nd
M17	61/M	S218X	Transitional	Yes	++	++	+++	+	nd	nd	nd
M18	45/M	S230G97	Meningothelial	Yes	++	+	++	++	+	+	nd
M19	60/M	S235P97	Atypical	Yes	nd	nd	nd	nd	nd	nd	nd
M20	88/M	S249V97	Spinal meningothelial	Yes	+	nd	nd	++	nd	nd	nd
M21	71/M	S264Y97	Meningothelial	Yes	++	+	+/++	+/++	nd	nd	nd
M22	-/F	S359F.97	Meningothelial	No	nd	nd	nd	nd	nd	nd	No mitosis
M23	71/F	S393N.97	Transitional	Yes	nd	nd	nd	nd	nd	nd	> 1%
M24	63/F	S443Q.97	Transitional	Yes	nd	nd	nd	nd	nd	nd	No mitosis
M25	67/F	S464H.97	Meningothelial	Yes	+?	++	+?	Weak +?	nd	nd	Low

Staining scaled '+' positive, '++' strong positive, '+++' very strong positive

'+/-' mixed result

'-' negative

'?' inconclusive result

'nd' not determined

CHAPTER 4 MENINGOCOCCAL INTERACTIONS WITH HUMAN MENINGIOMA CELLS

During infection, meningococci can invade the subarachnoid space to cause meningeal inflammation, but these bacteria are apparently unable to traverse the leptomeninges, as they are not found in the underlying brain cortex (Gray & Nordmann, 1997). Little is known about the role of leptomeningeal cells during meningitis, and attempts to study this process have so far been hampered by the inability to grow these cells in culture. However, it has been established that cultures of meningothelial cells derived from human meningiomas represent an *in vitro* model of the barrier properties of leptomeningeal cells (Feurer & Weller, 1991). In Chapter 3, the components of a model were developed using cultured meningioma cells and a panel of phenotypically variant meningococci, that could be used to investigate the interactions of meningococci with cells of human meningeal origin.

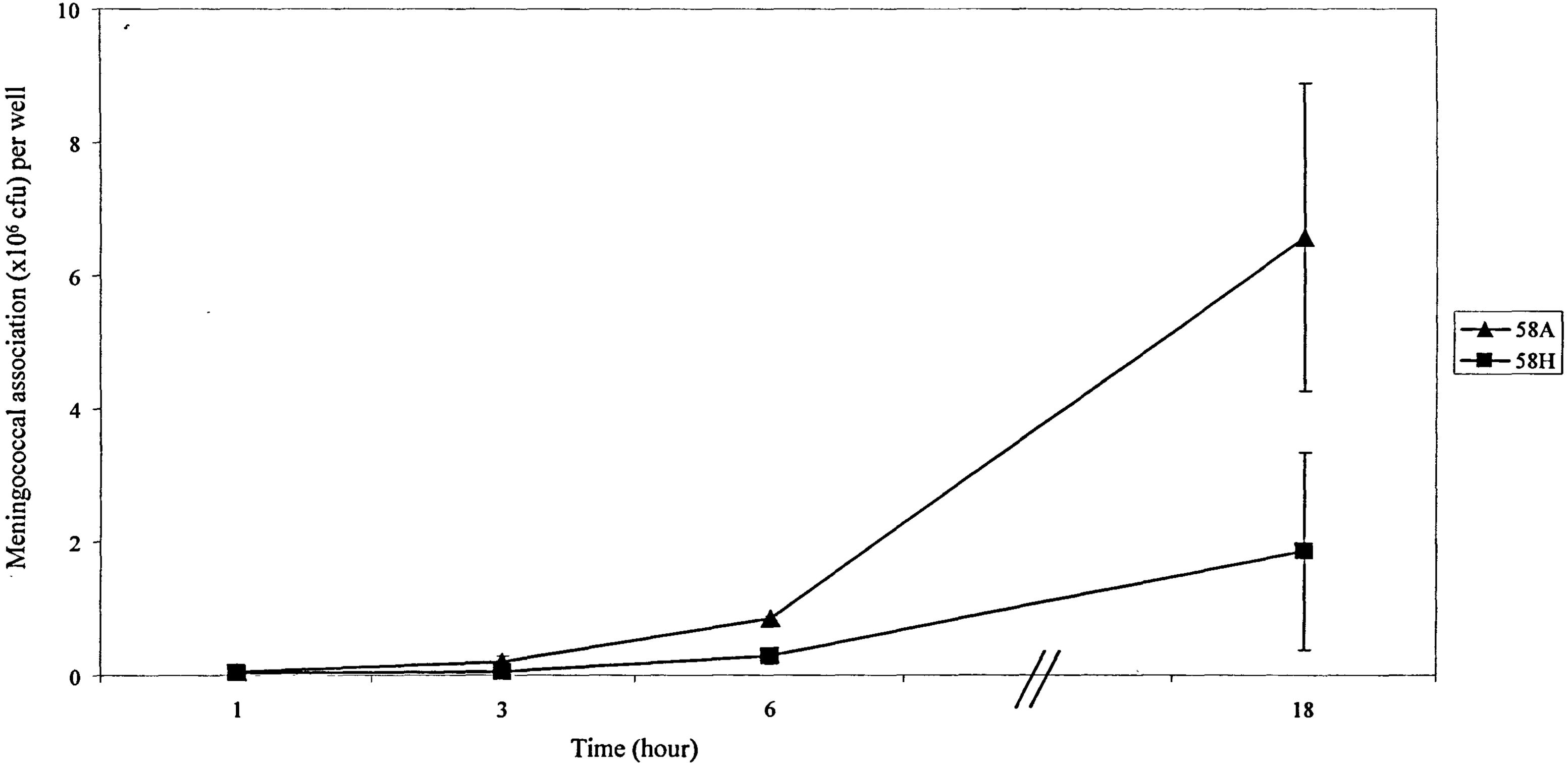
4.1 Establishment of infection protocols

Although meningioma cells grew well in culture, one individual sample could not provide an unlimited source of cells. Therefore, prior to investigating interactions of meningococci with these cells, working protocols were established using Chang conjunctival epithelial cells. This cell line was chosen as it is an immortalised cell line that is thus readily available and easy to grow, and there is a large amount of published data regarding the meningococcal infection of Chang cells.

4.1.1 Infection of Chang cells with piliated and non-piliated meningococci

The methods used in this study for the analysis of meningococcal association to human cells were adapted from those of Virji and colleagues (Virji & Everson, 1981; Virji *et al.*, 1991; Virji *et al.*, 1992b). These workers typically infected confluent monolayers of Chang cells, grown in 96 well plates, with $5x10^7$cfu of meningococci per well. For this study, Chang cells were seeded into 96 well tissue culture plates and typically used in infection experiments the day after confluence. To investigate bacterial association over time, meningococcal isolates differing only in their expression of pili ($Cap^+Pil_{Ia}{}^+Opa^+Opc^+$ and $Cap^+Pil^-Opa^+Opc^+$) were used. Approximately $5x10^7$ cfu in 200µl of each variant were added to separate monolayers for 1, 3, 6, 18h, and the cells were processed as

Figure 4.1: The effects of time on the infection of Chang cells with 58A ($Cap^+Pil_{Ia}{}^+Opa^+Opc^+$) and 58H ($Cap^+Pil^-Opa^+Opc^+$).
The error bars represent the standard deviation of triplicate wells in one experiment.



in the standard method (Section 2.12.1). Figure 4.1 shows that at all time points, piliated meningococci adhered to the Chang cells in greater numbers than did the non-piliated organisms. On average piliated bacteria associated with Chang cells in three times greater numbers than non-piliated counterpart bacteria.

Chang cells were also used to establish the gentamicin survival assay, to determine the likelihood of meningococci invading human cells. In this assay, infected Chang cells were treated with gentamicin to kill extracellular bacteria. It was assumed that surviving meningococci were protected from the actions of the antibiotic because they had been internalised by the cells. The Chang cell monolayers were infected with meningococci for time intervals as above, and after the bacteria were removed and the cells washed, they were treated with gentamicin for 1.5 hours. The monolayers were then washed and lysed, and the number of cfu surviving quantified by viable counting. Using encapsulated isolates expressing pili, no bacteria were found to survive gentamicin treatment and therefore these meningococci were deemed not invasive of this cell line.

The increased association of Pil^+ over Pil^- organisms correlated with the results of others (Virji *et al.*, 1992a) who have shown that piliated MC58 adhere to Chang cells in greater numbers than do the non-piliated MC58 variants. These methods were therefore used to investigate meningococcal interactions with meningioma cells.

4.2 Infection of human meningioma cells

Having validated the methods required for infection studies using other cell lines, meningioma cells were then used to study the nature of the interaction between meningococci and meningeal cells. Several meningioma cell lines were used, of which most experiments were carried out with two meningothelial (M18 and M21), one transitional (M17) and one anaplastic (M16) cell line.

4.2.1 Adherence of encapsulated meningococci to meningioma cells

The interaction of encapsulated meningococci with meningioma cells was initially investigated by quantitative viable count methods. Meningioma cells were infected with meningococci to study the dynamics of their association over time, the effects of differing bacterial inocula and of different phenotypic variants. In all infection experiments meningioma cells were typically used the day after achieving confluence, to mimic as closely as possible the near quiescent state of the adult human leptomeninges.

4.2.1.1 Time course comparison of piliated and non-piliated bacteria

The associative nature of Pil^+ and Pil^- meningococci with different meningioma cell lines was studied at different time intervals. Confluent monolayers of meningioma cells were infected with approximately $5x10^7$ cfu per well of either $Pil_{Ia}^+Opa^+Opc^+$ or $Pil^-Opa^+Opc^+$ meningococci for 1, 3, 6, 9 and 24h and processed as in the standard method (Section 2.12.1).

With a meningothelial cell line (M18) the association of both organisms increased with time (Figure 4.2). This was especially marked after 24h of infection where the association of Pil^+ bacteria had increased by approximately 90 times from the 1h levels. In contrast, the association of Pil^- bacteria over the same time course increased more gradually and to a much lesser extent. The Pil^+ bacteria always adhered in larger numbers than did the Pil^-; the difference became more marked by 6h of infection and increased with time. This experiment was repeated with another meningothelial cell line (M21) with essentially the same results; the total bacterial association increasing with time and Pil^+ organisms always associating in higher numbers than Pil^- (Figure 4.3).

The same experiment was done using transitional cells (M17). As with the meningothelial cells, overall bacterial association increased with time and Pil_{Ia}^+ organisms associated in greater numbers than the Pil^- ones (Figure 4.4). Unexpectedly however, the association of $Pil^+_{Ia}Opa^+Opc^+$ organisms did not increase between the 6 and 9h time points, but continued to rise steadily from 9 to 24h. The association of non-piliated organisms followed a continuous gradual increase over time, much as the results obtained with meningothelial cells. At all time points though, more piliated meningococci associated with transitional cells than with the meningothelial cell lines.

The time course experiment was also done using anaplastic cells (M16). Piliated meningococci always associated in higher numbers than non-piliated, and the association of both increased with time (Figure 4.5). However, between 9 and 24h, the increase in association of the $Pil_{Ia}^+Opa^+Opc^+$ organisms was more gradual than seen with meningothelial or transitional cells, and at all time points piliated organisms associated with anaplastic cells in less numbers than with the other cell lines.

In summary, with meningothelial, transitional and anaplastic meningioma cells over a time course of 24h, $Pil_{Ia}^+Opa^+Opc^+$ organisms always associated in higher numbers than $Pil^-Opa^+Opc^+$ bacteria, and the association of both increased with time. It is likely that each of the inoculum used in these experiments was a saturating dose, and also that meningococci were multiplying during the time course, so that there would be more bacteria present at 24h than at the start. Therefore it was possible that after a certain time all cellular binding sites for meningococci were filled, and then either adherent bacterial colonies replicated *in situ*, or that meningococci in the infection media

Figure 4.2: The effects of time on the association of $Pil_{Ia}^{+}Opa^{+}Opc^{+}$ and $Pil^{-}Opa^{+}Opc^{+}$ to M18 meningothelial cells.
The error bars represent the standard deviation of triplicate wells in a single experiment.

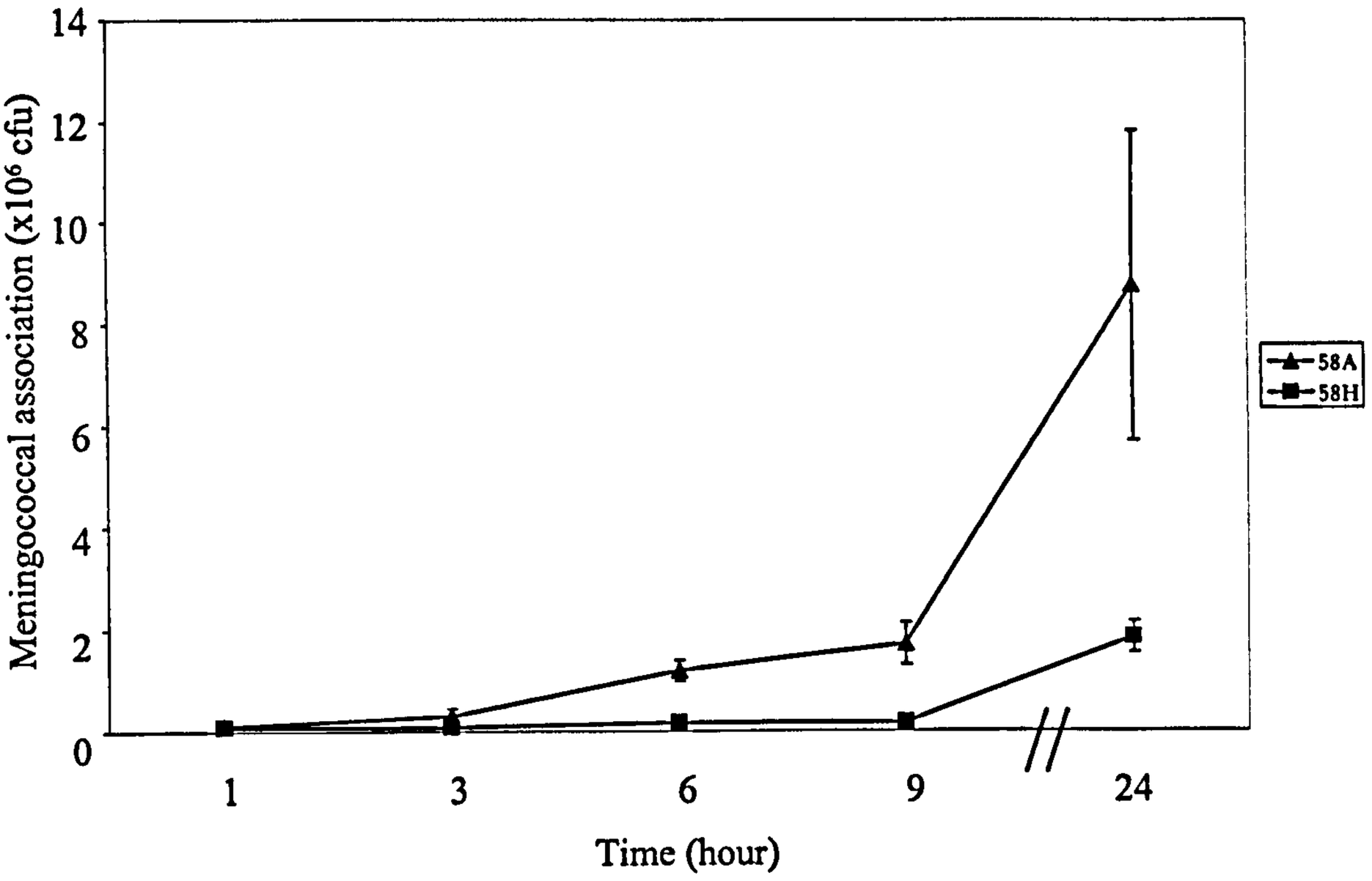


Figure 4.3: The effects of time on the association of $Pil_{Ia}^{+}Opa^{+}Opc^{+}$ and $Pil^{-}Opa^{+}Opc^{+}$ to M21 meningothelial cells.
The error bars represent the standard deviation of triplicate wells in a single experiment.

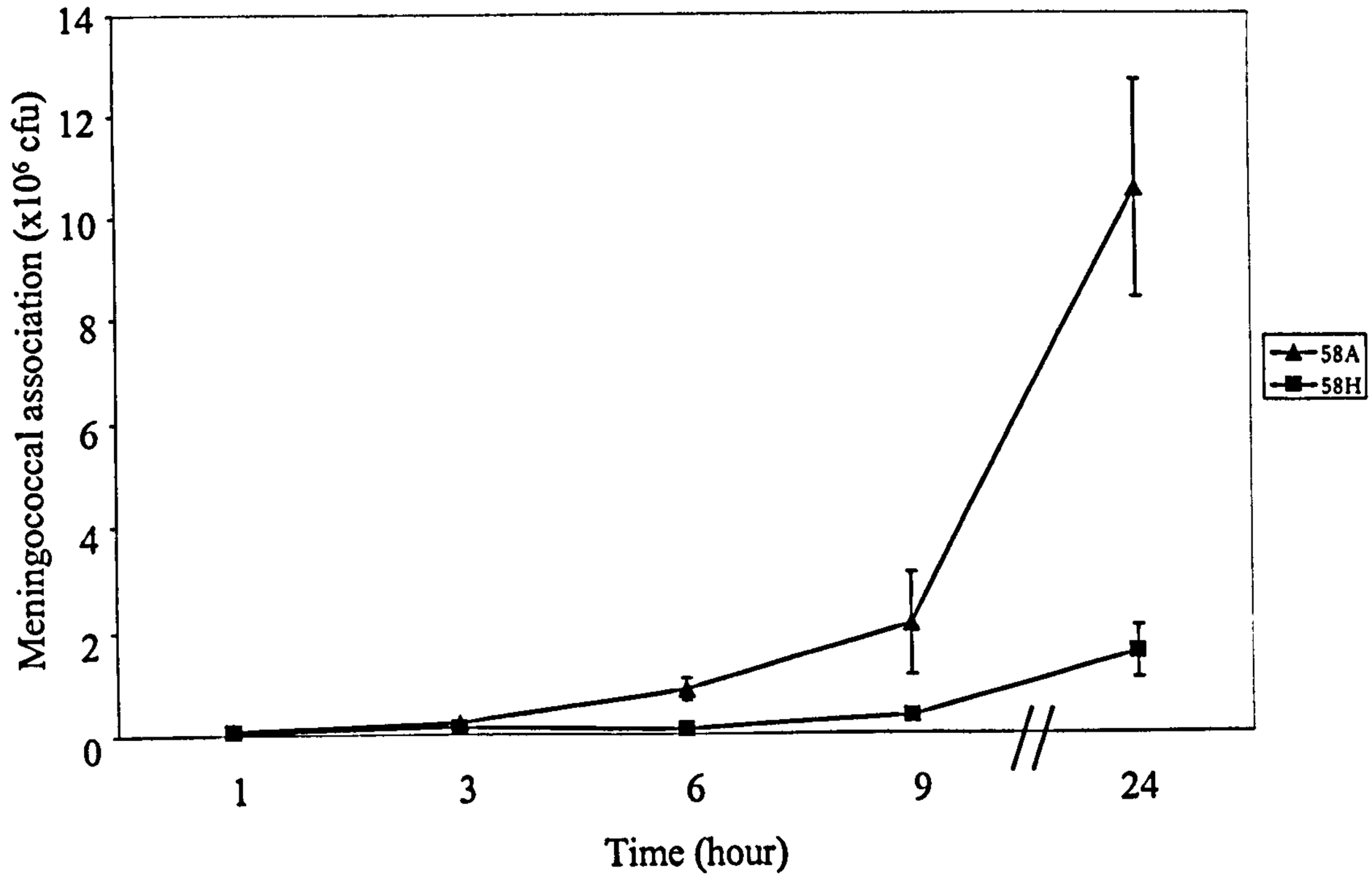


Figure 4.4: The effects of time on the association of $Pil_{Ia}^{+}Opa^{+}Opc^{+}$ and $Pil^{-}Opa^{+}Opc^{+}$ to M17 transitional meningioma cells.
The error bars represent the standard deviation of triplicate wells in a single experiment.

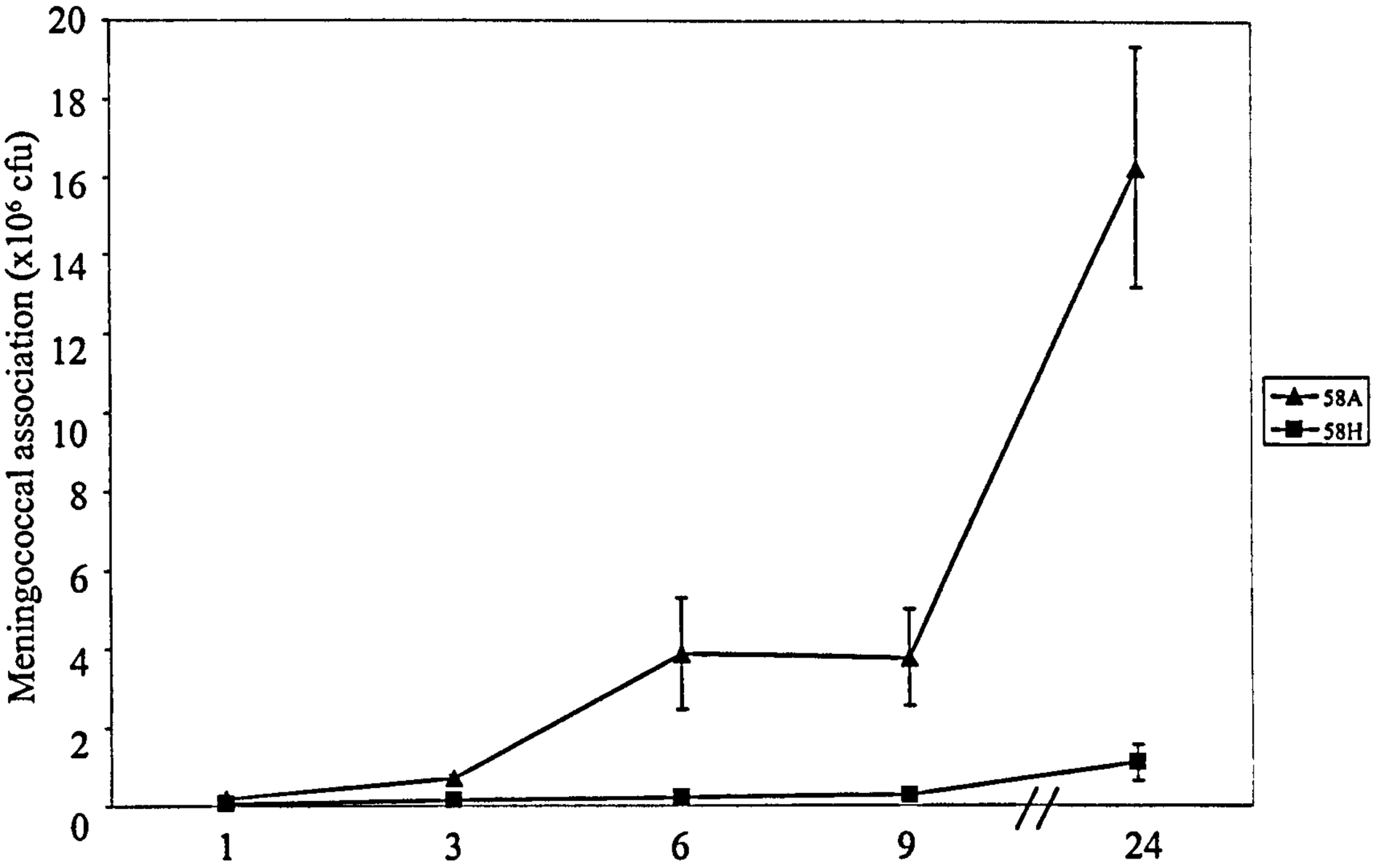
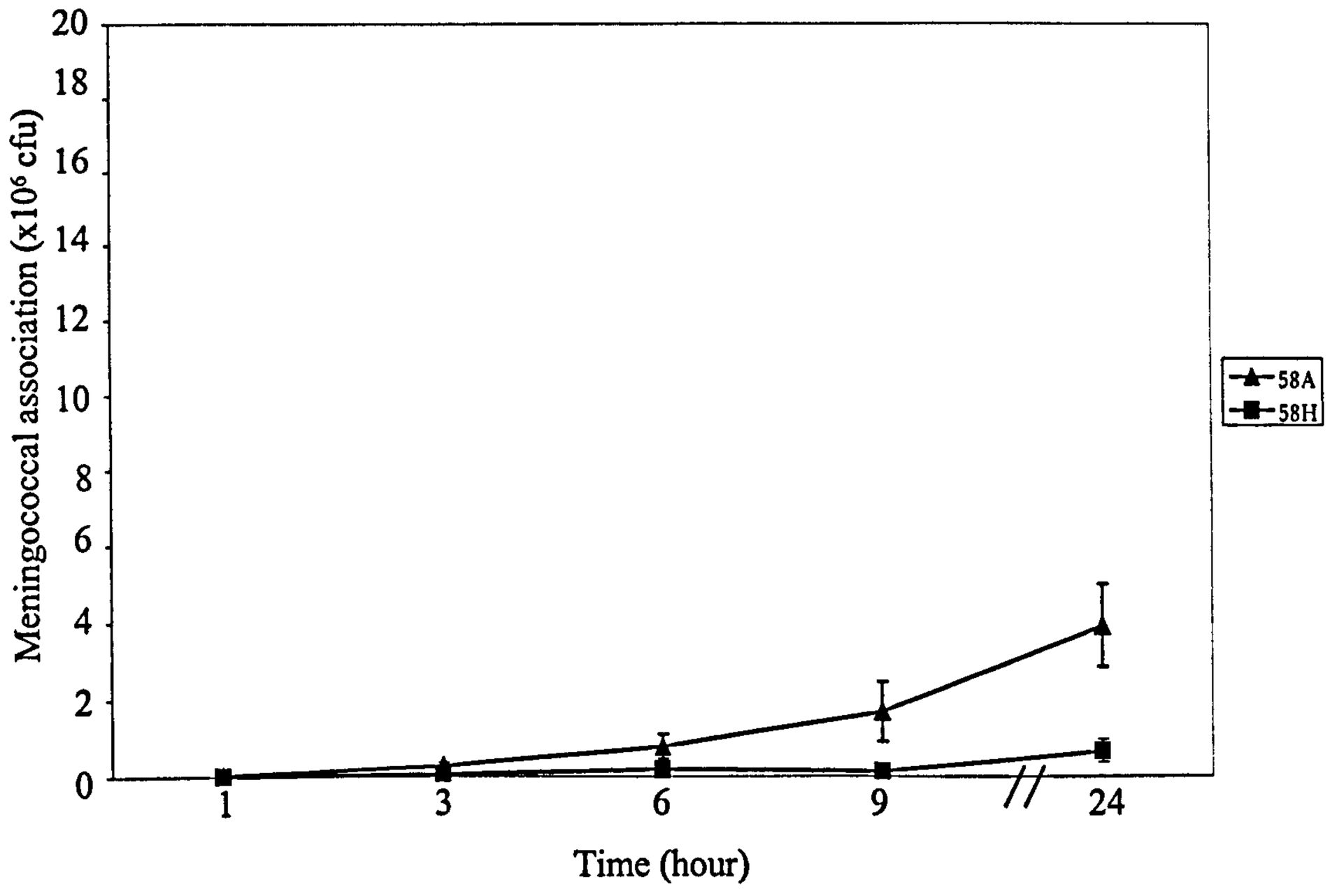


Figure 4.5: The effects of time on the association of $Pil_{Ia}^{+}Opa^{+}Opc^{+}$ and $Pil^{-}Opa^{+}Opc^{+}$ to M16 anaplastic meningioma cells.
The error bars represent the standard deviation of triplicate wells in a single experiment.



adhered to the previously bound organisms. This would create a situation in which some organisms that survived the washing steps were not actually associated with meningioma cells but stuck to each other, and meanwhile association would continue to increase. Meningococcal replication and aggregation could therefore explain the relatively sharp increase in association of $Pil_{Ia}^{+}Opa^{+}Opc^{+}$ meningococci with meningothelial and transitional cells between 9 and 24h, although it does not explain the static association with transitional cells between 6 and 9h. These experiments established that after 6h of infection many organisms had associated with meningioma cells and that clear differences in association were visible between piliated and non-piliated bacteria. This time point was therefore used in future experiments.

4.2.1.2 Variation of $Pil_{Ia}^{+}Opa^{+}Opc^{+}$ infecting dose

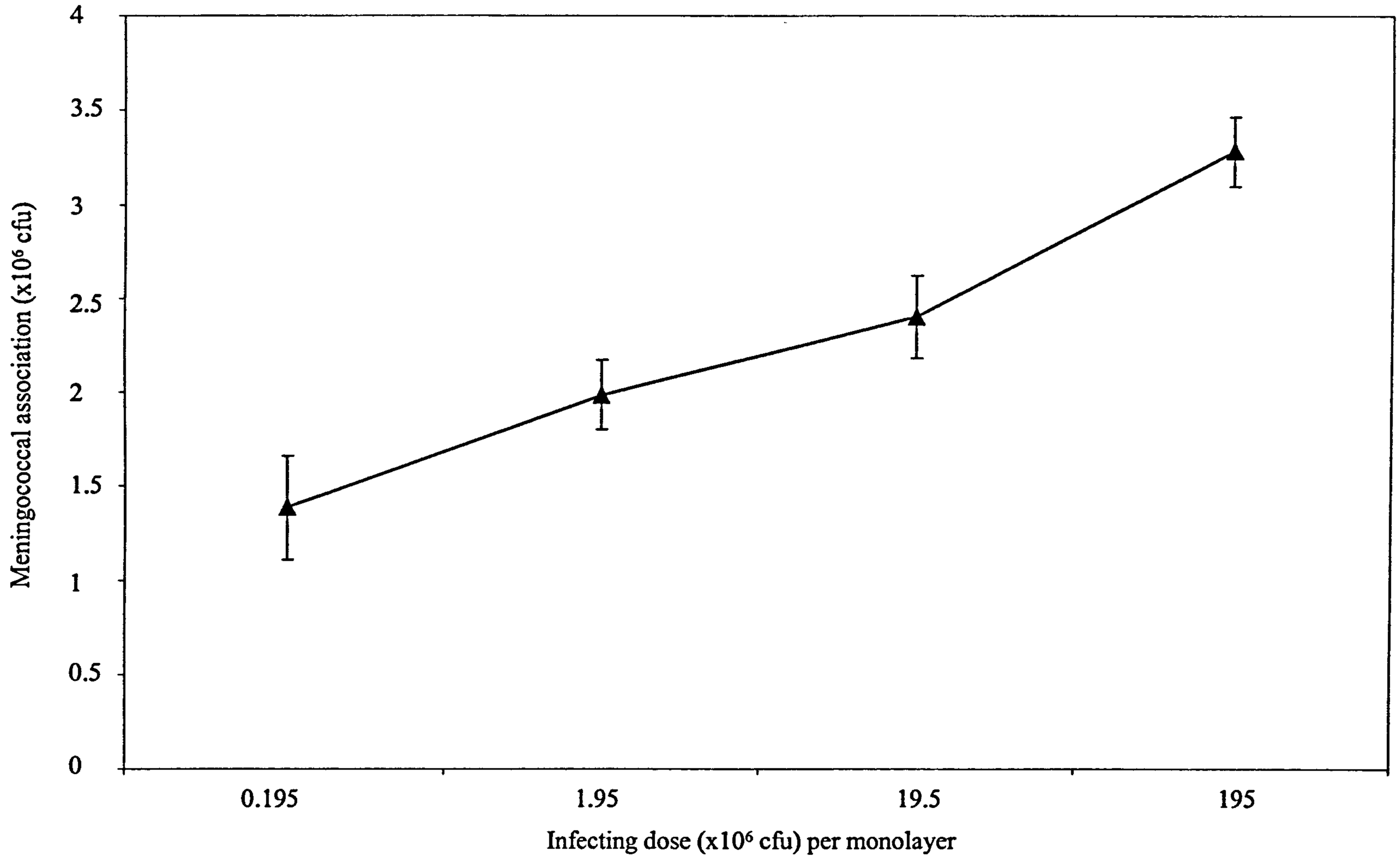
To study differences in meningococcal association with respect to different infecting doses, meningothelial cells were infected for 6h with quantities of $Cap^{+}Pil_{Ia}^{+}Opa^{+}Opc^{+}$ organisms that varied by ten-fold between $2x10^{5}$ and $2x10^{8}$ cfu. The monolayers were then processed as in the standard method (Section 2.12.1). The results in Figure 4.6 show that although the infecting dose was increased 1000-fold overall, the number of associated bacteria rose just 2.4 times from the lowest to the highest dose.

The standard infecting dose used in subsequent infection experiments was approximately $5x10^{7}$ cfu per monolayer. Although there was some day to day variation in the precise inoculum, it was evident that such deviation had little effect on the actual numbers of adherent bacteria.

4.2.1.3 Comparative association of meningococcal variants with meningioma cells

To examine the effect that pilus, Opa and Opc have on the association of encapsulated meningococci with meningioma cells, different variants of MC58/59 (as defined in Table 3.1) were used to infect monolayers in the same experiment. Meningioma cells were infected with these variants for 6h, as previous experiments had shown that differences in the association of phenotypic variants were evident by this time. Each variant was tested in at least three separate experiments, and although not all of them could be tested at the same time, isolate $Pil_{Ia}^{+}Opa^{+}Opc^{+}$ was included in every infection as a control. Between experiments there was some variation in the association of a given meningococcal isolate, but this was acceptable because on any particular day the association of the different bacterial variants followed the same overall trend. The relative association of different bacterial variants was compared statistically using one-way ANOVA analysis to compare the level of significance between the means taking values with $P=<0.05$ as significant.

Figure 4.6: The effect of different bacterial infecting doses on the association of meningococcal isolate 58A ($Cap^+Pil_{Ia}{}^+Opa^+Opc^+$) to M18 meningothelial cells.
Each point represents the average of triplicate wells in one experiment with the standard deviation.



Overall there was a wide range in the association of meningococcal variants with meningothelial cells (M18), but certain trends were discernible (Figure 4.7). Piliated organisms always associated in greater numbers than did non-piliated, and this was significant for the isolates $Pil_{Ia}^{+}Opa^{+}Opc^{+}$, $Pil_{Ia}^{+}Opa^{-}Opc^{+}$ and $Pil_{Ib}^{+}Opa^{+}Opc^{-}$ ($p<0.001$). The association of piliated meningococcal variants with meningothelial M18 cells ranged from $0.5x10^6$ cfu per monolayer for $Pil_{Ib}^{+}Opa^{-}Opc^{-}$, to $3x10^6$ for $Pil_{Ia}^{+}Opa^{+}Opc^{+}$, and from this it was observed that organisms expressing Ia pili associated in significantly higher numbers than those with Ib pili ($p\leq0.001$). However, these isolates also differed in their expression of Opc; Pil_{Ia}^{+} bacteria were always Opc^{+} whereas Pil_{Ib}^{+} bacteria were Opc^{-}. Unfortunately, it was not possible to investigate directly whether the loss of Opc in meningococci expressing the same pili resulted in lowered association, as spontaneous Opc^{-} variants of MC58 and Opc^{+} variants of MC59 could not be isolated. However, all of the non-piliated isolates associated in very similar numbers regardless of whether Opc was expressed. These results suggested that the increased association of Pil_{Ia}^{+} over Pil_{Ib}^{+} organisms is unlikely to be caused by difference in Opc expression but is more likely to be a result of pilin antigenic variation. It was possible to investigate the role of Opa in adherence. The two variants expressing Ia pili differed in their expression of Opa protein, and although $Pil_{Ia}^{+}Opa^{+}Opc^{+}$ associated in greater numbers than $Pil_{Ia}^{+}Opa^{-}Opc^{+}$, this difference was not significant ($p=0.08$). In contrast, with the variants expressing Ib pili, the loss of Opa expression from $Pil_{Ib}^{+}Opa^{+}Opc^{-}$ to $Pil_{Ib}^{+}Opa^{-}Opc^{-}$ resulted in a three-fold decrease in association with meningothelial cells which was significant ($p=0.012$). Indeed, variant $Pil_{Ib}^{+}Opa^{-}Opc^{-}$ only associated with meningothelial cells in marginally greater numbers than the non-piliated organisms. These results suggest that the expression of Opa protein promoted the association of isolates expressing low adherent pili (Pil_{Ib}^{+}), but that this was hidden in organisms with high adherent pili (Pil_{Ia}^{+}). Opa expression appeared to have no effect in the association of non-piliated isolates as all of these associated with meningothelial cells to very similar levels regardless of their Opa status.

The association of the different meningococcal variants with another meningothelial cell line (M21) yielded a similar pattern of results to those obtained with M18 cells (Figure 4.7). Piliated organisms always associated with M18 cells in slightly greater numbers than with M21 cells, although this difference was significant for the Pil^{+} isolates $Pil_{Ia}^{+}Opa^{+}Opc^{+}$ ($p<0.001$), $Pil_{Ib}^{+}Opa^{+}Opc^{-}$ ($p=0.019$) and $Pil_{Ib}^{+}Opa^{-}Opc^{-}$ ($p=0.019$). Non-piliated organisms associated with both cell lines in approximately equal numbers.

Figure 4.8 shows the association of meningococcal variants with the transitional meningioma cell line (M17), in comparison with meningothelial cells. Piliated variants associated with transitional cells in significantly greater numbers than with meningothelial cells ($p\leq0.007$), whereas non-piliated organisms associated with both cell types to similar low levels. With transitional cells, all of the piliated variants associated in significantly greater numbers than the non-piliated isolates ($p<0.001$), with a range of $1.7x10^6$ cfu per monolayer for $Pil_{Ib}^{+}Opa^{-}Opc^{-}$ to $4.4x10^6$ for

Figure 4.7: Comparative association of encapsulated MC58/59 phenotypic variants to the meningothelial cell lines M18 and M21 after 6 hours of infection.
The error bars represent the standard error of the mean of experiments repeated at least three times with triplicate wells.

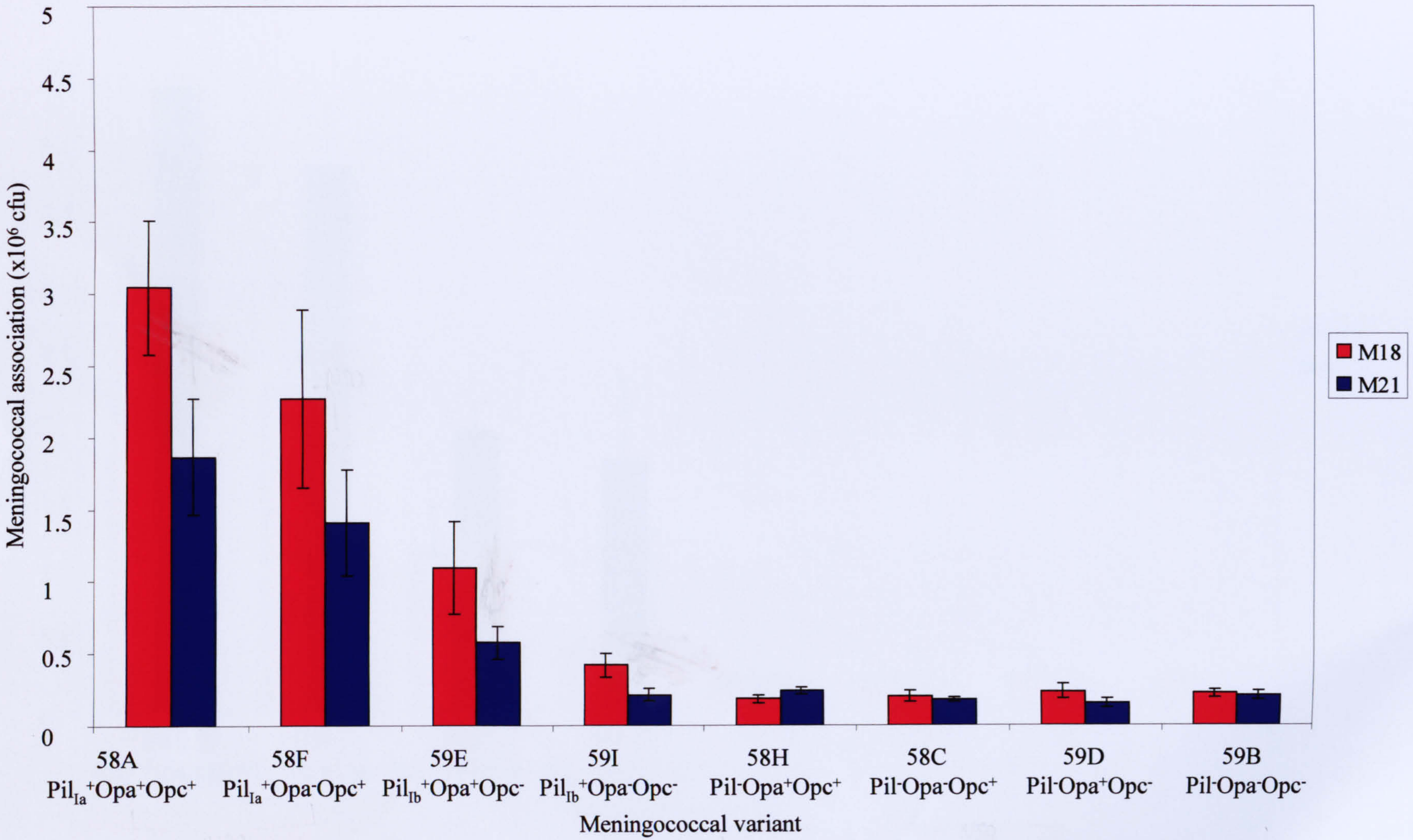
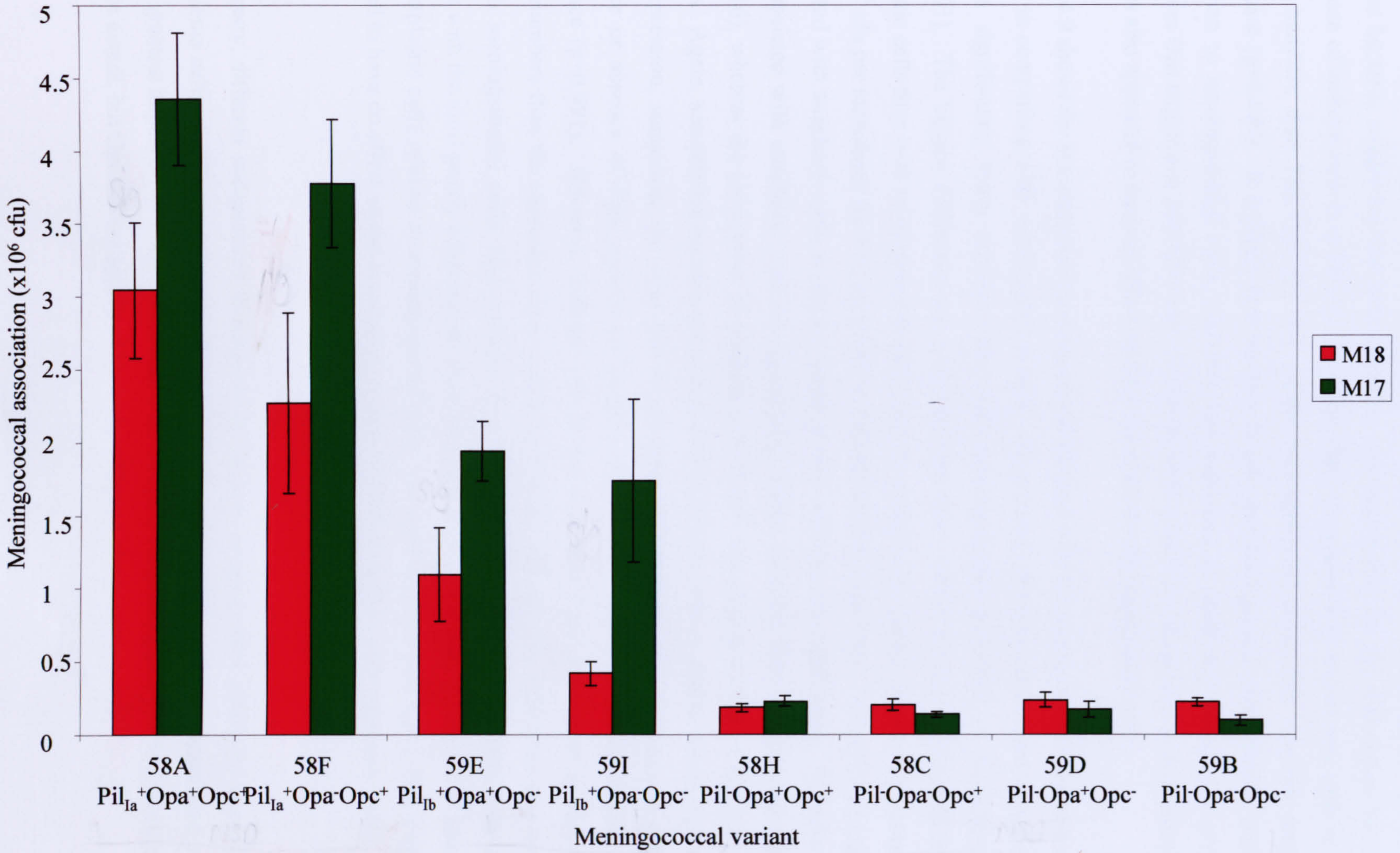


Figure 4.8: Comparative association of encapsulated MC58/59 phenotypic variants to the transitional cell line M17 and the meningothelial cell line M18 after 6 hours of infection.
The error bars represent the standard error of the mean of experiments repeated at least three times with triplicate wells.

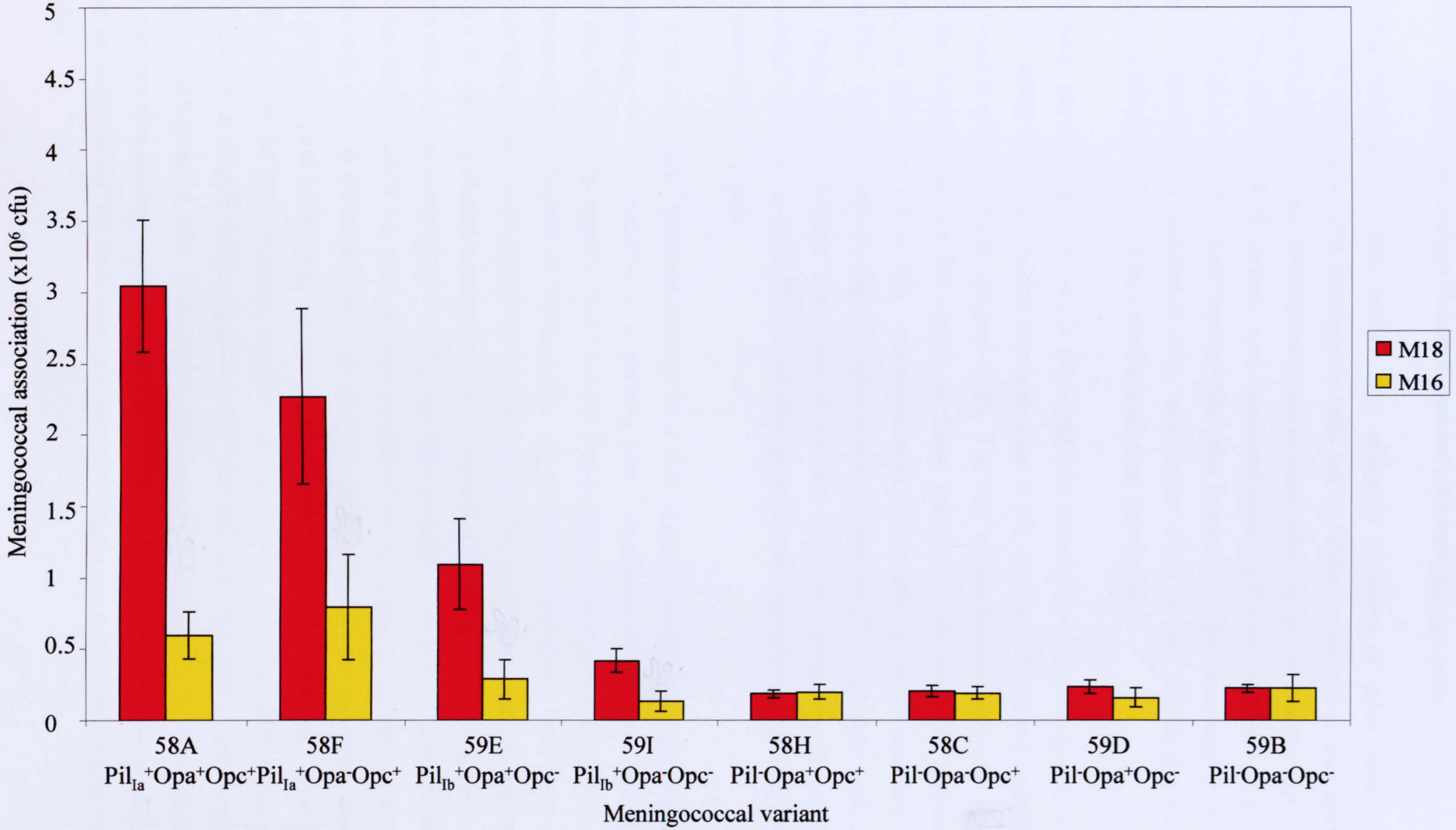


$Pil_{Ia}^{+}Opa^{+}Opc^{+}$. As with meningothelial cells, organisms expressing Ia pili associated with transitional cells in significantly higher numbers than those with Ib pili ($p<0.001$). Also, the presence or absence of Opc in non-piliated organisms appeared to have no effect on the numbers of adherent bacteria, suggesting that pilin variation was responsible for the differences between the adherence of isolates with Ia or Ib pili. $Pil_{Ib}^{+}Opa^{+}Opc^{-}$ associated with transitional cells in slightly higher numbers than $Pil_{Ib}^{+}Opa^{-}Opc^{-}$ but unlike meningothelial cells, this was not statistically significant ($p=0.409$). It is likely that because Ib pili promoted greater adherence to transitional cells than to meningothelial cells, that this was sufficient to mask any association promoting properties Opa expression may have had. In non-piliated organisms, the presence or absence of Opa proteins also appeared to have no effect on their association with transitional cells.

Figure 4.9 shows the association of meningococcal variants with the anaplastic meningioma cell line (M16), in comparison with meningothelial cells. All piliated organisms associated with anaplastic cells in significantly lower numbers than with meningothelial ($p\leq0.007$) or transitional cells ($p<0.001$). The biggest difference was with $Pil_{Ia}^{+}Opa^{+}Opc^{+}$, which associated five-fold less with anaplastic cells than with meningothelial cells, and the overall association range of Pil^{+} isolates was $1.3x10^{5}$ cfu per monolayer for $Pil_{Ib}^{+}Opa^{-}Opc^{-}$ to $7.9x10^{5}$ for $Pil_{Ia}^{+}Opa^{+}Opc^{+}$. Non-piliated organisms associated with anaplastic cells to similar levels as seen with the other cell types. Bacteria with Ia pili associated with anaplastic cells in significantly higher numbers than non-piliated organisms ($p\leq0.016$), whereas the association of bacteria with Ib pili was similar to that of the non-piliated variants. Again, non-piliated bacteria associated with anaplastic cells to similar levels regardless of Opc expression, supporting the view that Ia pili promoted greater adherence than Ib pili. The presence or absence of Opa proteins in bacteria expressing Ia pili had no significant role in adherence ($p=0.991$). However, isolates with Ib pili that were Opa^{+} associated in significantly higher numbers than the equivalent Opa^{-} variant ($p=0.022$), although the difference was not large. As with meningothelial cells, Opa proteins may promote adherence to anaplastic cells only in variants with the most poorly adherent pili, even although all pili were poor mediators of attachment with anaplastic cells relative to meningothelial cells. As with the other cell types, Opa expression appeared to have no effect on the association of non-piliated organisms with anaplastic cells.

In summary, different encapsulated meningococcal isolates varied in their ability to associate with meningioma cells of different subtypes. Phase and antigenic variation of pilus-associated proteins had the greatest impact on meningococcal association, Opa proteins may also promote association to a smaller extent, but Opc did not appear to have any effect on these interactions.

Figure 4.9: Comparative association of encapsulated MC58/59 phenotypic variants to the anaplastic cell line M16 and the meningothelial cell line M18 after 6 hours of infection.
The error bars represent the standard error of the mean of experiments repeated at least three times with triplicate wells.



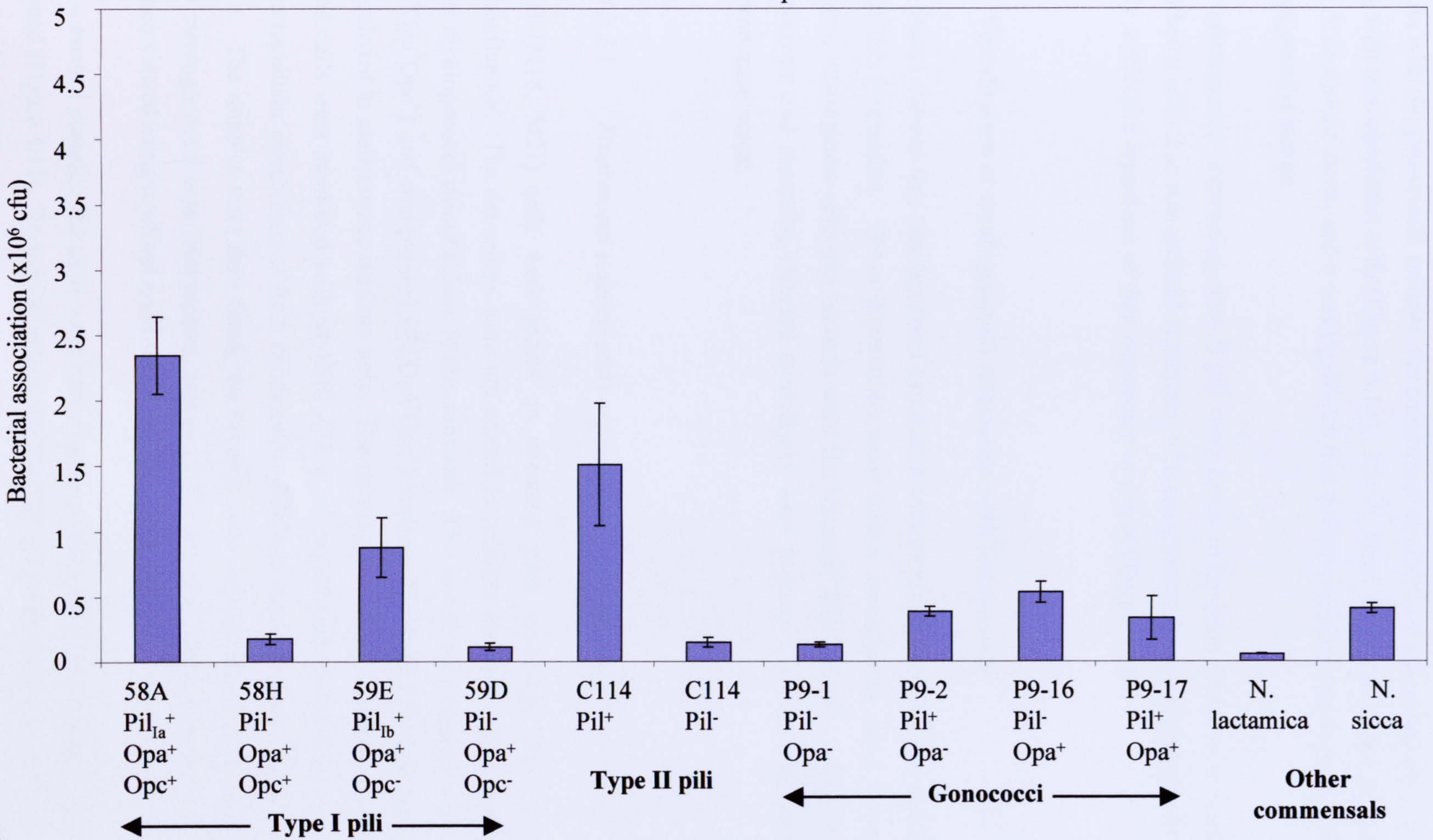
4.2.2 Adherence of other Neisseria species with meningioma cells

The finding from the previous section that antigenic variation of pilin subunits affected meningococcal association with meningioma cells led to further investigation into the effects of other types of pilus variation. Meningococci can express either one of two classes of pili, class I or class II. The Pil^+ MC58/59 variants used expressed class I pili (see Section 3.1.1). Virji and colleagues (Virji *et al.*, 1991) have reported that class I and class II pili were similar in promoting bacterial association with endothelial cells, and further (Virji *et al.*, 1992a) that class II pili mediated less effective adherence to epithelial cells than class I pili.

To investigate the efficiency of class II pilus-mediated association with meningothelial cells the serogroup C strain C114 Pil_{II}^+, which expresses class II pili, and its non-piliated equivalent C114 Pil^- were used to infect M18 cells (Figure 4.10). The Pil_{II}^+ organism associated with M18 cells in much higher numbers than the Pil^- variant, as found previously with other Pil^+ isolates. It was interesting to find that C114 Pil_{II}^+ associated with M18 cells in lower numbers than the $Pil_{Ia}^+Opa^+Opc^+$ organism but in slightly higher numbers than the $Pil_{Ib}^+Opa^+Opc^-$ isolate. Taken together, these results indicate that class II pili are important mediators of attachment to meningothelial cells for the strain C114, and that these pili are less effective than Ia pili, but are more effective than the Ib pili.

Gonococci are acapsulate *Neisseria* that can be a rare cause of meningitis, especially following disseminated gonococcal infection or in patients with complement deficiencies (Del Rio *et al.*, 1989). These bacteria can express class I pili and Opa proteins, both of which are known to have a role in gonococcal association to human cells. At present, no gonococci have been shown to produce the Opc protein, and none of the isolates used in this study were found to express it (see Section 3.1.1). Meningothelial meningioma cells were infected with gonococcal variants of the P9 strain that varied in their expression of pilus and Opa proteins. The piliated gonococcal variants express the class I α-pili on their surface (Lambden *et al.*, 1981). All gonococcal variants associated with meningothelial cells at much lower levels than the Pil^+ meningococci, regardless of the class of pili or pilin variant that the meningococci expressed. P9-1 (Pil^-Opa^-) associated with meningothelial cells in lower numbers than the other gonococcal variants, which expressed either pili or Opa or both (Figure 4.10), suggesting that both pili and Opa have a role in gonococcal adherence to meningothelial cells. It is possible that gonococci associated with meningothelial cells in lower numbers than meningococci because their pili were less effective adhesins or, alternatively, that other unidentified surface molecules present on meningococci but absent on gonococci were responsible.

Figure 4.10: Comparative association of meningococcal, gonococcal and commensal *Neisseria* strains with the meningothelial cell line M18 after 6 hours of infection. Bacterial strains varied with respect to pilus expression (Pil^+ or Pil^-), class of pili expressed (I or II), and intrastrain pilin variation (Pil_{Ia}^+ or Pil_{Ib}^+). The results of a single experiment with the standard deviation are shown. Variants $Pil_{Ib}^+Opa^+Opc^-$ and $Pil^-Opa^+Opc^-$ were tested in a separate experiment in which $Pil_{Ia}^+Opa^+Opc^+$ was also tested and found to associate in similar numbers to the same variant in this experiment.



Commensal strains of *Neisseria* are also acapsulate and class I or II pili have been identified on their surfaces, but these bacteria normally reside in the human nasopharynx and remain non-pathogenic. The strains *Neisseria lactamica* and *Neisseria sicca* were also used to infect M18 cells; the former was Pil_{II}^{+} whilst the latter did not express either class I or II pili, and both lacked Opa proteins (Table 3.2). As with the gonococcal variants, the commensal strains were also relatively ineffective at associating with meningothelial cells (Figure 4.10). The *N. sicca* strain associated more readily than the Pil^{+}_{II} *N. lactamica* strain, and it was significant that neither organism was anymore effective than Pil^{-} meningococcal strains.

In summary, meningococci expressing class II pili were found to be more effective at association with meningothelial cells than non-piliated organisms, whereas gonococci and commensal *Neisseria* were relatively ineffective regardless of their expression of pili or Opa proteins.

4.2.3 Visualisation of meningococcal association with human cells

Viable count assays showed that meningococci associated with meningioma cells, and enabled the quantitation of this interaction. These interactions were further investigated using microscopic methods. Firstly, meningioma cells and bacteria were fluorescently labelled and viewed using the confocal microscope and secondly, infected monolayers were processed for analysis under the scanning electron microscope.

4.2.3.1 Fluorescent staining and confocal microscopy analysis

Meningothelial (M18, M21) cells were grown on uncoated glass coverslips until they were approaching confluence. The coverslips were not coated in collagen because initial experiments had shown that meningococci would adhere to this substrate. The monolayers were incubated with piliated ($Pil_{Ia}^{+}Opa^{+}Opc^{+}$) and non-piliated ($Pil^{-}Opa^{+}Opc^{+}$) variants for 3, 6, 24h, and the control wells were incubated in maintenance medium only. The staining procedure was done in two steps. Firstly, unfixed cells were incubated with SP-$DiIC_{18}(3)$, a sulfonated carbocyanine that stains the exterior and intracellular membranes of both meningioma cells and meningococci, and is taken up by endocytosis. The samples were then fixed, the bacteria were counterstained with an antibody raised against meningococcal outer membranes, and an FITC-conjugated secondary antibody. The results were then viewed using confocal microscopy (see Section 2.13.2).

In the negative control samples, the cells were stained a deep orange/red colour with a very clean black background (Figure 4.11). To a small extent, the SP-$DiIC_{18}(3)$ stain caused the cells to shrink away from each other and fatten-up. The confocal microscope simulated fluorescence projection

Laser scanning confocal images of meningothelial meningioma cells infected with meningococci ($Cap^+Pil_{Ia}{}^+Opa^+Opc^+$ 58A or $Cap^+Pil^-Opa^+Opc^+$ 58H) over time.
Confocal images are representative images from an infection experiment carried out in triplicate.
The scale bar represents 20μm.

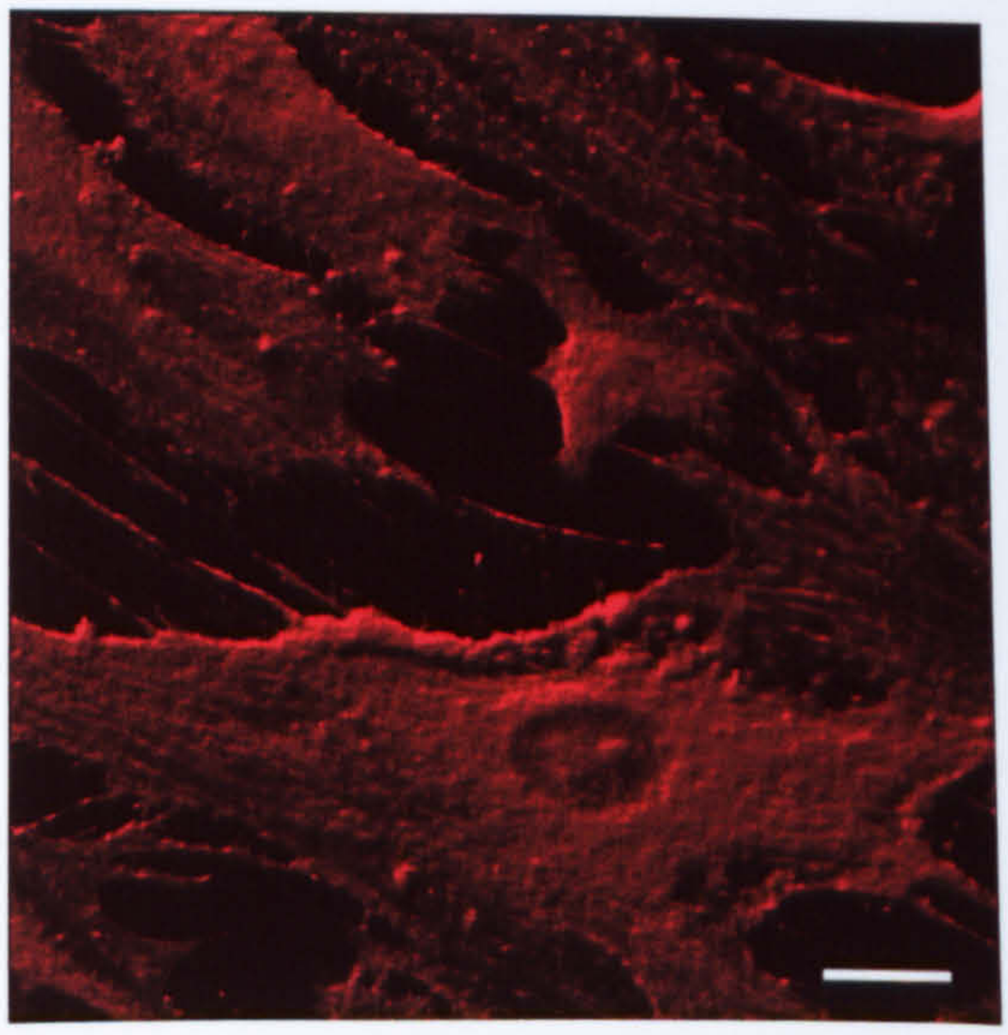


Figure 4.11: Uninfected M18 cells stained deep orange with SP-$DiIC_{18}(3)$. The cell processes are visible, as is the nucleus of the bottom cell, which appears as a raised circular pit.

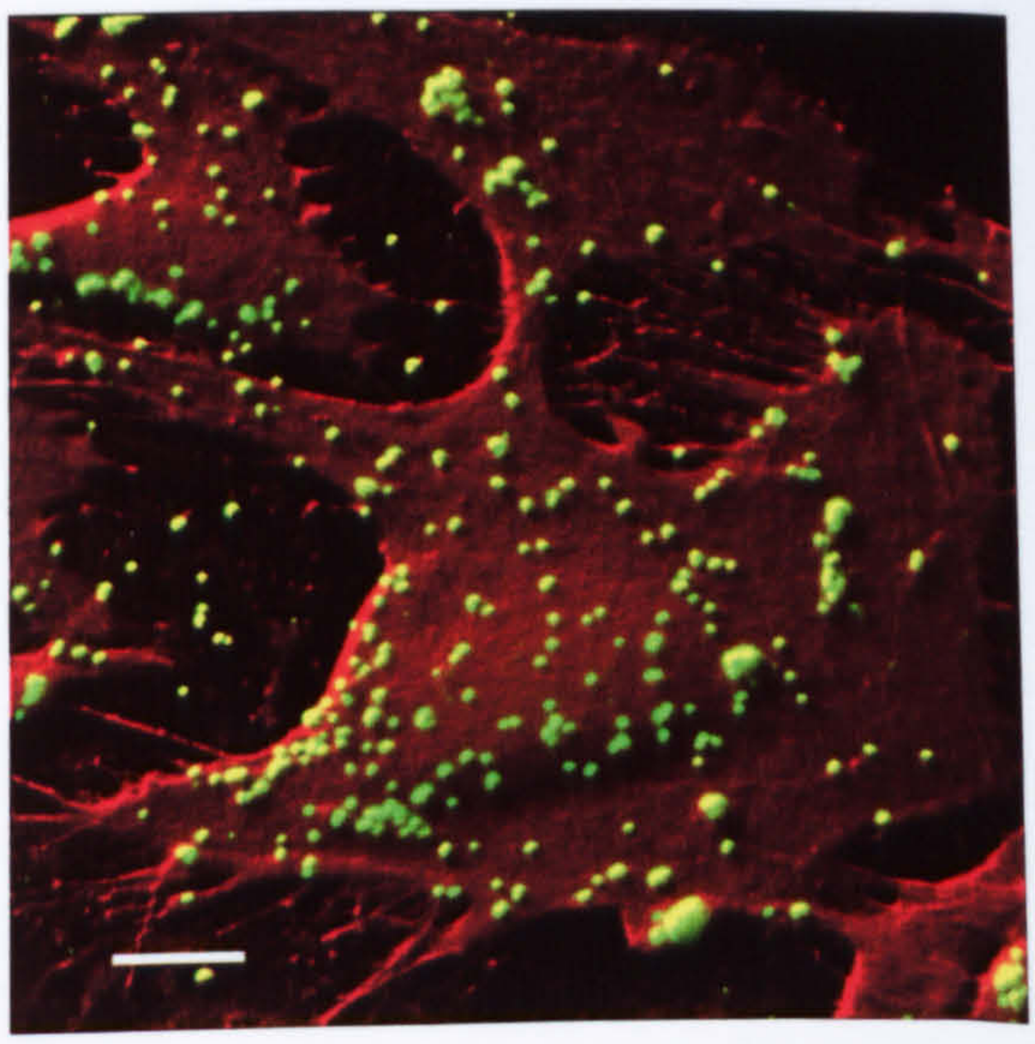


Figure 4.12: M18 cells infected with $Pil_{Ia}{}^+$ 58A for 3h. The human cell membranes are stained deep orange and the meningococci are the green dots. Diplococci are well spread over the cells and are visible over the raised nuclear region of the main cell in the field.

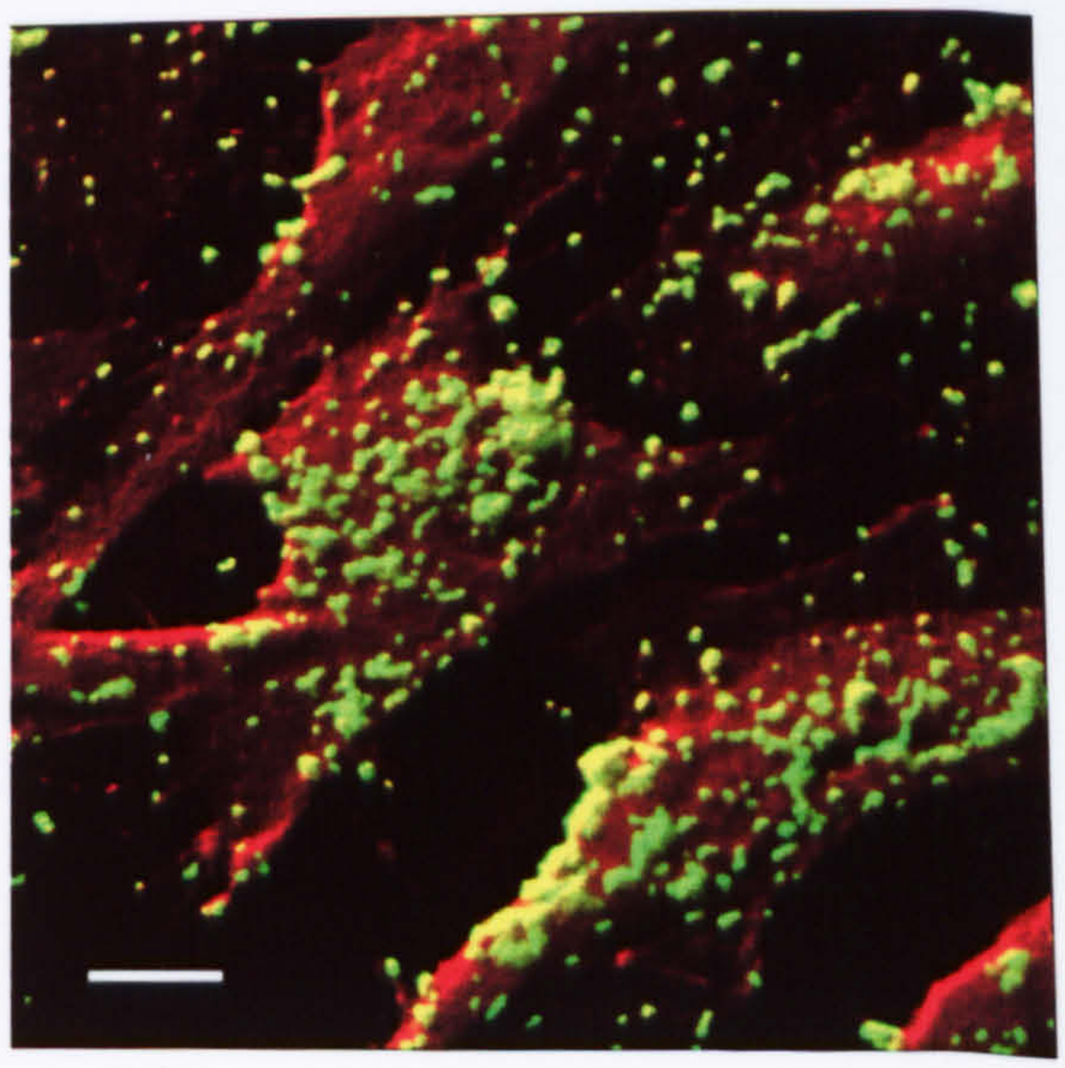


Figure 4.13: M18 cells infected with $Pil_{Ia}{}^+$ 58A for 6h. There are many more adherent bacteria and some clumps of meningococci are present, especially over the meningioma cell nuclei.

Figure 4.14: M21 cells infected with Pil_{Ia}^{+} 58A for 24h. The main cell in this image is nearly completely covered in clumps of adherent meningococci.

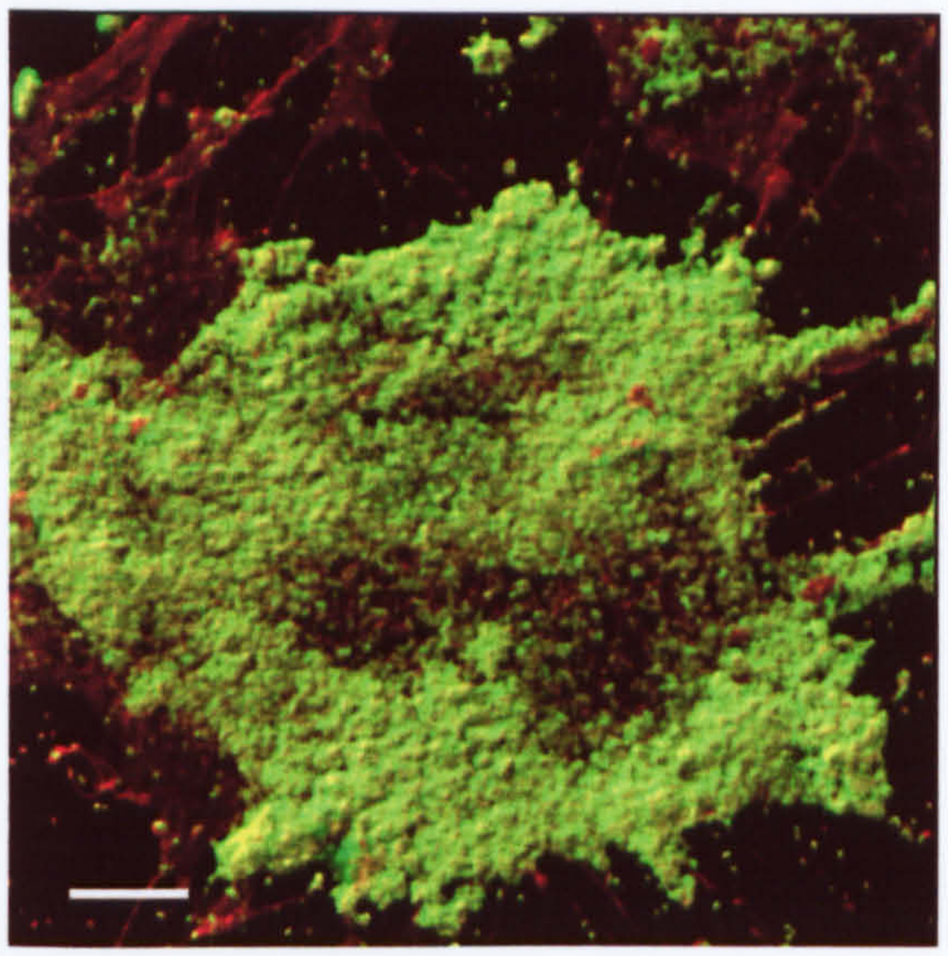


Figure 4.15: M18 infected with Pil_{Ia}^{+} 58A for 24h. The cells in this field were not completely covered in meningococci. The adherent organisms were mostly present as clumps over the cell nuclei, whilst in other areas the association was more diffuse.

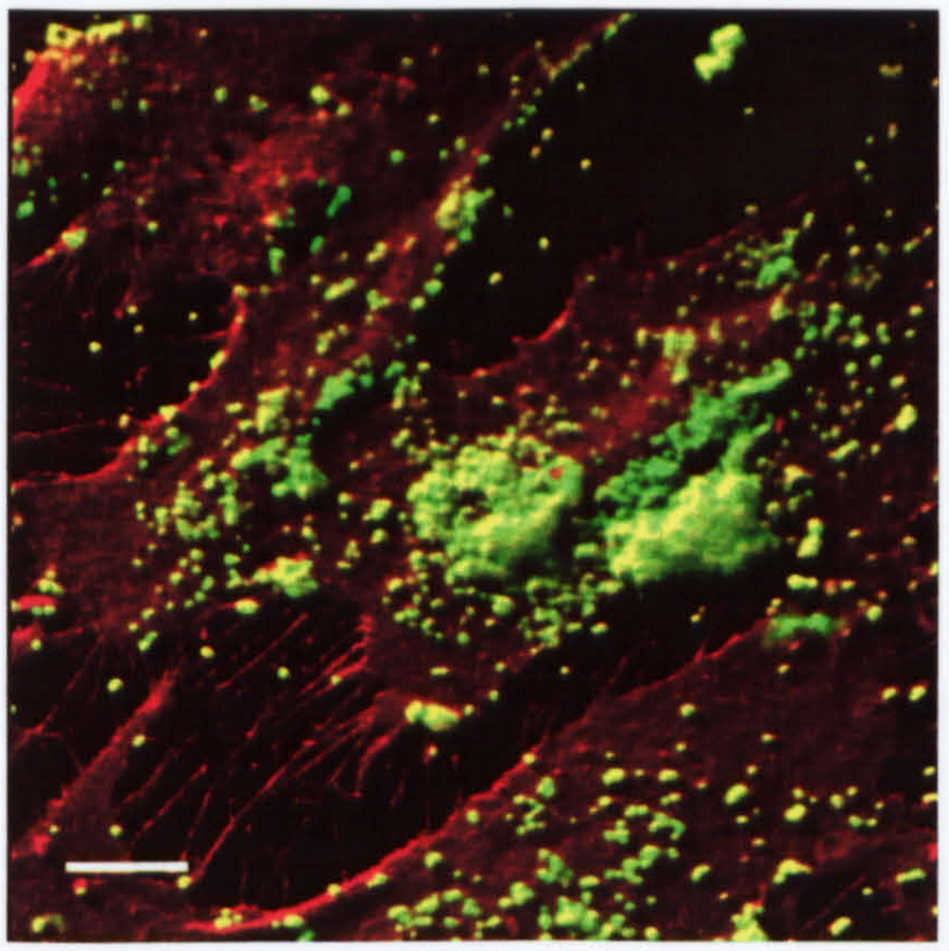


Figure 4.16: M18 cells infected with non-piliated 58H for 3h. There are few adherent meningococci per cell and their distribution appears as diplococci associating randomly with the meningioma cells.

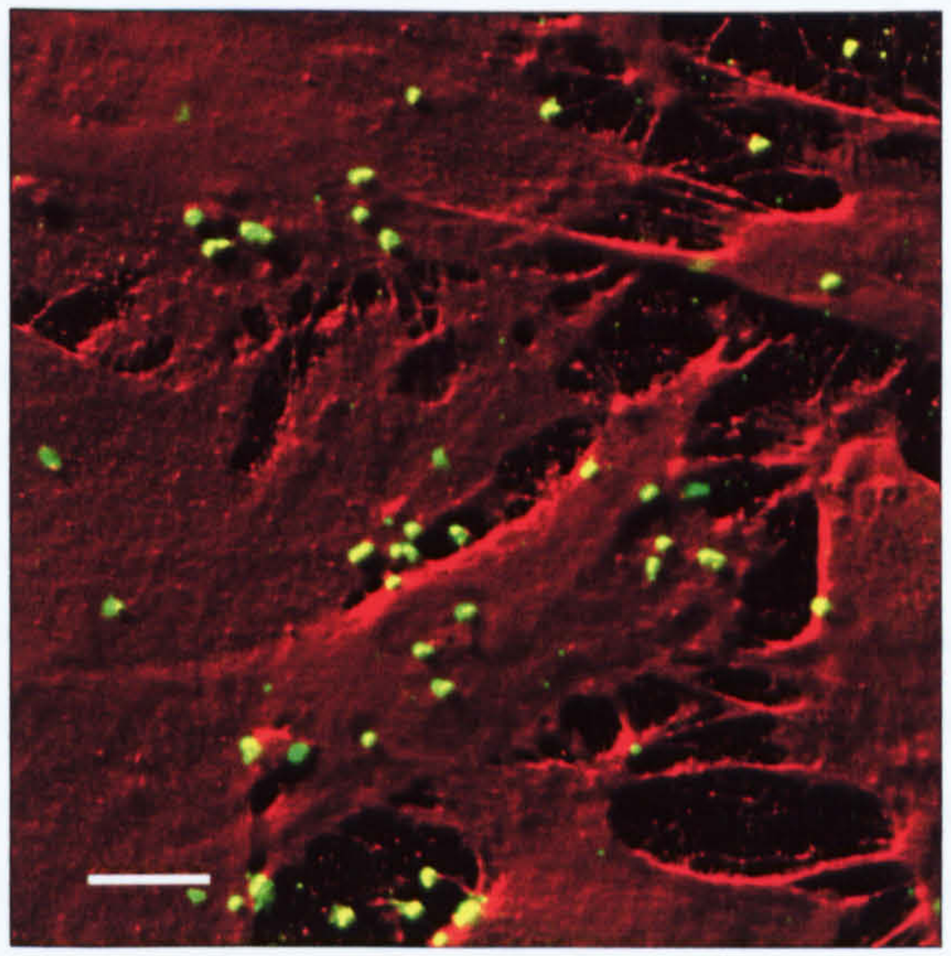


Figure 4.17: M18 infected with Pil⁻ 58H for 6h. Throughout the monolayers, meningococci associated with meningioma cells in a similar pattern to the 3h time point but with slightly more adherent bacteria.

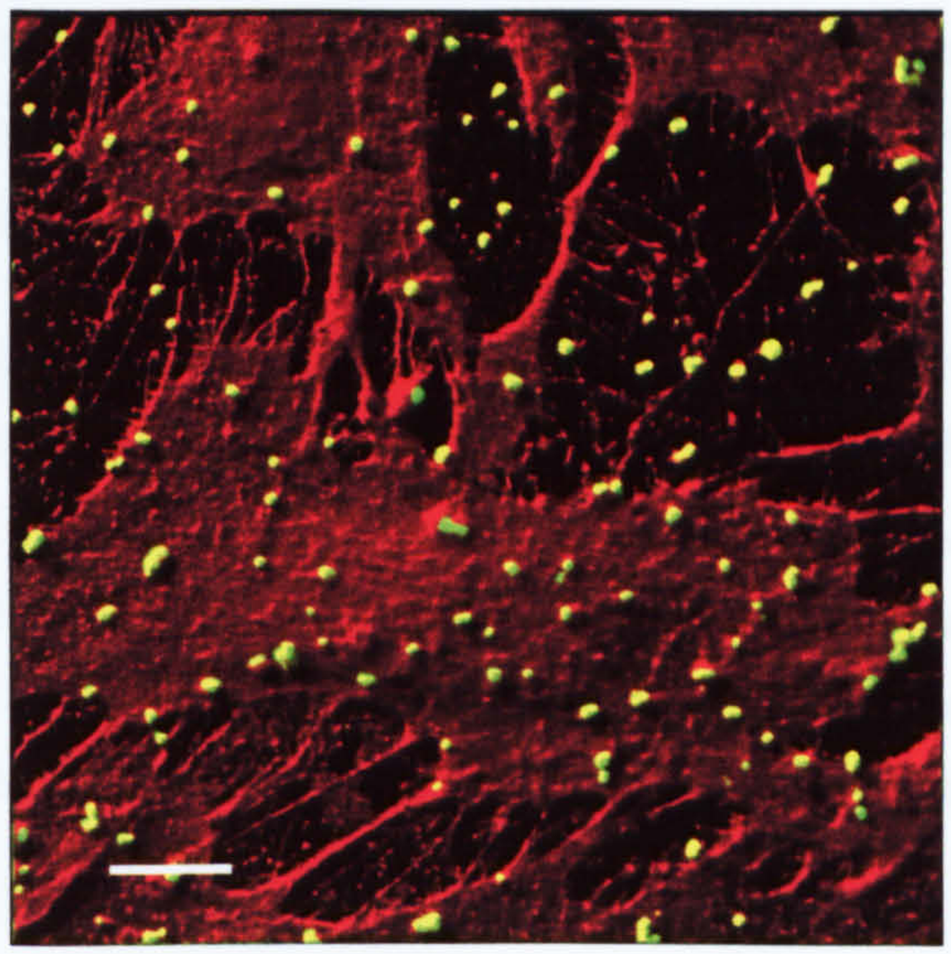
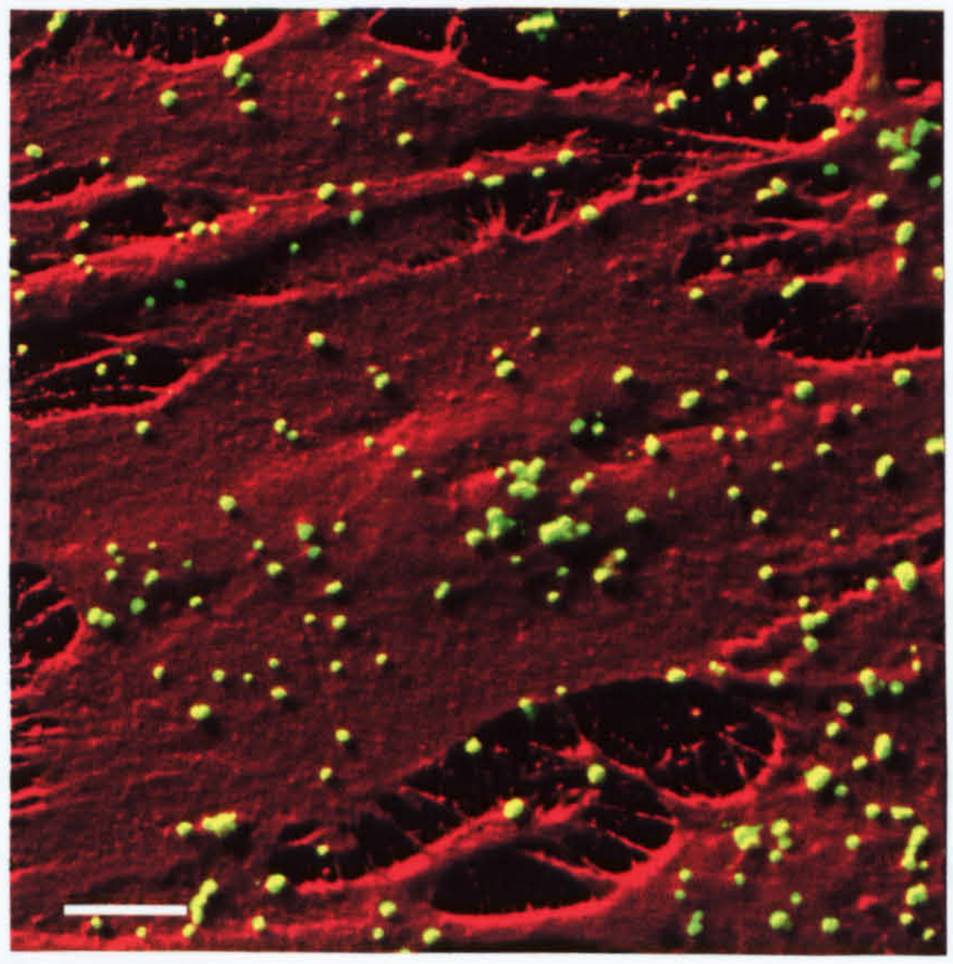


Figure 4.18: M18 cells infected with Pil⁻ 58H for 24h. There are more adherent meningococci per meningioma cell than at the other time points. Again, bacterial association appears to be non-specific, although meningococci are present over the raised nuclear region of the central cell in this image.



provided excellent detail of cellular processes and of the cell surface contours, including the location of the nucleus, which appeared as a raised circular pit. Individual cells were relatively easy to distinguish and cellular processes could be seen to reach out to contact other cells.

Meningococci were visible as spherical green cocci, usually existing as diplococci or clumps, and were found to adhere to meningioma cells only and not the glass coverslip. Experiments using both M18 and M21 meningothelial cells yielded similar results. Piliated meningococci were seen to associate with meningioma cells in greater numbers, and with more specificity for certain cellular structures than were the non-piliated variants. Analysis of the samples infected with the Pil^+ variant showed that at 3h (Figure 4.12) the adherent meningococci existed mostly as diplococci which were well spread over the cells. After 6h (Figure 4.13), considerably more meningococci had associated with the meningothelial cells than at 3h, and clumps of bacteria were present. Meningococci also showed some specificity for meningothelial cell structures, and on some cells there was especially dense binding over and around the nucleus and cellular processes, whilst on other cells there were fewer adherent bacteria overall. Whilst many of the cells that had been infected for 24h were almost completely covered in clumps of bacteria (Figure 4.14), others were less densely covered and meningococci again showed a predilection for the nuclear region (Figure 4.15).

In contrast, the samples infected with non-piliated bacteria showed far less binding at all time points than with Pil^+ organisms, and less specificity for the nucleus and cell processes. With the 3h samples (Figure 4.16) there were very few adherent bacteria; typically no more than about ten per cell distributed randomly. The 6h samples showed similar results to the 3h monolayers (Figure 4.17), with slightly higher numbers of attached meningococci. By 24h (Figure 4.18), there were greater numbers of adherent meningococci, although these were not clumped as they were in the equivalent Pil^+ time point.

4.2.3.2 SEM analysis

Meningioma 18 cells were grown to confluence on collagen coated glass coverslips and were infected with piliated ($Pil_{Ia}^+Opa^+Opc^+$) and non-piliated ($Pil^-Opa^+Opc^+$) meningococci for 3, 6, 24h, and the control wells were incubated with maintenance medium only. The coverslips were processed and viewed under SEM (see Section 2.13.3).

It was not possible to observe the cell boundaries within the confluent areas of the coverslip, apart from near the edges of the monolayer. In the negative control sample the cells appeared to be quite flat usually with smooth surfaces (Figure 4.19a), whilst in some areas the surfaces were quite bumpy with an uneven texture (Figure 4.19b). Cellular processes were visible, and the nuclei generally appeared more dome-like than in the confocal images.

Figure 4.19a: Low power SEM analysis of meningioma 18 negative control. The cells, grown on glass coverslips, appear to be quite flat but with a textured surface. In this field areas of just the coverslip are also visible.
The scale bar represents 20µm.

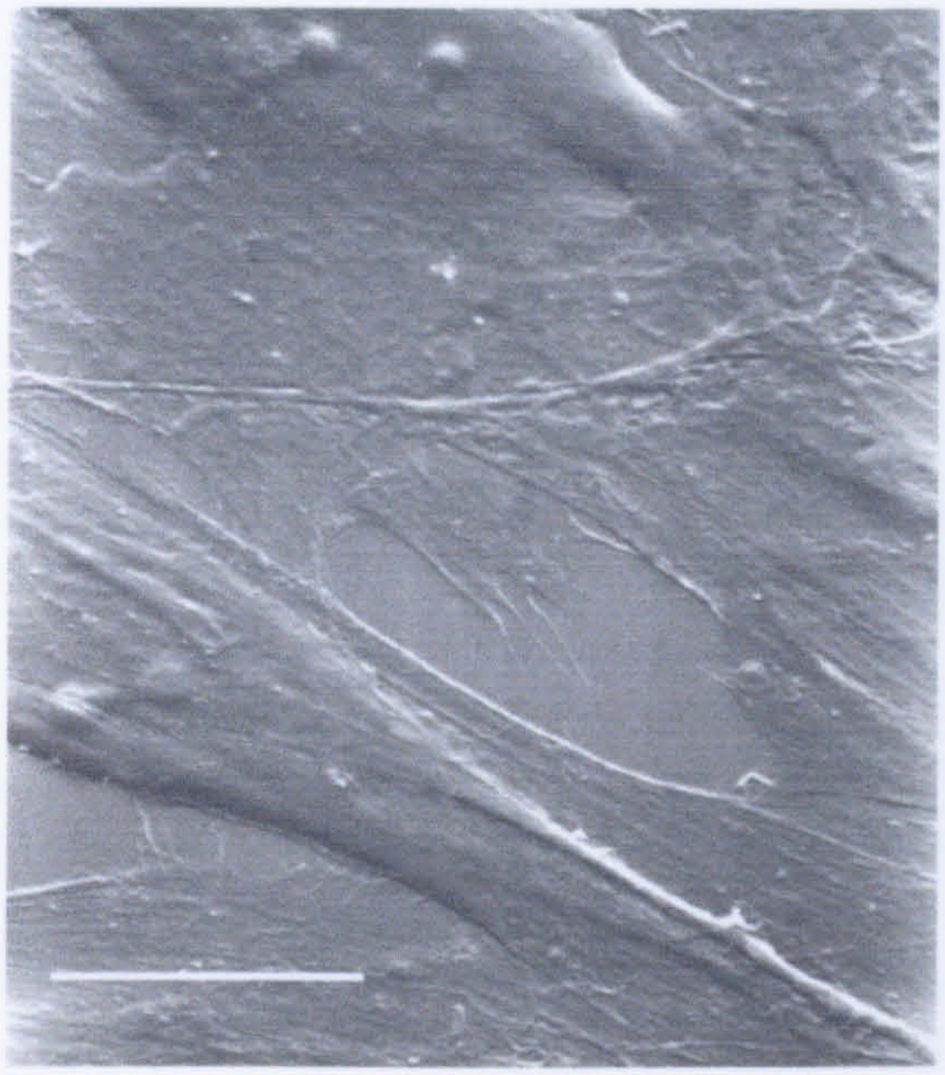


Figure 4.19b: Higher power SEM of Figure 4.19a showing meningioma cells with a more uneven texture.
The scale bar represents 10µm.

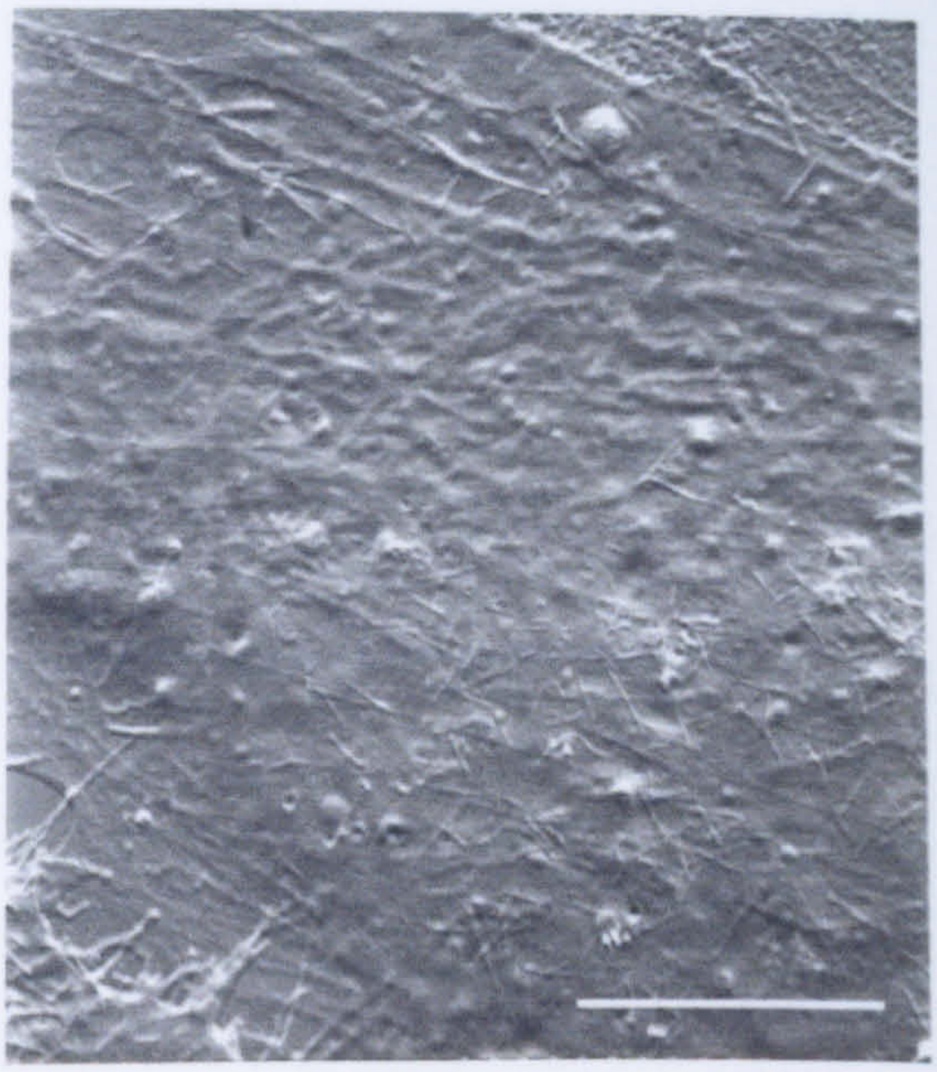


Figure 4.20a: M18 cells infected with 58A ($Pil_{Ia}^{+}Opa^{+}Opc^{+}$) for 3h. Meningococcal association with the M18 cells was uneven and some structures that were apparently pili bundles (arrow) were evident.
The scale bar represents 20µm.

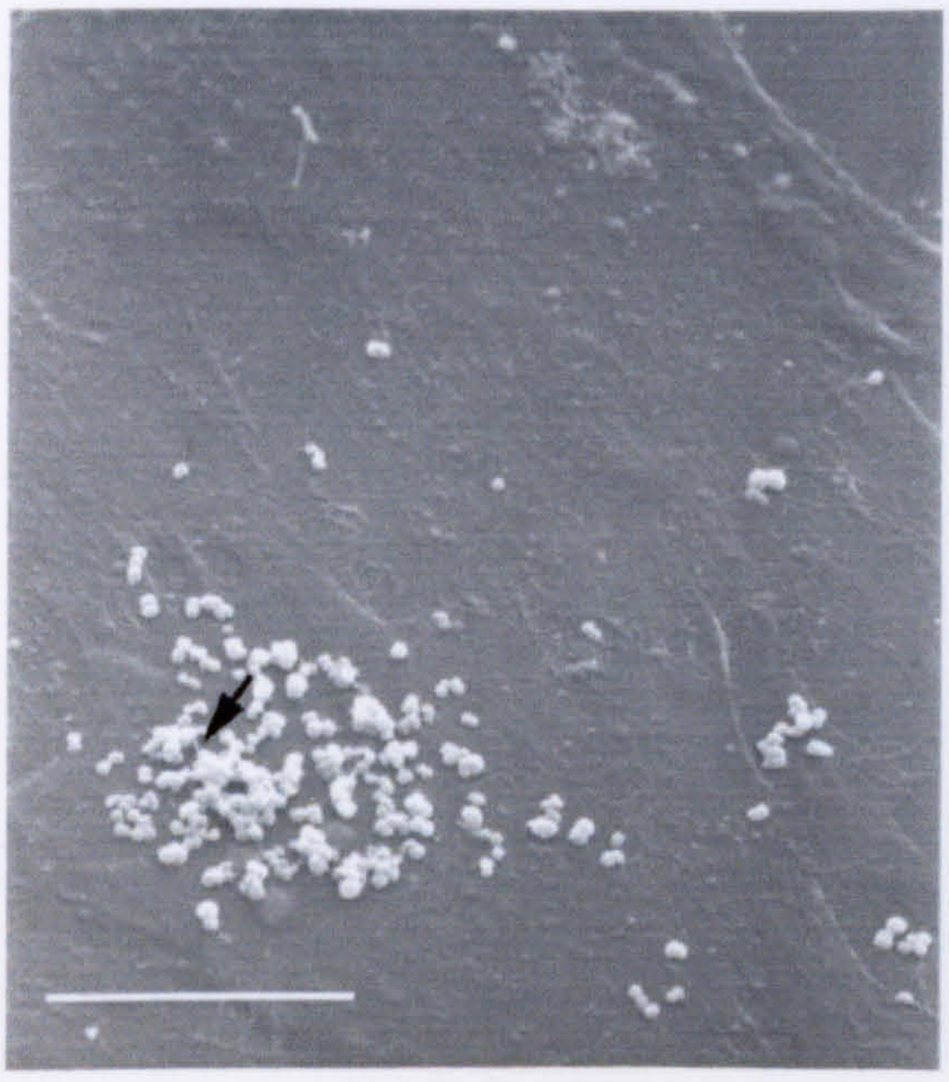


Figure 4.20b: Higher power of Figure 4.20a. Meningococci were occasionally seen sitting on small uprisings of the meningioma cell membrane. Pili are not apparent on these bacteria.
The scale bar represents 2µm.

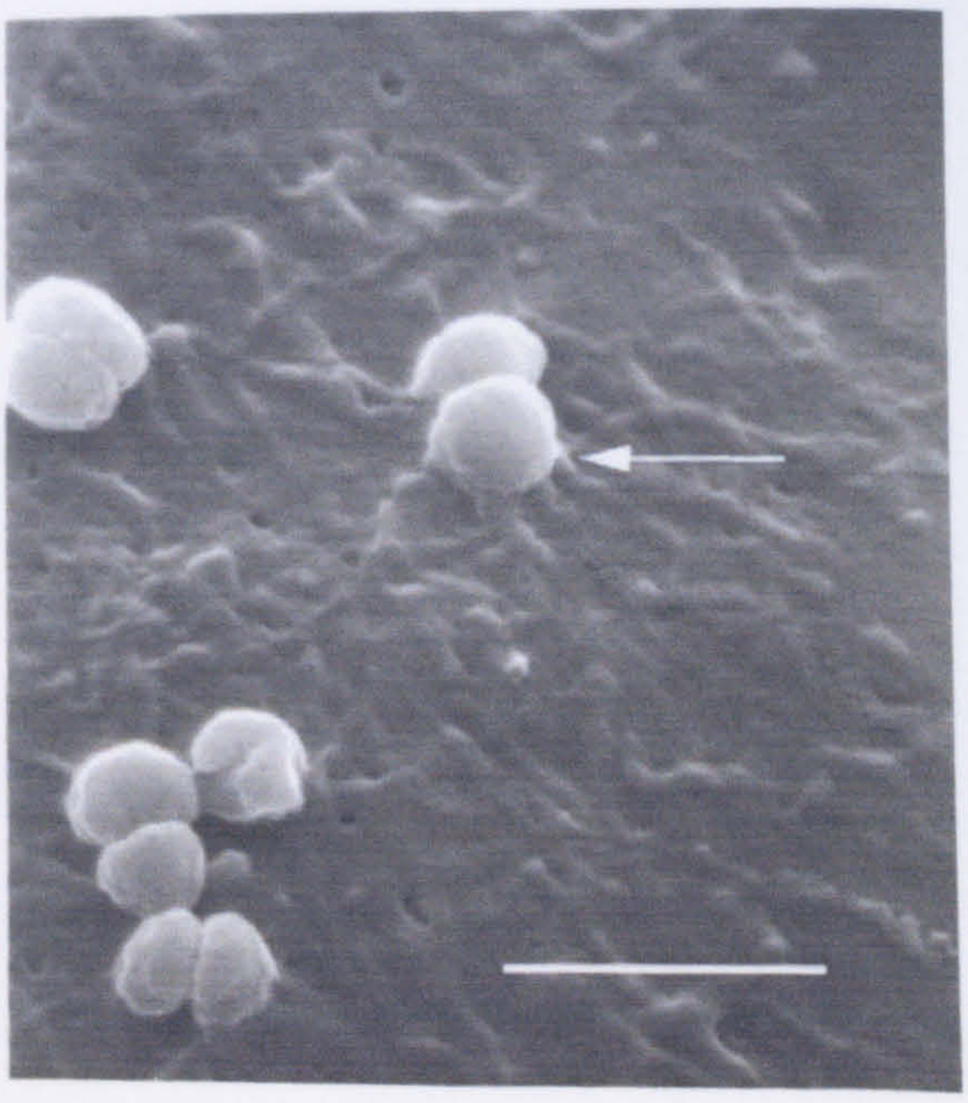


Figure 4.21a: M18 cells infected with 58A ($Pil_{Ia}^{+}Opa^{+}Opc^{+}$) for 6h. Meningococci are most densely associated around the nuclear region (arrow) of meningioma cells whilst they are sparse in other areas.
The scale bar represents 20μm.

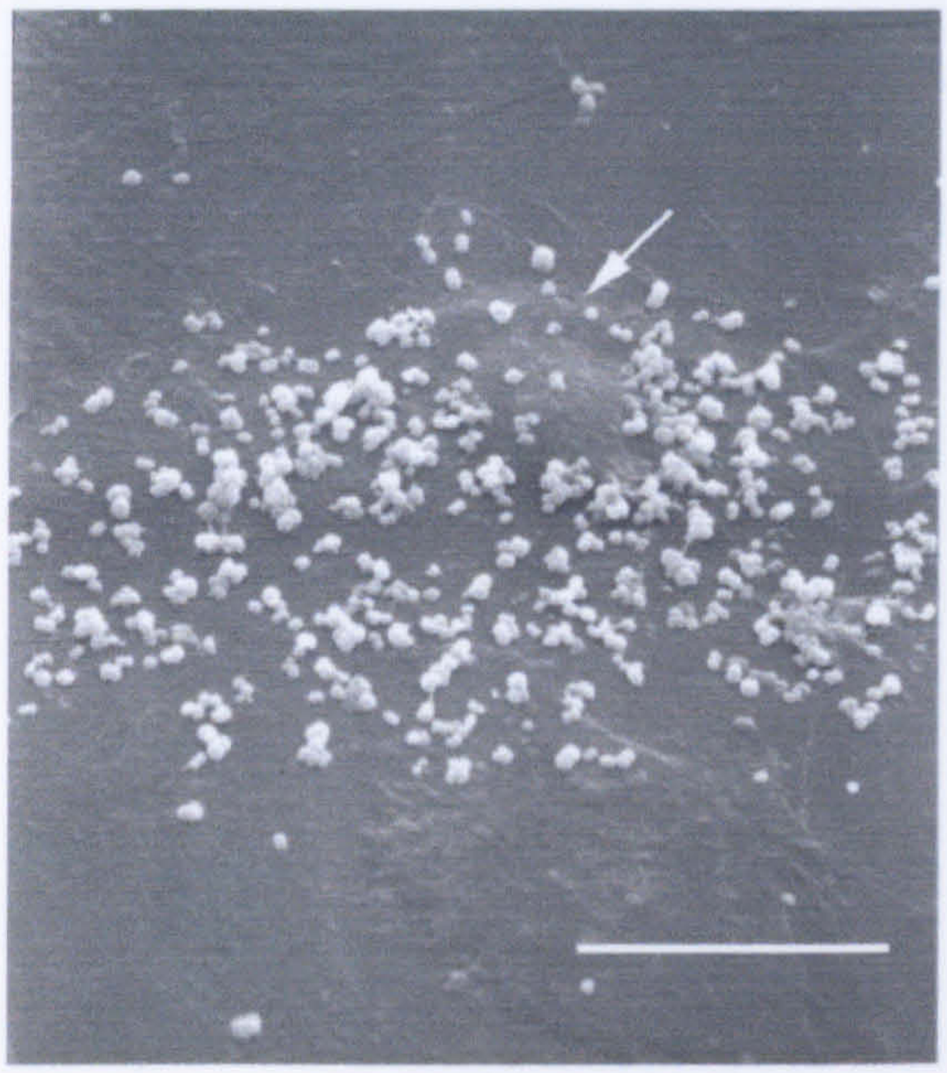


Figure 4.21b: Higher power of Figure 4.21a. Meningococci were also found to adhere to cellular processes (arrows), and pili are not visible in this field.
The scale bar represents 3μm.

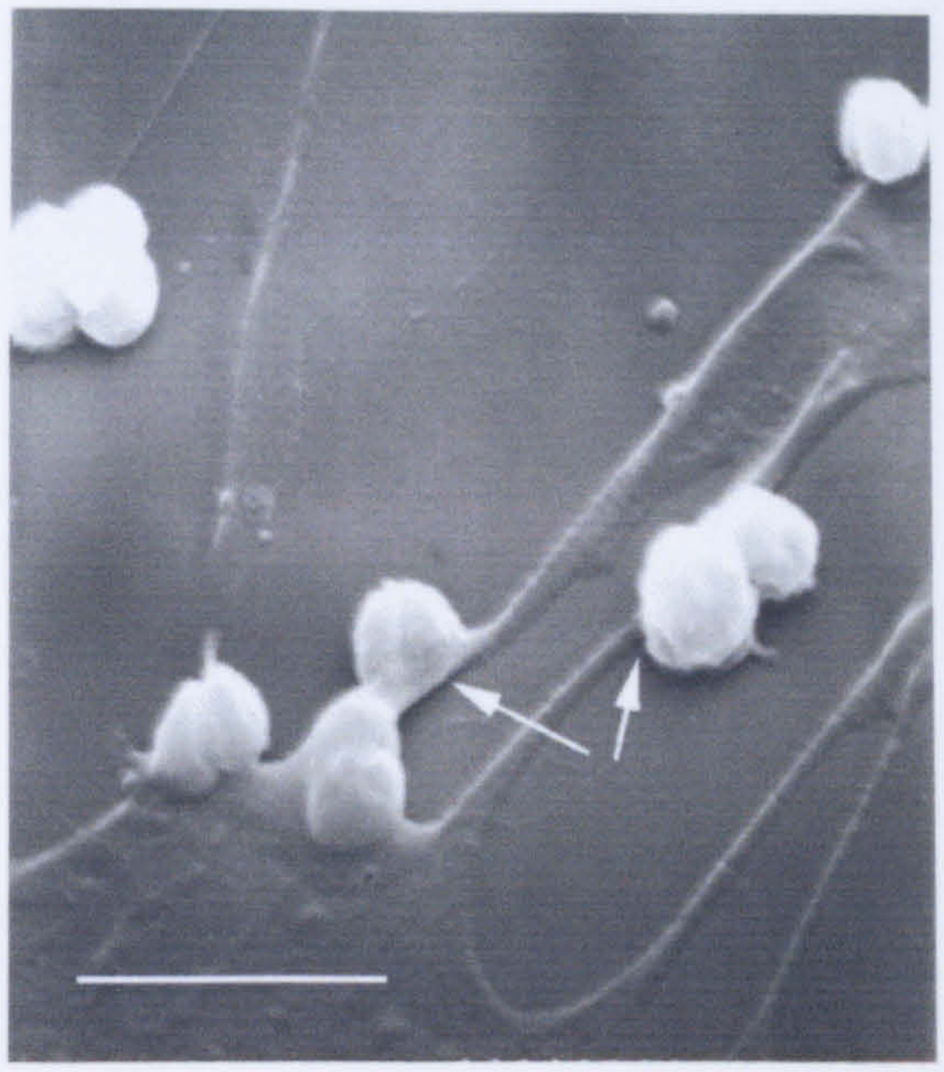


Figure 4.21c: Another view of Figure 4.21a. Infected meningioma cells were often characterised by the presence of cellular processes (arrow) that lied flat against the cell surface.
The scale bar represents 2μm.

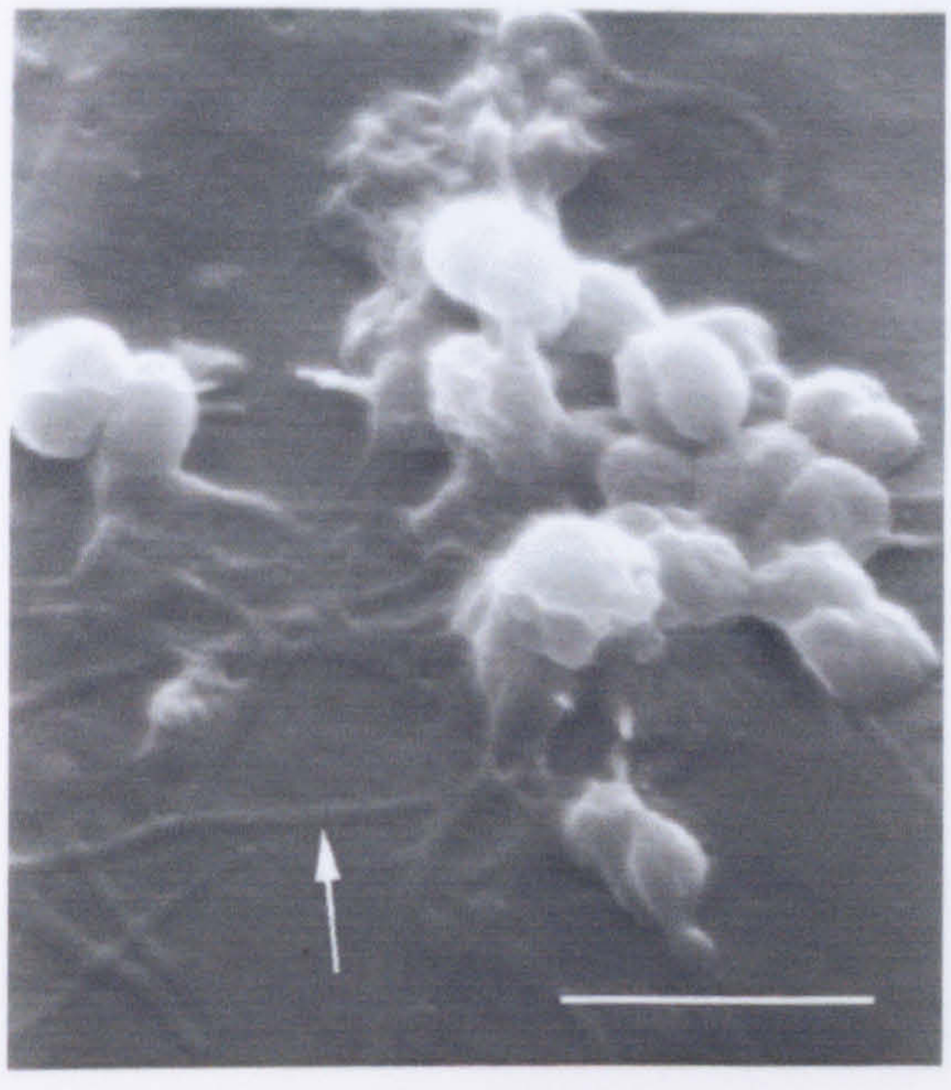


Figure 4.22a: M18 cells infected with 58A ($Pil_{Ia}^{+}Opa^{+}Opc^{+}$) for 24h. Many bacteria were associated with the human cells by this time point, and adherence was especially dense around the nuclear region.
The scale bar represents 20μm.

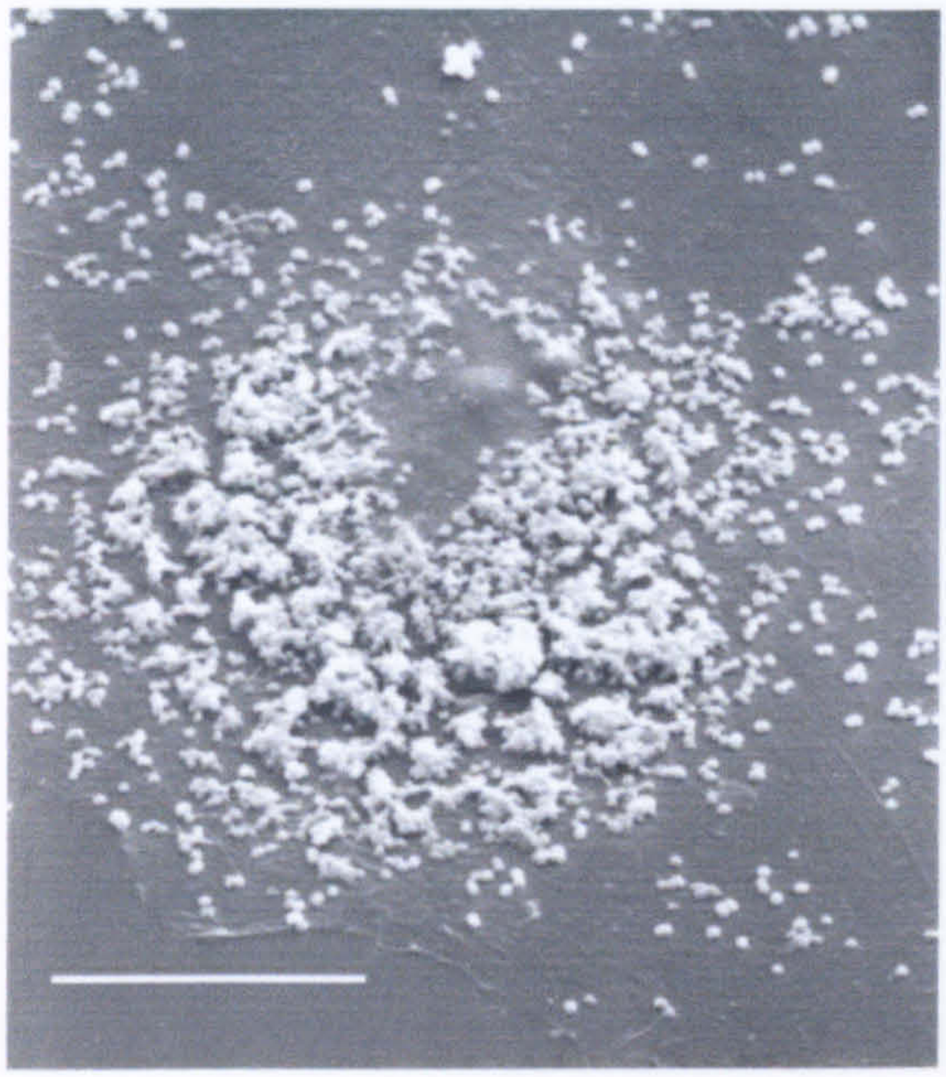


Figure 4.22b: Higher power of Figure 4.22a. The adherent bacteria were often highly aggregated.
The scale bar represents 3μm.

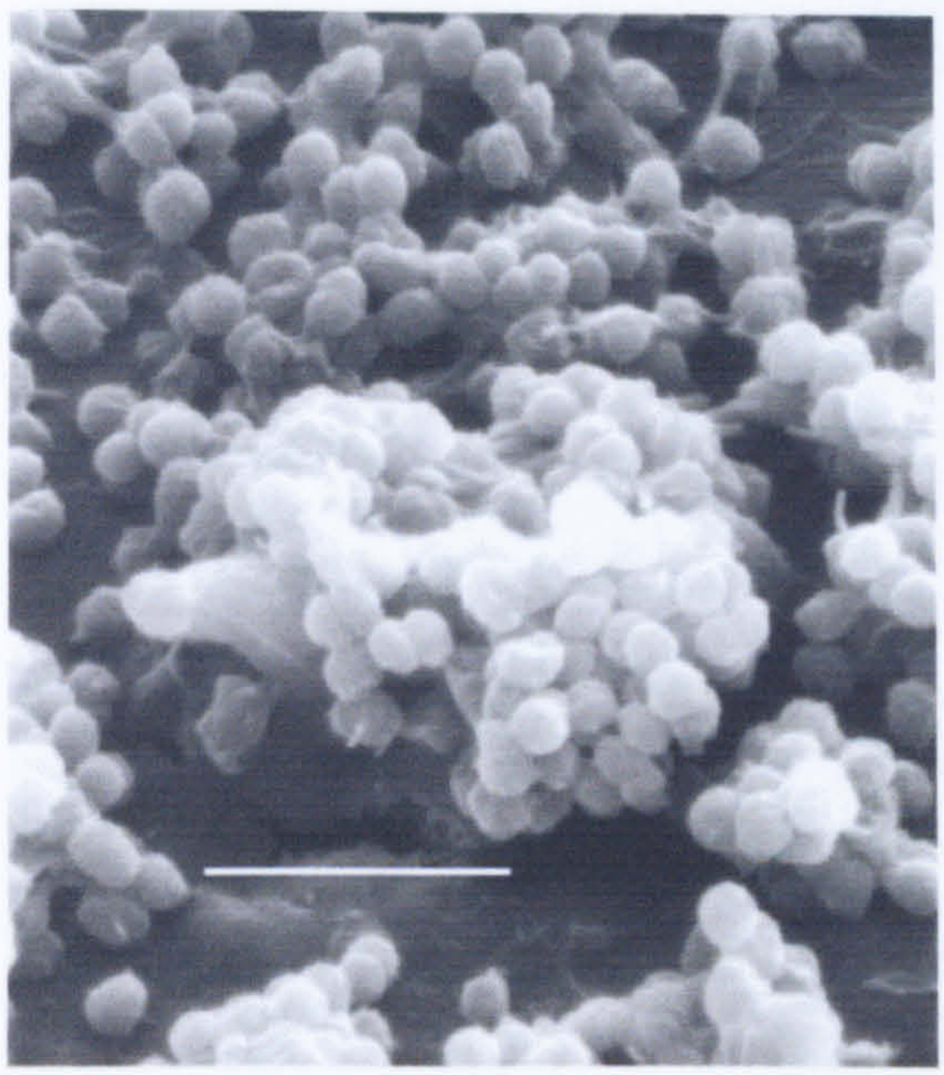


Figure 4.22c: Higher power of Figure 4.22a. Thick pili bundles (arrow) were commonly seen connecting adjacent meningococci and thinner ones (arrow) attaching bacteria to the surface of the meningioma cells.
The scale bar represents 2μm.

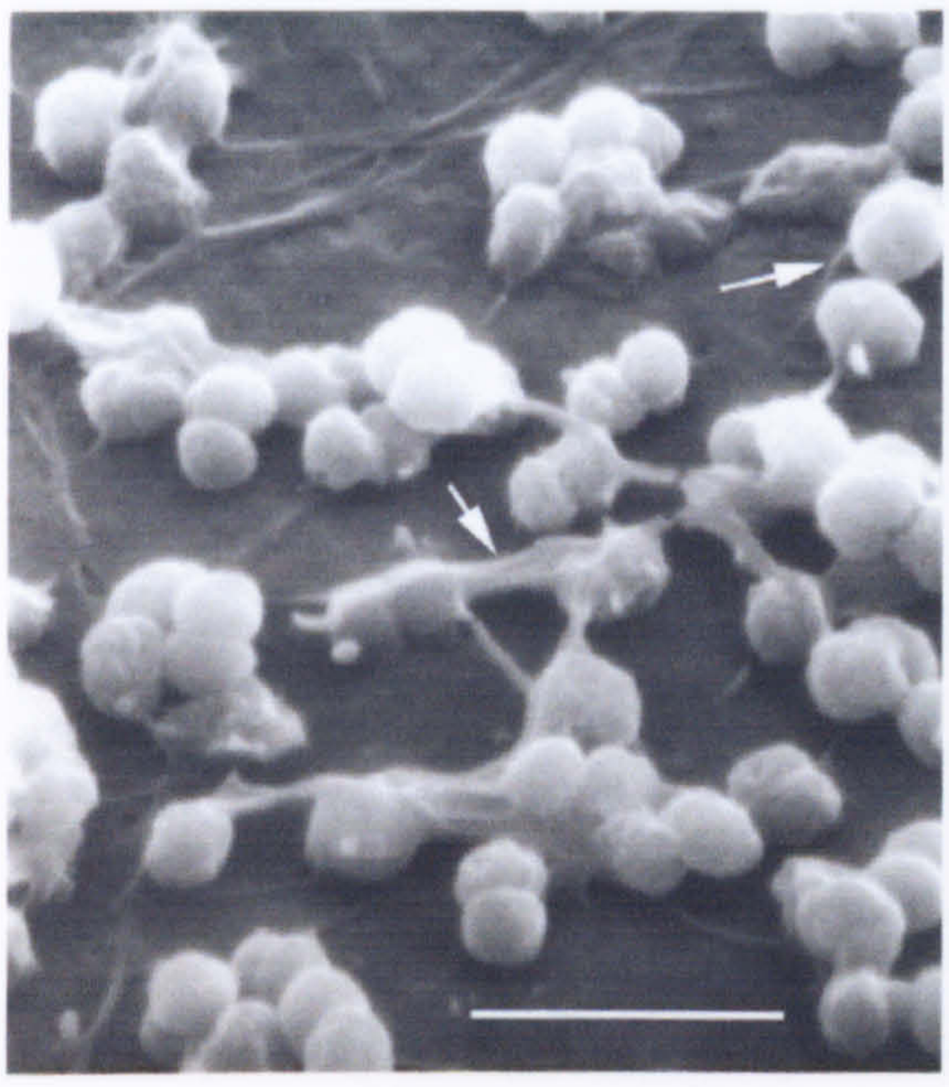


Figure 4.23: M18 cells infected with 58H ($Pil^{-}Opa^{+}Opc^{+}$) for 3h. Here meningococcal association was sparse.
The scale bar represents 20μm.

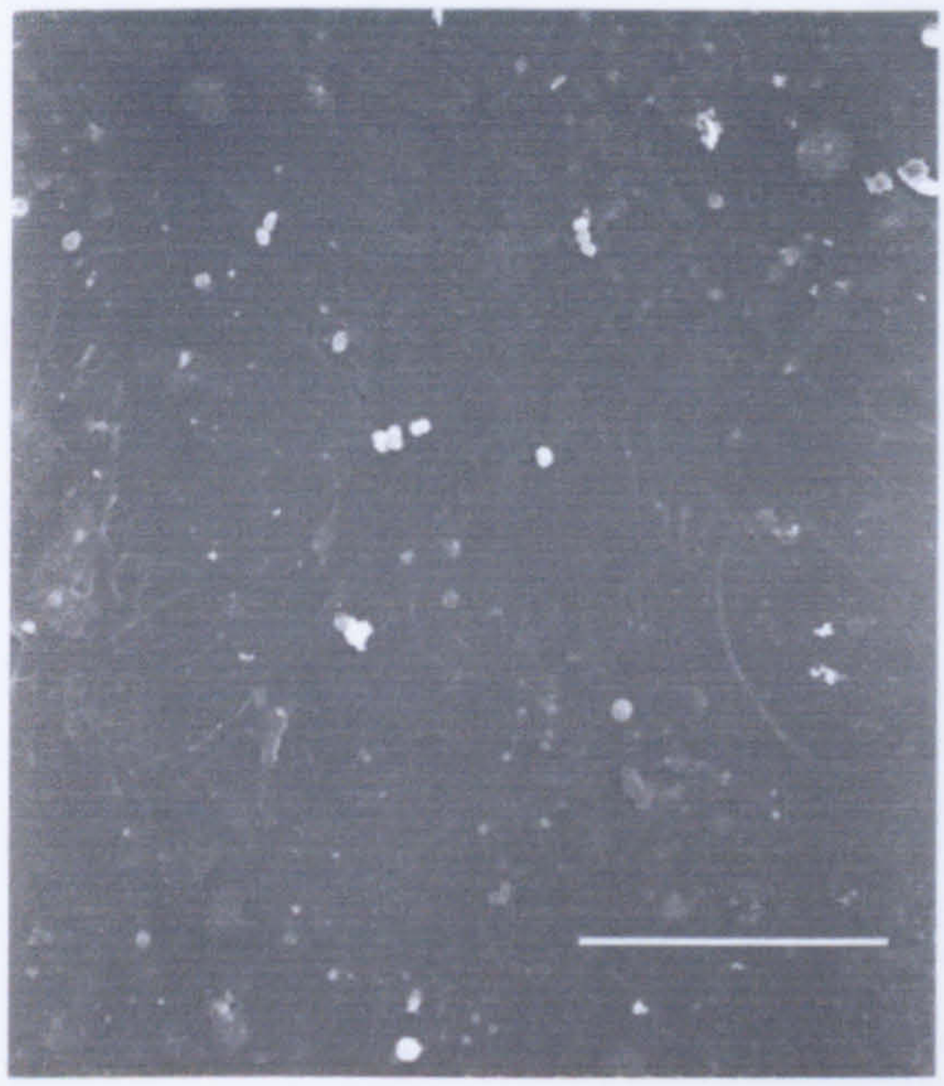


Figure 4.24: M18 cells infected with 58H ($Pil^{-}Opa^{+}Opc^{+}$) for 6h. These meningococci tended to be more evenly spread over the meningioma cell surface than 58A.
The scale bar represents 20μm.

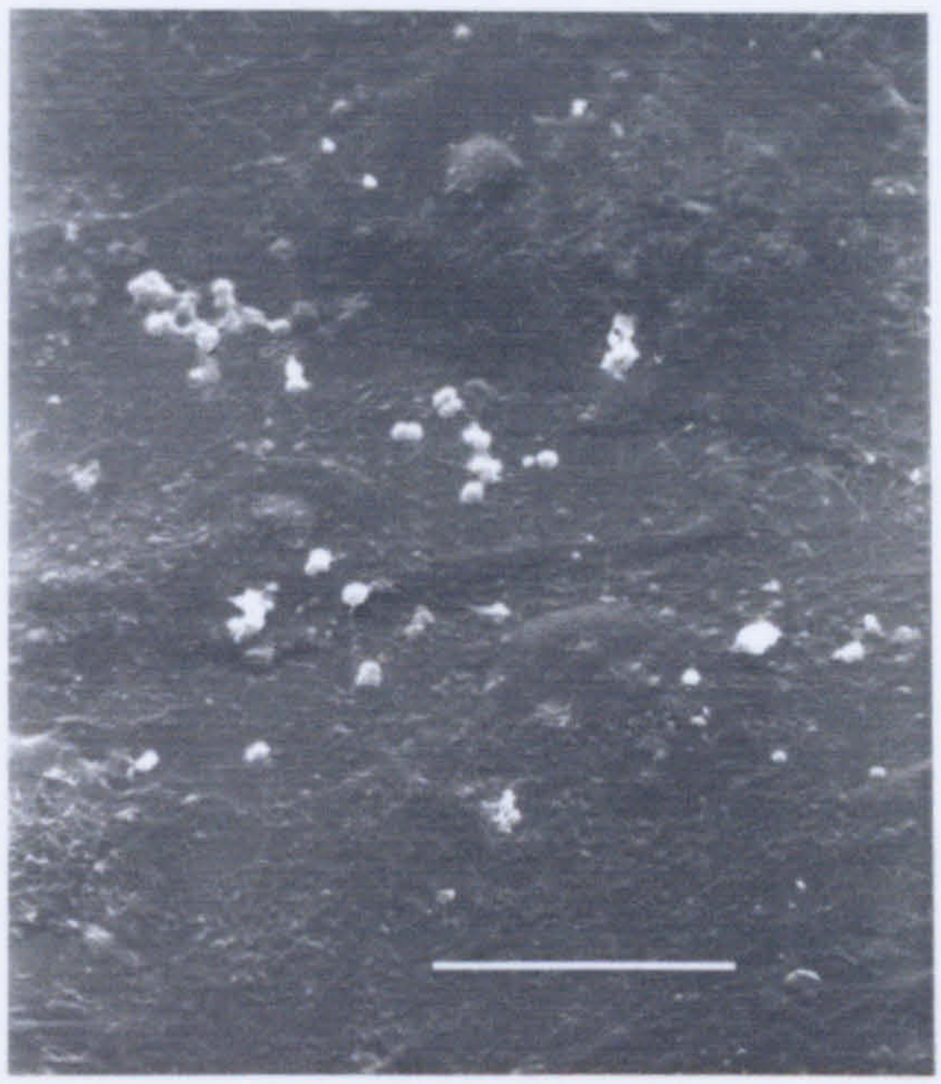


Figure 4.25a: M18 cells infected with 58H ($Pil^{-}Opa^{+}Opc^{+}$) for 24h. Some small clumps of meningococci adhered to the meningioma cells.
The scale bar represents 20μm.

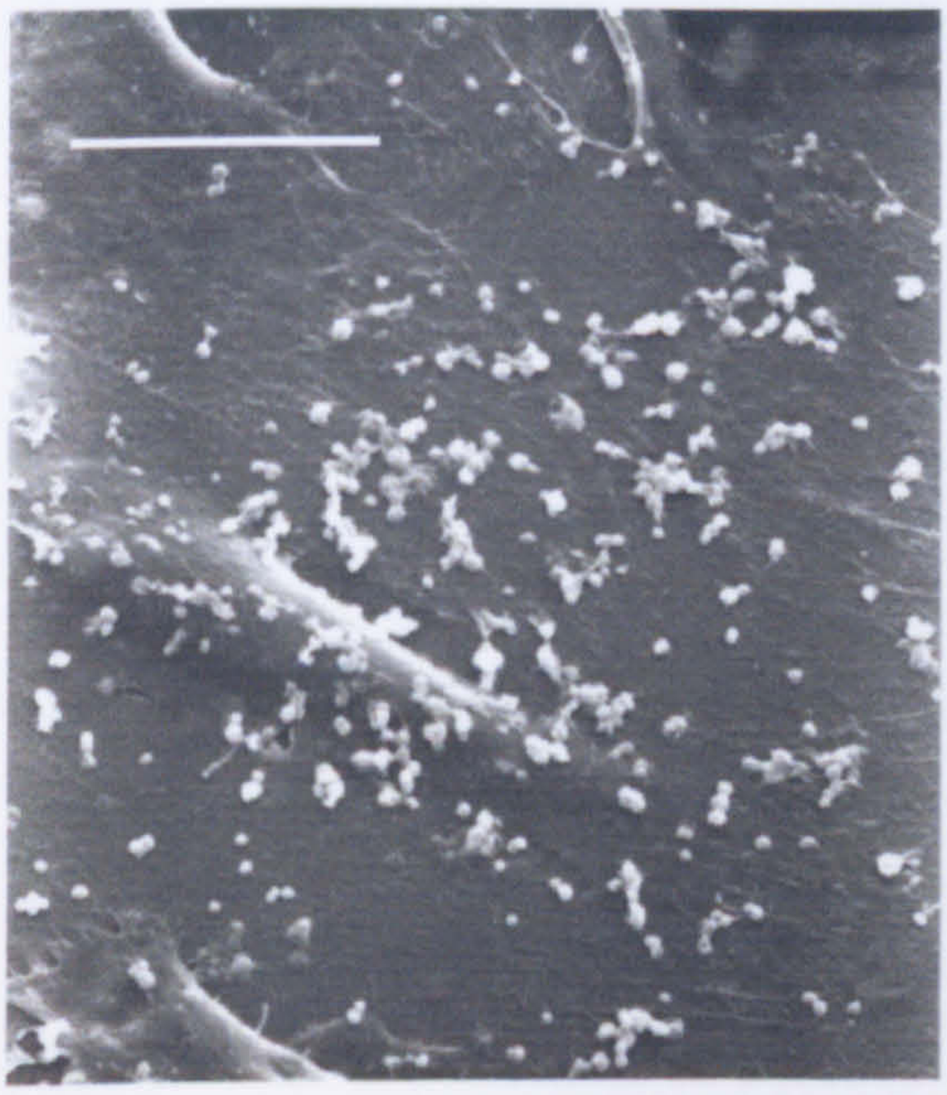
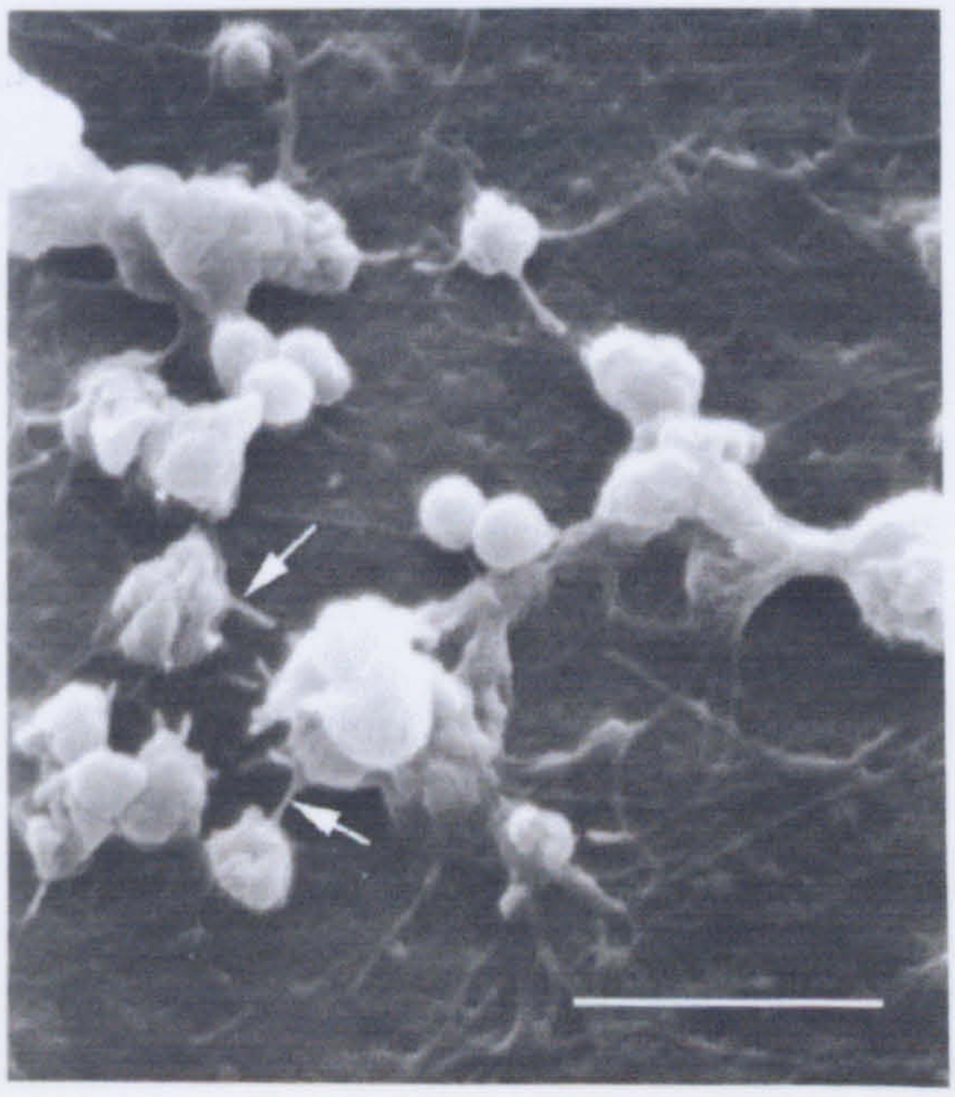


Figure 4.25b: Higher power of Figure 4.25a. Pilus-like structures (arrows) appeared to be present on some adherent bacteria.
The scale bar represents 3μm.



Throughout the time course, piliated ($Pil_{Ia}^{+}Opa^{+}Opc^{+}$) meningococci adhered to meningioma cells in greater numbers than the $Pil^{-}Opa^{+}Opc^{+}$ organisms and the pattern of association was similar to that obtained from the confocal images in the previous section. Analysis of the monolayers infected with $Pil_{Ia}^{+}Opa^{+}Opc^{+}$ revealed that at 3h the adherent meningococci were unevenly distributed over the cells. Figure 4.20a illustrates that in some areas there were groups of adherent organisms, sometimes just isolated diplococci were present, whereas in other areas there were no bacteria at all. Meningococci appeared to associate quite closely with the cells, and occasionally they could be seen sitting on very small uprisings of the meningioma plasmalemma around which the membrane appeared slightly ruffled (Figure 4.20b). Some pili bundles could be seen between adjacent bacteria and attached to the cell surface. After 6h of infection, many more meningococci were visible adhering to the cells, but again the bacteria were not present evenly over the monolayer. The results reflected those obtained in the confocal analysis, with many organisms associating in clumps around the nuclear region of some meningioma cells (Figure 4.21a), whilst elsewhere meningococci were present as diplococci in fewer numbers or even completely absent. In sub-confluent regions near the edge of the coverslip, meningococci could also be seen to associate with cell processes (Figure 4.21b). Cell processes were often present on infected meningothelial cells, but these did not protrude far and usually seemed to lie flat along the cell surface (Figure 4.21c). At 24h, there were considerably more adherent meningococci present than at other time points and again, these appeared to be unevenly distributed over the cells (Figure 4.22a). The bacteria were often highly aggregated (Figure 4.22b) and localised around the nuclear region of meningioma cells, although some areas of the monolayer had few adherent organisms that existed as diplococci rather than in clumps. Apparent pili bundles were commonplace and thick bundles were present connecting adjacent bacteria, with smaller pili aggregates attaching meningococci to the human cell surface (Figure 4.22c). However, throughout the time course it was not always clear whether the structures linking meningococci to meningothelial cells originated from the bacteria or the host cells, as cytoplasmic processes were often indistinguishable from meningococcal pili. In addition, pili were not always apparent on the bacteria that were most closely associated with the meningothelial cells.

After 3h of infection, non-piliated ($Pil^{-}Opa^{+}Opc^{+}$) meningococci associated to meningioma cells in very low numbers with probably no more than about ten adherent bacteria per cell, mostly as diplococci or occasionally as groups of diplococci (Figure 4.23). Samples infected for 6h were similar to those at 3h but with slightly more adherent bacteria (Figure 4.24). Variant $Pil^{-}Opa^{+}Opc^{+}$ meningococci did not appear to bind specifically to regions around meningioma cell nuclei, and they tended to be more evenly spread over the cell surface than the $Pil^{+}_{Ia}Opa^{+}Opc^{+}$ organisms. However, there were random areas of cells to which these organisms did not adhere. Cell processes were present on the meningioma cell surface at least to the same extent as with the cells infected with the Pil^{+} organisms. After 24h, more meningococci had associated with the M18 cells and some small clumps of bacteria were present (Figure 4.25a), although the Pil^{-} organisms did not clump to

the same extent as the Pil^+. Again these meningococci were more evenly spread over the cells and showed little specificity for any particular eukaryotic cell structures. Interestingly after 24h of infection of Pil^- organisms, some meningococci appeared to possess structures resembling pili that linked the bacteria to the meningioma cell surface (Figure 4.25b). Such structures were considerably thinner than the pili bundles produced by the Pil^+ organisms after the same period of infection (Figure 4.22c).

4.2.4 Investigation into the barrier properties of meningioma cells against meningococcal invasion

The invasive characteristics of certain meningococci for cultured epithelial and endothelial cells have been studied in some detail, but the possibility that these bacteria can invade meningioma cells has not been investigated previously. Cultured meningioma cells are known to endocytose and retain latex particles of 1μm diameter (Feurer & Weller, 1991) and so, on the basis of size, they are capable of internalising meningococci. To test for the possibility that meningioma cells could internalise meningococci, meningioma cells were subject to assays designed to measure and observe this interaction.

4.2.4.1 Gentamicin survival assay

a) Time Course Experiments

Meningioma cell lines of the subtypes meningothelial (M18, M21) transitional (M17) and anaplastic (M16) were infected with $Pil^+_{Ia}Opa^+Opc^+$ and $Pil^-Opa^+Opc^+$ meningococcal variants for 1, 3, 6, 9 and 24h (Section 2.12.1). Invading organisms were then quantified with a standard gentamicin survival assay, as described by other workers (Shaw & Falkow, 1988; Virji *et al.*, 1991); the rationale of this method is described in Section 2.12.1. The data were represented as the total number of cfu surviving gentamicin treatment (Figures 4.26-4.29).

After infection of meningothelial cells (M18; Figure 4.26 and M21; Figure 4.27) the number of meningococci that survived gentamicin treatment was consistently very low in comparison to the numbers associated (Section 4.2.1.1). The piliated and non-piliated isolates survived in similar numbers and overall the survival of both organisms increased with time. After six hours of infection with $Pil_{Ia}^+Opa^+Opc^+$, less than 900 cfu survived the antibiotic treatment, and this represented less than 0.09% of the total number of cell-associated cfu, which was greater than $3x10^6$ cfu per monolayer. The survival of $Pil_{Ia}^+Opa^+Opc^+$ organisms was similar between M18 and M21 cells until 24h, when this variant appeared to survive in greater numbers after infection of M21.

Meningococcal survival after gentamicin treatment as a measure of internalisation with meningothelial cells. Internalisation is represented as the total number of cfu surviving the antibiotic per monolayer. Each graph shows the mean values of triplicate determinations from a single experiment, and the error bars represent the standard deviation.

Figure 4.26: M18

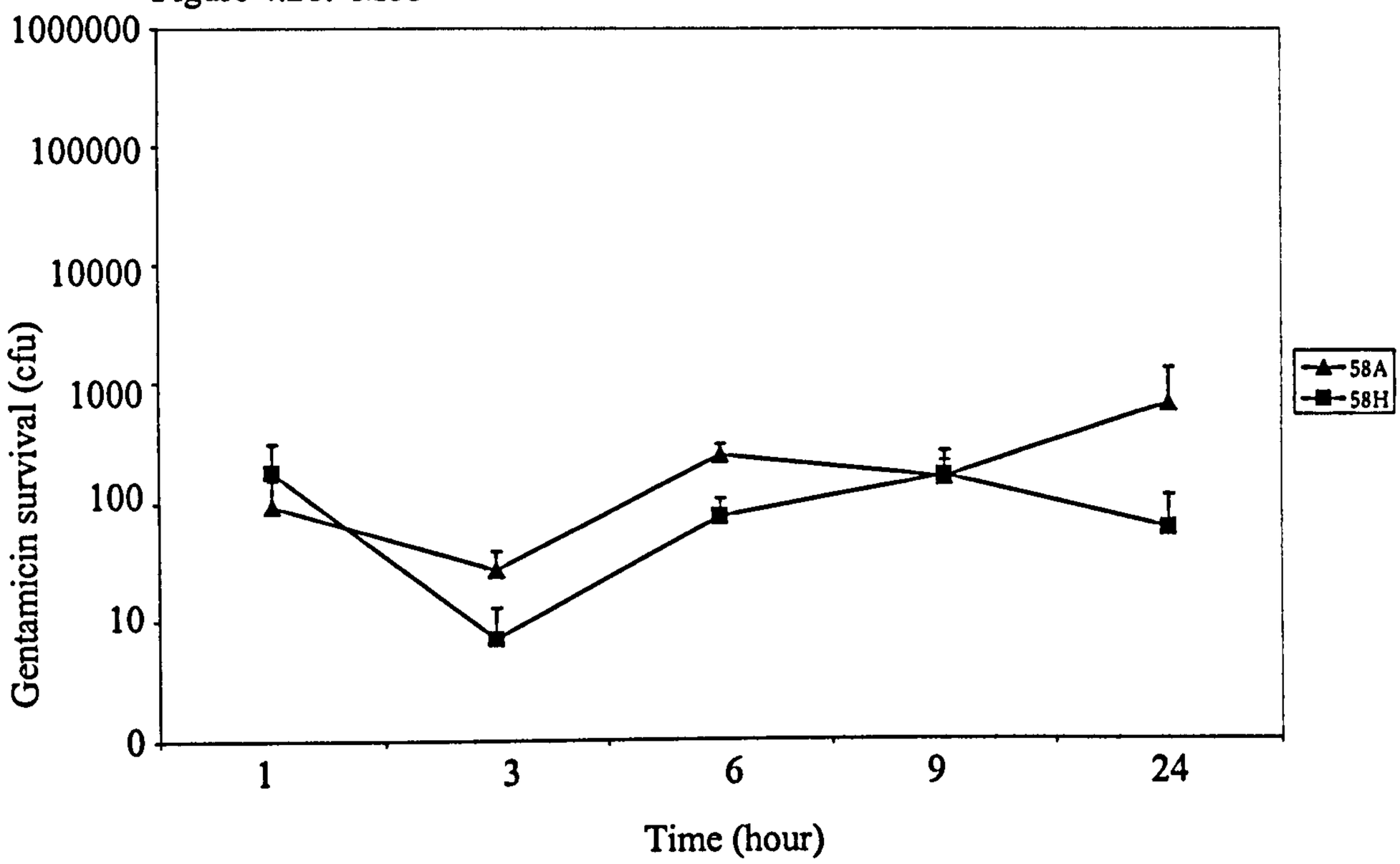
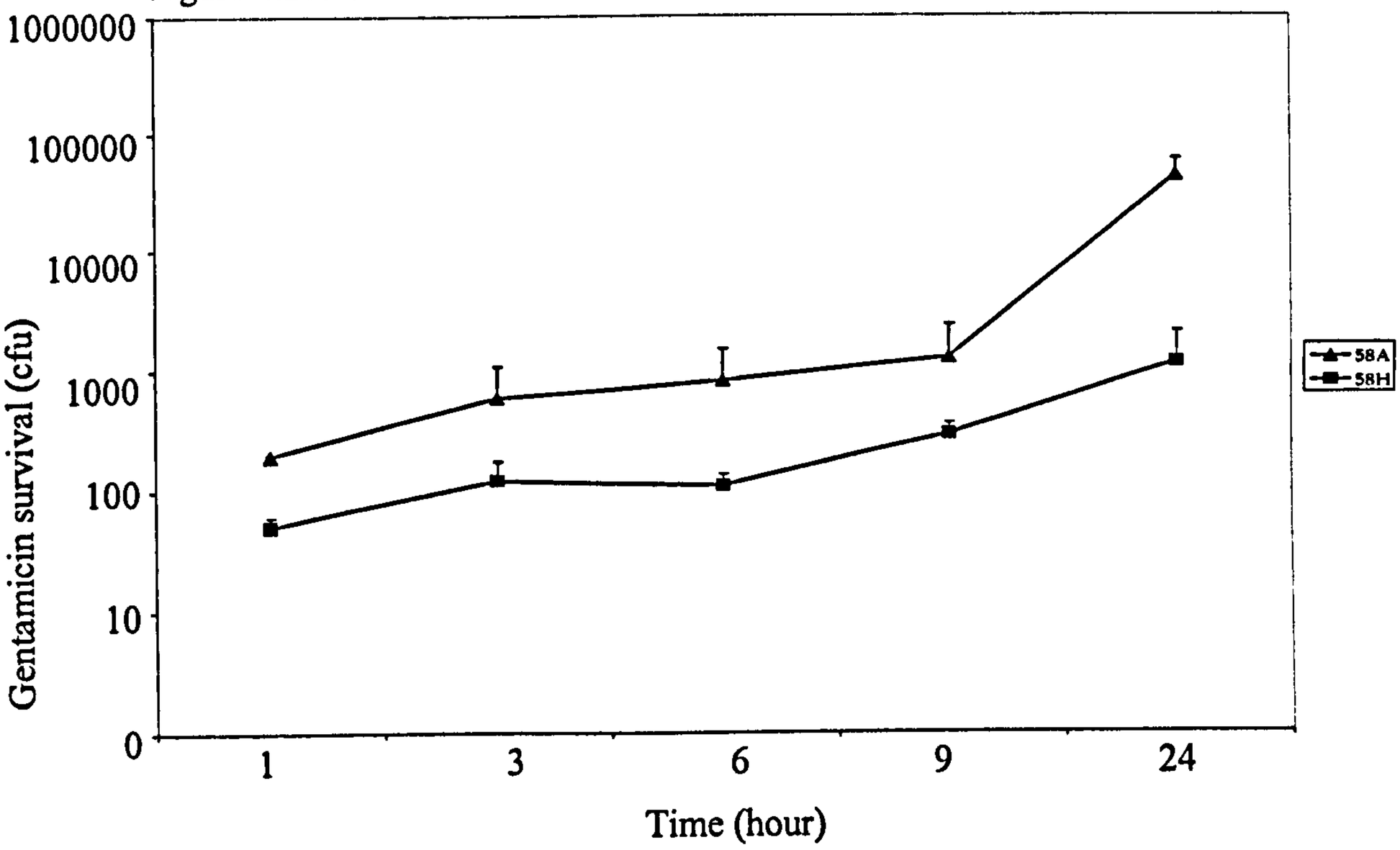


Figure 4.27: M21



However, at later time points in particular, the number of surviving organisms was variable between the triplicate wells, and also between repeated experiments. The total cfu surviving gentamicin treatment after 24h infection of M21 cells ranged from $2x10^3 - 4x10^4$ but overall, bacterial survival after infection of meningothelial cells for 24h was always less than 0.41% of the total number of cell-associated cfu. For each experiment with meningothelial cells, the survival of $Pil^-Opa^+Opc^+$ meningococci was less than $1.1x10^3$ cfu per monolayer at all time points, which represented maximum survival of less than 0.2% of the total cell-associated cfu.

The time course assay was repeated with transitional cells (Figure 4.28). Up to and including the 9h time point, the survival of the $Pil_{Ia}^+Opa^+Opc^+$ organism largely reflected the results obtained with meningothelial cells. However, after 24h nearly $3x10^5$ organisms survived gentamicin treatment which represented 1.8% of the total cell-associated cfu, and which was considerably greater than the survival found with meningothelial cells. The survival of $Pil^-Opa^+Opc^+$ organisms after infection of transitional was less than 0.21% of the total cell-associated cfu, which was very similar to survival with meningothelial cells.

The results obtained with the $Pil_{Ia}^+Opa^+Opc^+$ infection of anaplastic cells (Figure 4.29) also reflected the findings with meningothelial cells until the 24h time point. At this time, nearly $3.9x10^4$ cfu survived gentamicin and this was equivalent to just under 1% of the total cell-associated cfu. The survival of $Pil^-Opa^+Opc^+$ organisms after infection of anaplastic cells was less than 1.2% of the total cell-associated cfu which was higher than the survival seen with meningothelial and transitional cells.

These experiments suggest that after 24h of infection, meningococci of the $Pil_{Ia}^+Opa^+Opc^+$ phenotype were capable of invading meningioma cells, and that they were more capable of invading the transitional and anaplastic subtypes than meningothelial. However, it is also possible that the survival of apparently intracellular organisms could be attributed to experimental error; there was often variability between wells, a phenomenon that was more prominent with later time points (Figure 4.27-4.29), and which prevented statistical significance from being tested. There was also considerable variation between repeat experiments done on different days. Other workers have reported an experimental error inherent with this infection system (deVries *et al.*, 1996; Shaw & Falkow, 1988). Because meningococci tend to clump together and form large aggregates, extracellular bacteria within these clumps may have been protected from the action of gentamicin thus providing false counts of surviving organisms. In addition, the washing of the monolayers prior to antibiotic treatment could have been uneven, which could have exacerbated this problem. It is possible therefore that $Pil_{Ia}^+Opa^+Opc^+$ bacteria surviving gentamicin treatment are actually extracellular and that these meningococci do not invade meningioma cells. Some $Pil^-Opa^+Opc^+$ bacteria also survived gentamicin treatment, although there was less variation between triplicate

Meningococcal survival after gentamicin treatment as a measure of internalisation with transitional (Figure 4.28) and anaplastic (Figure 4.29) cells. Internalisation is represented as the total number of cfu surviving the antibiotic per monolayer. Each graph shows the mean values of triplicate determinations from a single experiment, and the error bars represent the standard deviation.

Figure 4.28: M17

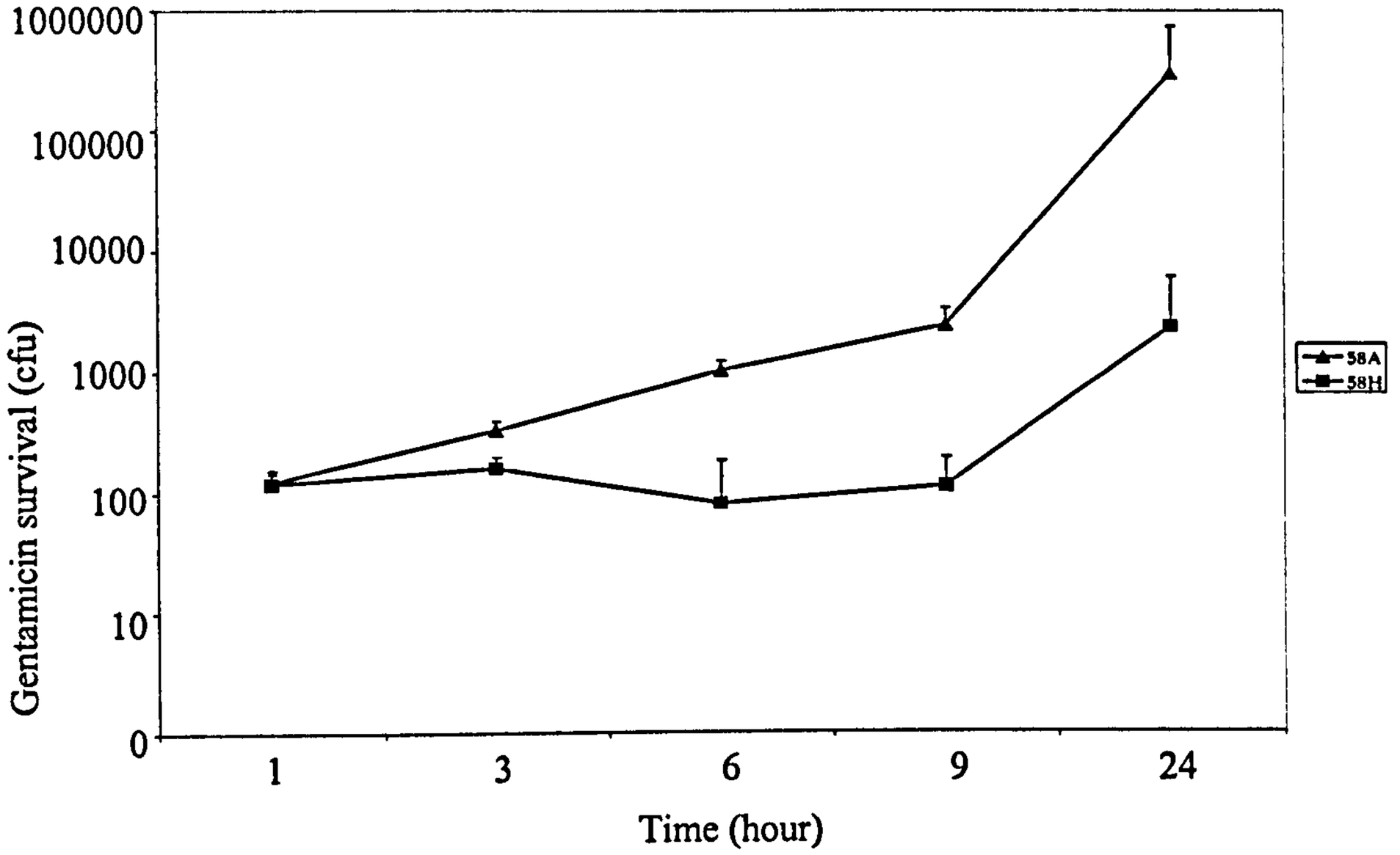
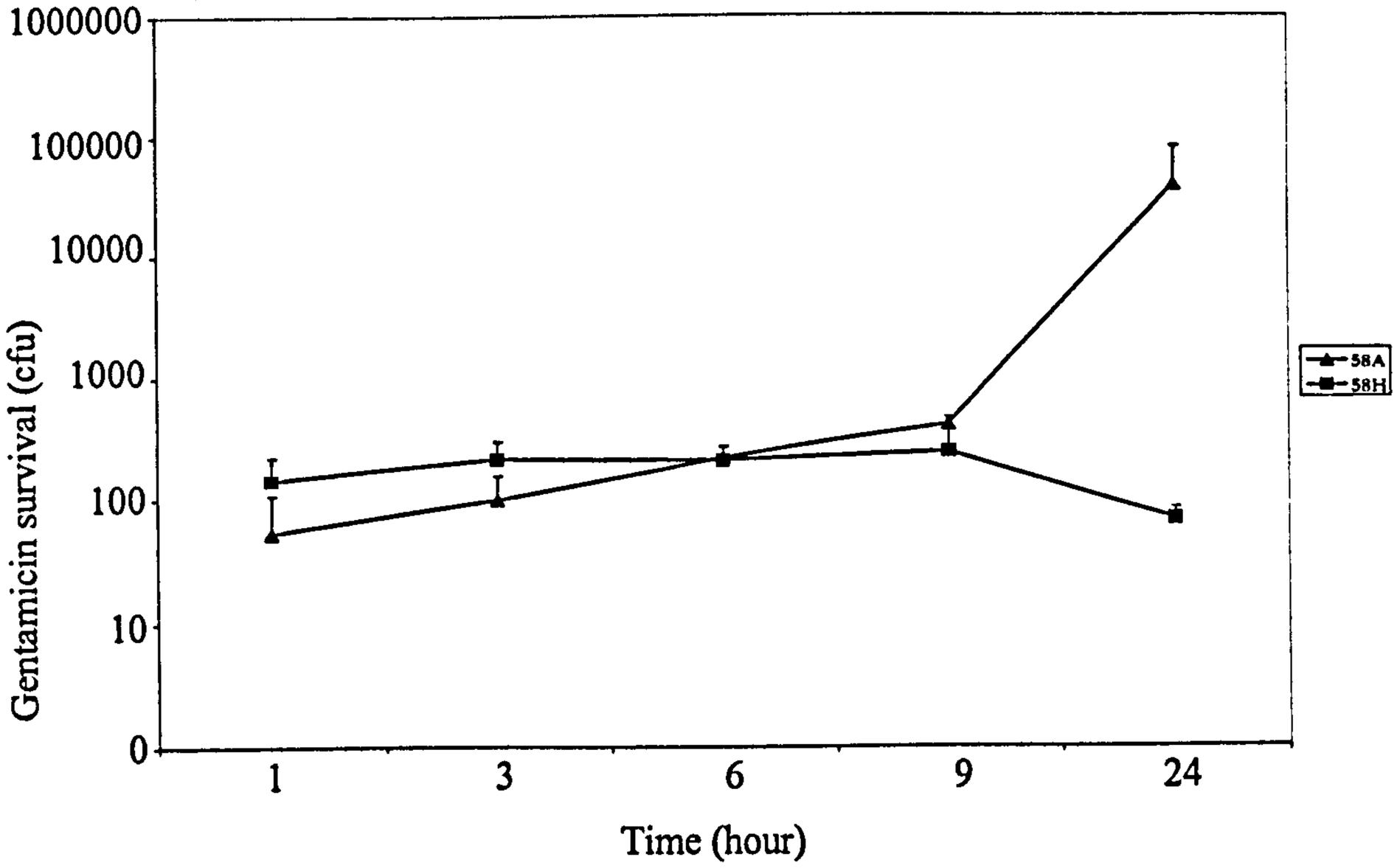


Figure 4.29: M16



wells than there was with the Pil^+ organisms. It is also quite possible that the Pil^- bacteria were extracellular for the reasons given above. The survival of non-piliated meningococci was highest with anaplastic cells after 24 h of infection. However, this survival rate was at an early time point and the actual number of surviving organisms did not increase throughout the time course, whilst total cell association continued to rise. Thus the survival rate decreased with time and it is possible that the numbers of organisms surviving at the beginning of the experiment represented background survival and were not truly invasive.

b) Comparison between phenotypic variants

To test whether variation in meningococcal pili and other surface antigens were capable of mediating invasion of meningioma cells, all of the capsulate MC58/59 variants (Table 3.1) were used in the gentamicin survival assay. Meningothelial (M18 and M21), transitional and anaplastic cells were infected with these variants for 6h, incubated with gentamicin and processed as in the standard method (Section 2.12.1). This time point was chosen as it had previously been used in the detailed analysis of total meningococcal association to meningioma cells (Section 4.2.1.3), and also experiments had shown less variation in survival between wells than at 24h.

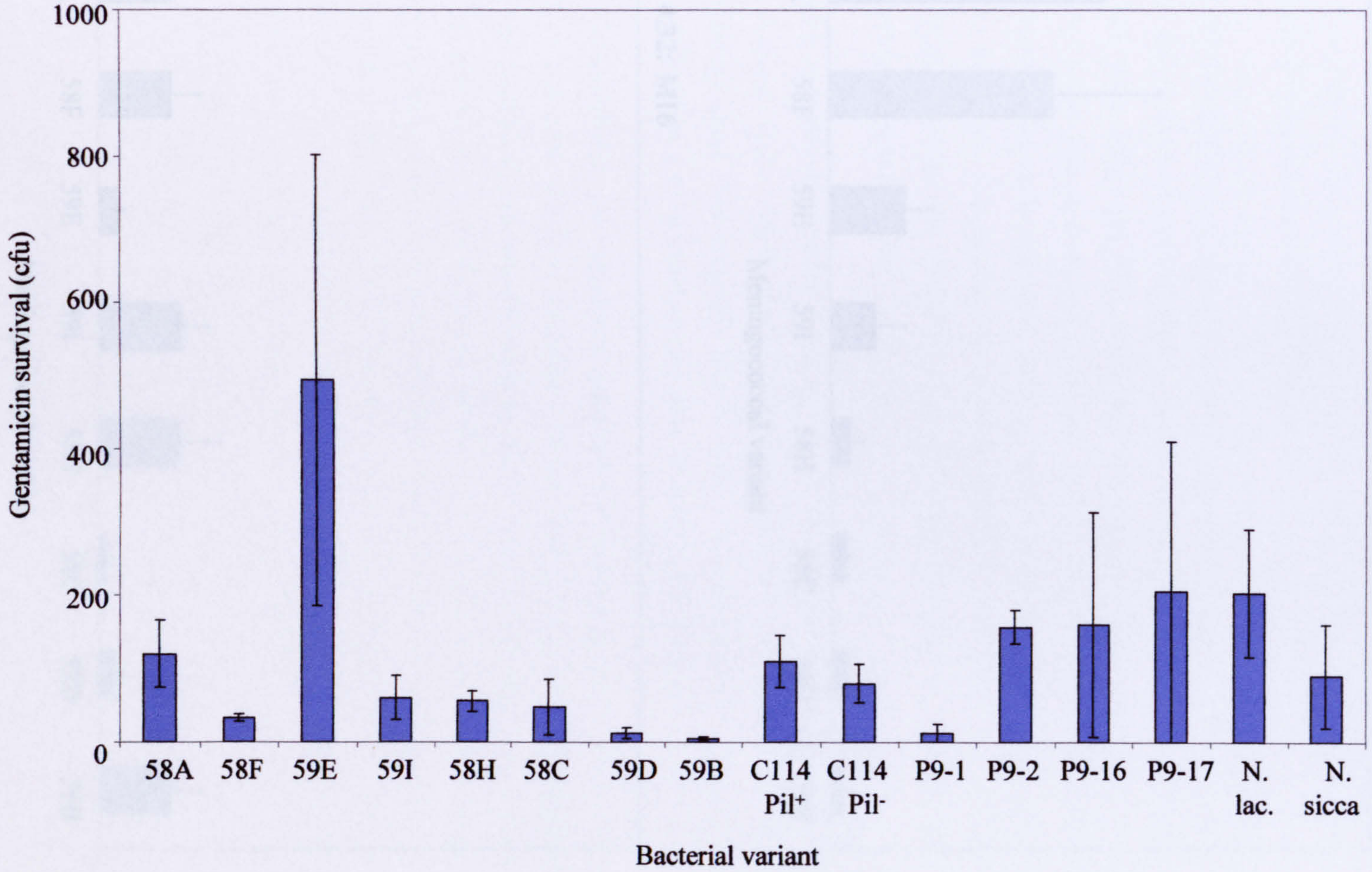
The results obtained with the M18 cell line were representative of the pattern for both of the meningothelial cell lines tested (Figure 4.30). The actual numbers of surviving bacteria were very low, always being less than 600 cfu per monolayer and, generally, piliated organisms survived in slightly higher numbers than the non-piliated variants. The variant $Pil_{Ib}^+Opa^+Opc^-$ survived the gentamicin treatment in the highest numbers, although the variation between wells was high and it is possible that this organism was not internalised any more than the other piliated isolates. The M18 cell line was also infected with meningococcal strain C114 (which expresses Pil_{II} pili), gonococci and commensal strains of Neisseria described in Table 3.2. These bacteria did not survive gentamicin treatment to significantly greater extent than the MC58/59 variants (Figure 4.30).

Invasion assays performed on transitional meningioma cells showed just a slightly different pattern of results from the meningothelial cells (Figure 4.31). Again piliated organisms survived in higher numbers than non-piliated, and although the isolates $Pil_{Ia}^+Opa^+Opc^+$ and $Pil^+_{Ia}Opa^-Opc^+$ were the best survivors, the actual counts were still less than 600 cfu per monolayer.

All of the meningococcal variants survived to a very low rate when invasion assays were done on anaplastic meningioma cells (maximum of 150 cfu per monolayer), and piliated organisms survived at a similar level to non-piliated (Figure 4.32).

Taken together these data suggest that after 6h there was no significant invasion of meningioma cells by encapsulated meningococci, gonococci or commensal strains regardless of the surface

Figure 4.30: Bacterial survival after gentamicin treatment comparing the meningococcal MC58/59 variants with meningococci expressing pili of a different class, with gonococcal variants and with commensal Neisseria. The graph shows the actual number of cfu surviving after infection with the meningothelial cell line M18.
Not all bacteria were tested on the same day but $Pil_{Ia}^{+}Opa^{+}Opc^{+}$ was included in every experiment as a control, and the mean values of at least triplicate estimations are shown. The error bars represent the standard error of the mean



Bacterial survival after gentamicin treatment comparing the meningococcal MC58/59 variants. The graph shows the actual number of cfu surviving after infection with the transitional cell line M17 (Figure 4.31) and the anaplastic cell line M16 (Figure4.32), with the SEM. Not all bacteria were tested on the same day but $Pil_{Ia}^{+}Opa^{+}Opc^{+}$ was included in every experiment as a control, and the mean values of at least triplicate estimations are shown.

Figure 4.31: M17

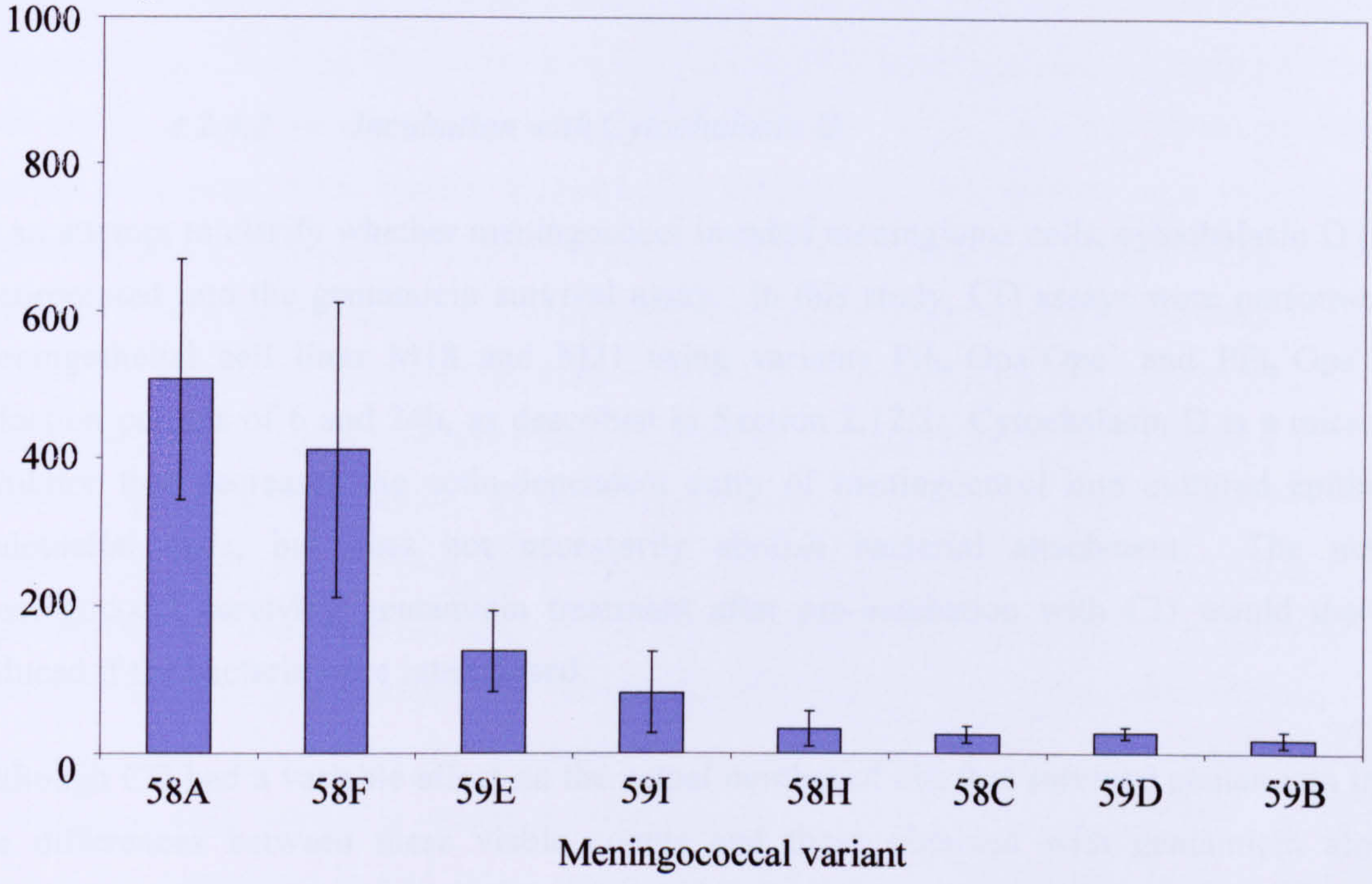
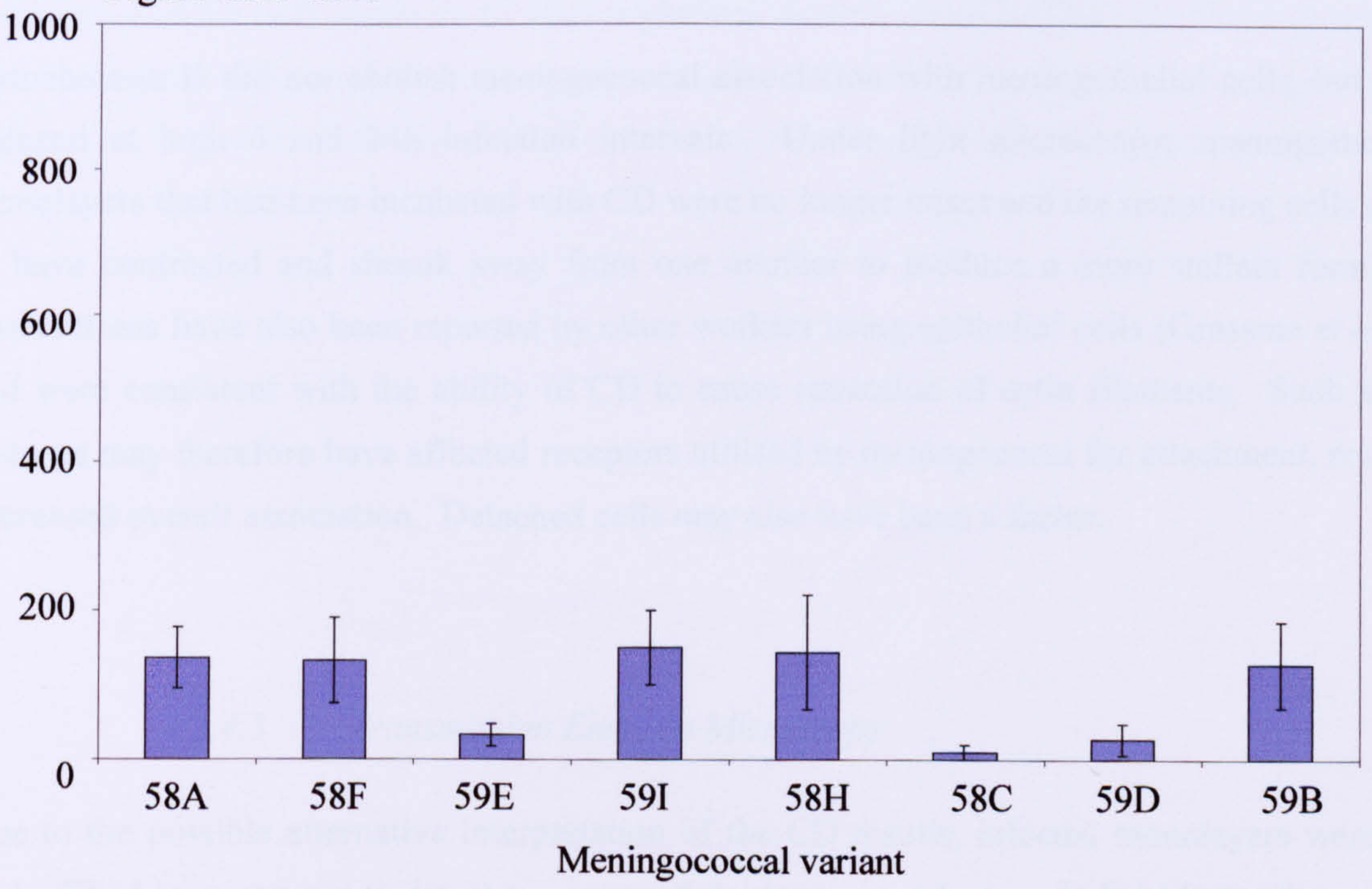


Figure 4.32: M16



structures present on these bacteria. With meningothelial and transitional cells, it is likely that Pil^+ bacteria survive in higher numbers than Pil^- because of their greater tendency to clump together during the experiment, and they could therefore be protected from the actions of the antibiotic. These results also suggest that no significant invasion of meningothelial cells was achieved by certain gonococci, commensal *Neisseria* and meningococci expressing a different class of pili and a different capsule.

4.2.4.2 Incubation with Cytochalasin D

In an attempt to clarify whether meningococci invaded meningioma cells, cytochalasin D (CD) was incorporated into the gentamicin survival assay. In this study, CD assays were performed on the meningothelial cell lines M18 and M21 using variants $Pil_{Ia}^+Opa^+Opc^+$ and $Pil_{Ia}^+Opa^-Opc^+$ for infection periods of 6 and 24h, as described in Section 2.12.2. Cytochalasin D is a microfilament inhibitor that decreases the actin-dependent entry of meningococci into cultured epithelial and endothelial cells, but does not necessarily abolish bacterial attachment. The number of meningococci surviving gentamicin treatment after pre-incubation with CD would therefore be reduced if the bacteria were internalised.

Although CD had a variable effect on the actual number of cfu that survived gentamicin treatment, the differences between these viable counts and those obtained with gentamicin alone were insignificant. Although these data suggested that invasion did not occur, the CD assay could not eliminate the possibility that some organisms may have invaded meningothelial cells.

Cytochalasin D did not abolish meningococcal association with meningothelial cells, but this was reduced at both 6 and 24h infection intervals. Under light microscopy, meningothelial cell monolayers that had been incubated with CD were no longer intact and the remaining cells appeared to have contracted and shrunk away from one another to produce a more stellate form. These observations have also been reported by other workers using epithelial cells (Grassme *et al.*, 1996) and were consistent with the ability of CD to cause retraction of actin filaments. Such structural changes may therefore have affected receptors utilised by meningococci for attachment, resulting in decreased overall association. Detached cells may also have been a factor.

4.2.4.3 Transmission Electron Microscopy

Due to the possible alternative interpretation of the CD results, infected monolayers were viewed under TEM in an attempt to detect any intracellular bacteria. Meningothelial M18 cells were grown to confluence on collagen coated Thermanox coverslips and then infected with piliated

$Pil_{Ia}^{+}Opa^{+}Opc^{+}$ meningococci for 24h; the control wells were incubated with maintenance medium only. The coverslips were processed and viewed as in Section 2.13.5.

Examination of more than 20 grids (each containing at least 1 section) showed no evidence of internalised meningococci. Overall, infected meningothelial cells, in the planes of view available, appeared to possess less rough endoplasmic reticulum and more cellular processes than the control cells. In infected samples bacteria were often seen in close association with the surface of the meningioma cells, and pili-like structures could sometimes be seen to mediate this interaction (Figure 4.34). However, the absence of pili in a section could not rule out their presence as they will not be present in every plane of view (Figures 4.34,4.36). In areas where the bacteria and human cells were most closely apposed, the meningioma cell surface was sometimes slightly depressed into a very shallow cup at the site of the attached organism (Figure 4.34). Occasionally, diplococci were closely associated with small protrusions of the plasmalemma (Figure 4.35) which looked like small cell processes as seen in the SEM samples (Section 2.2.3.2). Coated pits were present on both the apical and basolateral surfaces of infected cells, evidence that the cells were still performing metabolic processes. The apical pit shown in Figure 4.35 is at the base of a cell process, which is folding over. Earlier SEM analysis also showed cell processes that seemed to lie flat against the cell surface. The depth of the cell monolayer was generally thin, and sometimes the meningococci had greater depth than the distance between the apical and basolateral surfaces of the meningothelial cells, especially in regions of the cell processes. Even after 24h of infection, the meningioma cells seemed to remain relatively inert and indifferent to the bacterial challenge. Most cells in the monolayer looked intact, but in some areas the cells appeared to have lost membrane integrity and disintegrated (Figure 4.37). Likewise not all bacteria remained viable after 24h of infection, and the only structure of dead organisms to stain was the outer membrane (Figure 4.34). Meningococcal blebs are known to contain components toxic to human cells and in this experiment blebs were frequently seen emanating from the bacterial surface and free in the immediate vicinity of the monolayer (Figure 4.35).

Figure 4.33: Transmission electron micrograph of an uninfected meningioma 18 cell.
This field shows the end of the nucleus, mitochondria and rough endoplasmic reticulum. The apical surface lacks processes and is quite smooth. Processes from other cells can be seen running below the main cell on its basolateral side.
Magnification x 22800.

Figure 4.34: TEM of an infected meningioma 18 cell.
The M18 cells were infected with 58A ($Pil_{Ia}^{+}Opa^{+}Opc^{+}$) for 24h. Pili appear to attach a diplococcus to the M18 surface; and at points of close association there are slight depressions of the plasmalemma.
Magnification x 22420.

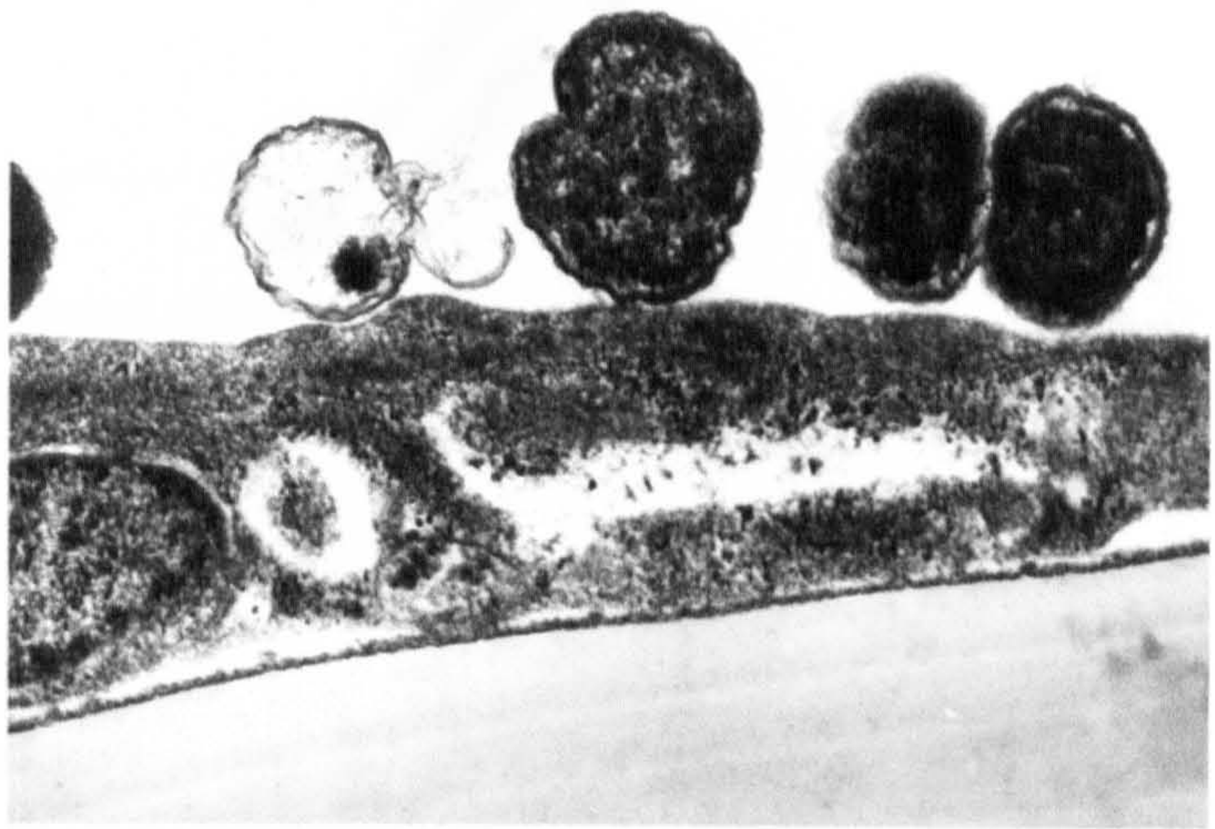


Figure 4.35: TEM of M18 cells infected with 58A for 24h.
A diplococcus is seen closely associated with a small protrusion of the M18 plasmalemma, at the base of which is a coated pit. Another coated pit is present on the basolateral side of the cell. Outer membrane blebs are visible (arrows) both emanating from the bacteria and free around the cells.
Magnification x 19000.

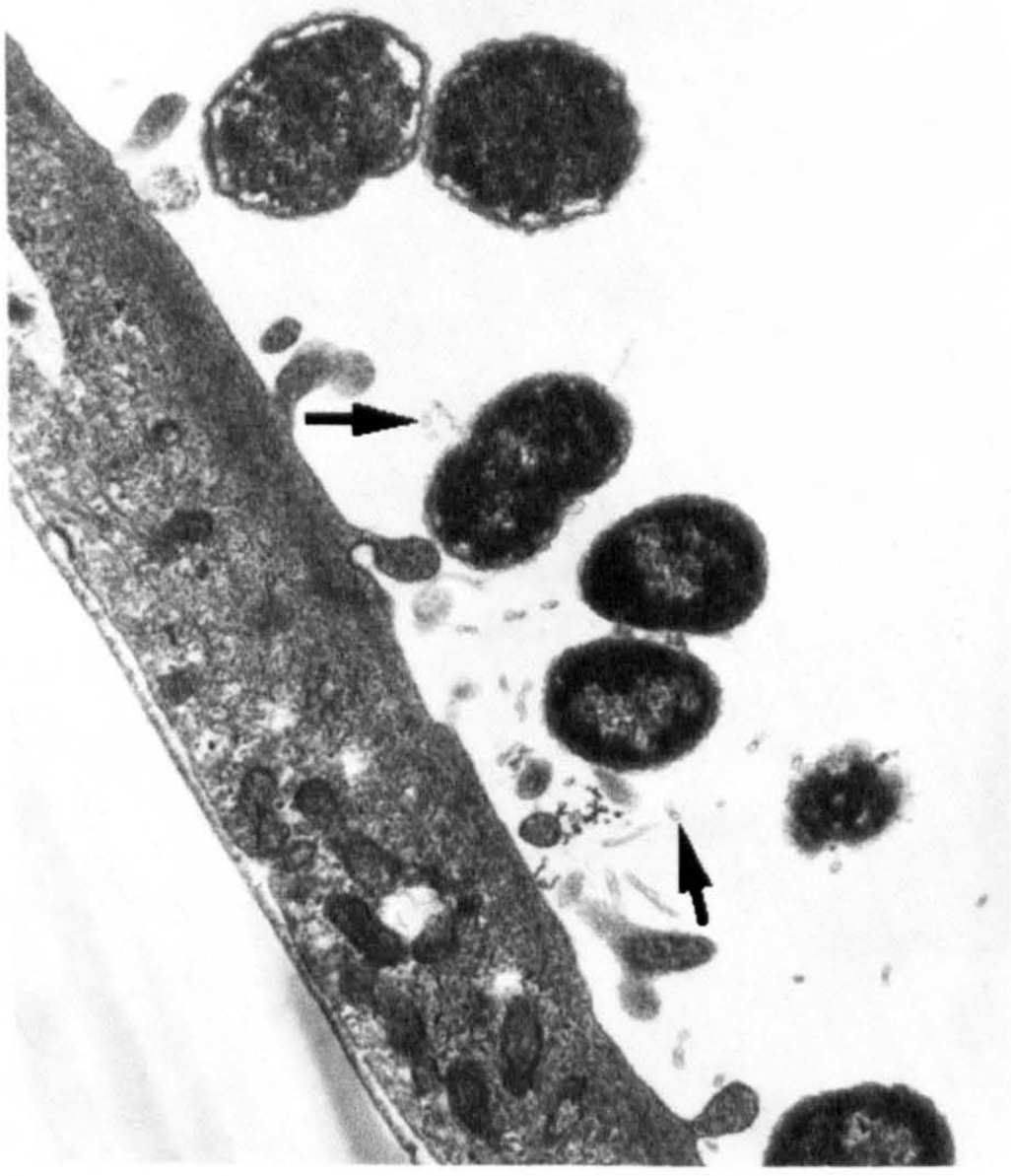


Figure 4.36: TEM of M18 cells infected with 58A for 24h.
A meningococcus is attached to the thin process of an M18 cell by an unknown mechanism.
The distance between the apical and baso-lateral surfaces of the human cell is less than the depth of the bacterium.
Magnification x 28000.

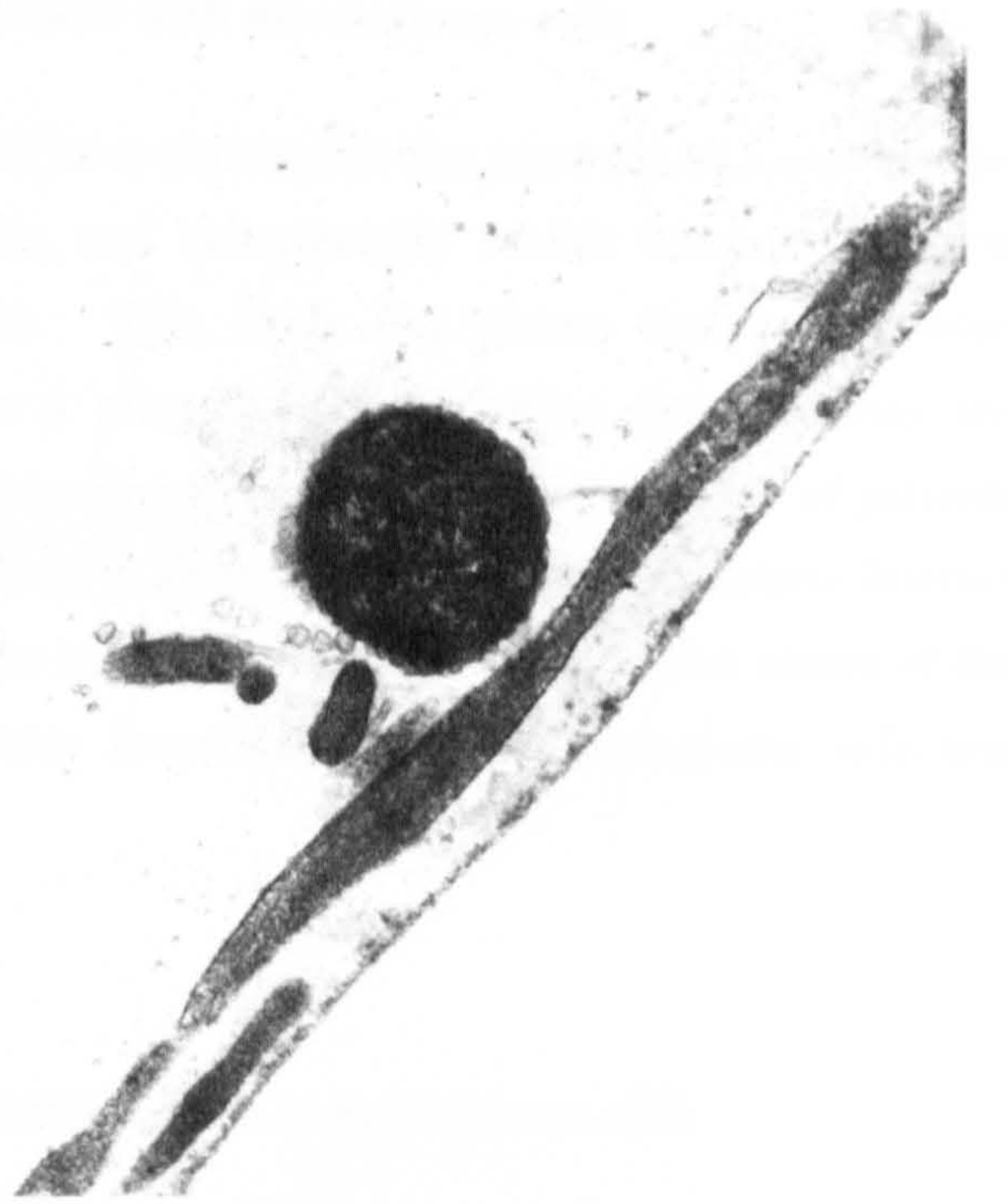
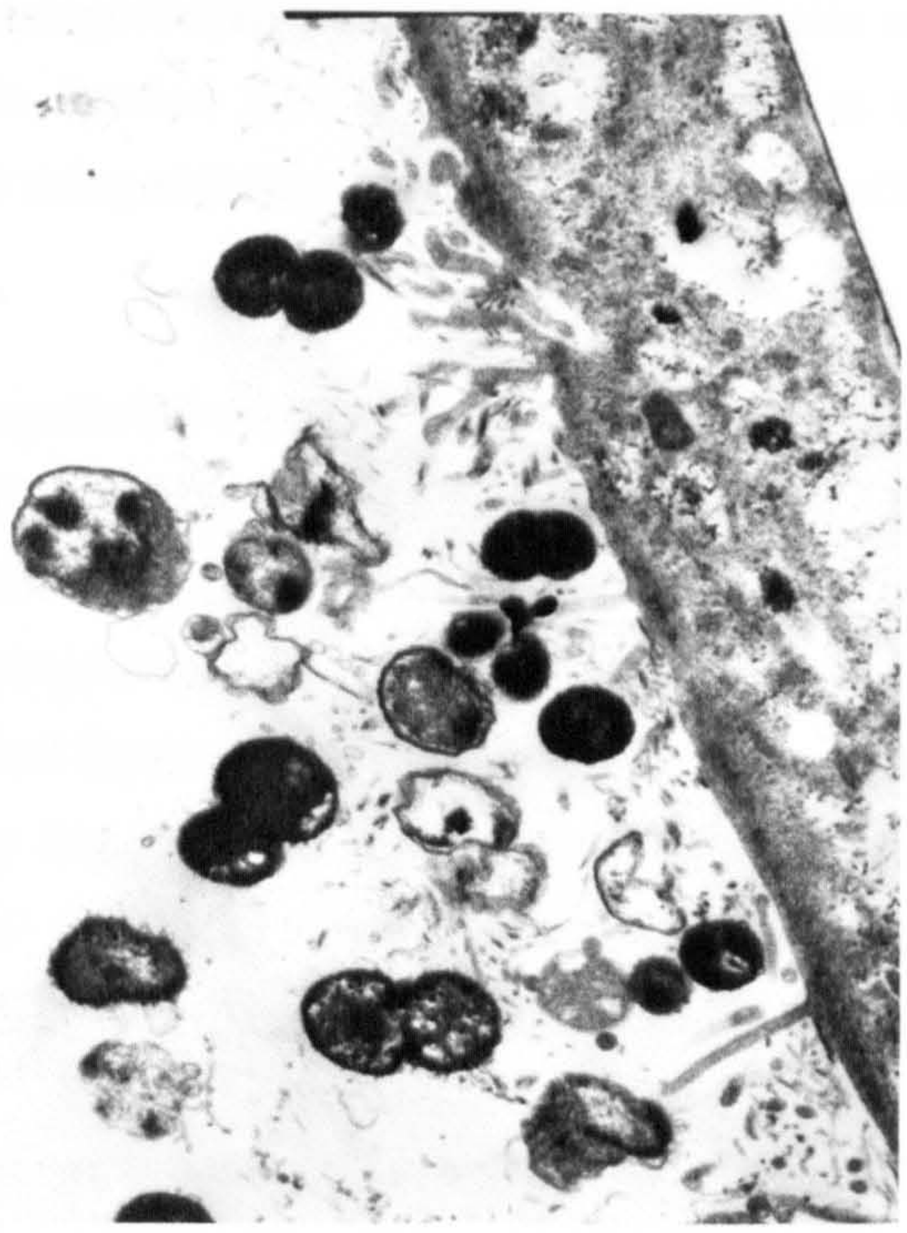


Figure 4.37: TEM of M18 cells infected with 58A for 24h.
After 24h of infection, some bacteria and meningioma cells were no longer viable. This field shows a structurally impaired meningioma cell that appears to be falling apart. Also present are a cluster of meningococci, some of which are viable as typified by black staining, and dead bacteria that appeared less electron dense than live cells.
Magnification x 10000.



4.2.5 Interactions of acapsulate meningococci with meningioma cells

Studies with cultured epithelial and endothelial cells have shown that the presence of a capsule can reduce meningococcal association with these cells, and subsequently inhibit bacterial invasion (McGee & Stephens, 1984; Virji *et al.*, 1995b). This study has shown that encapsulated meningococci do adhere to meningioma cells, in a pilus-dependent fashion, but that they are probably do not invade these cells. Although most meningococci isolated from the CSF of patients with meningitis express a capsule, it is still important to know how acapsulate organisms interact with human meningioma cells in comparison to other cells. The adhesive and invasive nature of the acapsulate MC58/59 variant $Cap^{-}Pil_{Ia}^{+}Opa^{-}Opc^{+}$ was investigated in the meningioma infection model.

4.2.5.1 Adherence of acapsulate meningococci to meningioma cells

To establish whether acapsulate meningococci adhere to meningioma cells, the human cells were infected with these bacteria in the viable count assay, a method that also allowed quantification of the interaction. Adherence was also analysed visually by SEM.

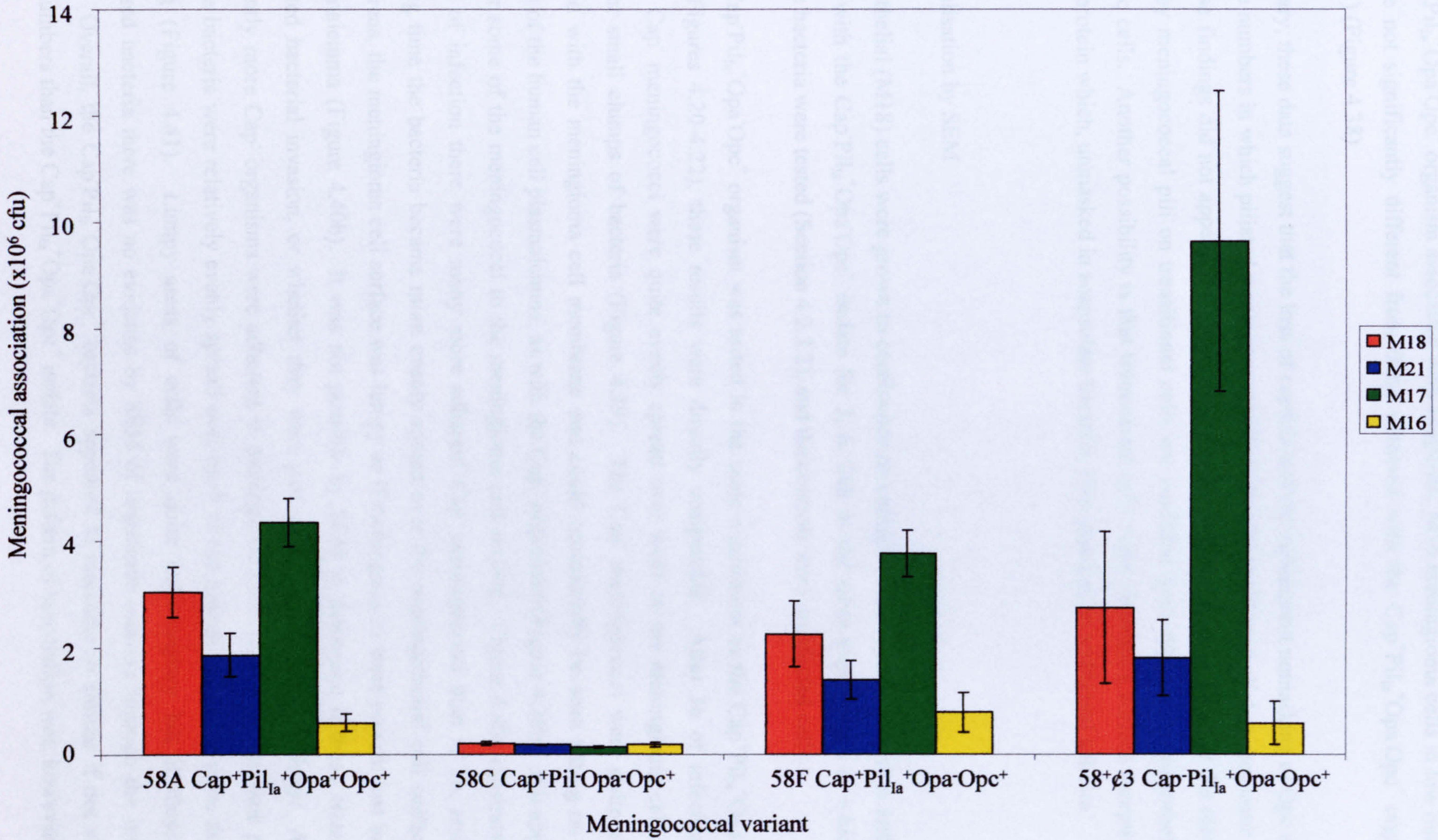
a) Viable Count Assay

The variant $Cap^{-}Pil_{Ia}^{+}Opa^{-}Opc^{+}$ was used to infect meningothelial, transitional and anaplastic cell lines for 6h in the viable count assays described in Section 2.12.1, at the same time as the encapsulated organisms tested. The data was analysed using one-way ANOVA as described earlier (Section 2.12.3).

The association of the acapsulate organism with meningothelial cells (M18, M21) was very similar to the results obtained with the phenotypically identical, but encapsulated, organism $Cap^{+}Pil_{Ia+}^{+}Opa^{-}Opc^{+}$, as well as the $Cap^{+}Pil_{Ia}^{+}Opa^{+}Opc^{+}$ isolate. These results suggest that capsule does not affect the efficiency of the pilus-mediated interaction of meningococci with meningothelial cells. As the acapsulate organism expressed the Opc protein but lacked Opa expression, these results also suggest that the loss of capsule did not unmask any capability that Opc may have to promote association with meningothelial cells. The acapsulate isolate associated with meningothelial cells in significantly higher numbers than any of the encapsulated non-piliated organisms ($p \leq 0.001$).

Previously (Section 4.2.1.3), $Cap^{+}Pil_{Ia/b}^{+}$ meningococci were shown to associate with transitional cells in significantly higher numbers than with meningothelial cells. This was also true with the $Cap^{-}Pil_{Ia}^{+}$ organism, and the loss of capsule in this piliated isolate resulted in a striking and

Figure 4.38: Comparative association of Cap^+/Cap^- phenotypic variants to meningothelial (M18, M21), transitional (M17) and anaplastic (M16) cell lines after 6 hours of infection.
The error bars represent the standard error of the mean of experiments repeated at least three times with triplicate wells.



significant increase in meningococcal association with transitional cells ($p \leq 0.001$); Acapsulate organisms adhered nearly three times more than their $Cap^+Pil_{Ia}{}^+Opa^-Opc^+$ counterpart (Figure 4.38).

The $Cap^-Pil_{Ia}{}^+Opa^-Opc^+$ organism associated with anaplastic M16 meningioma cells in low numbers that were not significantly different from those obtained with the $Cap^+Pil_{Ia}{}^+Opa^-Opc^+$ organism ($p=0.161$) (Figure 4.38).

In summary, these data suggest that the loss of capsule and the subsequent unmasking of Opc do not affect the numbers in which piliated meningococci associate with meningothelial or anaplastic cells. That these findings did not appear to hold true for transitional cells may be because the receptors utilised by meningococcal pili on transitional cells are modified from those of meningothelial or anaplastic cells. Another possibility is that transitional cells were expressing surface receptors for the Opc protein which, unmasked in acapsulate bacteria, then acted as an additional adhesin.

b) Visualisation by SEM

Meningothelial (M18) cells were grown to confluence on collagen coated glass coverslips and were infected with the $Cap^-Pil_{Ia}{}^+Opa^-Opc^+$ isolate for 3, 6, 24h in the same experiments as which the capsulate bacteria were tested (Section 4.2.3.2), and the controls were uninfected cells.

As the $Cap^-Pil_{Ia}{}^+Opa^-Opc^+$ organism was tested in the same experiment as the $Cap^+Pil_{Ia}{}^+Opa^+Opc^+$ isolate (Figures 4.20-4.22), these results were directly comparable. After 3h of infection, the adherent Cap^- meningococci were quite evenly spread over most of the meningioma cells, with infrequent small clumps of bacteria (Figure 4.39). The Cap^- meningococci were quite closely associated with the meningioma cell membrane and could occasionally be seen sitting on small uprisings of the human cell plasmalemma, as with the Cap^+ organisms (Figure 4.20b). Pili appeared to anchor some of the meningococci to the meningioma cell surface. Figure 4.40a illustrates that upon 6h of infection there were many more adherent Cap^- meningococci than at 3h, and with increasing time the bacteria became more evenly spread over the meningothelial cell surface. In certain areas, the meningioma cell surface was lumpy as if meningococci were present just beneath the plasmalemma (Figure 4.40b). It was not possible by SEM to determine if these areas truly represented bacterial invasion, or whether they were just cells of altered morphology. At 24h significantly more Cap^- organisms were adherent to meningioma cells than at earlier time points, and these bacteria were relatively evenly spread over most of the human cells with some areas of clumping (Figure 4.41). Lumpy areas of cells were quite commonplace but if these were internalised bacteria there was no evidence by SEM of organisms midway through the invasion process. Overall, the $Cap^-Pil_{Ia}{}^+Opa^-Opc^+$ bacteria appeared to associate in similar if not slightly greater numbers than the $Cap^+Pil_{Ia}{}^+Opa^+Opc^+$ isolate. The pattern of association was, however,

Figure 4.39: Low power SEM analysis of meningothelial M18 cells infected with the Cap^- $Pil_{la}{}^+Opa^-Opc^+$ isolate for 3h. This field shows an area of meningioma cells over which meningococci are quite evenly spread, and an area where the bacteria are absent. Structures that appear to be pili are visible on a few meningococci.

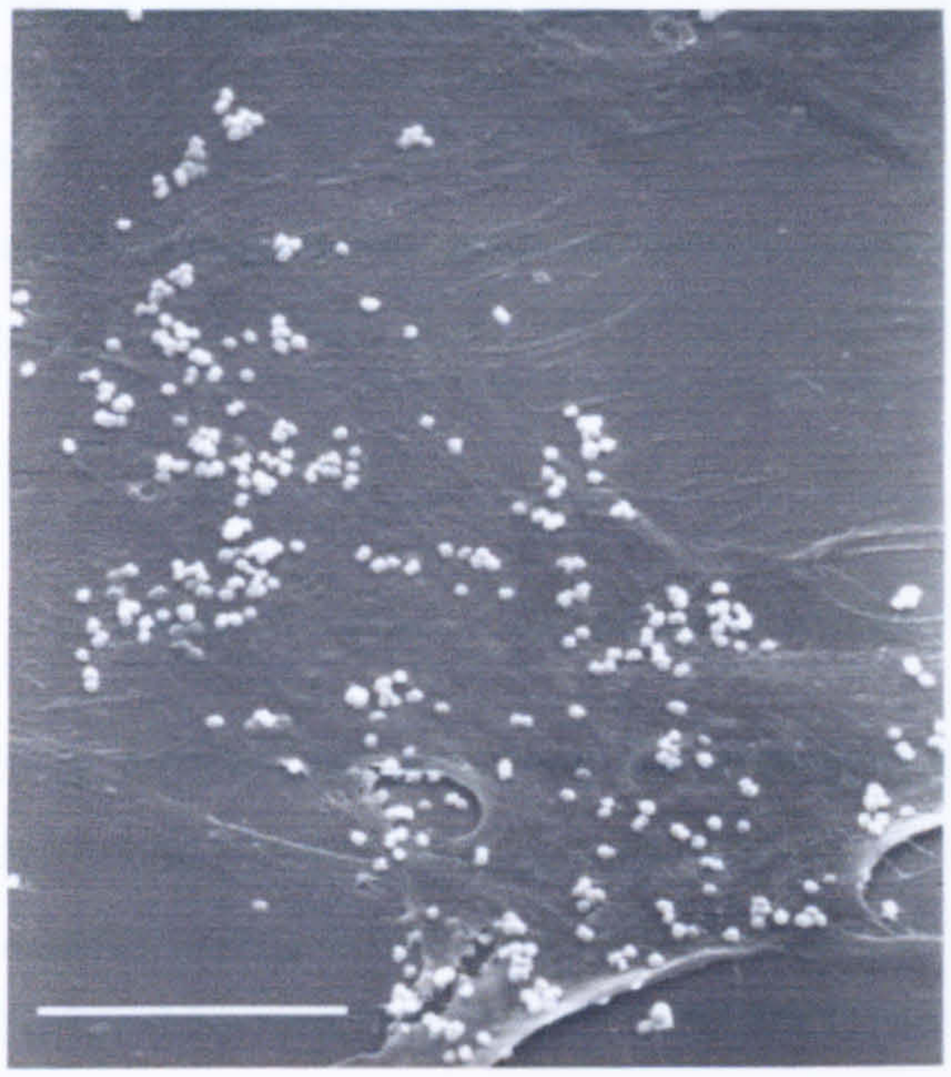


Figure 4.40a: M18 cells infected with Cap^- $Pil_{la}{}^+Opa^-Opc^+$ for 6h. Meningococci were spread relatively evenly over areas of the monolayer, with occasional small aggregates. The scale bar represents 20µm.

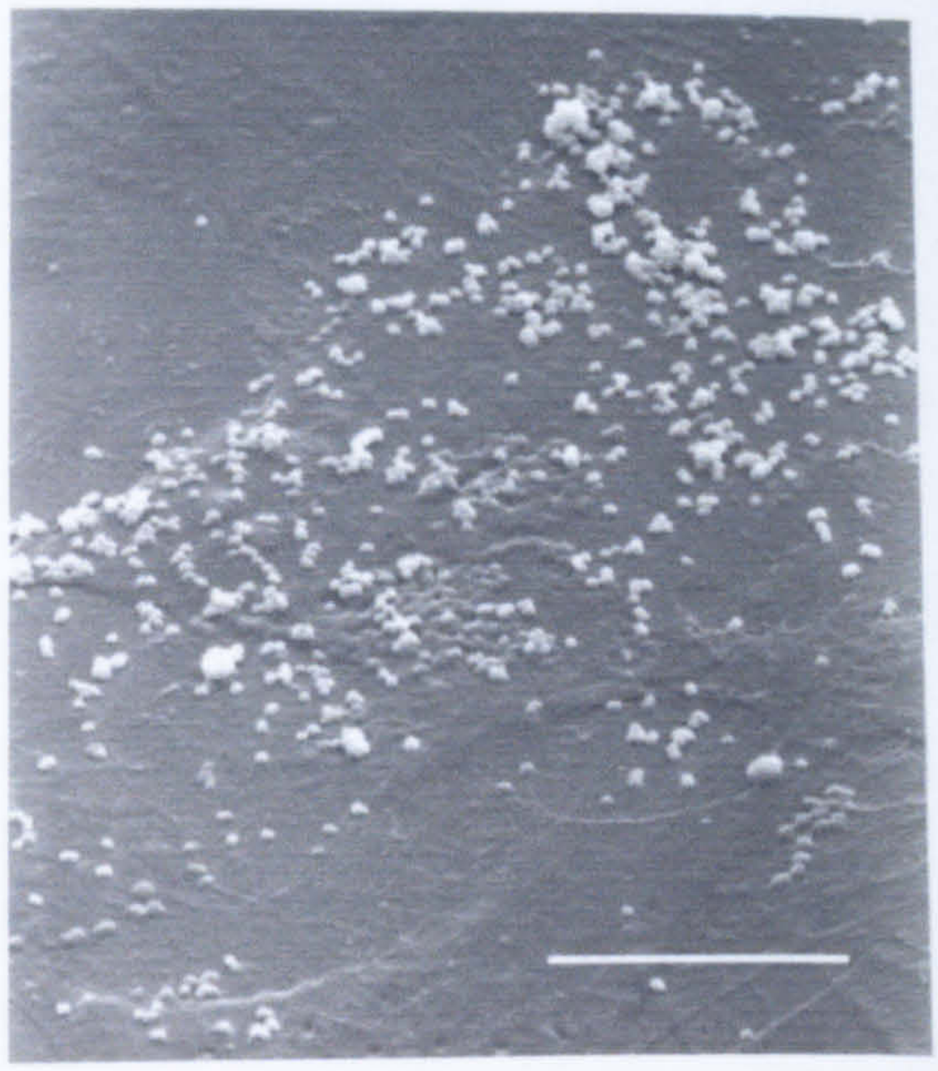


Figure 4.40b: Higher power SEM of Figure 4.40a. In some areas the meningothelial cell surface had a lumpy appearance as if the Cap^- bacteria were present underneath.
The scale bar represents 4.3µm.

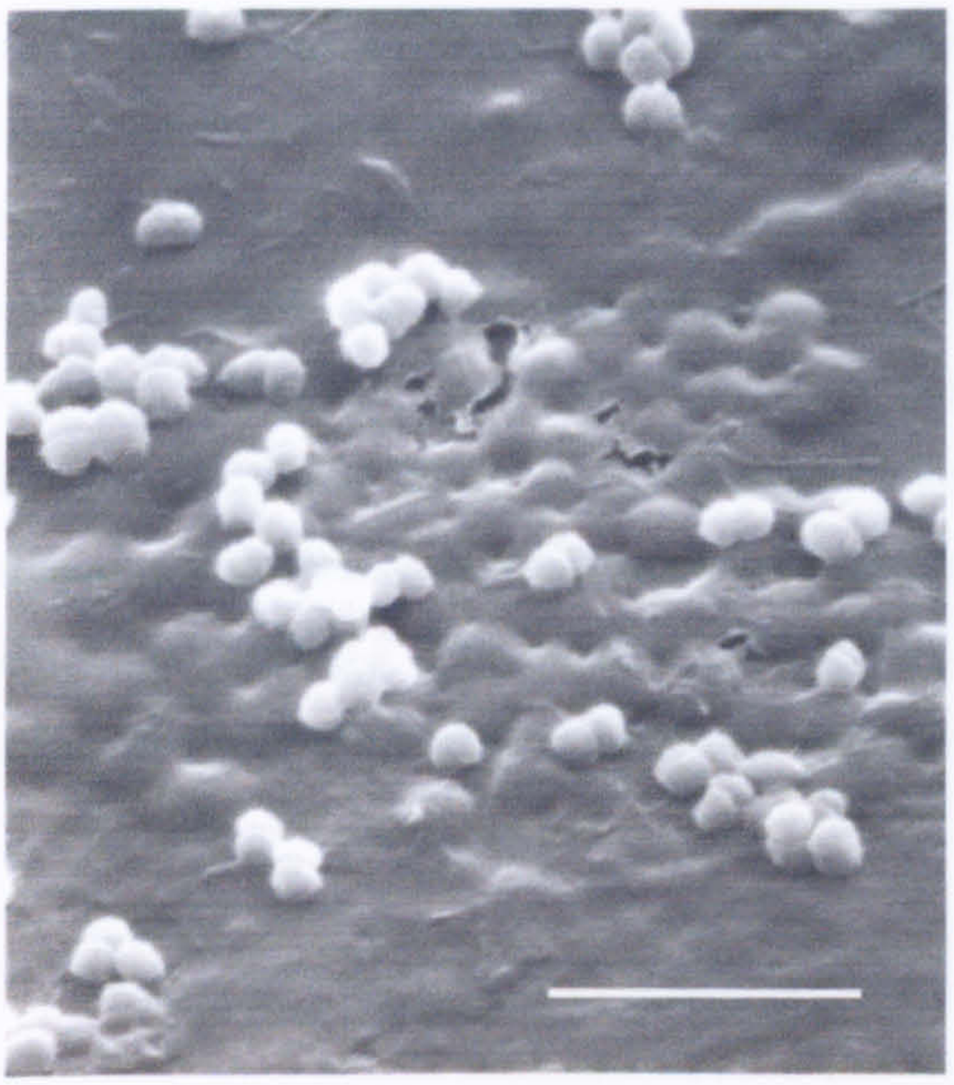


Figure 4.41: M18 cells infected with Cap^- $Pil_{la}{}^+Opa^-Opc^+$ for 24h. Many bacteria were evenly associated with most of the meningothelial cells by this point and the apical surface of the human cells often appeared uneven.
The scale bar represents 20µm.

different between the two organisms; acapsulate meningococci were much more evenly spread across most of the meningothelial monolayer and less prone to aggregation than the encapsulated bacteria. However, at all time points there were still areas of meningioma cells to which no or very few bacteria were associated. It was not obvious whether the $Cap^{-}Pil_{Ia}^{+}Opa^{-}Opc^{+}$ bacteria had a predilection for the perinuclear region of the meningioma cells as the $Cap^{+}Pil_{Ia}^{+}Opa^{+}Opc^{+}$ organisms did. Overall, there seemed to be fewer pili visible with the Cap^{-} infection than with the Cap^{+}. This could be due to the apparently closer association of Cap^{-} meningococci with meningothelial cells, thus masking the pili from sight. Alternatively, it could be as a result of pilus down-regulation which is reported by some workers to occur prior to meningococcal or gonococcal invasion of other human cells (Pujol *et al.*, 1997; Ilver *et al.*, 1998).

4.2.5.2 Invasion of meningioma cells by acapsulate meningococci

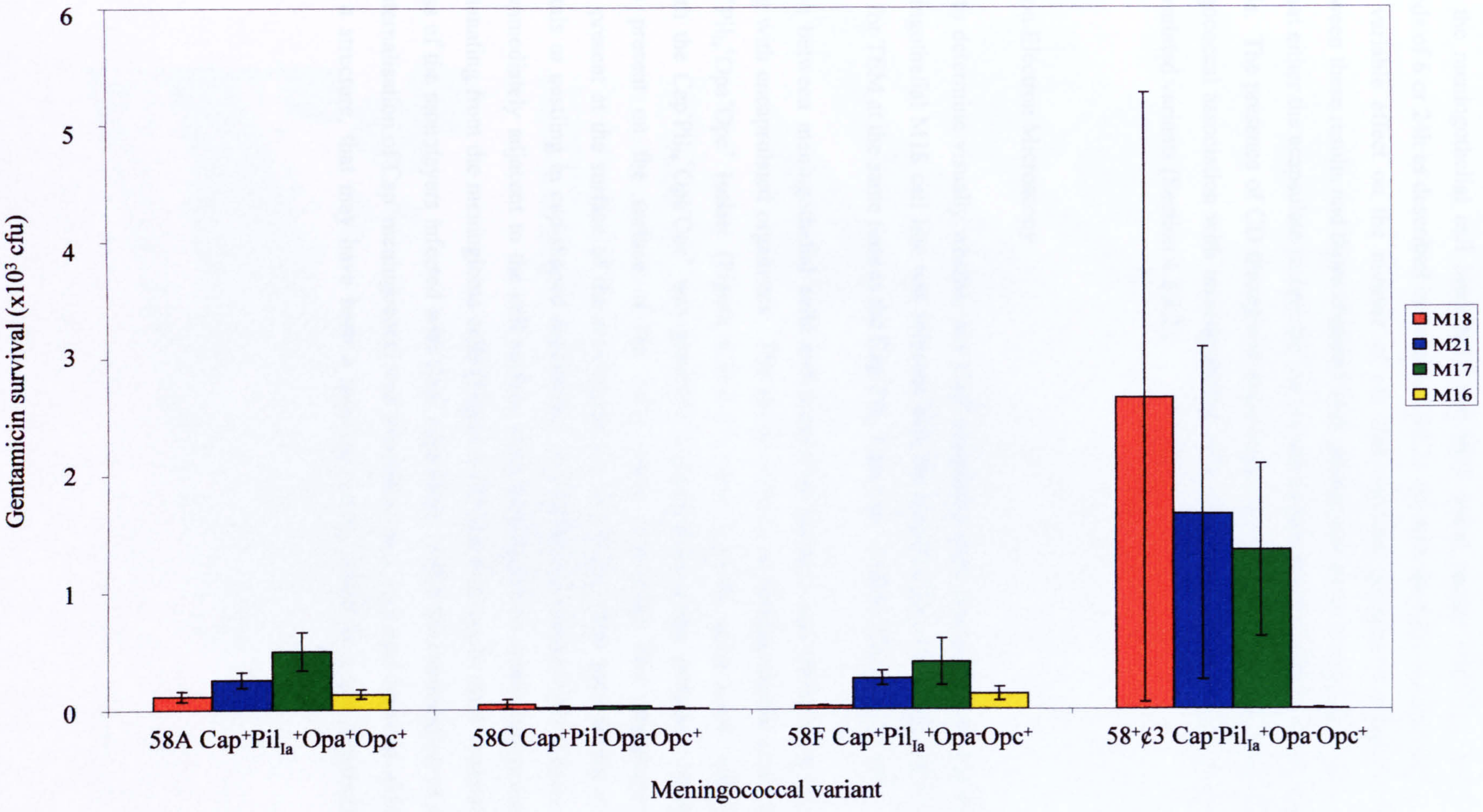
It is largely accepted that the meningococcal capsule prevents these bacteria from invading epithelial and endothelial cells, and that acapsulate meningococci can invade these cells. Since acapsulate organisms were found to adhere to meningioma cells, the possibility that they could also invade them was investigated using the gentamicin survival assay and TEM.

a) Gentamicin survival

The $Cap^{-}Pil_{Ia}^{+}Opa^{-}Opc^{+}$ variant was used to infect the meningioma cell lines M18, M21, M17 and M16 for 6h in the gentamicin survival assays described in Section 2.12.1, at the same time as the encapsulated organisms tested.

After infection of meningothelial and transitional cells, Figure 4.42 shows that more $Cap^{-}Pil_{Ia}^{+}Opa^{-}Opc^{+}$ organisms survived gentamicin treatment than the equivalent $Cap^{+}Pil_{Ia}^{+}Opa^{-}Opc^{+}$ isolate, or other encapsulated variants regardless of pilus expression. However, survival of the acapsulate isolate was still less than 2700 cfu per infected monolayer, and only represented 0.1% of the total cell-associated cfu with meningothelial cells, and 0.014% with transitional cells (compared to $Cap^{+}Pil_{Ia}^{+}Opa^{-}Opc^{+}$ survival of 0.001% and 0.01% respectively). There was much variation between triplicate wells, in a given experiment, with the survival of $Cap^{-}Pil_{Ia}^{+}Opa^{-}Opc^{+}$. It is likely that this was due to meningococcal clumping and thus escape from the actions of the antibiotic as described earlier. The number of $Cap^{-}Pil_{Ia}^{+}Opa^{-}Opc^{+}$ organisms surviving gentamicin treatment after infection of anaplastic cells was negligible (Figure 4.42).

Figure 4.42: Survival of Cap$^-$ and Cap$^+$ isolates after 6h infection of meningothelial (M18, M21), transitional (M17) and anaplastic (M16) cell lines with gentamicin treatment.
The graph shows the mean number of cfu that survived the antibiotic with at least triplicate estimations, and the error bars represent the SEM.



b) Incubation with Cytochalasin D

Although the number of organisms surviving gentamicin treatment was very low, the action of CD was investigated to try to establish whether these bacteria were truly internalised. CD assays were performed on the meningothelial cell lines M18 and M21 using variant $Cap^{-}Pil_{Ia}^{+}Opa^{-}Opc^{+}$ for infection periods of 6 or 24h as described in Section 2.12.2. As with the Cap^{+} isolates, the presence of CD had a variable effect on the number of cfu that survived gentamicin treatment but the difference between these results and those obtained with gentamicin alone was insignificant. These data suggest that either the acapsulate isolate did not invade meningothelial cells, or that it did so at very low levels. The presence of CD throughout these experiments was also found to consistently reduce meningococcal association with meningothelial cells after 6 and 24h of infection, as it did with the encapsulated variants (Section 4.2.4.2).

c) Transmission Electron Microscopy

In an attempt to determine visually whether any Cap^{-} organisms were internalised by meningioma cells, the meningothelial M18 cell line was infected with the $Cap^{-}Pil_{Ia}^{+}Opa^{-}Opc^{+}$ organism for 24h and processed for TEM at the same time as the $Cap^{+}Pil_{Ia}^{+}Opa^{+}Opc^{+}$ isolate (Section 2.13.5).

The interaction between meningothelial cells and acapsulate bacteria was different to that seen when infecting with encapsulated organisms. The apical surfaces of meningothelial cells infected with the $Cap^{+}Pil_{Ia}^{+}Opa^{+}Opc^{+}$ isolate (Figure 4.34-4.37) were typically quite inert, whereas the interaction with the $Cap^{-}Pil_{Ia}^{+}Opa^{-}Opc^{+}$ was generally a much more active process. Many Cap^{-} bacteria were present on the surface of the cells, often more than four organisms deep. Meningococci present at the surface of the meningothelial cells were often seen sitting on small cellular pedestals or nestling in cup-shaped depressions formed by the human cells (Figure 4.43). Bacteria not immediately adjacent to the cell surface were surrounded by many cell process-like projections emanating from the meningioma cells (Figure 4.44) and this was in stark contrast to the relative flatness of the monolayers infected with Cap^{+} organisms. After the examination of at least 30 sections, internalisation of Cap^{-} meningococci was deemed to be a very rare event as on just one occasion was a structure, that may have been a meningococcus, found in a meningothelial cell (Figure 4.45).

Figure 4.43: TEM of M18 cells infected with 58G ($Cap^{-}Pil_{Ia}{}^{+}Opa^{+}Opc^{+}$) for 24h.
Meningococci were observed in close association with the apical surface of meningothelial cells, and in this field a meningococcus is seen sitting on a small cellular pedestal enclosed on 2 sides by short cell processes.
Magnification x 21200.

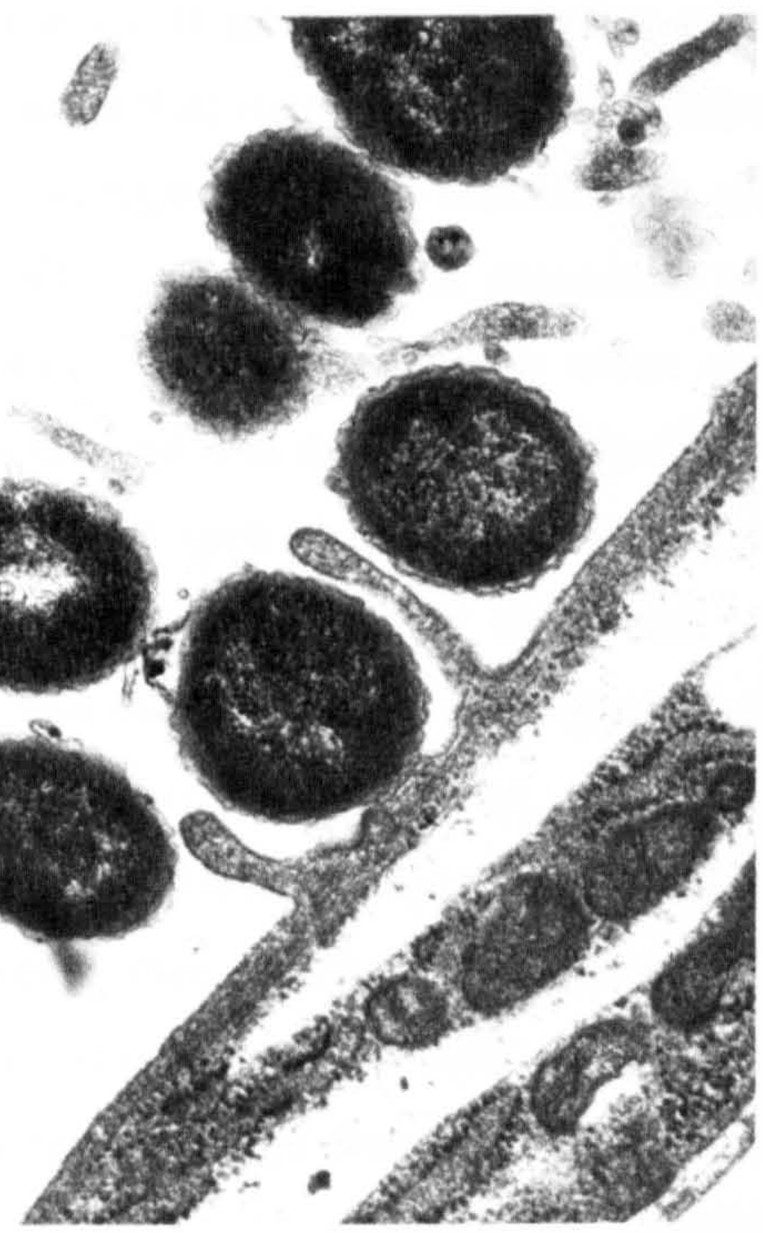


Figure 4.44: TEM of M18 after infection with 58G for 24h.
Aggregates of bacteria can be seen associated with and surrounded by protrusions from the apical surface of the meningothelial cell.
Magnification x 30000.

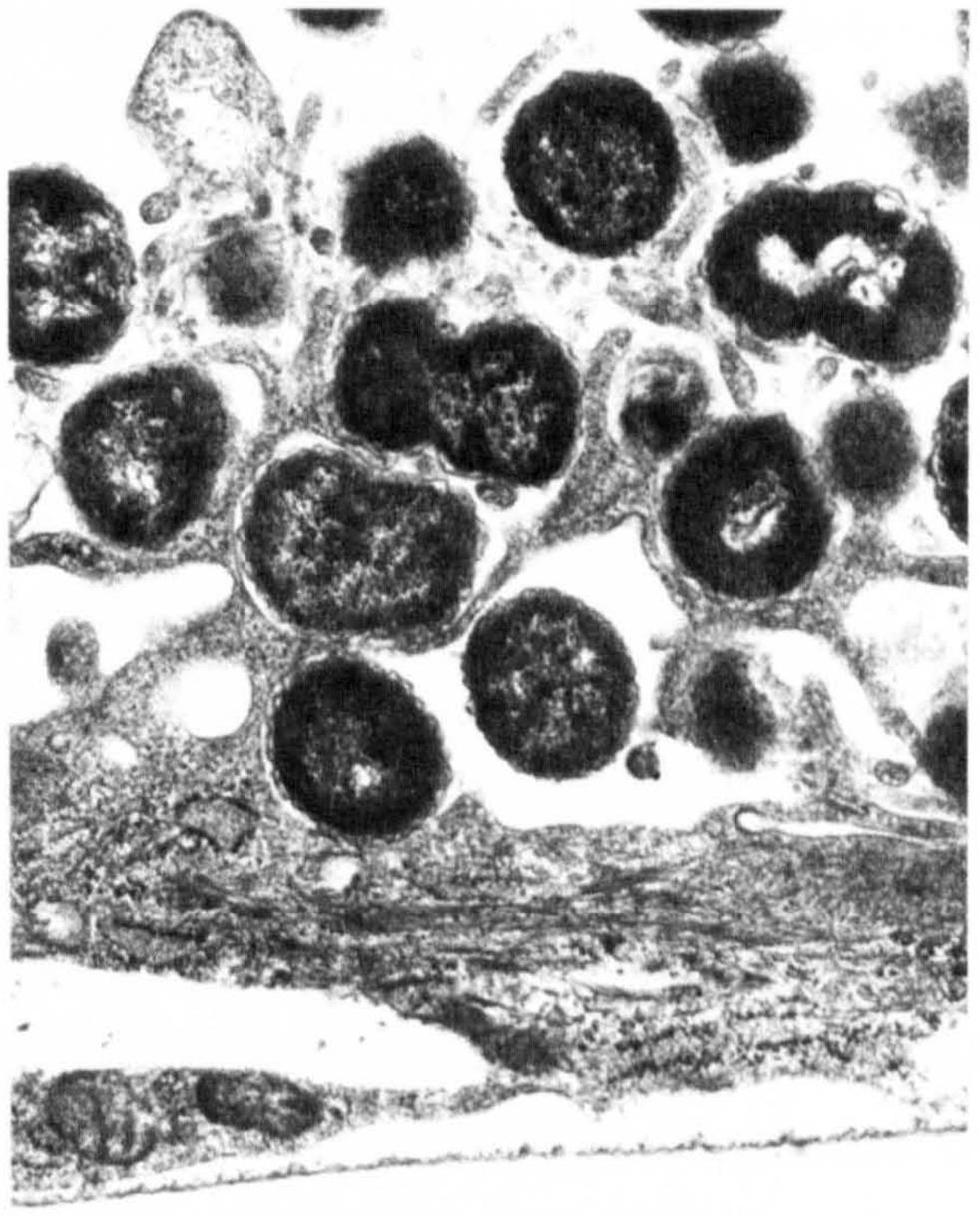
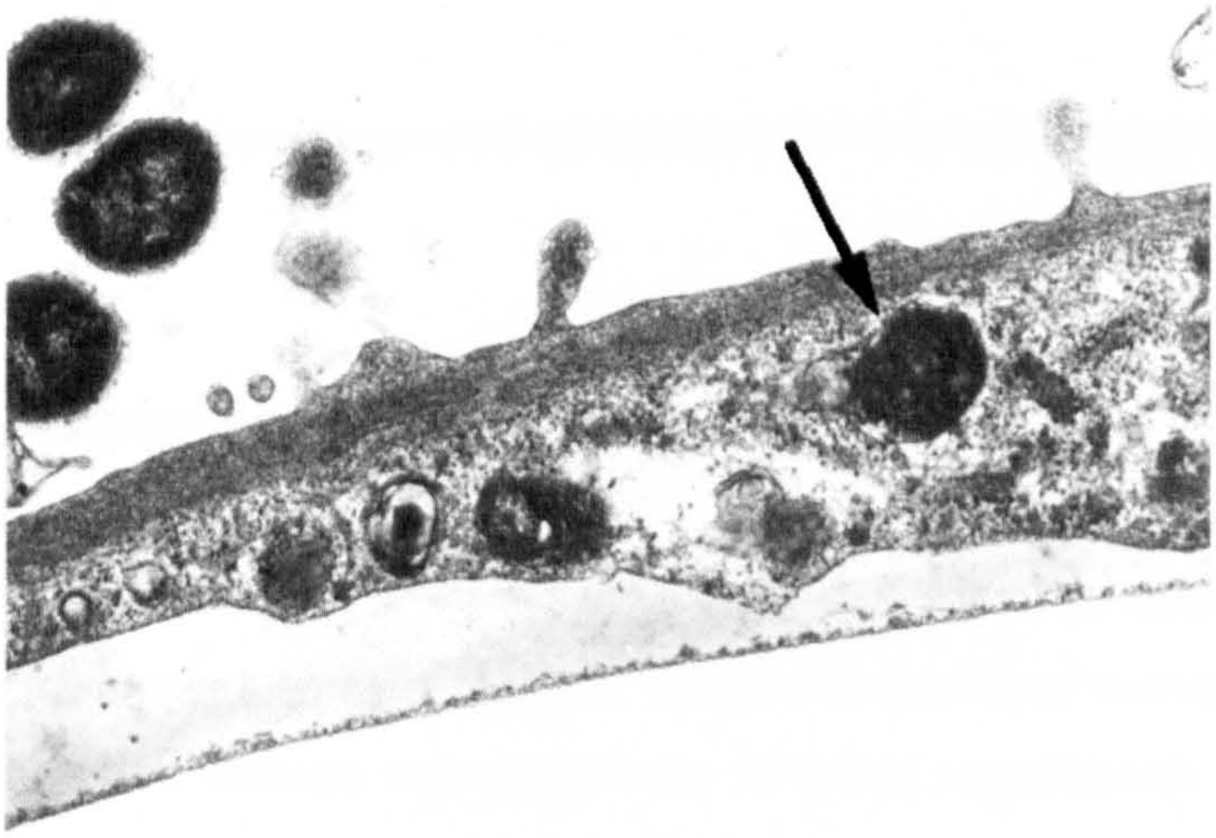


Figure 4.45: TEM of M18 after infection with 58G for 24h, showing a potential meningococcal structure within a meningioma cell.
The dense circular structure inside this cell may be a meningococcus in a membrane-bound vacuole (arrow).
Magnification x 20000.



4.2.6 Observations and cytopathic effect of meningococcal infection

Neisseria meningitidis is known to cause damage to human cells both *in vivo* and *in vitro* (Brandtzaeg, 1995; Virji *et al.*, 1991). Using an inverted light microscope, meningioma cell monolayers were monitored throughout infection experiments to observe the meningococcal-meningioma cell interaction including possible bacterial cytopathic effects. By 6h of infection it was apparent that meningococci that were encapsulated and piliated had clumped together, to form circular aggregates over the monolayers, and this was especially prominent with bacteria expressing type Ia pili. The acapsulate organisms also formed aggregates, which were larger than with the encapsulated isolates, and these often concealed most of the monolayer from view. As SEM analysis showed that the $Cap^{-}Pil_{Ia}^{+}Opa^{-}Opc^{+}$ isolate associated quite evenly over the cell monolayer, it is likely that such clumps of bacteria are present free within the culture medium and are mostly removed during the washing steps that precede cell lysis. The clumping phenomenon was not entirely due to contact with meningioma cells as bacteria incubated in wells just coated in collagen also formed circular aggregates, although these were smaller than wells containing meningioma cells. During infection experiments, cell monolayers were intact after 6h of infection, but by 24h there were often patches of missing cells and the media were yellow, indicating an acidic environment presumably resulting from the metabolism of the increased number of bacteria present. Piliated bacteria were found to cause greater damage to the integrity of the monolayer than non-piliated variants. In addition, the cytopathic effect of meningococci on meningioma cells was affected by the bacterial dose, with the higher doses causing more damage to the integrity of the monolayer than the lowest dose. Interestingly, the aggregates formed by piliated meningococci during an infection experiment were smaller in the presence of CD. This observation may suggest that CD had some ability to decrease meningococcal adhesiveness, which would be in accord with the earlier finding that CD decreased meningococcal attachment to meningioma cells.

4.3 Phenotypic analysis of meningococci recovered from infection experiments

Up to and including the bacteraemic stage of *in vivo* infection, meningococcal surface antigens are known to be subject to phase and/or antigenic variation, presumably to afford these bacteria with structures required for different stages of infection. Such variation is also known to occur with meningococci used to infect human cells *in vitro*. To monitor potential meningococcal variations in the meningioma cell model, bacteria recovered from infection experiments were analysed for the expression of surface antigens likely to undergo such changes.

The bacteria recovered from a representative infection experiment of each meningioma cell line were screened. Meningococci obtained from saponin lysis of the meningioma cell monolayers, with and without the addition of gentamicin, were cultured on PP agar and prepared for screening of surface antigens, as described in Section 2.5.1.3.

Capsule expression was determined by immunodot blot using the mAb 2-2-B as described in Section 2.9.2. None of the encapsulated meningococci recovered from infection experiments showed any differences in capsule expression from the bacteria with which the meningioma cells were initially infected. In addition, some of the meningococci that survived gentamicin treatment were tested using the latex agglutination assay (Section 2.9.1). There were no differences between post-infection meningococci and the parent strains.

Pilin expression was detected by immunoblotting and staining with the mAb SM1 as described in Section 2.6.2. From samples measuring the total association of meningococci to meningioma cells, most of the variants were identical to their respective parent strains at any time point tested (Figure 4.46). After infection of anaplastic cells for 6h, there was a pilin band in the sample recovered from the wells infected with $Cap^{+}Pil^{-}Opa^{-}Opc^{-}$, although this was not as strong as pilin bands from known piliated isolates (data not shown). Likewise, after infection of meningothelial cells (M18) for 6h, there was a small pilin band present in the wells infected with $Cap^{+}Pil^{-}Opa^{+}Opc^{+}$ organisms (Figure 4.47). From samples measuring possible internalisation of meningococci by meningioma cells, after infection of meningothelial cells (M21) there was a pilin band present in the sample infected with $Cap^{+}Pil^{-}Opa^{+}Opc^{+}$ for 3h (data not shown). These were the only cases of suspected pilus up-regulation after infection of meningioma cells.

Opa and Opc expression were detected by immunoblotting and staining with the mAbs 4B12 and B306 respectively, as described in Section 2.6.2. Most of the variants were identical to their respective parent strains at any time point tested (Figure 4.46). Sometimes, when samples of Opc expressing meningococci were reacted with this antibody, a faint lower band was also seen which was presumed to be slight cross-reactivity of the mAb with Opc. In experiments measuring total meningococcal association with meningothelial (M21) and anaplastic cells, post-infection samples from $Cap^{+}Pil_{Ib}^{+}Opa^{-}Opc^{-}$ showed a very faint band when reacted with the 4B12 Opa mAb (data not shown). This observation may indicate slight up-regulation of Opa expression after infection of meningioma cells. No differences in Opc expression were observed from between parent isolates and recovered meningococci from any infection experiment (Figure 4.46).

The migratory pattern of LPS on low molecular weight page, with silver staining, was used to indicate possible differences in immunotype between parent isolates and meningococci recovered from infected meningioma cells. The recovered bacteria always migrated to the same distance as their parent isolate regardless of the infection time, suggesting that their immunotype did not alter during the course of infection (Figure 4.48).

Figure 4.46: Immunostaining for expression of pilin, Opa and Opc using mAbs SM1, 4B12 and B306 respectively. The samples are the total associated meningococci recovered from a time course infection of the meningothelial cell line M21 infected with variants 58A ($Cap^+Pil_{Ia}{}^+Opa^+Opc^+$) and 58H ($Cap^+Pil^-Opa^+Opc^+$).
The relevant parent isolates are shown alongside each set of recovered bacteria. The pilin bands of the top blot have been omitted to avoid duplication.

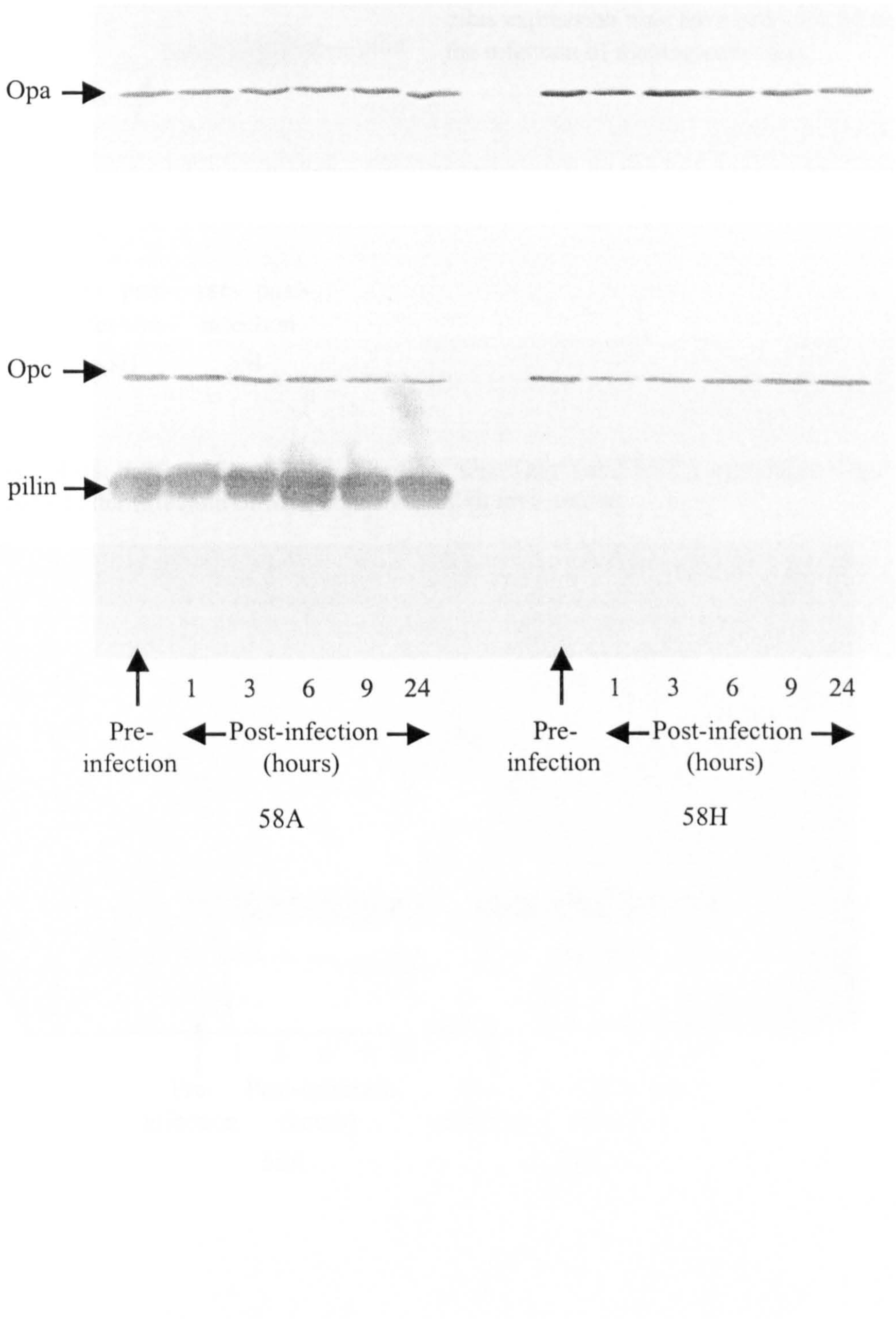
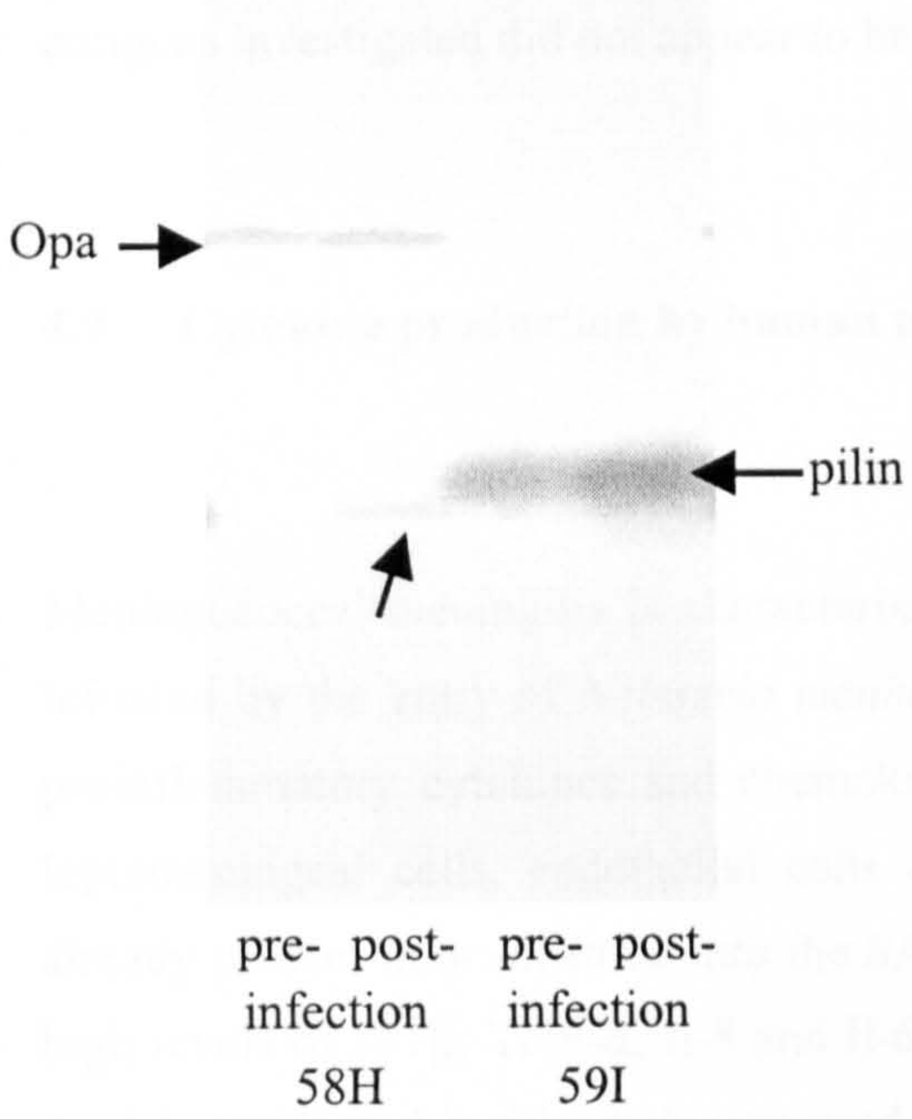
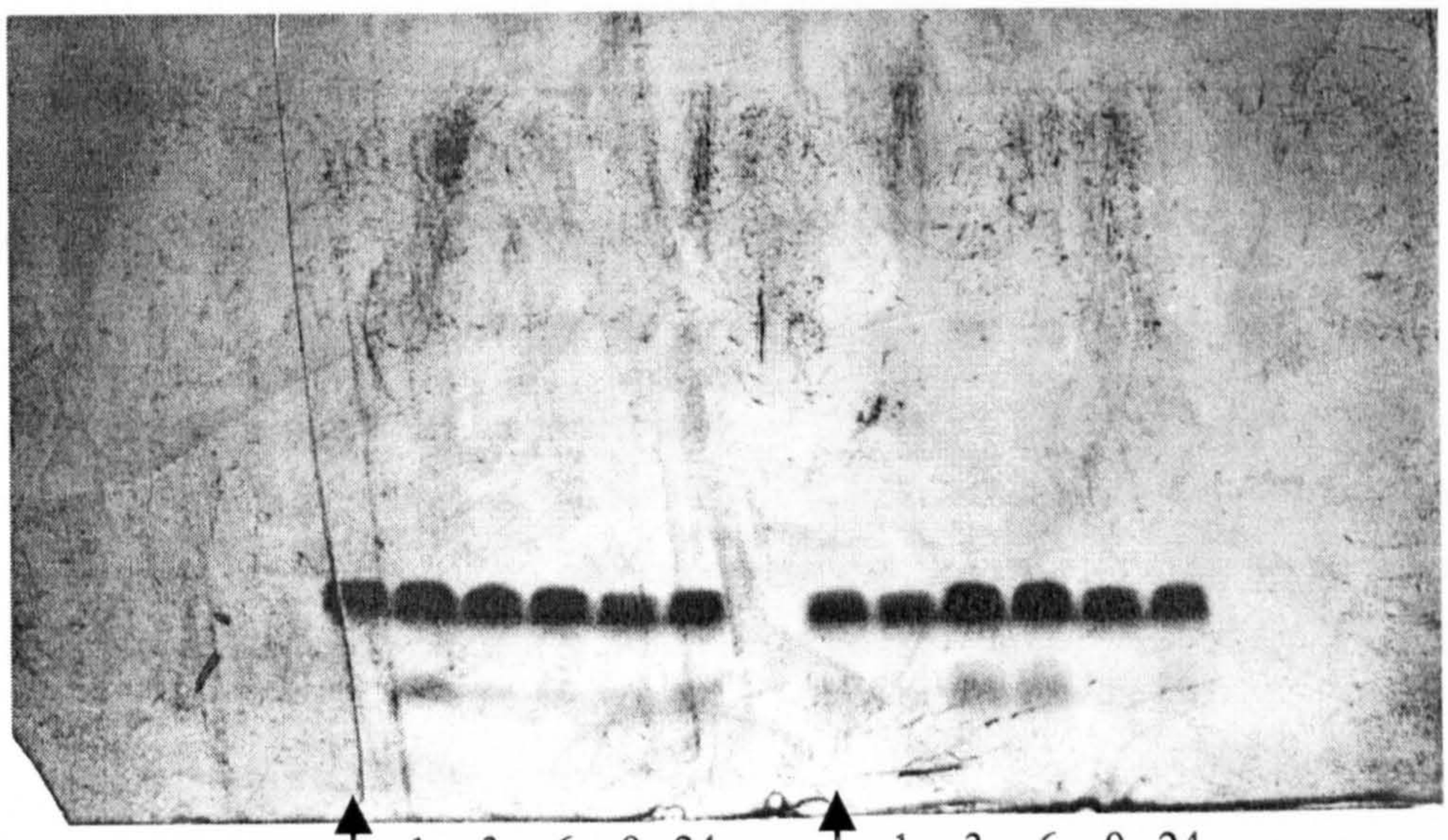


Figure 4.47: Immunostaining for pilin and Opa from an infection experiment of meningothelial cells (M18) comparing different meningococcal variants.



The results shown are the parental strain (pre-) and post-infection samples of the variants 58H ($Cap^+Pil^-Opa^+Opc^+$) and 59I ($Cap^+Pil_{Ib}{}^+Opa^-Opc^-$). The 6h post-infection sample of 58H shows a small pilin band (unlabelled arrow) which is absent from the parental sample, indicating that pilus expression may have been turned on during the infection of meningioma cells.

Figure 4.48: LPS profiles of 58A ($Cap^+Pil_{Ia}{}^+Opa^+Opc^+$) and 58H ($Cap^+Pil^-Opa^+Opc^+$) obtained after infection of M16 cells over a 24h time course.



In summary, with respect to capsule, Opc and LPS expression, no differences were observed between the parental strain and meningococci recovered after infection of meningioma cells. In a few isolated cases, pili or Opa appeared to be up regulated after meningioma infection, and upon checking with the viable counts for each case, none showed unexpectedly high association values. Overall, upon infection of meningioma cells, up- or down-regulation of the meningococcal surface antigens investigated did not appear to be commonplace.

4.4 Cytokine production by human meningioma cells upon meningococcal challenge

Meningococcal meningitis is characterised by an acute compartmentalised inflammatory response initiated by the entry of *Neisseria meningitidis* into the SAS. The mediators of this response are proinflammatory cytokines and chemokines, but it is not known whether these are produced by leptomeningeal cells, endothelial cells of blood vessel walls or by macrophage that are either already present in or infiltrate into the SAS. In the CSF of patients with meningococcal meningitis, high levels of Il-1β, TNF-α, Il-8 and Il-6 are seen (Brandtzaeg *et al.*, 1992). The meningioma cell model established in this study was used in preliminary experiments to investigate the regulation of expression of proinflammatory cytokines and chemokines produced during meningitis.

Experiments were carried out using meningothelial M18 cells grown to confluence on collagen coated 24 well plates and growth arrested as described in Section 2.14. The cells were challenged with $Cap^{+}Pil_{Ia}^{+}Opa^{+}Opc^{+}$, $Cap^{+}Pil^{-}Opa^{+}Opc^{+}$ or no bacteria for 3h, after which time RNA was extracted and cDNA synthesised. Any cytokine message produced by infected meningioma cells was then investigated by RT-PCR using specific primers (Table 4.1).

Cells in control samples (uninfected cells) showed little or no constitutive expression of cytokines. Challenge with piliated meningococci induced significant increases in mRNA transcripts for Il-6, Il-8 and Il-1β compared to controls, but no significant increases in the levels of other cytokines or chemokines. In contrast, there was no significant difference in the levels of mRNA transcripts detected in cells challenged with non-piliated bacteria, compared to control, unchallenged cells.

Table 4.1: Upregulation of cytokine mRNA transcripts following infection of meningothelial M18 cells after 3h, with piliated and non-piliated meningococci.

Cytokine message is ranked '+, ++, +++' to denote mRNA signals of increasing intensity, '+/-' weak signal, and '-' no signal.

Cytokine	Control	Pil+	Pil-
Il-6	-	++	+
Il-8	+/-	+++	+/-
TNF-α	-	-	-
Il-1α	+/-	+	+/-
Il-1β	+/-	++	+
RANTES	+/-	+	-
TGF-1β	+	+	+
MCP-1	+	+	+/-
MIP-1α	+/-	+/-	-
GM-CSF	+/-	+	+/-

Note: Il = interleukin; TNF-α = tumour necrosis factor α; RANTES = regulated on activation, normal T cell expressed and secreted; TGF-1β = transforming growth factor 1β; MCP-1 = macrophage chemotactic protein 1; MIP-1α = macrophage inflammatory protein 1α; GM-CSF = granulocyte macrophage colony stimulating factor.

CHAPTER 5 DISCUSSION

5.1 Introduction

Neisseria meningitidis is known to colonise and invade the human nasopharynx before entering the bloodstream. In patients who develop meningitis, the meningococci travel via the bloodstream to the SAS, where they initiate an acute inflammatory response. Many studies have focused on the use of *in vitro* epithelial and endothelial cell models, to elucidate the molecular mechanisms behind meningococcal-host cell interactions in the early stages of infection. However, little is known about the subsequent events occurring in the SAS in the latter stages of meningococcal disease; in particular the way in which this organism interacts with cells of the human meninges, which is likely to be pivotal in the progression of bacterial meningitis.

This thesis tested the hypothesis that meningococci associate with leptomeningeal cells, and that this interaction is mediated by specific ligands. The species-specificity of meningococci for human infection precludes the use of animal models in the study of meningococcal meningitis, and obviously experimental *in vivo* infection of the human meninges is not possible. In addition, human leptomeningeal cells do not grow reliably in culture (Feurer & Weller, 1991), and although the establishment of an immortalised leptomeningeal cell line has been reported (Murphy *et al.*, 1991), these cells are not now available to other workers. However, meningiomas are the neoplastic derivatives of normal leptomeninges, and as such they share many characteristics with the normal cells, whilst retaining the ability to survive *in vitro*. The aims of this project were to circumvent the problems highlighted above by using human meningioma cells as an *in vitro* model to study the interactions of meningococci with leptomeningeal cells.

5.2 Summary of results

Meningiomas of the meningothelial, transitional and anaplastic subtypes were taken at biopsy and meningioma cell lines were readily cultured from them. The cell lines represented pure populations of meningothelial cells, and they exhibited characteristics of normal leptomeningeal cells with respect to the expression of desmosomes and intermediate filaments of cytokeratin and vimentin.

A defined panel of encapsulated meningococcal variants was established that varied in their expression of the surface antigens pili, Opa and Opc proteins. These bacteria were used to infect meningioma cells and the interaction was monitored. The adherence of encapsulated meningococci to human meningioma cells was predominantly pilus-mediated and subject to antigenic variation of

the pilin subunit. In organisms with less adherent pili, the presence of Opa protein did increase adherence slightly, but Opc had no effect. The experimental evidence suggests that neither encapsulated or acapsulate meningococci invade meningioma cells.

5.3 Characterisation of meningioma cell cultures

The culture of cells from clinical samples was very successful, with growth from nine out of ten confirmed meningiomas. Although the cell lines were not immortal, they could be expanded sufficiently to perform a large number of experiments, thus eliminating inter-sample variation to some extent and allowing experimental replication to test for statistical significance. In addition, it was convenient that meningioma cells could be cryogenically preserved and stored until needed without significant loss of viability. Upon confluence, the meningioma cells reflected the zero proliferation state of normal leptomeningeal cells because they were contact inhibited, and they could be held in this state for some time. Not all of the cell lines grew at the same rate, in particular the anaplastic M16 line was the slowest grower. Since the survival of tumour cells *in vivo* is often reliant on the presence of appropriate growth factors, it is possible that the lack of these factors in culture could slow the proliferation of some meningiomas.

It was obviously very important that these cells reflected leptomeningeal cells as far as possible with respect to cellular markers known for the normal cells. Within the brain and meninges, desmosomes are only present in the leptomeninges and their meningiomas. The strict criterion applied by (Feurer & Weller, 1991) for characterisation of meningioma cells was to identify the presence of desmosomes, and this was adhered to in the current study; cultured meningioma cells were considered to be of meningeal origin if they expressed desmosomal desmoplakin. The majority of the meningioma cultures expressed desmosomes, and positive cultures were homogeneous for desmoplakin staining. EMA is also considered a marker for leptomeningeal origin, although not as reliably as desmoplakin, and the cell lines used for further investigations in this study were both desmoplakin and EMA positive. Most of the meningioma cell lines tested represented the dual epithelial and mesenchymal nature of leptomeningeal cells by coexpression of cytokeratin and vimentin intermediate filaments, although the staining was not homogeneous for either marker. The coexpression of these intermediate filaments has been widely reported by other workers, and is thought to reflect the dual embryological origins of the leptomeninges from both neuroectodermal and mesenchymal elements.

It was also important to ensure that meningioma cultures were pure populations so that during infection experiments, the results could confidently be ascribed to interactions of meningococci with meningeal cells. The possibility of meningioma cultures being contaminated with differentiated cells such as macrophages, endothelial cells or smooth muscle cells was easily

eliminated by using cultures that had been sub-cultured at least three times. After this split number, direct staining for macrophage using CD68 antibody was negative, and the homogeneous staining of cells for desmoplakin provided an indirect method of demonstrating that fibroblasts and other non-meningioma cell types were not present.

The histological classification of tumours of the meninges describes a large variety of meningioma variants (Lantos *et al.*, 1997). Examples of the most common types were used for infection experiments in the present study, namely the benign meningothelial and transitional meningioma subtypes, which are most similar to normal leptomeningeal cells (Feurer & Weller, 1991). For comparison, cells were also cultured from the less common anaplastic meningioma, which bear less resemblance to normal leptomeningeal cells.

5.4 Establishment of meningococcal panel

Meningococcal surface membrane antigens are known to play a major role in the association of these bacteria with human epithelial and endothelial cells. Pili are the primary mediators of attachment but other bacterial surface ligands have also been reported to determine the interactions of meningococci with human cells, and these include the Opa (Virji *et al.*, 1993a; Dehio *et al.*, 1998) and Opc (Virji *et al.*, 1992b; Virji *et al.*, 1994; deVries *et al.*, 1996; deVries *et al.*, 1998) proteins, LOS (Porat *et al.*, 1995a; Porat *et al.*, 1995b) and capsule (Virji *et al.*, 1995b). It is likely that these antigens are also important in meningococcal interactions with leptomeningeal cells and so, a panel of meningococcal variants which varied in their expression of pilus, Opa and Opc proteins was established by single colony isolation. Meningococci isolated from the CSF of meningitis patients are reported to be encapsulated (Devoe & Gilchrist, 1975) suggesting that the presence of a capsule is required for bacterial survival at this site, and most of the meningococci used in this study were encapsulated. However, for comparison a non-capsulate phenotype was also used. Since attempts to obtain a spontaneous Cap^- variant did not succeed, a genetically modified Cap^- mutant (MC58¢3) was used.

5.5 Establishment of infection protocols

Before meningioma cells were infected with meningococci, infection experiments were established by comparing the association of encapsulated piliated and non-piliated variants of meningococcal strain MC58 with Chang epithelial cells. These cells were ideal for initial experiments as they are an immortalised cell line and easily cultured, and their interactions with meningococci have been characterised previously (Virji *et al.*, 1992a). The current study found that Cap^+Pil^+ organisms associated with Chang cells in greater numbers than did the equivalent non-piliated bacteria, and

that these organisms did not invade epithelial cells, and these findings were in agreement with the published observations.

5.6 Association of meningococci to meningioma cells

5.6.1 Pilus-mediated interactions

In the current study, viable count assays comparing the total cell association of the panel of Cap^+ meningococcal variants to meningioma cells, found that these isolates exhibited different patterns of adherence. The pilus protein was the primary ligand to mediate this interaction, and confocal microscopy and SEM clearly demonstrated the specific adherence of Pil^+ meningococci to meningioma cells, with the significantly lower adherence of Pil^- variants. However, antigenic variation in the class I pilin protein resulted in a significantly reduced ability of Pil_{Ib}^+ bacteria to associate with meningioma cells even though, by TEM analysis, the Pil_{Ib}^+ variants appeared to be more piliated than the Pil_{Ia}^+ variants. Similar observations have been reported in previous studies with epithelial cells, which demonstrated that the type of pilin variants expressed by a strain was a crucial factor in the determination of bacterial adherence (Nassif *et al.*, 1993; Virji *et al.*, 1992a). In contrast, some workers (Virji *et al.*, 1992a) have found that such pilin variation apparently had no effect on pilus-associated adherence to endothelial cells. It is possible that meningococcal interactions with different human cell types is mediated by distinct ligands but, with regards to pilus-mediated adherence, meningioma cells may resemble epithelial cells.

The mechanism behind how pilin variation influences the adherence of meningococci to meningioma cells is not known, but several hypotheses have been proposed by other studies using different cells. Marceau and colleagues (Marceau *et al.*, 1995) have reported that meningococci expressing highly adherent variants of pilin were able to form bundled pili, and that aggregated pili promoted high adherence of capsulated bacteria with epithelial cells. Since isolates from patients with meningococcal disease are reported to be more likely to produce bundled pili than those isolated from carriers (Greenblatt *et al.*, 1988) it is possible that such aggregates of pili are required during invasive disease. Within the current study using SEM analysis of infected meningioma cells, apparent pili bundles were present on meningococci expressing the more adherent Pil_{Ia} pili, although the less adherent Pil_{Ib} phenotype was not tested. However, under TEM after negative staining, meningococcal pili bundles were not seen with either type Ia or Ib pili. Although pilus bundling may be a phenomenon associated with meningioma cell contact, this would be in contrast to the study by Marceau (Marceau *et al.*, 1995) which found bundling of pili regardless of the presence of host cells. These workers also suggested that the bundling of pili of highly adherent variants caused these bacteria to aggregate and grow as colonies on infected monolayers, whereas low-adhesive derivatives generally adhered as single cells. Despite the lack of bundling seen under

TEM, when viewed under the inverted light microscope, meningococcal variants expressing type Ia pili formed aggregates over the meningioma monolayers that were more prominent than those with the less adherent type Ib pili. Admittedly though, the magnification was not sufficient to observe actual pili bundles, but the presence of bacterial aggregates suggests that they may be present. Other studies using endothelial cells infected with meningococci have shown that these bacteria existed as clustered masses which were not confined to the cell junctions but were often spread over the entire surface of the cells (Virji *et al.*, 1994). When a human cell microfilament inhibitor was added, the clustered appearance was lost, and this led the authors to suggest that meningococcal aggregation is due to a host-cell receptor driven phenomenon rather than just to bacterial agglutination.

Meningococcal pili are subject to post-translational modification, including glycosylation of conserved amino acid residues, and such glycosylations have been described in detail (Stimson *et al.*, 1995). It was originally proposed by Virji and co-workers (Virji *et al.*, 1993b) that functional variations in pili may result from changes in primary amino acid sequences within pilin, which create or remove glycosylation sites on the pilin subunit. However, more recent studies have shown that removal or substitution of the serine residue (Ser^{63}) believed to be the site of *O*-glycosylation, makes no difference to pilus-mediated adhesion (Marceau *et al.*, 1998). In fact, in meningococcal strains producing non-glycosylated pilin variants, piliation was slightly increased with a concurrent raise in adhesiveness for epithelial cells. When pilin proteins are deglycosylated, their migration on SDS-PAGE increases, and so variations in the extent of glycosylation may result in differences of pilin apparent molecular weight. In the current study, variable glycosylation may account for the different migratory pattern of type Ia and type Ib pilin on SDS-PAGE, but probably does not account for differences in adhesion to meningioma cells. There are several other theories as to the role of pilin glycosylation. Studies by Marceau (Marceau *et al.*, 1998) have shown that in meningococci, glycosylation is required for the production of soluble pilin (S pilin) monomers which are not assembled into pili but are secreted. A function for S pilin has yet to be defined but it is possible that a cell-binding domain, hidden in assembled pili, is available for binding in the soluble monomer. In this context, the cell-binding domain may interact with host components in some way, and therefore contribute to the pathogenesis of meningococcal infection (Marceau & Nassif, 1999). It has been reported that variation in meningococcal and gonococcal pilin is responsible for variation in tropisms of these bacteria for different cell types (Jonsson *et al.*, 1994; Virji *et al.*, 1991; Virji *et al.*, 1992a; Virji *et al.*, 1993b). It is therefore possible that such cellular tropism is mediated by a post-translational modification such as glycosylation. An additional substituent, α-glycerophosphate, has been found attached to Ser^{93} by a phosphodiester linkage, although its function in meningococcal pilin has not been defined (Stimson *et al.*, 1996). Another modification to pilin which may influence attachment to human cells is the phosphorylcholine (ChoP) epitope, which has been identified on a number of respiratory pathogens, including Neisserial pilus proteins, where it is subject to phase variation. In summary, post-translational

modifications may modulate bacterial adherence by either altering the interaction between the pilus ligand and the host cell receptor, or by effecting changes in charge distribution.

Although viable count assays showed that the association of non-piliated meningococci with meningioma cells was to a significantly lower level than the Pil^+ isolates, nevertheless some bacteria from the Pil^- pool did associate with these cells, and this data was supported by observations from LSCM and SEM. The Pil^+ isolates seemed to attach to meningioma monolayers with some specificity for certain areas of the cells, whereas the Pil^- organisms seemed to adhere randomly across the cells. It is possible that the adherent bacteria from the Pil^- stock represents non-specific adhesion, or that other ligands can mediate adherence to meningioma cells, albeit to a low level. However, SEM analysis of meningothelial monolayers infected with the $Cap^+Pil^-Opa^+Opc^+$ isolate also revealed that pilus-like structures appeared to be present on some adherent bacteria from the Pil^- infecting stock, although these were only apparent after 24h of infection, and were much thinner than such structures present on the comparable Pil^+ isolate. Due to the dynamic on-off nature of meningococcal pilus production, in a non-piliated population there will always be some organisms that spontaneously turn on pilus production, and vice versa. It is possible that in the presence of human cells, Pil^- meningococci turn on pilus production to provide themselves with a survival advantage. Immunoblot analysis of associated bacteria recovered from infection experiments revealed just two examples of possible pilus up-regulation from a Pil^- stock; however viable count data showed that these bacteria associated with the meningioma cells to the same low levels as always found with non-piliated organisms. In support of the possibility that non-pilus structures may be responsible for association of Pil^- organisms, other workers have found that greater than 95% of the bacteria recovered after infecting HUVECs with Pil^- meningococci were still Pil^- (Virji *et al.*, 1991).

In general, the same pattern of association was seen for all of the meningioma subtypes tested, although the actual numbers of associated meningococci varied between subtype. Since all of the piliated variants associated with transitional cells (M17 cell line) in significantly higher numbers than with meningothelial cells (M18 and M21 cell lines), the suggestion is that M17 cells may express more, or different pilus receptors than M18 or M21 cells. In contrast, all of the Pil^+ isolates associated with anaplastic cells (M16 cell line) in significantly lower numbers than with meningothelial or transitional cells, suggesting that there may be less, or different pilus receptors on M16 cells than on cells of the other meningioma subtypes. Human CD46 is a receptor for meningococcal pili, the putative bacterial ligand is PilC, and meningothelial M18 cells were shown to express CD46, albeit less intensely than the epithelial Chang cells. However, the diffuse pattern of CD46 distribution on M18 cells does not correlate with the specific localisation of Pil^+ meningococci to the nuclear regions and cell processes of these cells seen under SEM and LSCM; such tropism could be suggestive of specific receptors for bacterial ligands being present at these locations. However, the cellular distribution of receptors may be different before and after bacterial

attachment and thus, it may be more relevant to stain infected rather than uninfected cells. In addition, although the other meningioma cell lines used in this study were not tested for CD46, this information may reveal altered receptor expression between meningioma subtypes which could account for the disparity observed in meningococcal association with the different cell lines.

In addition to the pilus, both *Neisseria meningitidis* and *Neisseria gonorrhoeae* produce low quantities of a phase-variable PilC protein which has been found at the tip of the pilus (Rudel *et al.*, 1995) where it is proposed to act as a meningococcal (Nassif *et al.*, 1994) and gonococcal (Rudel *et al.*, 1992) adhesin for human cells. PilC is also associated with the meningococcal cell surface regardless of piliation status (Rahman *et al.*, 1997). Two PilC proteins, designated PilC1 and PilC2 are usually present in both pathogens, but in meningococci only PilC1 has been implicated in the adhesion to human epithelial and endothelial cells (Nassif *et al.*, 1994; Rahman *et al.*, 1997). Bacteria that differed in their expression of PilC levels but were otherwise isogenic, have been shown to associate with human cells at different levels; increased PilC levels correlate with enhanced meningococcal adherence to Chang epithelial cells. In addition, the pili from Pil^+ meningococci of strain MC58 that expressed low levels of PilC were easily detached from the surfaces of the bacteria, with the pili being left attached to the infected HUVEC monolayer (Virji *et al.*, 1995a). All of the variants of MC58/59 used in the current study expressed the same low levels of PilC1 protein, suggesting that variable PilC1 expression did not play a role in modulating the adherence of the pilin variants. This is in accord with the studies of Virji and colleagues, who showed that for variants of capsulated *Neisseria meningitidis* strain C311, which expressed similar levels of PilC protein, adherence to endothelial and epithelial cells correlated only with pilin variation (Virji *et al.*, 1993b). Interestingly, with isolates obtained from a fatal case of meningococcaemia, PilC expression has been found to be higher in bacteria isolated from the CSF and associated with the choroid plexus than from the blood (Pron *et al.*, 1997). In addition, the isolates with higher PilC expression were found to be more adhesive with cultured human endothelial cells than the blood isolates. The authors suggested that during disease, raised PilC expression is probably selected for its ability to increase meningococcal adhesiveness. The group B MC58/59 isolates used in the current study expressed low levels of PilC, but bacteria recovered from meningioma infection experiments were not analysed for differences in PilC expression. However, strain C114 expressed high levels of PilC and yet this organism still associated with meningioma cells in lower numbers than the MC58/59 isolates. Since it has recently been reported that PilC1 expression is transiently induced by bacterial cell contact even in the absence of assembled pili, analysis of recovered bacteria from the present study may reveal differences in PilC expression as a result of contact with human meningioma cells (Taha *et al.*, 1998).

A substantial number of meningococcal strains do not express class I pili, but rather class II pili, which are of a distinctly different antigenic type (Diaz *et al.*, 1984; Aho *et al.*, 1997). Virji and workers (Virji *et al.*, 1991; Virji *et al.*, 1992a) have reported that class I and class II pili were

similar in promoting bacterial association with endothelial cells, and further that class II pili mediated less effective adherence to epithelial cells than class I pili. This study also found that the same strains of meningococci with class II pili did not adhere equally well to all epithelial cell lines, and suggested that these bacterial ligands may require distinct receptors which may not be present on some cell types. In the present study, meningococci expressing either class I (MC58/59) or class II pili (strain C114) showed high levels of adherence to cultured meningioma cells, although isolates with class II pili were less effective than those with Ia pili. In contrast, the commensal *Neisseria lactamica*, which also expressed class II pili, adhered to meningioma cells at a very low level, suggesting that perhaps other bacterial surface structures, or variation in class II pilin itself, may determine the interactions of pathogenic *Neisseria* with meningeal cells. Strain C114 is a serogroup C organism, and although the association of meningococci with HUVEC is not consistently altered by different capsules, it is possible that the type of capsule has a profound effect on bacterial association with meningioma cells.

Neisseria meningitidis is closely related to the pathogen *Neisseria gonorrhoeae*, which is the causative agent of gonorrhoea, and the class I pili of meningococci are structurally and antigenically related to gonococcal pili (Virji & Heckels, 1983; Diaz *et al.*, 1984). Despite the fact that both of these pathogens expressed the same class of pili, and although gonococci did adhere to meningioma cells, this was at much lower levels than with piliated meningococci, suggesting that gonococcal pili were less effective adhesins than meningococcal pili. The observation that gonococci adhere to meningioma cells is not surprising since leptomeningitis caused by *Neisseria gonorrhoeae* has been reported to occur as a rare event during disseminated gonococcal infection (Del Rio *et al.*, 1989). Size differences between the pilin proteins of the gonococcal and meningococcal strains used in the current study are indicative of structural variations between the two organisms, which could account for altered adhesion patterns as discussed for meningococci above.

5.6.2 Role of LPS

In studies with endothelial cells, pili are reported to be the only effective adhesin in Cap^+ meningococci, and in phenotypes with the L3 sialylated LPS immunotype (Virji *et al.*, 1995b). The panel of Cap^+ isolates used in the current study were all of the L3 immunotype, and although it has been reported that in Cap^+ bacteria the LPS type has negligible effect on the association with human endothelial cells (Virji *et al.*, 1995b), it has not been established that this holds true for interactions with meningioma cells. Additionally, since it is possible that there are multiple types of receptor for LPS on human cells, including CD14, ASGP-R and integrins it would be interesting to see whether meningioma cells expressed any of these. Studies on rats (Lacroix *et al.*, 1998) have shown that under basal conditions there are low levels of CD14 mRNA in the leptomeninges, choroid plexus and along blood vessels of the brain microvasculature. After systemic injection of bacterial LPS there was a marked increase in the expression of the CD14 gene within these structures thus

providing evidence that LPS has a direct role on specific cell populations in the CNS. However, it is also possible that leptomeningeal cells could lack LPS receptors but that LPS could still mediate interactions with these cells indirectly via a soluble CD14 receptor present in serum.

5.6.3 Role of capsule

The presence of a capsule has been shown to reduce the pilus-mediated adherence of meningococci to human epithelial and endothelial cells, although the reason for this is unclear. In the current study, the similarity in adherence of Cap^+ and Cap^- meningococci to meningothelial and anaplastic meningioma cells suggested that the presence of capsule did not interfere with pilus-mediated adherence. However, the loss of capsule did appear to significantly increase the association of meningococci with transitional meningioma cells. It is possible that the receptors expressed on transitional cells for the pilus protein are different to, or had higher affinity for their ligand than those expressed on the meningothelial cells, and that the polysaccharide capsule somehow selectively interfered with the transitional receptor resulting in higher association in Cap^- organisms. During the meningococcal infection of meningothelial cells, results from SEM analysis revealed that there were also differences in the way Cap^- meningococci associated with meningothelial cells in comparison to Cap^+ organisms. The Cap^- bacteria appeared to be much more evenly spread over the cells than seen with the Cap^+ isolates, even though in the culture media, Cap^- isolates formed more and larger clumps than the Cap^+ isolates. In addition, these experiments also revealed that pili were often not apparent on the bacteria that were most closely associated with the cells and that overall, there seemed to be fewer pili visible on adherent Cap^- bacteria than on the Cap^+ isolates. It has been suggested that after the initial pilus-mediated association of meningococci with human cells, that pilus production is down-regulated before attachment becomes more intimate, and before the bacteria are internalised by the cells (Pujol *et al.*, 1997). It is proposed that after meningococci have adhered to human cells as microcolonies surrounded by microvillus extensions of the host cell, they subsequently spread across the cells apical surfaces, to form a single layer of bacteria, and that this process does not involve Opa or Opc proteins or the pilus itself. Pathogenic *Neisseria* produce a protein called PilT, located in the bacterial cytoplasm and which is responsible for pilus twitching motility. Pujol and colleagues (Pujol *et al.*, 1999) have recently reported that PilT plays an essential role in the dispersion of Cap^+ meningococci over human cells, and in the subsequent loss of piliation and concurrent intimate attachment to the cells. Apparently, bacteria which lack PilT remain attached to the cell monolayer in a pilus-mediated manner, surrounded by microvillus extensions, and are unable to spread across the cells and form intimate contact with them. In the current study it is possible that there is some down-regulation of pili after attachment to meningothelial cells. Since the Cap^+ bacteria tended to adhere in clumps over meningothelial monolayers, then according to the PilT hypothesis, these results suggest that the bacterial strains used are $PilT^-$, although microvillus extensions of the cells were not prominent. If capsule has no role in the action of PilT, then the more diffuse adherence of the Cap^- organism to meningothelial

cells, coupled with the more intimate adherence seen under TEM, suggest that these bacteria are PilT$^+$. However, none of the variants used in the current study were tested for the presence of PilT and this area requires further investigation.

5.6.4 Opa- and Opc-mediated interactions

Opa proteins are known to have adhesive and invasive roles with gonococci, which are acapsulate organisms (Makino *et al.*, 1991; Lambden *et al.*, 1979b). In meningococci, expression of Opa proteins has been reported to increase association of Cap$^-$, but not Cap$^+$ bacteria with human cells, via binding to CD66 molecules expressed on the cell surface. Although the meningococcal capsule is typically known to be inhibitory of Opa proteins, using COS cells transfected with cDNA for CD66a, Virji and co-workers (Virji *et al.*, 1996) have shown that Cap$^+$ meningococci can adhere to these animal cells via the CD66a receptor, if it is expressed at a high level. In the current study, expression of Opa protein did not influence the association of Cap$^+$ meningococci expressing the high affinity Pil_{Ia} with meningothelial cells, presumably because the efficiency of the pilus-mediated interaction masked the potential adhesive role of Opa. However, Opa expression did increase the association with meningothelial cells of meningococci that expressed the low adhesive Pil_{Ib}. These observations were also seen with anaplastic meningioma cells, but not with transitional cells. Presumably, because the association of all Pil$^+$ isolates with these cells was high, then any effect of Opa expression was masked. Meningioma cells were not tested for their expression of receptors such as those in the CD66 family, but reports in the literature have found that secretory meningiomas occasionally express CEA (CD66e). Although this molecule appears to have a role in meningococcal interactions with epithelial and endothelial cells, bacterial-meningioma cell interactions have not yet been investigated regarding this molecule (Partington *et al.*, 1995). It is not known whether normal leptomeningeal cells express CD66 surface molecules; it would be worthwhile to stain sections of leptomeningeal tissue for expression of the members of this family as potential candidates of receptors for meningococcal ligands. It is possible that variable expression of such receptors on meningioma cells of different histological subtypes may in part explain the significantly lower adherence of meningococci to anaplastic meningioma cells, compared with meningothelial and transitional meningioma cells. It might be expected that in the absence of pili, the role of Opa as an adhesin would be more evident, and the Pil$^-$ variants used for infection experiments differed with respect to their expression of these proteins. However, all of the Pil$^-$ isolates associated with meningioma cells to very similar levels, although this would not be surprising if pili were required to mediate the first step in meningococcal association with these cells.

The Opc protein is implicated in the invasion of cultured epithelial and endothelial cells, but, as with Opa proteins, it has been reported that Opc is only effective at increasing these interactions in Cap$^-$ bacteria with unsialylated LPS immunotypes (Virji *et al.*, 1995b). Using Pil$^-$ isolates, and in

contrast to Opa protein, the Opc protein did not appear to play a role in increasing the association of $Cap^{+}Pil^{+}$ meningococci with meningioma cells. Since all of the isolates used in the current study are of the sialylated L3 immunotype, it maybe that Opc is inhibited in these bacteria. Also, as pili are thought to be essential in mediating the first attachment to human cells, then Opc may have a role in the more intimate association of meningococci to meningioma cells, which cannot be seen with Pil^{-} organisms. Efficient Opc-mediated bacterial interactions require meningococci to express Opc at high levels, and since the isolates used in the current study showed high Opc expression, then Opc density was not an issue. Opc has been shown to bind to human cells via integrins (using serum-derived RGD bridging molecules) and heparan sulphate proteoglycans, much like the Opa_{50} protein of the gonococcal strain MS11. Some studies have shown that the expression of integrins on meningioma cells does not differ consistently from the pattern of expression seen on normal leptomeningeal cells (Paulus *et al.*, 1993). It would therefore be of great relevance to investigate the expression of integrins on the meningioma cells used in the current study. It would also be of value to look at the expression of heparan sulphate proteoglycans on these cells.

Interestingly, cytochalasin D (CD) was found consistently to decrease the association of Cap^{+} and Cap^{-} meningococci to meningothelial cells. CD severely disrupts the organisation of the network of cellular actin filaments and causes disruption of cell structure (Schliwa, 1982). Under light microscopy, meningothelial cell monolayers that had been incubated with CD were no longer intact and the remaining cells appeared to have contracted and shrunk away from one another into star-shaped cells. These observations have also been reported by other workers using epithelial cells (Grassme *et al.*, 1996) and were consistent with the ability of CD to cause retraction of actin filaments. It is therefore possible that such changes in meningothelial cells mask or modify the receptors used by meningococcal ligands for attachment, resulting in decreased association. In similar experiments with endothelial cells, it has been reported that with Cap^{-} organisms, if Opa is present then adherence is actually increased, suggesting that Opa associative interactions with host cells are not dictated by host cytoskeletal function (Virji *et al.*, 1994). After the addition of CD to meningothelial cells, with Cap^{+} organisms there were no differences in adhesion between Opa^{+} and Opa^{-} isolates, but the only Cap^{-} organism available was Opa^{-}. It would be interesting to see, in the absence of capsule, whether Opa has a role as an adhesin to these meningeal cells. Additionally, with Cap^{-} bacteria in the absence of Opa, CD has been shown to decrease Opc mediated association with endothelial cells (Virji *et al.*, 1994); it would be interesting to see if this holds true for meningococcal interactions with meningioma cells.

5.6.5 Cytopathic effects of meningococcal infection

In accordance with observations by other studies using human endothelial cells, piliated bacteria were found to cause greater damage to the integrity of the meningioma monolayer than non-piliated variants (Virji *et al.*, 1991).

5.7 Meningococcal invasion of meningioma cells

Although meningococci are present in the SAS of meningitis victims they do not actually invade the underlying cerebral tissue, even though the brain may become oedematous and haemorrhagic. This suggests that the leptomeninges acts as a barrier to prevent the passage of meningococci into the underlying neurophil. Meningioma cells were therefore tested to see if they exhibited such barrier properties. Despite high levels of adherence, $Cap^{+}Pil^{+}$ meningococci did not invade meningothelial meningioma cells. Although a greater number of Cap^{-} meningococci survived treatment with gentamicin, pre-incubation of meningioma cells with CD did not reduce the number of bacteria that survived treatment with antibiotic, suggesting that if any invasion did occur that it was not dependent on cytoskeletal activity of the cells, which is necessary for invasion of epithelial and endothelial cells (Virji *et al.*, 1991). In addition, there was no evidence of internalisation of the Cap^{-} meningococcus by TEM, despite the intimate contact between the outer membrane of the meningococcus and the meningioma cell membrane. It is likely that this intimate association, coupled with the apparent increased activity of the host cell membrane, created an environment that enabled the bacterium to evade the action of the antibiotic. Similar observations have been reported by Bessen and Gotschlich (Bessen & Gotschlich, 1986) and Virji and co-workers (Virji *et al.*, 1991) with epithelial and endothelial cells. Meningothelial cells also did not internalise certain variants of gonococci, commensal strains of *Neisseria* or a serogroup C meningococcal strain. It has been reported that gonococci are internalised by endothelial cells in relatively larger numbers than Cap^{+} meningococci (Virji *et al.*, 1991). This difference in internalisation has been attributed to the lack of capsule on the gonococcus. However, since neither Cap^{+} or Cap^{-} meningococci or gonococci were internalised by meningioma cells, this provides further evidence that meningioma cells have barrier properties against bacteria. Endothelial cells obviously do not form such a barrier or else there would not be invasive meningococcal disease. Since cultured meningioma cells have been shown to take up inert latex beads of 1μm diameter (Feurer & Weller, 1991), the lack of internalisation of meningococci by these cells is not due to an inability to phagocytose foreign material. This notable lack of invasion of meningioma cells by meningococci *in vitro*, is consistent with the barrier properties of the leptomeninges to *N. meningitidis* observed *in vivo*, where capsulated bacteria are found only in the SAS and in association with leptomeningeal cells, with little or no spread of the organism to the underlying brain.

It is still a matter of debate, with no direct evidence either way, whether meningococci gain access to the SAS by crossing the B-CSFB at the site of the choroid plexus or leptomeningeal coated vessels. Although reports concerning a fatal case of meningococcaemia have shown that more bacteria were present at the endothelium of the choroid plexus than of the meninges, if the bacteria were to cross at the choroid plexus, they would first enter the ventricles of the brain and initiate ventriculitis before meningitis. Ventriculitis is a rare complication of meningitis and thus occurs

after the onset of meningitis, supporting the notion that meningococci enter the SAS at the site of the leptomeninges.

5.8 Meningioma cytokine response to meningococcal challenge

The damage to the CNS resulting from meningococcal infection of the SAS is a result of a pathogen-induced acute host inflammatory response. In addition, the CNS is very ineffective at eliminating pathogens. In response to bacterial stimuli, the CNS mounts an inflammatory reaction, which is dependent upon the local production of soluble mediators, including cytokines and chemokines (Brandtzaeg *et al.*, 1992). As mentioned previously, the presence of certain cytokines and chemokines within the CSF is associated with meningococcal meningitis, but it is unclear precisely which cells are responsible for the production of these inflammatory mediators during this disease. Amongst these, TNF-α, Il-1β, Il-8, Il-6 and Il-10 have perhaps received the most scrutiny, although others have been reported on. In this study, preliminary experiments were carried out to assess the regulation of expression of mRNA transcripts for certain cytokines and chemokines by meningioma cells challenged with meningococci. Only one time point was done (3h) so the results cannot exclude message for cytokines that are produced very early on in the experiment, and then decline rapidly, or those produced at later time points. As FCS contains factors such as LPS that could induce the expression of cytokines from meningioma cells even in the absence of meningococci, the concentration of FCS used in these experiments was reduced from 2% to 0.1% (v/v). The uninfected meningioma cells used in this study did not constitutively express message for any of the cytokines tested, and so the detection of cytokine message in infected samples was attributed to the presence of meningococci. Meningococci from the CSF of meningitis sufferers are predominantly piliated, and significantly in this study, only piliated bacteria were capable of upregulating cytokine mRNA transcription in meningioma cells. From the panel of cytokines tested, message for Il-1β, Il-6 and Il-8 were found to increase upon Pil^+ meningococcal challenge. These cytokines play important roles in the inflammatory response. In particular, the Il-8 chemokine is likely to be the major signal for the influx of neutrophils into the SAS during meningitis. The up-regulation of message for Il-8 in cultures of infected meningioma cells may reflect an *in vivo* situation whereby leptomeningeal cells express this chemokine in response to meningococcal challenge to attract inflammatory cells. These experiments also raise the possibility that leptomeningeal cells are also capable of expressing the pro-inflammatory cytokines Il-1β, which is known to increase in the presence of bacterial LPS, and Il-6. Interestingly message for TNF-α, the other major pro-inflammatory cytokine present at high levels during meningitis, was not produced by meningioma cells. In addition, no message was detected for the chemokines MCP-1 and MIP-1α, which are known to be chemotactic for monocytes and macrophages. Whilst it is possible that these cytokines are produced by other host cells, just as Il-8, Il-1β and Il-6 are

probably not exclusively expressed by leptomeningeal cells, it must be stressed that these are preliminary observations. However, other workers have reported that some meningiomas express mRNA and protein for cytokines that were negative in this study, including MCP-1 (Sato *et al.*, 1995). As meningioma cells are the neoplastic derivatives of normal leptomeningeal cells, prior to their use in meningococcal infection experiments, they would first have to be characterised with respect to constitutive expression of the cytokines of interest. In summary, meningothelial meningioma cells were found capable of producing mRNA for several cytokines known to be important in the pathophysiology of meningitis. Clearly the inflammatory response needs to be investigated in greater detail and, in particular, the analysis of actual cytokine protein production is necessary. An interesting point regards patients with occult meningococcal disease. These patients do not present with the typical sudden life threatening disease, but instead seem to have mild symptoms of disease as if their bodies do not produce the inappropriate immune response seen in most cases. Perhaps further investigation into the immune systems of these individuals would provide clues as to how to over-ride the lethal reaction to pathogenic meningococci.

5.9 Further support of the meningioma model

Within this laboratory, an additional model of the human meninges has been established that utilises cryostat sections of fresh human brain with overlying leptomeninges (Hardy *et al.*, 2000). When incubated with these sections, Pil^+ meningococci showed a predilection for binding to the leptomeninges and meningeal blood vessels and not to the cerebral cortex. In addition, the pattern of adherence of the meningococcal variants closely correlated with that seen with cultured meningioma cells, thus validating the use of these cells for meningococcal-meningeal cell interaction studies.

5.10 Conclusions

Many efforts are concerned with the development of a vaccine against group B meningococcal disease. Current avenues of treatment include the application of antimicrobial and anti-inflammatory principles to kill the pathogens and reduce the body's response to their presence. Obviously, additional problems are encountered as candidate drugs must first cross the B-CSFB to reach the SAS. A greater understanding of the mechanisms that occur during the interaction of meningococci with leptomeningeal cells may highlight possible avenues for therapeutic strategies to alleviate the sequelae associated with meningococcal meningitis, especially as a vaccine against group B disease still eludes us. This project has established the use of meningioma cells to permit *in vitro* studies of the acute intra-cranial inflammatory response at the molecular level, and the

potential for testing new anti-inflammatory therapies for the treatment of meningococcal meningitis. A working hypothesis has now been established that states that meningococci do associate with leptomeningeal cells, and that this interaction is mediated primarily by pili and to a lesser extent by Opa proteins. In addition, leptomeningeal cells probably act as a barrier against meningococcal invasion.

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