UNIVERSITY OF SOUTHAMPTON

THE ROLE OF THE HUMAN LEPTOMENINGES IN THE INFLAMMATORY RESPONSE TO BACTERIAL PATHOGENS

by Mark Ian Fowler

Submitted for the degree of Doctor of Philosophy

DEPARTMENT OF MOLECULAR MICROBIOLOGY AND INFECTION

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

APRIL 2004

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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Despite the introduction of effective bactericidal antibiotics and supportive care, pyogenic bacterial meningitis still remains a substantial cause of morbidity and mortality worldwide. At present and exclusive of epidemics, over one million cases of bacterial meningitis are estimated to occur annually worldwide resulting in 135,000 deaths, with up to 50% of survivors presenting with serious psycho-neurological sequelae. Bacterial meningitis is characterised by an acute compartmentalised inflammatory response induced by the presence of bacteria in the cerebrospinal fluid in the subarachnoid space between the arachnoid and pia mater. Although recent studies have begun to elucidate the molecular mechanisms underlying the interactions of bacterial pathogens with human epithelial and endothelial cells, there is little information available on the later stages of infection involving cells of the leptomeninges. In the current study, an *in vitro* model of the human leptomeninges based on the culture of meningioma cells, was used to investigate the interactions of the meningeal pathogens *Neisseria meningitidis, Escherichia coli* K1, *Haemophilus influenzae* and *Streptococcus pneumoniae* and the commensal *N.lactamica.*

All of the meningeal pathogens exhibited a tropism for human meningioma cells. However, there were significant differences between the pathogens in the numbers of bacteria adhering to the cells *in vitro*. *N.meningitidis* MC58-7 and *E.coli* strains DSM and IH3080 adhered to meningioma cell monolayers in the greatest numbers, closely followed by *H.influenzae* strain RD. *N.meningitidis* MC58-9 and *S.pneumoniae* associated with meningioma cells at levels approximately ten-fold lower than those observed with MC58-7 and both *E.coli* strains, with an additional ten-fold reduction in adherence exhibited by *N.lactamica* and *H.influenzae* strain Eagan. Gentamicin-viable counting assays and transmission electron microscopy demonstrated that only *E.coli* IH3080 could invade meningioma cells. This ability to overcome the barrier properties of the leptomeninges may allow the spread of this particular pathogen into the subpial space and the underlying cerebral tissue and may be associated with the high morbidity and mortality of neonatal meningitis. In addition, the association of meningeal pathogens was investigated with Chang cells as a representative epithelial cell line. The numbers of *N.meningitidis* MC58-7 and MC58-9 associating with Chang cell monolayers were consistent with those observed with meningioma cells. However, the levels of association of *H.influenzae*, *E.coli*, *S.pneumoniae* and *N.lactamica* to Chang cells were approximately 10-20 fold higher than the corresponding values with meningioma cells. The association of meningeal pathogens with a representative epithelial cell line at equally high levels is consistent with the early colonisation of the nasopharynx.

Meningioma cell lines challenged with meningococci secreted large amounts of the pro-inflammatory cytokine IL-6, the CXC chemokine IL-8, the CC chemokines MCP-1 and RANTES, and the cytokine growth factor GM-CSF. Challenge of meningioma cell lines with the other bacteria also resulted in the secretion of these inflammatory mediators: however the type and concentration of cytokines and chemokines induced was specific to each meningeal pathogen. By contrast, Chang epithelial cells challenged with pathogenic bacteria did not secrete MCP-1, and the overall protein secretion levels of IL-6, IL-8, RANTES and GM-CSF were greatly reduced compared to meningioma cell lines. Thus the current study demonstrates that the leptomeninges is an active participant in the acute inflammatory response characteristic of bacterial meningitis, through the early secretion of pro-inflammatory cytokines and chemokines in response to bacterial challenge.

PUBLICATIONS

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

Fowler, M.I., Weller, R.O., Heckels, J.E., and Christodoulides, M. (2004) Different meningitis-causing bacteria induce distinct inflammatory responses on interaction with cells of the human meninges. *Cellular Microbiology. In Press.*

Christodoulides, M., Makepeace, B.L., Partridge, K.A., Kaur, D., Fowler, M.I., Weller, R.O., and Heckels, J.E. (2002). Interaction of *Neisseria meningitidis* with human meningeal cells induces the secretion of a distinct group of chemotactic, proinflammatory, and growth-factor cytokines. *Infect.Immun.* **70**, 4035-4044.

Fowler, M.I., Heckels, J.E., and Christodoulides, M. (2002) The interactions of bacterial pathogens causing meningitis induce suignificant differences in the inflammatory responses of leptomeningeal cells. 13th International Pathogenic *Neisseria* Conference, Oslo.

Fowler, M.I., Heckels, J.E., and Christodoulides, M. (2000) Expression of β-defensins in epithelial and meningeal cells following challenge with *Neisseria meningitidis*. 12th International Pathogenic *Neisseria* Conference, Texas.

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ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. Myron Christodoulides and Professor John Heckels for their overall guidance, continued advice and support throughout this project. I would also like to acknowledge the Medical Research Council for providing the studentship to carry out this study.

Many thanks go to my friends past and present for making my time in Southampton such an enjoyable one: Holly, Jenny, Kris & Lucas, Davi, Omar & Lindsay, Sarah & James, Carrie, Deng, Silvia, Lesley, Stuart & Helen, Annabelle, Ian, Paul, Steve G, and my buddy (for a year anyway!) Steve H. Special thanks go to Paddy for always having the time for a chat and his unnerving accuracy in pcv estimates, and Mark Pickett for his technical knowledge on TaqMan and Mac computers. In addition, I would like to thank 'my girls' from 166 Dale Valley Rd, for taking in a stranger and making him feel so welcome: Laura, Ruth, Amy, Sarah and Julia. Thanks also to my big brother Paul, in the words of a great, albeit evil, Doctor "You complete me".

I would also like to thank the canteen sausage chef and the MARS Corporation for providing me with the sustenance and the variation in diet necessary to complete this project.

Most of all, special thanks and a lot of love go to Annie B, for not only leaving her native country to join me in Southampton but also for the unconditional love and support she has bestowed upon me regardless of my many mood swings during the last four years.

Finally, I would like to express my fondest gratitude to my late granddad, Griffith John Davies, whose quick thinking and imaginative use of my mother's tea towels many years ago saved the life of a very grateful eight-year-old boy. Thanks Gramps.

DEDICATION

This thesis is dedicated to my parents, Lynda and Tony, whose endless love and support have allowed me to get where I am today. Thanks for believing in me, especially during the times when I doubted myself.

ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
AIF	apoptosis-inducing factor
ANOVA	analysis of variance
BBB	blood-brain barrier
B-CSFB	blood-cerebrospinal fluid barrier
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BMEC	brain microvascular endothelial cells
β-NAD	β-nicotinamide adenine dinucleotide
BSA	bovine serum albumin
CD	cytochalasin D
cDNA	complementary DNA
cfu	colony forming unit
CbpA	choline binding protein A
CF	cystic fibrosis
ChoP	phosphorylcholine
CMVEC	cerebral microvascular endothelial cells
CNF1	cytotoxic necrotizing factor 1
CNS	central nervous system
CRP	C-reactive protein
CSF	cerebrospinal fluid
dFCS	decomplemented foetal calf serum
dH_2O	distilled water
DIC	disseminated intravascular coagulation
DMEM	Dulbecco's modification of eagles medium
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides

DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EMA	epithelial membrane antigen
ETCC	European Type Culture Collection
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage colony stimulating factor
GPI	glycosylphosphatidylinositol
GRO-α	growth related protein alpha
H_2O_2	hydrogen peroxide
Hap	*Haemophilus* adherence and penetration protein
hBD-1/2/3	human β-defensin 1/2/3
HBEC	human brain endothelial cells
HBSS	hanks balanced salt solution
HbuB	haemoglobin binding protein B
HD5/6	human defensin 5/6
HIV	human immunodeficiency virus
HNP1-4	human neutrophil defensins 1-4
HSV	herpes simplex virus
HTM	*Haemophilus* test medium
HUVECs	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule 1
ICP	intracranial pressure
IL	interleukin
IFN-γ	interferon gamma
IRAK	interleukin-1 receptor-associated kinase
KDa	kilodalton

LbpA	lactoferrin binding protein A
LOS	lipooligosaccharide
LPS	lipopolysaccharide
mAb	monoclonal antibody
MCP-1	monocyte chemoattractant protein 1
$MgCl_2$	magnesium chloride
MIP-1/2	macrophage inflammatory protein 1/2
MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
NBT	nitro blue tetrazolium
NCI	National Cancer Institute
NCTC	National Collection of Type Cultures
NF-κβ	nuclear factor kappa beta
NO	nitric oxide
OM	outer membrane
OMP	outer membrane protein
PAF	platelet activating factor
PAMP	pathogen-associated molecular patterns
PBS	Dulbecco's phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
pIgR	polymeric immunoglobulin receptor
PIPES	piperazine-NN'-bis-2-ethanesulphonic acid
PMNL	polymorphonuclear leukocyte
PMSF	phenylmethylsulfonylfluoride
PP	proteose peptone
PspA/C	pneumococcal surface protein A/C
RANTES	regulated on activation, normal T cell expressed and secreted
RBC	red blood cell
RNA	ribonucleic acid

ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SAS	sub-arachnoid space
SDS-PAGE	sodium-dodecyl sulphate polyacrylamide gel electrophoresis
sIgA	secretory immunoglobulin A
TBE	tris-borate-EDTA buffer
TbpA/1/2	transferrin binding protein A/1/2
TEM	transmission electron microscopy
TEMED	N,N,N',N'-Tetramethyllethylenediamine
TGF-β	transforming growth factor beta
TLR	toll-like receptor
TNF-α	tumour necrosis factor alpha
TRAF6	tumour necrosis factor receptor-associated factor 6
Tris	tris(hydroxymethyl)methylamine
TBS	tris buffered saline
TTBS	tween-tris buffered saline
UHQ	ultra high quality water
UNG	uracil *N*-glycosylase
VCAM-1	vascular intercellular adhesion molecule 1

CHAPTER 1 INTRODUCTION

1.1 The human meninges

1.1.1 Structure and function of the human meninges

The meninges is a thin anatomical structure, consisting of three closely associated yet distinct layers or membranes that cover the brain and spinal cord intimately (Figure 1.1). These layers are the dura mater (pachymenix), the 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). Although the arachnoid and pia mater are separated by the sub-arachnoid space (SAS), they are connected by trabeculae of leptomeningeal cells, which traverse this fluid containing space (Al-Rodhan & Laws, 1991; Gray & Fedorko, 1992; Weller, 1995). Overall, the meninges are composed primarily of meningothelial cells and varying amounts of extracellular connective tissue (Al-Rodhan & Laws, 1991).

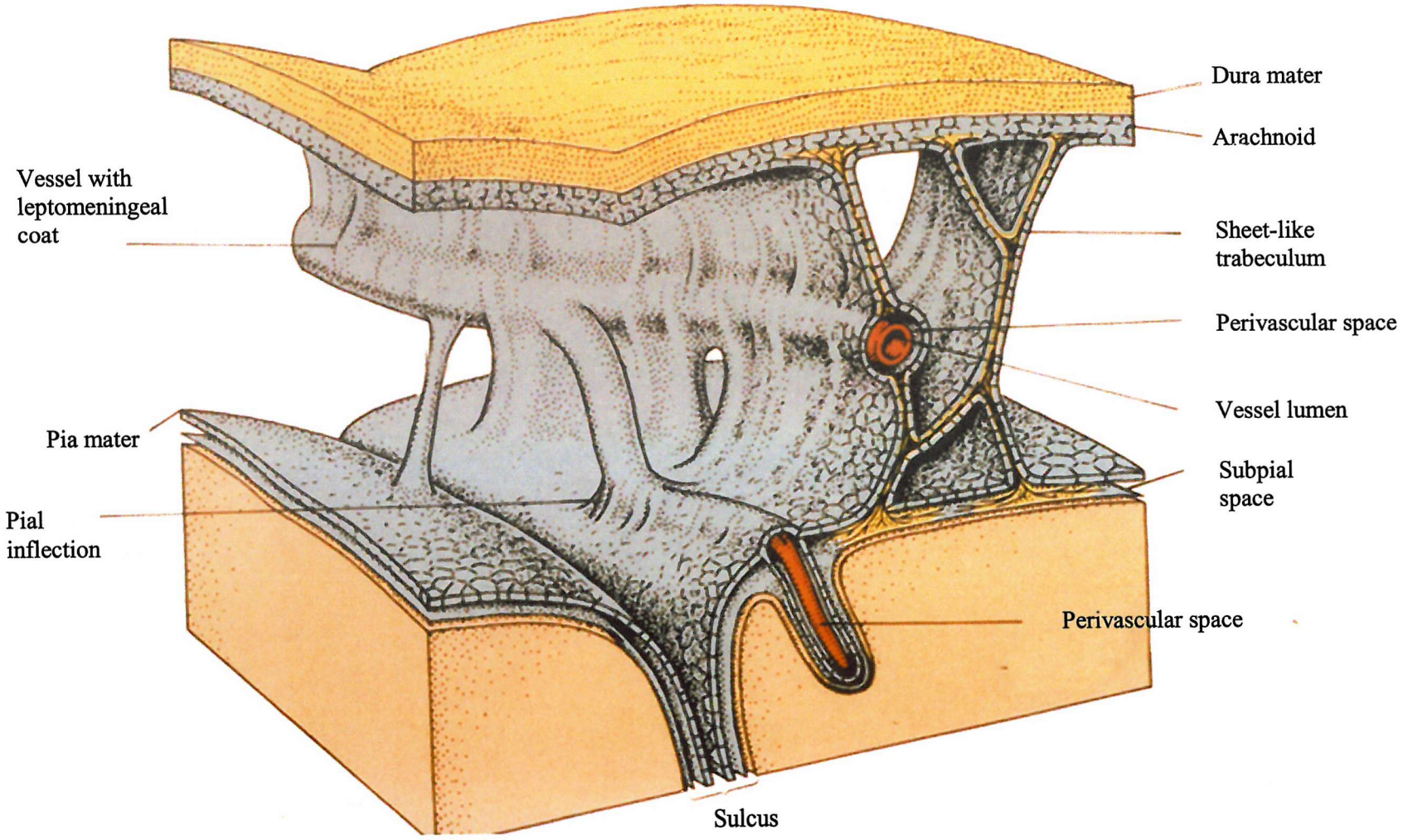
1.1.1.1 The dura mater

The dura mater, "hard mother", is intimately associated with the skull and is a dense, fibrous sheet, composed of tough, non-elastic connective tissue. The dura is a relatively thick membrane composed of two histologically similar layers, an outer or endosteal region and an inner or meningeal region (Al-Rodhan & Laws, 1991; Weller, 1995). The innermost meningeal layer is attached to the arachnoid and there is no sub-dural space (Al-Rodhan & Laws, 1991).

1.1.1.2 The arachnoid

In contrast to the thick dura layer, the arachnoid is a delicate membrane consisting of several layers of translucent, tightly packed barrier cells that adhere tightly to the dura mater. In addition, arachnoid trabeculae consist of loosely organised, 'spindly' cells that traverse the sub-arachnoid space connecting the arachnoid to the pia mater (Al-Rodhan & Laws, 1991). The SAS contains cerebro-spinal fluid (CSF) and the larger blood vessels that traverse the surface of the brain. The human arachnoid is similar to the arachnoid of other mammals, but it is considerably thicker (Alcolado *et al.*, 1988), and the presence of desmosomes and tight junctions are characteristic of the meningothelial cells within the arachnoid (Nabeshima *et al.*, 1975; Alcolado *et al.*, 1988). The presence of tight junctions is unique to these cells in the meninges and confers upon the arachnoid membrane the role of a physiological barrier, since these junctions are impermeable to CSF, protein macromolecules, water, ions and other soluble materials (Waggener & Beggs, 1967; Nabeshima *et al.*, 1975).

Figure 1.1: Structure of the human meninges and their inter-relationships (Adapted from Weller (Weller, 1995))



Arachnoid trabeculae in the form of sheets or narrow cords traverse the SAS at irregular intervals from the deep layers of the arachnoid to the pia mater. These trabeculae enclose the blood vessels of the SAS within a collagen core and they are coated in leptomeningeal cells, which become continuous with the pia mater at the interface of these cells (Alcolado *et al.*, 1988). These cells appear to be specialised fibroblasts with long cytoplasmic processes and they attach to the arachnoid layer and pial cells with desmosomes and gap junctions (Lopes & Mair, 1974; Schachenmayr & Friede, 1978).

1.1.1.3 The pia mater

The pia mater, "soft mother", is a delicate membrane formed from a layer of leptomeningeal cells often only one cell thick, which completely covers the surface of the brain and spinal cord (Alcolado *et al.*, 1988; Weller, 1995). Cells of the pia mater share a number of morphological similarities with arachnoid trabecular cells, being flattened and joined by desmosomes and gap junctions (Alcolado *et al.*, 1988; Al-Rodhan & Laws, 1991; Weller, 1995). However, in contrast to the arachnoid layer there are few, if any, tight junctions present (Alcolado *et al.*, 1988). In addition to the barrier role of the arachnoid layer, the pia mater, despite its delicate and thin nature, appears to form a regulatory interface between the CSF and the surface of the brain. The pia mater separates the SAS from the subpial space, the space between the pia and the basement membrane of the glia limitans on the surface and the perivascular spaces of the brain (Alcolado *et al.*, 1988; Zhang *et al.*, 1990) The pia mater could have a role in protecting the brain from metabolites and particulate matter in the CSF (Hutchings & Weller, 1986). In addition, pial cells contain the neurotransmitter-degrading enzymes glutathione S-transferase (Carder *et al.*, 1990) and catechol-O-methyl-transferease (Kaplan *et al.*, 1981), which suggests an enzymatic barrier function.

1.1.2 Meningioma cells as an *in vitro* model of the human leptomeninges

In order to study the molecular pathogenesis of bacterial meningitis, appropriate models are essential. While most studies have concentrated on the interactions of bacteria at the mucosal epithelia and vascular endothelia, there has been little work on the entry of meningeal pathogens into the circulation and subsequent interaction with components of the blood-cerebrospinal fluid barrier (B-CSFB) and brain. Cell lines such as human brain endothelial cells (HBEC) (Pron *et al.*, 1997), human brain microvascular endothelial cells (BMEC) (Ring *et al.*, 1998; Stins *et al.*, 2001; Kim, 2002) and cerebral microvascular endothelial cells (CMVECS) (Townsend & Scheld, 1995) have provided important models of pathogen-B-CSFB interactions. However, information on the latter stages of meningococcal disease, following entry into the CSF, involving interactions with the leptomeninges is still vague. One reason is that the primary culture of human leptomeningeal cells *in vitro*, despite occasional reports in the literature (Murphy *et al.*, 1991; Motohashi *et al.*, 1994), is unreliable (Feurer & Weller, 1991). However, cells can be cultured from benign tumours, known as meningiomas, of the human leptomeninges. Meningiomas are derived specifically from cells of the arachnoid membrane and they have many similarities to cells of the

pia mater (Feurer & Weller, 1991). Therefore, they are suitable for studying pia mater functions *in vitro* (Feurer & Weller, 1991). They can be further classified according to their histological characteristics, with the meningothelial subtype considered to most closely resemble its non-neoplastic counterpart. Meningioma cells exhibit all of the major characteristics of leptomeningeal cells, with essentially the same cytological structure and expression of the same cell markers, notably desmosomal desmoplakin, intermediate filaments of cytokeratin and vimentin, and epithelial membrane antigen (EMA) (Alcolado *et al.*, 1988; Feurer & Weller, 1991; Kleihues & Cavenee, 1997; Hardy *et al.*, 2000). Recently, meningioma cells have been used as a model to investigate the interactions of *N.meningitidis in vitro* (Hardy *et al.*, 2000) and the mechanisms involved in the generation of the compartmentalised immune response, characteristic of bacterial meningitis (Wells *et al.*, 2001; Christodoulides *et al.*, 2002).

1.2 Inflammation of the human leptomeninges

Inflammation of the human leptomeninges is referred to as leptomeningitis, and it can be caused by a wide variety of different pathogens including many bacteria, viruses and fungi. Viral meningitis is usually a relatively mild, self-limiting condition, with symptoms including headache, fever and depression (McCance & Huether, 1998). Since most patients recover without specific medical treatment, the incidence of viral meningitis is not known with any accuracy, but it is considered to be more common than the bacterial form (Cartwright, 1995a; Peate, 1999). By contrast, fungal meningitis is rare and is mainly associated with immunocompromised individuals, such as meningitis caused by *Cryptococcus neoformans* in patients with AIDS or organ transplantation recipients (Mehta & Pollard, 1999; Menezes *et al.*, 2002; Vilchez *et al.*, 2002). However, the main focus of the current study is meningitis caused by pyogenic bacteria.

1.2.1 Bacterial meningitis

Despite the introduction of effective bactericidal antibiotics and supportive care over the last fifty years, pyogenic bacterial meningitis still remains a substantial cause of morbidity and mortality worldwide (Schuchat *et al.*, 1997; Anonymous, 2000). At present and exclusive of epidemics, over one million cases of bacterial meningitis are estimated to occur annually worldwide resulting in 135,000 deaths (Anonymous, 1998). Although the bacterial form of meningitis is principally a disease of early childhood with over fifty percent of all cases occurring in children under five years of age, it can also affect all age groups including the newborn (neonate) and the elderly (Mehta & Pollard, 1999; Choi, 2001). In the current study, the four pathogens that have been responsible for the majority of cases of bacterial meningitis reported during the last century will be investigated, namely *Neisseria meningitidis*, *Haemophilus influenzae*, *Escherichia coli* and *Streptococcus pneumoniae* (Koedel *et al.*, 2002).

1.2.1.1 Neisseria meningitidis

Bacteria of the genus *Neisseria* are gram-negative diplococci, and include the obligate human pathogens, *N.meningitidis* (meningococci) and *N.gonorrhoeae*, and the non-pathogenic commensal bacterium, *N.lactamica*. The only known reservoir of meningococci is the human nasopharynx, and at any given time approximately 10% of the population are carriers, with respiratory droplets thought to be responsible for the transmission of bacteria between close contacts (Cartwright, 1995b). The organism is classified into serogroups based on the composition of its polysaccharide capsule, and of the thirteen or more serogroups responsible for human disease, three serogroups (A, B, and C) account for 90% of meningococcal disease worldwide (Spach & Jackson, 1999). Recently, the complete genomes of a serogroup A and a serogroup B meningococcal strain have been made available, facilitating comparisons between pathogenic isolates and the identification of virulence-associated genes and their molecular mechanisms (Tettelin *et al.*, 2000; Parkhill *et al.*, 2000). The meningococcus is capable of synthesising various virulence factors, detailed in Table 1.1, that may mediate the interaction with host cell surfaces and play a role in the disease process in man.

In England and Wales, *N.meningitidis* is the most common cause of bacterial meningitis, accounting for over 50% of the total cases each year (Table 1.2). The distribution of serogroup B (~60%) and serogroup C (~40%) meningococcal infections in England and Wales was similar to that of the rest of Europe (Anonymous, 2000). However, the incidence of meningococcal meningitis in England and Wales has fallen over the last few years, from 1164 cases in 2000 to just over 700 cases in 2002 (Table 1.2). The decrease was mainly due to a fall in the number of serogroup C cases, attributable to the successful impact of the meningococcal group C conjugated vaccine program introduced in the last quarter of 1999 (Anonymous, 2000; Miller *et al.*, 2001; Balmer *et al.*, 2002). In the United States, serogroups B and C of *Neisseria meningitidis* are responsible for approximately 25% of all cases of bacterial meningitis, with mortality rates of approximately 3-7% consistent with those observed in Western Europe (Stuart, 1996; Schuchat *et al.*, 1997). In addition, *N.meningitidis* differs from other leading causes of bacterial meningitis, because of its potential to cause large-scale epidemics with considerable frequency in developing countries. Meningococcal meningitis epidemics regularly sweep across the "meningitis belt" of sub-Saharan Africa from Ethiopia to Senegal; the most recent epidemic that began in 1996 has resulted in 300,000 cases and 30,000 deaths (Tikhomirov *et al.*, 1997; Chonghaile, 2002). In contrast to the United States and Europe, the predominant serogroup causing disease is group A, with mortality rates approaching 50% in some areas (Chonghaile, 2002).

Table 1.1: Virulence factors of meningeal pathogens involved in the pathogenesis of meningitis

Bacterial virulence factor	Role in pathogenesis	References:
Neisseria meningitidis		
Polysaccharide capsule	Avoidance of the alternative complement pathway and phagocytosis by neutrophils.	(Joiner, 1988)
Type IV pili	Adhesion to epithelial and endothelial cells via interaction with CD46	(Virji *et al.*, 1992a)
Lipopolysaccharide (LPS)	Cytotoxicity of endothelial cells in a serum CD14-dependent manner	(Arditi *et al.*, 1995)
Opa & Opc	Intimate adhesion to and invasion of epithelial cells through interactions with CD66 and heparan sulfate proteoglycan receptors respectively	(Virji *et al.*, 1996; de Vries *et al.*, 1998)
PorB	Intracellular survival by preventing the maturation of phagosomes	(Rudel *et al.*, 1996)
IgA1 protease	Inactivates mucosal secretory IgA and promotes intracellular survival	(Ayala *et al.*, 1998)
TbpA, LbpA & HpuB	Survival in systemic circulation through acquisition of iron from human transferrin, lactoferrin and haemoglobin	(Schryvers, 1989; Chen *et al.*, 1996)
Haemophilus influenzae		
Polyribosyl phosphate capsule	Avoidance of the classical complement pathway and phagocytosis by neutrophils.	(Levine *et al.*, 1983)
Haemagglutinating fimbriae	Adhesion to sialic containing lactosylceramide structures on epithelial cells	(van Alphen *et al.*, 1991)
Lipopolysaccharide (LPS)	Inhibits ciliary activity of human respiratory epithelial cells	(Johnson & Inzana, 1986)
Surface fibrils (hst)	Adhesion to epithelial cells following down-regulation of capsule	(St.Geme JW *et al.*, 1996)

(cont.)

Table 1.1: Continued.

Bacterial virulence factor	Role in pathogenesis	References:
Hap autotransporter	Adhesion and invasion of epithelial cells	(Fink *et al.*, 2003)
Phosphorylcholine (ChoP)	Adhesion to airway epithelium and endothelial cells via interactions with platelet-activating factor (PAF)	(Schweda *et al.*, 2000)
IgA1 protease	Inactivates mucosal secretory IgA	(Plaut, 1983)
Tbp1 & Tbp2	Survival in systemic circulation through acquisition of iron from human transferrin	(Schryvers, 1989)
Escherichia coli		
Polysaccharide capsule	Avoidance of the classical complement pathway and phagocytosis by neutrophils, and intracellular survival in endothelial cells.	(Joiner, 1988) (Hoffman *et al.*, 1999)
Type 1 fimbriae	Adhesion to nasopharyngeal epithelia via interactions with α-D-mannosides	(Duguid & Old, 1980)
S-fimbriae	Adhesion to vascular endothelia and choroidal epithelia via interactions with sialic acid containing glycoconjugates	(Parkkinen *et al.*, 1988)
Hek	Adhesion and invasion of gastrointestinal epithelial cells via interactions with heparan sulfate proteoglycans	(Fleckenstein *et al.*, 1996)
Omp-A	Serum resistance	(Prasadarao *et al.*, 2002)
Haemolysin	Secreted cytolytic toxin	(Stanley *et al.*, 1998)
IgA1 protease	Inactivates mucosal secretory IgA	(Plaut, 1983)
Enterobactin	Secreted siderophore promoting survival in circulation through acquisition of iron	(Bagg & Neilands, 1987)

Table 1.1: Continued.

Bacterial virulence factor	Role in pathogenesis	References:
Streptococcus pneumoniae		
Polysaccharide capsule	Avoidance of the classical complement pathway and phagocytosis by neutrophils.	(Brown *et al.*, 1983b)
Choline binding protein (Cbp)-A	Adhesion and invasion of epithelial and endothelial cells via interactions with polymeric immunoglobulin receptor (pIgR) and PAF	(Rosenow *et al.*, 1997; Ring *et al.*, 1998; Koedel *et al.*, 2002)
Pneumococcal surface protein (Psp) A and C	Resistance against complement activation and complement-dependent opsonophagocytosis	(Tu *et al.*, 1999; Dave *et al.*, 2001)
Phosphorylcholine	Intimate adhesion and invasion of endothelial cells via interactions with PAF	(Cundell *et al.*, 1995b)
Pneumolysin	Inhibition of ciliary beating in mucosa and reduction of serum opsonic activity, through the activation of the classical complement pathway in the absence of specific antibodies. Liberation of haemin-containing compounds via the lysis of erythrocytes	(Steinfort *et al.*, 1989; Tai *et al.*, 1993; Rubins *et al.*, 1996)
Neuraminidase (NanA)	Decreases viscosity of mucus	(Tong *et al.*, 2000)
IgA1 protease	Inactivates mucosal secretory IgA	(Janoff *et al.*, 1999)
Autolysin (LytA)	Local release of immunogenic subcapsular bacterial components including pneumolysin	(Mitchell *et al.*, 1997; Koedel *et al.*, 2002)
Haemin binding proteins	Survival in systemic circulation through acquisition of iron from human haemin	(Tai *et al.*, 1997)

Table 1.2: Statutory notifications of meningitis in England and Wales, 1992-2003

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003*
Meningitis:												
N.meningitidis	1067	1053	938	1146	1164	1220	1152	1145	1164	1020	709	353
S.pneumoniae	248	239	220	241	297	300	243	237	218	201	166	138
H.influenzae	484	168	52	51	57	38	29	29	39	74	61	43
Viral	280	196	214	315	509	260	214	211	547	811	-	-
Other	492	426	376	532	659	527	434	472	464	517	610	300
Total meningitis:	2571	2082	1800	2285	2686	2345	2072	2094	2432	2623	1546	834

* - Provisional data up to June 2003 only.

Data compiled from annual and quarterly reports of Notification of Infectious Disease (NOIDS), PHLS

Meningococcal disease most commonly presents as acute purulent meningitis, accounting for up to 50% of cases, whilst fulminant septicaemia, characterised by endotoxic shock and diffuse intravascular coagulation, alone is responsible for 10% of cases (Brandtzaeg *et al.*, 1989; Hart & Rogers, 1993; Steven & Wood, 1995). However, meningitis and septicaemia are not mutually exclusive and the syndromes often overlap. One report has shown that 75% of children in the UK with meningococcal disease had septicaemia, with or without meningitis (Thomson *et al.*, 1991). Indeed, 33% of patients with meningococcal disease in the UK now present with septicaemia alone, with just under 2000 cases of meningococcal septicaemia reported in England and Wales every year (Riordan *et al.*, 1995; Anonymous, 2000). By contrast, less than 5% of children with meningococcal disease in epidemics in Africa have evidence of septicaemia (Salih *et al.*, 1990). Whether these differences are due to variation in the release of endotoxin between serogroup A and B meningococci, or due to differences in host genetic susceptibility remains unclear (Hart & Cuevas, 1997).

1.2.1.2 Haemophilus influenzae

H.influenzae is a gram-negative, commensal bacterium of the human nasopharynx, which is responsible for a variety of localised respiratory infections, such as bronchitis, otitis media, and pneumonia, and invasive disease, including meningitis and septicaemia (Moxon, 1986; Gilsdorf, 1987; St.Geme *et al.*, 1993). While 50-80% of healthy individuals are colonised with acapsular, non-typeable *H.influenzae* organisms (Turk, 1982), only 2-15% harbour encapsulated strains of *H.influenzae* type b, which are responsible for systemic disease (Mhlanga-Mutangadura *et al.*, 1998). The avirulent *H.influenzae* strain Rd has been fully sequenced, facilitating comparisons with pathogenic clinical isolates and the identification of virulence-associated genes (Fleischmann *et al.*, 1995). Several virulence factors are expressed on the cell surface of the bacterium that may mediate interactions with host cell surfaces and play a role in the disease process in man, and these are detailed in Table 1.1.

Over the past fifteen years the epidemiology of acute bacterial meningitis has changed dramatically with the introduction of the *H.influenzae* type b conjugate vaccines. The conjugation of *H.influenzae* type b capsular polysaccharides to a carrier protein adjuvant, which transforms the T-cell-independent antigen (plain polysaccharide) into a T-cell-dependent one, confers immunological memory and greater immunogenicity in infancy (Schneerson *et al.*, 1980; Peltola, 2000). Since 1992, the incidence of *H.influenzae* meningitis in England and Wales has fallen by approximately 94% since the introduction of routine vaccination of children against *H.influenzae* type b and has remained at low levels (Table 1.2). Similar trends have been seen in the United States and Western Europe, where the introduction of the conjugate vaccine has resulted in a ninety percent reduction in the incidence of invasive *H.influenzae* disease (Schuchat *et al.*, 1997; Peltola, 2000; Koedel *et al.*, 2002). Although, there has been a slight increase in the number of cases of *H.influenzae* meningitis during the past few years in England and Wales, this may be explained by a reduction in the uptake of the vaccine from 1997 onwards, when concerns were raised about the safety of the MMR triple vaccine and childhood vaccines in general (Mason &

Donnelly, 2000; Elliman & Bedford, 2002). However, the number of global cases of *H.influenzae* meningitis has only fallen by 6% to approximately 400,000 per year, mainly due to the lack of available conjugate vaccine in developing countries (Peltola, 2000). In addition, *H.influenzae* is responsible for approximately 1.7 million cases of pneumonia worldwide, with mortality rates of 10-20% (Peltola, 2000). Consequently, it is estimated that there are in the excess of 2.2 million cases worldwide of *H.influenzae* infection each year, resulting in the deaths of approximately 600,000 children (Peltola, 2000; Scheld *et al.*, 2002).

1.2.1.3 Streptococcus pneumoniae

S.pneumoniae is a gram-positive, commensal bacterium of the human nasopharynx. Recently, both avirulent and pathogenic pneumococcal strains have been fully sequenced, facilitating comparisons between clinical isolates and the potential identification of novel virulence-associated genes (Hoskins *et al.*, 2001; Tettelin *et al.*, 2001). Table 1.1 gives details of the various virulence factors expressed by *S.pneumoniae* that may mediate interactions with host cell surfaces and play a role in the disease process in man.

S.pneumoniae continues to be the most common pathogen causing acute bacterial meningitis of adults, with one million cases estimated annually worldwide (Quagliarello & Scheld, 1992; Peltola, 2001). Since the routine introduction of the *H.influenzae* type b vaccine in the United States and Western Europe, *S.pneumoniae* is now the most frequent cause of childhood meningitis in these regions, although *N.meningitidis* continues to dominate in the UK (Table 1.2) (Spellerberg & Tuomanen, 1994). In addition, bacterial meningitis has radically changed in recent years to become a disease largely of older adults and the elderly (≥60 years), with *S.pneumoniae* the most common cause (Choi, 2001). The increased risk for meningitis in the older population is likely to be multifactoral and includes both a greater propensity for underlying diseases and immunosenescence (Hoen *et al.*, 1993; Choi, 2001). In England and Wales the incidence of pneumococcal meningitis has remained constant over the past decade, with approximately 200 cases annually, which represents 8-10% of the total cases of bacterial meningitis (Table 1.2). In addition, *S.pneumoniae* is the most common cause worldwide of community acquired bacterial pneumonia amongst children, adults and the elderly, accounting for a quarter of all cases of pneumonia (~500,000 cases annually) and a mortality rate of 10-20% in the United States alone (Shann, 1986; Fedson *et al.*, 1999; Jedrzejas, 2001). Pneumococcal pneumonia is a particular problem in developing countries and is responsible for the deaths of an estimated 1 million young children each year, with a similar number of deaths also occurring in older adults and the elderly (Cundell *et al.*, 1995a; Wuorimaa & Kayhty, 2002).

The normal habitat of *S.pneumoniae* is the human nasopharynx with the carriage rates at any one time being as high as 60% (Austrian, 1986). Compared with infection caused by other meningeal pathogens and despite the use of antibiotics, the prognosis of pneumococcal meningitis is poor, with mortality rates approaching 20-25% (Durand *et al.*, 1993; Schuchat *et al.*, 1997). The mortality rate in developing countries is dramatically higher than in industrialised countries,

reaching a level of approximately 60% (Goetghebuer *et al.*, 2000). Of the survivors, 30-50% experience major and permanent neurological sequelae including hearing loss, seizures, pareses, and learning and cognitive defects (Durand *et al.*, 1993; Pfister *et al.*, 1993; van de Beek *et al.*, 2002). Indeed, both the incidence of infection and resulting sequelae are increasing, due to the failure of chemotherapy and the emergence of antibiotic-resistant strains (Spika *et al.*, 1991). Although a polysaccharide vaccine has been available for over three decades, its use has been limited due to poor immunogenicity in individuals at the extremities of age (≤2 and ≥60 years) (White, 1988; Mitchell, 2000). However, new conjugate vaccines based on capsular polysaccharides linked to diphtheria toxoid, have recently demonstrated high immunogenicity (>97%) and safety in children under 2 years during efficacy trial in the United States (Obaro, 2002; Darkes & Plosker, 2002).

1.2.1.4 Escherichia coli K1

In the first month after birth, newborns are susceptible to infections acquired via the vertical transmission of commensal organisms from the maternal genital tract *in utero* or during passage through the birth canal (Sarff *et al.*, 1975; Mehta & Pollard, 1999). Worldwide, *E.coli* K1 is the leading cause of gram-negative meningitis in the neonatal period (Feigin, 1977; Siegel & McCracken, Jr., 1981), but it is rarely encountered in similar infections in older children (Broome & Schlech, 1985). Infections caused by the bacteria associated with childhood meningitis (*Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*) are less common in the neonatal period due to the presence of protective maternal antibodies acquired *in utero* via the placenta (Mehta & Pollard, 1999). Although approximately 100 different *E.coli* capsular antigens have been recognised, strains possessing the K1 capsular antigen are responsible for 80% of the cases of neonatal meningitis (Schiffer *et al.*, 1976). Recently, the *E.coli* K12 and O157:H7 genomes have been fully sequenced, providing insights into pathogenic *E.coli* strains and potentially identifying novel virulence-associated genes, some of which are detailed in Table 1.1 (Blattner *et al.*, 1997; Perna *et al.*, 2001).

E.coli K1 causes approximately 30-50% of the cases of neonatal meningitis in the USA and UK (Bell *et al.*, 1989) and it is also the major pathogen associated with an estimated 130,000 cases and 50,000 deaths annually in the developing nations of the world (Stoll, 1997). Despite the use of bactericidal antibiotics and supportive care, the case fatality rates of *E.coli* neonatal meningitis remain high at approximately 15-40%, with over half the survivors sustaining permanent neurological sequelae (McCracken *et al.*, 1974; Gladstone *et al.*, 1990; Unhanand *et al.*, 1993). The lack of knowledge of the pathogenesis and pathophysiology of this disease and the inability of the neonatal immune system to localise and fight infection are both likely to contribute to this significant mortality and morbidity.

1.2.2 Clinical characteristics of bacterial meningitis

The characteristic signs and symptoms of bacterial meningitis are high fever, headaches, and neck stiffness, which usually develop suddenly over a few hours, but may also occur over one to two days. Other common symptoms include vomiting and photophobia (Carpenter & Petersdorf, 1962; Pfister *et al.*, 1993; Peate, 1999). In addition, positive Kernig's and Brudzinski's signs, which are observed in approximately 50% of all cases, present as a resistance to leg extension from a seated position and the passive flexion of the neck resulting in the flexion of hips and knees, respectively (Verghese & Gallemore, 1987). However, bacterial meningitis is often difficult to diagnose as the signs and symptoms are 'flu-like' and do not occur in any particular sequence or frequency, presenting in certain individuals and not in others (Peate, 1999; Bleck & Greenlee, 2000). As the disease progresses, signs of cerebral dysfunction *i.e.* confusion, lethargy, decreased consciousness and seizures, may develop as a result of increasing intracranial pressure and cerebral ischaemia (Tunkel *et al.*, 1990a; Quagliarello & Scheld, 1992). In addition, certain signs and symptoms may also suggest a specific aetiological diagnosis in patients with bacterial meningitis. For example, approximately 50% of patients with meningococcaemia, with or without meningitis, present with a prominent petechial rash located principally on the extremities (Cartwright, 1995b; Roos *et al.*, 1997).

However, in the newborn and small infants the classical symptoms of fever and headache may be absent or difficult to detect. Instead, the signs of meningitis are likely to be present as temperature instability (hypo- or hyperthermia), an altered state of alertness or mood, irritability or a high-pitched cry (Bleck & Greenlee, 2000). In addition, confusion and lethargy with no fever or signs of meningeal inflammation are the most common presentation of bacterial meningitis in elderly patients, especially those with underlying conditions (Gorse *et al.*, 1984).

Survivors of bacterial meningitis are frequently left with permanent neurological sequelae resulting mainly from raised intracranial pressure and cerebral herniation. These sequelae include hearing impairment, seizures, pareses, myopia, behavioural problems and learning and cognitive defects (Unhanand *et al.*, 1993; van de Beek *et al.*, 2002). Approximately 15-20% of patients that survive meningococcal and *H.influenzae* type b meningitis present with serious psycho-neurological sequelae (Coggins *et al.*, 1993; Naess *et al.*, 1994; Daoud *et al.*, 1995), whereas the corresponding figure for survivors of both pneumococcal and *E.coli* meningitis is approximately 50% (Gladstone *et al.*, 1990; Unhanand *et al.*, 1993; van de Beek *et al.*, 2002). This adverse outcome in pneumococcal meningitis has been suggested to result from prolonged inflammation in the SAS due to a slower clearance of bacteria from the CSF (Roine *et al.*, 1995; Kanegaye *et al.*, 2001). In addition, the permanent loss of neurons in the hippocampus through apoptosis, induced by the pneumococcal toxins pneumolysin and H_2O_2, is likely to contribute to the high percentage of survivors of pneumococcal meningitis suffering from neurological sequelae (Zysk *et al.*, 1996; Tauber *et al.*, 1997; Braun *et al.*, 2002).

1.3 Pathogenesis of bacterial meningitis

Through the careful selection of organ cultures, cells and cell lines from various anatomical sites, it has been possible to investigate the pathogenesis of bacterial meningitis *in vitro*. Invasive disease is likely to occur when various pathogenic virulence determinants overcome host immune defence mechanisms. Specifically, the bacterial pathogens must initially colonise and invade a mucosal epithelial barrier, e.g. of the nasopharynx or intestine, before crossing the vascular endothelium and surviving in the blood. Following dissemination through the bloodstream, the meningeal pathogens must traverse the blood-CSF barrier to gain entry into the SAS, with the subsequent survival and proliferation of bacteria within the CSF inducing an acute compartmentalised inflammatory response characteristic of bacterial meningitis.

1.3.1 Mucosal colonisation

Initiation of bacterial infection begins with the acquisition of bacteria by colonisation of the upper respiratory tract, primarily the nasopharynx and tonsils. *N.meningitidis*, *H.influenzae* and *S.pneumoniae* are thought to be transmitted between individuals through respiratory aerosols or by direct contact with nasopharyngeal secretions (Tzeng & Stephens, 2000). For all three pathogens, asymptomatic carriers are believed to act as a natural reservoir. By contrast, *E.coli* K1 is acquired by newborns via the vertical transmission of commensal organisms from the maternal genital tract *in utero* or during passage through the birth canal (Sarff *et al.*, 1975; Mehta & Pollard, 1999). In addition, the primary site of *E.coli* K1 colonisation of the neonate is not the nasopharynx but the gastrointestinal epithelium (Burns *et al.*, 2001).

However, to successfully colonise and survive in the nasopharynx, pathogenic bacteria must overcome various host defence mechanisms including the mucous barrier, the presence of secretory IgA antibody and ciliary clearance (Stephens & Farley, 1991). Although the marked affinity for, and non-specific association with mucus is characteristic of the initial phases of *H.influenzae* colonisation, meningococci are rarely seen associated with mucus (Stephens & Farley, 1991; Rayner *et al.*, 1995). The evasion of host mucosal sIgA, which is actively secreted by plasma cells in the mucous blanket, is an important first step in the colonisation of the nasopharynx. Virtually all clinical isolates of *N.meningitidis*, *H.influenzae* type b, *S.pneumoniae* and *E.coli* secrete IgA1 proteases (Table 1.1), which cleave the heavy-chain hinge region of sIgA to produce non-functional antibody (Plaut, 1983; Kilian *et al.*, 1988; Janoff *et al.*, 1999). Furthermore, the pneumococcal neuraminidase NanA, which cleaves N-acetylneuraminic acid from mucin, can further enhance colonisation by decreasing the viscosity of mucus (Tong *et al.*, 2000). Infection of cultured human nasopharyngeal tissue with meningococci and *H.influenzae* consistently results in injury to ciliated epithelial cells and the subsequent sloughing of cilia (Stephens & Farley, 1991; Quagliarello & Scheld, 1992). Although this loss of ciliary action required the presence of viable bacteria, it did not require the attachment of these organisms to ciliated cells, implying that a soluble bacterial factor induced ciliostasis, either directly or through

the induction of host cytokines (Stephens & Farley, 1991). Indeed, the lipopolysaccharide (LPS) of *H.influenzae* has been shown to inhibit the ciliary activity of human respiratory epithelial cells (Johnson & Inzana, 1986), although meningococcal LPS has no affect on ciliary function *in vitro* (Stephens *et al.*, 1986). In addition, the pneumococcal toxin, pneumolysin, has been shown to inhibit the beating of cilia in the respiratory epithelium (Steinfort *et al.*, 1989).

Once past the mucus and ciliary barriers, the meningeal pathogens bind selectively to the non-ciliated epithelial cells of the mucosal surface, an interaction facilitated by a range of bacterial surface expressed proteins (Stephens & Farley, 1991; Quagliarello & Scheld, 1992). The filamentous pili and fimbriae are the most important surface components mediating this initial attachment of meningococci and *E.coli*, respectively, to human epithelial cells and nasopharyngeal mucosa (Stephens & McGee, 1981; Stephens *et al.*, 1983; Stins *et al.*, 1994). The type IV pili of *N.meningitidis* have been shown to be essential for the adherence of encapsulated meningococci to non-ciliated epithelial cells (Stephens *et al.*, 1983). The membrane cofactor protein (MCP or CD46), an epithelial transmembrane glycoprotein present on virtually every human cell and tissue type, has been identified as the potential pilus receptor for pathogenic *Neisseria* species (Virji *et al.*, 1992a; Kallstrom *et al.*, 1997; Kallstrom *et al.*, 2001). *E.coli* express many types of fimbriae, which are characterised by their carbohydrate binding specificity (Hacker, 1990). Type 1 fimbriae bind to α-D-mannosides and are found on most *E.coli* stains (Duguid & Old, 1980), whereas S-fimbriae bind to sialic acid-containing glycoconjugates and are common in *E.coli* strains isolated from the CSF of neonates with meningitis (Korhonen *et al.*, 1985; Selander *et al.*, 1986) (Korhonen *et al.*, 1985). Such sialyl galactoside structures occur widely in human tissues, including the brain, and could potentially act as receptors for the S-fimbriae (Ledeen & Yu, 1982). Although the primary site of *E.coli* colonisation is the gastrointestinal tract, interaction with the nasopharyngeal mucosa is essential for the dissemination of orally ingested bacteria through the stomach to the intestine (Bloch *et al.*, 1992). The type 1 fimbriae-mediated adherence to the nasopharyngeal mucosa is believed to allow the multiplication of low numbers of orally ingested *E.coli* K1 to a level that has an increased chance of surviving exposure to the acidic contents of the stomach (Clegg *et al.*, 1984; Bloch *et al.*, 1992).

The haemagglutinating pili of *H.influenzae* have also been shown to mediate adhesion to human nasopharyngeal epithelial cells (Gilsdorf *et al.*, 1996), via interactions involving sialic containing lactosylceramide structures on the epithelial cell surface (van Alphen *et al.*, 1991). However, the role of pili in the pathogenesis of *H.influenzae* is not fully understood, since both piliated and non-piliated strains have been shown to bind to non-ciliated epithelial cells of nasopharyngeal organ cultures, suggesting a role for non-pilus adhesins (Farley *et al.*, 1990). One such non-pilus adhesins is phosphorylcholine (ChoP), which is expressed on the lipooligosaccharide (LOS) of *H.influenzae* type b and allows binding to the platelet-activating factor (PAF) receptor on human airway epithelial cells (Townsend & Scheld, 1994; Schweda *et al.*, 2000). Although *S.pneumoniae* also binds avidly to cells of the upper and lower respiratory tract, pathogen-host cell interactions do not involve pili or fimbriae or invoke actin polymerisation

as seen with the other meningeal pathogens (Tuomanen, 1997). Initial pneumococcal adhesion involves the interaction with certain carbohydrates displayed on epithelial cells such as GalNAc(β1-3)Gal, GalNAc(β1-4)Gal, and sialic acid (Andersson *et al.*, 1983; Cundell *et al.*, 1995b). However, in the absence of the capsule spanning pili or fimbriae, the down-regulation of capsule and subsequent exposure of surface structures is critical for the colonisation of the nasopharynx. *S.pneumoniae* displays various proteins on its surface, which are non-covalently linked to the phosphorylcholine of the cell wall (Tuomanen & Masure, 1997). The most abundant of these choline-binding proteins (Cbps) is CbpA, an important adherence factor for *S.pneumoniae* that appears to be essential for the colonisation of the nasopharyngeal mucosa (Rosenow *et al.*, 1997). Other members of the Cbp family, including CbpD, CbpE and CbpG may also be involved in adherence, although their molecular interactions are still unclear (Gosink *et al.*, 2000).

1.3.2 Mucosal invasion

Following a period of localised multiplication and formation of microcolonies on the epithelial surface, the meningeal pathogens must cross the epithelia to gain access to the sub-mucosa and blood stream. In the case of meningococci, further intimate contact with the host cell is established via the Opa and Opc outer membrane proteins (OMP), which recognise the carcinoembryonic antigen CD66 (Virji *et al.*, 1996; Chen *et al.*, 1997; Bos *et al.*, 2002) and heparan sulfate proteoglycan receptors (de Vries *et al.*, 1998) respectively. However, these interactions are hindered by the presence of capsular polysaccharide, a virulence factor essential for the transmission of the bacteria (Virji *et al.*, 1995b; van Deuren *et al.*, 2000). Consequently, down-regulation of capsular polysaccharide (Virji *et al.*, 1996; Klein *et al.*, 1996; de Vries *et al.*, 1998) and the binding of these now exposed proteins to their receptors stimulates the engulfment of the meningococci by the nasopharyngeal epithelial cells via parasite-mediated endocytosis. The meningococci are subsequently transported via the transcellular pathway within a phagocytic vacuole across the non-ciliated columnar epithelial cells (Stephens *et al.*, 1983; Mason *et al.*, 1985; Dehio *et al.*, 1998). By contrast, Birkness and colleagues presented microscopic evidence of long columns of encapsulated meningococci between epithelial cells rather than in intracellular vacuoles (Birkness *et al.*, 1995). Indeed, this intercellular pathway may be an alternative route for encapsulated meningococci to traverse the mucosal epithelial barrier.

The capsule of *H.influenzae* type b also hinders the further intimate adhesion to non-ciliated epithelial cells, with non-encapsulated bacteria exhibiting enhanced adhesion to and invasion of epithelial cells (St.Geme & Falkow, 1991). This interference was either indirect, due to the negative charge of the ribosyl-ribitol phosphate capsule (Davies, 1984), or direct, through the masking of specific adhesins present on the bacterial surface (Virji *et al.*, 1991b). The latter is especially true of *H.influenzae* type b surface fibrils (hst), which are short, thin surface fibrils distinct from HA pili (Gilsdorf *et al.*, 1992) that promote the pili-independent adherence to epithelial cells only in the absence of capsular polysaccharide (St.Geme & Cutter, 1995; St.Geme

et al., 1996). However, *H.influenzae* type b bacteria do not invade non-ciliated epithelial cells but actively break down the apical tight junctions of columnar epithelial cells and invade by means of a paracellular route (Stephens & Farley, 1991). The secreted *Haemophilus* adherence and penetration protein (Hap), which has considerable homology to meningococcal and *H.influenzae* IgA1 proteases (St.Geme *et al.*, 1994), LOS (Kimura & Hansen, 1986) and the European OMP subtype 1 of *E.coli* (Takala *et al.*, 1987), may contribute to the invasion and virulence of *H.influenzae*.

The ability of pneumococci to invade non-ciliated epithelial cells correlates with the presence of the polymeric immunoglobulin receptor (pIgR) on human cell surfaces and CbpA on the pneumococcus (Koedel *et al.*, 2002). The interaction of CbpA and pIgR allows the pneumococcus to commandeer the pIgR transcytosis machinery, which is normally used for transporting antibodies across mucosal epithelial cells (Kaetzel, 2001), and traverse the mucosal barrier (Zhang *et al.*, 2000). In addition, the activation of epithelial cells by the local generation of inflammatory factors, such as those induced by a concurrent viral infection, changes the concentration and type of receptors available to the pneumococcus (Cundell *et al.*, 1995a). *S.pneumoniae* engages one of these up-regulated receptors, the platelet-activating factor (PAF) receptor, via interactions with the phosphorylcholine of the pneumococcal cell wall (Cundell *et al.*, 1995b). Within minutes of the PAF receptor appearance, the pneumococci undergo waves of enhanced intimate adhesion and invasion of the epithelia, traversing the activated epithelial cells in intracellular vacuoles (Geelen *et al.*, 1993; Cundell *et al.*, 1995b). The pneumococcal hyaluronate lyase may contribute to bacterial spreading within the host, by degrading hyaluronic acid in the extracellular matrix of connective tissues (Mitchell, 2000; Li *et al.*, 2000).

By contrast, the molecular mechanisms that describe the initial colonisation of the nasopharynx, dissemination to and secondary colonisation of the gastrointestinal tract, and the subsequent entry into the blood stream by *E.coli* K1 have received less attention. However, the colonisation of gastrointestinal epithelial cells has been shown to be independent of type 1 fimbriae, suggesting a role for other non-fimbrial adhesins (Bloch *et al.*, 1992). Recently, an *E.coli* K1 outer membrane protein, Hek, has been identified and demonstrated to share homology with the enterotoxigenic invasion protein A (TiA) and the Neisserial, Opa protein (Smith, 2003). TiA has been shown to mediate the adhesion to, and invasion of cultured human gastrointestinal epithelial cells via cell surface heparan sulfate proteoglycans (Fleckenstein *et al.*, 1996; Fleckenstein *et al.*, 2002), suggesting that *E.coli* may cross the mucosal barrier via the transcellular pathway.

1.3.3 Survival within the bloodstream and bacteraemia

Within 24 hours of mucosal colonisation and invasion, pathogenic bacteria have gained access to the sub-epithelial tissues. The direct invasion of sub-mucosal blood vessels is one possible route of entry for bacteria into the bloodstream, which in the case of meningococci is supported by the

presence of bacteria in close proximity to local blood vessels (Stephens *et al.*, 1983). However, the lymphatic drainage of sub-epithelial tissues offers an alternative route, with the bacteria transferring to regional lymph nodes and subsequent passage to the blood via the efferent lymphatics (Rubin & Moxon, 1983).

Although the exact mechanisms for pathogen invasion into the blood are not known, the bacteria must evade additional host defences to sustain intravascular survival and promote bacteraemia. The major host defence against bacteraemia is circulating complement, primarily through the alternative pathway that does not require specific antibodies for activation (Quagliarello & Scheld, 1992). The most important bacterial virulence factor for avoiding lysis by the alternative complement pathway and phagocytosis by neutrophils is expression of capsular polysaccharide (Joiner, 1988; Klein *et al.*, 1996). Accordingly, surface encapsulation is a feature shared by the clinical isolates of all the pyogenic bacteria, although the molecular basis for complement evasion differs for each bacterium (Table 1.1). For *N.meningitidis* and *E.coli* K1, capsular sialic acid facilitates the binding of the complement regulatory protein factor H to C3b, thereby preventing the binding of factor B to C3b and subsequent activation of the alternative pathway (Joiner, 1988). In addition to the inefficient binding of C3b to factor B present on pneumococcal capsular surfaces (Brown *et al.*, 1983a), the pneumococcal capsule also prevents the interaction of any C3b bound to surface proteins with receptors on phagocytic cells (Morona *et al.*, 2000). By contrast, the polyribosyl phosphate capsule of *H.influenzae* is incapable of serving as a receptor for C3 (Levine *et al.*, 1983).

In addition to the polysaccharide capsule, the meningeal pathogens have other proteins that have been implicated in promoting their intravascular survival. During invasion, the meningococcal PorB OMP translocates into the target cell membrane of monocytes affecting the maturation of phagosomes (Rudel *et al.*, 1996) and IgA1 protease may stimulate the degradation of a membrane glycoprotein in endosomes and lysosomes, thereby promoting intracellular survival (Ayala *et al.*, 1998). Phosphorylcholine has been reported to modulate interactions with the innate defence component, C-reactive protein (CRP), which serves to opsonise bacteria expressing choline on their surface (Weiser *et al.*, 1998). Since the surface expression of choline is phase variable in *S.pneumoniae*, *N.meningitidis* and *H.influenzae*, its down-regulation promotes survival in the blood (Weiser *et al.*, 1997). The outer membrane protein A (OmpA) of *E.coli* also contributes to serum resistance through the binding of C4b binding protein (C4bp), a complement fluid phase regulator (Prasadarao *et al.*, 2002). Further virulence factors of *S.pneumoniae* include the pneumococcal surface proteins (Psp) A and C, which are involved in resistance against complement activation and complement-dependent opsonophagocytosis. PspA interferes with the complement factor B, thereby preventing the deposition and processing of surface bound C3b (Tu *et al.*, 1999). PspC is structurally similar to PspA, but binds the complement regulatory factor H that in turn catabolises bound C3b to its inactive form (Neeleman *et al.*, 1999; Dave *et al.*, 2001). In addition to the cytotoxic properties of pneumolysin, the pneumococcal toxin is capable of

activating the classical complement pathway in the absence of specific antibodies resulting in a concurrent reduction of serum opsonic activity (Rubins *et al.*, 1996).

1.3.4 Microbial entry into the CNS

Meningitis may be complication of trauma, surgery or developmental malformations. Indeed, fractures of the base of the skull or penetrating head injuries provide a direct means of entry for bacteria from the air sinuses to the SAS. However, the majority of cases of pyogenic meningitis are associated with the haematogenous dissemination of pathogenic bacteria to the leptomeninges (Gray & Nordmann, 1997).

A sustained or high-grade bacteraemia is thought to be necessary, but not solely sufficient, for the entry of pathogens into the subarachnoid space (Tuomanen, 1996; Huang *et al.*, 2000). Indeed, many other organisms, such as viridans *Streptococci*, which produces continuous bacteraemia during infective endocarditis rarely produces bacterial meningitis (Tunkel & Scheld, 1993). In the case of *E.coli* K1, susceptibility to sustained bacteraemia and subsequent meningeal invasion is dependent on the age of the individual. Glode and colleagues observed that the susceptibility of infant rats to *E.coli* bacteraemia fell from 68% at 5 days, to only 10% at 15 days and 0% at 30 days old, results that appear to parallel the incidence of disease in human infants (Glode *et al.*, 1977). However, the mechanisms and precise site of pathogen entry into the CSF remain unclear, but in order to invade the SAS and colonise the meninges, it is necessary for the bacteria to cross the physiological barrier between the bloodstream and the CSF, the blood-CSF barrier (B-CSFB).

The microenvironment of the brain is maintained by the presence of a cellular barrier, restricting the passage of circulating inflammatory cells, macromolecules and ions into the central nervous system (CNS). The CNS is effectively isolated from the intravascular space by two main barriers, the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (B-CSFB).

1.3.4.1 The blood-brain barrier

The BBB exists at the cerebral capillaries of the brain where the endothelium differs from that lining other capillaries by the presence of intercellular tight junctions with high electrical resistance (>1000 Ω cm^{-2}) (Butt *et al.*, 1990; Kniesel & Wolburg, 2000). In addition, the endothelial cells of cerebral capillaries have few plasmalemmal vesicles and thus rarely exhibit pinocytosis (Townsend & Scheld, 1995). Together, these properties constitute the principle barrier to the passive movement of fluids, electrolytes and macromolecules into and out of the brain via both paracellular and transcellular pathways. It was previously assumed that the entry of leukocytes into the CNS was prevented by the endothelial tight junctions and only occurred following damage of the BBB or B-CSFB (*e.g.* from the inflammatory response associated with meningitis). However, it has since been shown that leukocytes traverse the BBB and B-CSFB as

part of normal physiology, although the exact route through the endothelium is unclear (Perry *et al.*, 1997).

1.3.4.2 The blood-CSF barrier

The B-CSFB exists at the choroid plexus epithelium and at the endothelial cells of blood vessels present within the SAS (Strazielle & Ghersi-Egea, 2000). In addition to the presence of intercellular tight junctions between the vascular endothelial cells, the large cerebral arteries and veins present in the SAS are coated in a thin layer of leptomeningeal cells, often only one cell thick (Zhang *et al.*, 1990), which also possess gap junctions and desmosomes (Alcolado *et al.*, 1988). The choroid plexi are microscopic, finger-like projections of the vascular system located in the lateral, third and forth cerebral ventricles, and they consist of a core of blood vessels surrounded by a single layer of epithelial cells (Weller, 1995). In contrast to the brain microvasculature, with its intercellular tight junctions with high electrical resistance, the endothelial cells of the choroid plexi are highly fenestrated with a structure similar to those of peripheral capillaries. The homeostasis integrity in this region is maintained by the presence of tight junctions at the ventricular surface of the epithelial cells that restrict the paracellular movement of substances into the CSF. In addition, the epithelial cells of the choroid plexi actively secrete CSF and allow the controlled transcellular transport of micronutrients between the bloodstream and the CSF (Pron *et al.*, 1997; Strazielle & Ghersi-Egea, 2000).

It is not known whether meningococci primarily invade the SAS at a preferred anatomical site of the B-CSFB, but the most likely portals of entry are those areas of minimal resistance such as the choroid plexi and meningeal vessels. Indeed, Pron and colleagues (1997) identified meningococci associating strongly to both the endothelial cells of meningeal capillaries and choroidal epithelial cells. Although during bacteraemia more bacteria are delivered to the choroid plexi than to any other anatomical location in the CNS, the absence of bacteria inside or between choroidal epithelial cells suggests that meningococci enter the CSF directly through the blood vessels in the SAS and meninges (Pron *et al.*, 1997). The preferential concentration of *S.pneumoniae* around leptomeningeal blood vessels in the infant rat model of bacterial meningitis also suggests the cerebral microvascular endothelium as the primary site for pneumococcal entry (Zwijnenburg *et al.*, 2001). By contrast, the higher density of *H.influenzae* type b in the ventricular CSF of monkeys compared to the lumbar CSF, suggests, but does not prove unequivocally, that these bacteria may enter the SAS through the choroid plexi (Smith, 1987). The route of CSF invasion by *E.coli* K1 is still unclear, although S-fimbriated bacteria have been shown to bind to the luminal surfaces of the vascular endothelium and the epithelial lining of the choroid plexi in the infant rat (Parkkinen *et al.*, 1988).

Regardless of the preferred site of entry into the CSF, the meningeal pathogens must associate with and penetrate endothelial and/or epithelial cells. As with nasopharyngeal colonisation, type IV pili have an important role in the interactions of meningococci with vascular

endothelial cells, with piliated meningococci exhibiting increased adherence to endothelial cells compared to non-piliated strains (Virji *et al.*, 1992a; Townsend & Scheld, 1995). The Opc outer membrane protein is also associated with increased adherence to endothelial cells *in vitro*, following the down-regulation of capsular polysaccharide (Virji *et al.*, 1992c; Virji *et al.*, 1995b). Although Opc proteins have been shown to interact with human cells directly via heparan sulphate proteoglycans, they can also bind to integrins using serum-derived bridging molecules that contain the RGD motif (Virji *et al.*, 1994). In contrast to events in the nasopharynx, the adhesion of meningococci to microvascular endothelial cells is not a prelude to invasion; instead the intimate association between pathogen and host increases the cytopathic effects of various microbial components on the endothelial cells (Townsend & Scheld, 1995; Klein *et al.*, 1996). Indeed, LPS has been shown to induce gaps between cerebral endothelial cells both directly, most probably in a serum CD14-dependent manner, and indirectly through the local induction of the proinflammatory cytokines TNF-α and IL-1β (Quagliarello *et al.*, 1991; Patrick *et al.*, 1992; Arditi *et al.*, 1995). The disruption of microvascular tight junctions may thus allow entry of meningococci into the CSF via a paracellular route through the widened intercellular junctions. By contrast, Nassif and colleagues (2002) have suggested recently that meningococci utilise a transcellular route to cross the B-CSFB, demonstrated by the presence of intracellular bacteria within endothelial cells *in vivo* and the non-destructive traversal of tight-junction forming cell monolayers *in vitro* (Merz *et al.*, 1996; Pujol *et al.*, 1997; Nassif *et al.*, 2002).

However, the interactions of *H.influenzae*, *E.coli* K1 and *S.pneumoniae* with human endothelial cells did not result in the extensive cytopathic effects seen with meningococci, which suggest a transcellular mechanism involving active or passive transcytosis (Virji *et al.*, 1991b; Tunkel & Scheld, 1993; Ring *et al.*, 1998; Koedel *et al.*, 2002). *H.influenzae* may enter the CSF through the direct invasion of endothelial cells by parasite-mediated phagocytosis (Virji *et al.*, 1991b). The bacteria are believed to transcytose through the endothelial cells within membrane-bound intracellular vacuoles, which subsequently fuse with the basal plasmalemma and release *H.influenzae* into the CSF (Patrick *et al.*, 1992; Virji *et al.*, 1992b). Although, non-capsulated *H.influenzae* associated to and invaded endothelial cells more rapidly than encapsulated strains, these differences progressively decreased over time (Virji *et al.*, 1991b; Virji *et al.*, 1992b). The transcellular invasion of human brain microvascular endothelial cells (BMEC) has been demonstrated to occur for both *E.coli* K1 and *S.pneumoniae* without altering the integrity of the monolayer (Ring *et al.*, 1998; Prasadarao *et al.*, 1999c; Stins *et al.*, 2001). Indeed, the binding and invasion of endothelial cells by *E.coli* is specific to BMEC and is not observed with endothelial cells of non-brain origin (Koedel *et al.*, 2002). The binding and invasion of BMEC by *E.coli* K1 is believed to occur via two independent mechanisms; S-fimbriae mediate the initial adhesion to BMEC surface glycoproteins and sulfated glycolipids, but play no part in the subsequent internalisation of the bacteria (Parkkinen *et al.*, 1988; Prasadarao *et al.*, 1993; Stins *et al.*, 1994). Next, the parasite-mediated endocytosis of *E.coli* K1 by BMEC is multifactoral, and is believed to involve ligand-receptor interactions of a number of outer membrane proteins including OmpA

(Prasadarao *et al.*, 1996), IbeA & B (Huang *et al.*, 1995; Huang *et al.*, 1999), and CNF1 (Badger *et al.*, 2000). The host receptors for S-fimbriae, IbeA and OmpA are specific to the surface of BMEC, which may in part explain the meningeal tropism of *E.coli* K1 (Prasadarao *et al.*, 1996; Prasadarao *et al.*, 1997; Prasadarao *et al.*, 1999b). The viability of the bacteria inside the BMEC vacuole is maintained by the K1 polysaccharide capsule, which protects against the host cell defences such as nitric oxide (NO), superoxides and other oxygen radicals (Hoffman *et al.*, 1999).

Invasion of the CSF by *S.pneumoniae* is also believed to be multifactoral, and includes the activation of BMEC by pneumococcal cell wall components (Freyer *et al.*, 1999) and the expression of the pneumococcal adhesin, CbpA, which is also active in the nasopharynx (Ring *et al.*, 1998). Components of the pneumococcal cell wall released by growing bacteria are capable of initiating invasion by stimulating the production of cytokines from endothelial cells via the cell surface receptor CD14 (Tomasz & Saukkonen, 1989; Saukkonen *et al.*, 1990; Pugin *et al.*, 1994). This local production of cytokines activates the BMEC resulting in the up-regulation of the surface expressed PAF receptor, which is recognised by pneumococcal phosphorylcholine and CbpA (Cundell *et al.*, 1995b; Tuomanen, 1999). Since the interaction of PAF with its natural ligand results in its rapid internalisation, *S.pneumoniae* bacteria appear to invade BMEC in a vacuole together with the PAF receptor and transcytose through the endothelial cell barrier (Ring *et al.*, 1998; Koedel *et al.*, 2002). The ability of pneumococci to transcytose through endothelial cell monolayers is associated with the down-regulation of capsular polysaccharide and PspA expression, and the increased surface expression of CbpA (Ring *et al.*, 1998; Kim & Weiser, 1998). In contrast to the invasion of nasopharyngeal epithelial cells, the pneumococcal enzyme, hyaluronidase, does not influence bacterial passage into the CSF (Zwijnenburg *et al.*, 2001).

1.3.5 Inflammation in the SAS

Once the meningeal pathogen has penetrated through the B-CSFB and entered the SAS, the absence of humoral and cellular host defences provides an environment for unchecked bacterial proliferation (Simberkoff *et al.*, 1980; Quagliarello & Scheld, 1992; Brandtzaeg *et al.*, 1992). The dominant host defence mechanisms, such as polymorphonuclear neutrophils (PMNL), immunoglobulins and complement components, are virtually absent in CSF. Although meningeal inflammation leads to the increased concentration of various complement components in the CSF, the levels remain below the optimal concentration for opsonic activity and subsequent phagocytosis (Simberkoff *et al.*, 1980; Tunkel *et al.*, 1990b; Stahel *et al.*, 1997). Following significant bacterial proliferation in the CSF, the increasing liberation of cell wall components, including LPS, peptidoglycan and teichoic acid, elicit the rapid compartmentalised inflammatory response characteristic of bacterial meningitis (Tuomanen *et al.*, 1985; Brandtzaeg *et al.*, 1992; Leib & Tauber, 1999). Indeed, in the case of pneumococcal meningitis, activation of the major pneumococcal autolysin, LytA, and subsequent autolysis contributes to this inflammation through the release of subcapsular bacterial components and cell wall degradation products including

peptidoglycan, lipoteichoic acid and pneumolysin (Tuomanen *et al.*, 1985; Mitchell *et al.*, 1997; Koedel *et al.*, 2002).

Bacterial meningitis is characterised by the presence of pro-inflammatory cytokines and chemokines in the CSF and the influx of large numbers of PMNLs into the SAS, with little spread of infection or inflammation to the underlying brain (Brandtzaeg, 1995; Gray & Nordmann, 1997). Observations in various experimental models have confirmed that the LPS / LOS of Gram-negative bacteria (Syrogiannopoulos *et al.*, 1988; Mustafa *et al.*, 1989b) and the peptidoglycan-teichoic acids of Gram-positive bacteria (Tuomanen *et al.*, 1985; Tuomanen & Masure, 1997) are potent stimulators of cytokine and chemokine induction in different cell types. Indeed, the concentrations of LPS in the CSF of patients with meningococcal meningitis positively correlate with the intensity of the inflammatory response and, ultimately, clinical severity and neurological outcome (Mertsola *et al.*, 1991; Brandtzaeg *et al.*, 1992; Brandtzaeg *et al.*, 2001). Cytokines and chemokines have an important regulatory role in the initiation, maintenance and, eventually, the termination of this inflammatory response.

1.3.5.1 Pro-inflammatory cytokines in bacterial meningitis

Bacterial components such as LPS and peptidoglycan are capable of stimulating a variety of cell types within the CNS to synthesise and release pro-inflammatory cytokines (Burroughs *et al.*, 1992; Szelenyi, 2001). High levels of the pro-inflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 have been detected in the CSF of patients with meningitis caused by different Gram-negative and Gram-positive bacteria, but not in patients with viral meningitis or controls (Waage *et al.*, 1989; Nadal *et al.*, 1989; Mustafa *et al.*, 1989a; Arditi *et al.*, 1990; Lopez-Cortes *et al.*, 1993; van Furth *et al.*, 1995; Diab *et al.*, 1997; van Deuren *et al.*, 1997; Tauber & Moser, 1999). In pneumococcal meningitis, the pneumolysin toxin can also stimulate the production of inflammatory mediators including TNF-α, IL-1β and IL-6 (Houldsworth *et al.*, 1994; Braun *et al.*, 1999). These three pro-inflammatory cytokines are regarded as major early response cytokines that, often in synergy, trigger a cascade of inflammatory mediators including other interleukins, adhesion molecules and chemokines (Tauber *et al.*, 1997). However, the absence of these cytokines in plasma, suggests their local production in the CSF, with possible sources including cerebromicrovascular endothelial cells, activated astrocytes, microglial cells and meningeal cells (Chao *et al.*, 1992; Medana *et al.*, 1997; Tauber & Moser, 1999; Szelenyi, 2001).

One of the hallmarks of bacterial meningitis is the development of a neutrophillic pleocytosis within the CSF, in which TNF-α, IL-1β and IL-6 have critical roles. IL-6 is a pleiotrophic cytokine, capable of inducing acute phase proteins and leukocytosis, and its expression is strongly correlated with fever (Hirano *et al.*, 1990; Rothwell, 1994). TNF-α and IL-1β play synergistic roles in the massive recruitment of PMNLs, predominantly neutrophils, into the CSF during meningitis (Fassbender *et al.*, 1997) through the up-regulation of cerebral endothelial-expressed adhesin molecules such as P- and E-selectins (Bevilacqua *et al.*, 1989; Saukkonen *et al.*, 1990; Gotsch *et al.*, 1994; Dinarello, 2000). In addition, they trigger the release

of other cytokines and inflammatory mediators (Waage *et al.*, 1989; Dinarello, 1997) and promote increases in the permeability of blood vessels by direct toxic effects on microvascular endothelial cells, thereby widening intercellular junctions (Saukkonen *et al.*, 1990; Quagliarello *et al.*, 1991). Upon entry into the SAS, 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 aggravating the inflammation and blood vessel damage (Tunkel *et al.*, 1990b; Kim *et al.*, 1992). In addition to the high levels of TNF-α, IL-1β and IL-6, other pro-inflammatory cytokines have been detected in the CSF of individuals with bacterial meningitis, including interferon (IFN)-γ (Frei *et al.*, 1988) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Frei *et al.*, 1992). GM-CSF is a pleiotrophic cytokine that can stimulate the proliferation, maturation and function of granulocyte and macrophage progenitors (Fleischmann *et al.*, 1986; DiPersio *et al.*, 1988), prolong the survival of mature neutrophils and eosinophils (Coxon *et al.*, 1999), and induce the secretion of other pro-inflammatory cytokines from monocytes and neutrophils (Takahashi *et al.*, 1993).

The cytokine-induced widening of intercellular tight junctions results in increased permeability of the B-CSFB, allowing the influx of protein, particularly albumin, into the CSF (Lindquist *et al.*, 1987). Vasogenic cerebral oedema ensues, which hampers the efficiency of venous drainage, and since the skull cannot expand this oedema results in raised intracranial pressure, decreased cerebral blood flow and subsequent diffuse CNS ischaemic injury (DeVoe, 1982; Ashwal *et al.*, 1990; Tunkel *et al.*, 1990b; Brandtzaeg, 1995; Perry *et al.*, 1997).

1.3.5.2 Chemokines in bacterial meningitis

Based on the position of conserved cysteine residues within the amino acid sequence, the chemokine superfamily can be divided into four subfamilies, the two main ones being CC and CXC (Lahrtz *et al.*, 1998; Mackay, 2001). The CC subfamily consists of chemokines bearing two adjacent cysteines near the N-terminus, and are mainly chemotactic for monocytes and T lymphocytes. In the CXC subfamily, the corresponding cysteine residues are separated by one amino acid and these chemokines are predominantly neutrophils attractants and activators, but can also attract activated T-cells and NK cells (Mahalingam & Karupiah, 1999). Two additional chemokine subfamilies, C and CXXXC, are predominantly involved in stimulating the migration of mononuclear inflammatory cells (Ransohoff, 2002). The C subfamily consists of chemokines with fewer conserved cysteine residues, while the CXXXC subfamily are anchored to the cellular plasma membranes by a transmembrane domain and a cytoplasmic tail (Tauber & Moser, 1999). Recently, Zlotnik and Yoshie (2000) have proposed an updated nomenclature for the classification of chemokines, based on the arrangement of the two N-terminal cysteine residues; however, in the current study the chemokines will be referred by their original names with the new designations included in brackets (Zlotnik & Yoshie, 2000).

Significantly elevated levels of CXC chemokines, namely IL-8 (CXCL8) and growth related protein (GRO)-α (CXCL1) were detected in the CSF of patients with bacterial meningitis (Van Meir *et al.*, 1992; Lopez-Cortes *et al.*, 1993; Spanaus *et al.*, 1997). In addition, high levels of the CC chemokines monocyte chemotactic protein (MCP)-1 (CCL2), macrophage inflammatory protein (MIP)-1α (CCL3) and MIP-1β (CCL4), and low levels of regulated-upon-activation, normal-T-cell expressed and secreted-protein (RANTES) (CCL5) (Sprenger *et al.*, 1996; Spanaus *et al.*, 1997; Inaba *et al.*, 1997), were observed. Due to their target cell selectivity, the CXC and CC chemokines prominent in bacterial meningitis are likely to contribute to the pronounced initial influx of neutrophils into the CSF, and later the infiltration of monocytes and T-cells (Sprenger *et al.*, 1996; Spanaus *et al.*, 1997; Tauber & Moser, 1999). MCP-1, MIP-1α, MIP-1β, IL-8 and RANTES have all been reported to up-regulate cell adhesion molecules on vessel endothelia, enhancing the trafficking of PMNL from the vascular space into the CSF (Inaba *et al.*, 1997; Adams & Lloyd, 1997). In addition, MIP-1α, in a co-ordinated manner with IL-8 and G-CSF, has been reported to induce the accumulation of neutrophils into the CSF from the blood (Inaba *et al.*, 1997).

1.3.5.3 Anti-inflammatory cytokines in bacterial meningitis

In addition to the pro-inflammatory cytokines produced during bacterial meningitis, high levels of IL-10 and transforming growth factor (TGF)-β have been observed in the CSF of patients with bacterial meningitis (van Furth *et al.*, 1995; Lehmann *et al.*, 1995; van Deuren *et al.*, 1995; Huang *et al.*, 1997). Both TGF-β and IL-10 may stimulate negative feedback signals during leptomeningitis to limit the production and effects of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 (Paris *et al.*, 1997; Letterio & Roberts, 1998) and the chemokine IL-8 (Ehrlich *et al.*, 1998). Other anti-inflammatory activities of IL-10 include the inhibition of chemokine production by PMNL (Kasama *et al.*, 1994) and endothelial cells (van Furth *et al.*, 1996), and the down-regulation of the endothelial adhesion molecule ICAM-1 (Jones *et al.*, 1994). In addition, the CSF of patients with pneumococcal meningitis also contained the anti-inflammatory cytokine IL-4, a molecule that was not detected in patients with gram-negative bacterial meningitis (Diab *et al.*, 1997). The biological activities of IL-4 include the ability to down-regulate the secretion of IL-1, IL-6, IL-8 and TNF-α from monocytes (Paul, 1991), and the enhancement of VCAM-1 expression on endothelial cells that promotes the influx of T-cells and monocytes but not neutrophils (Thornhill *et al.*, 1990; Elliott *et al.*, 1991) .

1.3.5.4 Other inflammatory molecules in bacterial meningitis

Although the generation of proinflammatory cytokines and chemokines by leptomeningeal cells and immune effector cells is likely to be critical in leptomeningitis, other important host cell defence mechanisms and pathophysiological mediators of injury are also apparent. The identity, source and function of these other inflammatory molecules found in the CSF of patients with bacterial meningitis have been reviewed recently by Christodoulides and colleagues (2001), and

they are summarised in Table 1.3. The aim of the current study is to focus on the role of proinflammatory cytokines and chemokines in the pathogenesis of bacterial meningitis.

1.4 Host cellular defence in the SAS

Despite the pronounced entry of leukocytes into the CSF during inflammation, host defence mechanisms remain inefficient and bacterial proliferation is unchecked (Brown *et al.*, 1981). The minimal levels of complement components and specific immunoglobulins in the CSF measured during meningitis are below the optimal concentration required for opsonic activity, resulting in inefficient phagocytosis and killing of invading encapsulated bacteria (Smith & Bannister, 1973; Simberkoff *et al.*, 1980; Stahel *et al.*, 1997). However, the innate, or non-specific immune response may potentially play an important role at this immunologically privileged site, since non-specific effector molecules are synthesised within hours of microbial recognition, and many of them possess the ability to neutralise a number of microorganisms (Fearon, 1997).

1.4.1 The role of β-defensins in innate immunity

One particular mechanism of innate cell defence is provided by the defensins. Defensins are cationic, cysteine-rich antimicrobial peptides that are highly conserved across many animal classes, insects and plants, which lack a comparable adaptive immune system (Schroder, 1999; Lee *et al.*, 2000). Defensins are classified as either α- or β-defensins based on the sequence relationship of three intramolecular disulfide bonds (Ganz & Lehrer, 1994). Six human α-defensins are presently known in humans, of which four are produced by neutrophils (HNP-1,2,3, and 4) and two by small intestinal Paneth cells (HD5 and 6), which contribute to the oxygen-independent killing of ingested organisms (Jones & Bevins, 1992; Ganz & Lehrer, 1994; Lehrer & Ganz, 1996). Currently, three of the structurally related but epithelia-derived β-defensins have been identified in humans. Human β-defensin-1 (hBD-1) was originally isolated from blood filtrate obtained from end stage renal disease patients (Bensch *et al.*, 1995), and hBD-2 peptide was identified in skin and is induced in keratinocytes in response to infection and inflammation (Harder *et al.*, 1997). A third β-defensin, hBD-3, has been identified recently and its expression can be induced by inflammatory stimuli from various human epithelial cells (Harder *et al.*, 2001; Jia *et al.*, 2001).

Table 1.3: The nature and role of key inflammatory mediators in bacterial leptomeningitis (Adapted from Christodoulides *et al.* (Christodoulides *et al.*, 2001))

Molecule	Source	Function	References:
Adhesion molecules	Vessel endothelium; Choroid plexus epithelium; arachnoid cells	Families of homologous adhesion molecules, including ICAM-1 and ELAM-1, responsible for adhesion and migration of leukocytes into SAS and CNS	(Rieckmann *et al.*, 1993; Fassbender *et al.*, 1997; Endo *et al.*, 1998)
Complement factors (*e.g.* C3, factor B, and C5a)	Infiltrating myeloid cells or resident macrophages; neurons; astroglial cells	Chemoattractant for leukocytes; induce synthesis of IL-1, IL-6, IL-8 and TNF-α by monocytes / macrophages	(Yancey *et al.*, 1989; Daffern *et al.*, 1995; Morgan *et al.*, 1997)
Degranulating factor for neutrophils	Endothelial cells	LPS stimulated; activates PMNL, initiating or propagating vascular damage	(Gill *et al.*, 1998)
Eicosanoids (*eg.* Prostaglandin E2)	Derivatives of arachidonic acid; secreted by inflamed tissues and vascular endothelium	Modulation and function of barrier permeability; neutrophil migration; augment surface expression and secretion of MMP-2 and MMP-9; LPS-induced vasodilatation in concert with NO	(Mertz *et al.*, 1994; Leppert *et al.*, 1995; Jaworowicz, Jr. *et al.*, 1998)
Leukotriene B_4	PMNL	Potentates inflammatory response; causes transendothelial migration, aggregation, activation and degranulation of neutrophils	(Santer *et al.*, 1996)
Matrix metalloproteinases (MMP) *e.g.* MMP-9	Infiltrating PMNL and macrophages; meninges; endothelial cells	Process TNF-α to its soluble form; contributes to disruption of BBB and B-CSF barriers by proteolytic activity on extracellular basement membranes	(Gijbels *et al.*, 1992; Leppert *et al.*, 1995; Paul *et al.*, 1998; Kieseier *et al.*, 1999; Leib *et al.*, 2000)

(cont.)

Table 1.3: Continued.

Molecule	Source	Function	References:
Nitric oxide (NO)	Macrophages; neutrophils; vascular smooth muscle cells; glial cells; vascular endothelial cells; neurons	Derived from conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS). Critical role in regulation of vascular tone and cerebral blood flow, contributing to CSF pleocytosis, and alterations in barrier permeability. Microbicidal.	(Shenep & Tuomanen, 1998; Leib & Tauber, 1999; Hobbs *et al.*, 1999; Koedel & Pfister, 1999)
Reactive oxygen species (ROS)	Primarily in PMNL in SAS and vessel endothelium	Central role in development of intracranial complications and brain damage through loss of cell membrane function and integrity. Oxidative injury associated with simultaneous production of superoxide and NO.	(Leib & Tauber, 1999; Koedel & Pfister, 1999)
Platelet activating factor (PAF)	Endothelial cells; PMNL; macrophage; neurons; glial cells	Primes PMNL to low concentrations of bacterial components; potent vasodilator; involved in ischaemic and neuronal injury	(Arditi *et al.*, 1990; Townsend & Scheld, 1994)
Soluble (s)CD14	Leukocytes; macrophages	Binding of sCD14 to bacteria competitively hinder phagocytosis	(Cauwels *et al.*, 1999)
Soluble FAS (CD95)		Apoptotic removal of leukocyte infiltrates form SAS in order to control overwhelming inflammatory response	(Fassbender *et al.*, 1999)

1.4.1.1 Tissue distribution and expression of β-defensins

The β-defensins hBD-1, hBD-2 and hBD-3 have been identified in many human tissues (Table 1.4). The overall pattern of β-defensin expression and the absence of abundant mucosal phagocytic cells suggests that their primary role may be the defence of epithelial cells and various mucosal surfaces from microbial invasion. Significantly, β-defensins have been identified in the bovine brain (Stolzenberg *et al.*, 1997) and hBD-1, but not hBD-2 or hBD-3, has been identified in the human choroid plexi, potentially being secreted into the CSF (Bals *et al.*, 1998; Nakayama *et al.*, 1999). In addition, constitutive hBD-1 mRNA expression was detected in cultures of human astrocytes, microglia, meningeal fibroblasts and neurons, while hBD-2 was identified in cultured astrocytes stimulated with either *E.coli* LPS, IL-1β or TNF-α (Hao *et al.*, 2001).

In contrast to most α-defensins, which are myeloid of origin and expressed constitutively in leukocytes, the synthesis of many β-defensins can be induced from epithelial cells during inflammation and infection (Zhang *et al.*, 1999b). All of the data so far collected on hBD-1 suggest that it is constitutively expressed in all of the tissues in which it has been identified (Table 1.4). O'Neil (1999) reported a minor increase in hBD-1 peptide secretion from IL-1α stimulated Caco-2 cells, suggesting that IL-1α has an effect on the translation and/or release of hBD-1 (O'Neil *et al.*, 1999). By contrast, the expression of hBD-2 and hBD-3 in epithelial cells has been shown to be inducible by stimulation with physiologically relevant concentrations of pro-inflammatory cytokines such as TNF-α and IL-1β (Harder *et al.*, 1997; Harder *et al.*, 2000; Harder *et al.*, 2001; Jia *et al.*, 2001) and by stimulation with *E.coli* and *Pseudomonas aeruginosa* (O'Neil *et al.*, 1999; Harder *et al.*, 2001). The presence of the transcriptional regulator NF-κβ consensus sequence in the proximal promoter region of the hBD-2 gene is potentially one explanation for this behaviour (Harder *et al.*, 1997; Mathews *et al.*, 1999). However, IL-1β markedly induced the expression of hBD-3 mRNA in gingival keratinocytes, despite the absence of NF-κβ consensus sequences, suggesting the involvement of other transcriptional regulatory pathways (Liu *et al.*, 1998).

1.4.1.2 Molecular structure and function of β-defensins

The β-defensins represent an unusual class of antimicrobials due to their three stabilising disulphide bonds, in a 1-5, 2-4, 3-6 linkage pattern, and a β–hairpin as their principal structural feature (Selsted *et al.*, 1993). They are 36-42 amino acids in length, amphipathic and cationic molecules, *i.e.* contain excess numbers of lysine and arginine residues. Despite the lack of similarity in nucleotide and amino acid sequence between the α- and β-defensins, their genes are all clustered on the same chromosome, 8p23, suggesting evolution from a common ancestral defensin gene (Liu *et al.*, 1998).

Table 1.4: Human tissue distribution of hBD-1, hBD-2 and hBD-3 peptides

Tissue	hBD-1[1]	hBD-2[2]	hBD-3[2]	References
Kidney	+	-	+	(Bensch *et al.*, 1995; Zhao *et al.*, 1996; Harder *et al.*, 2001)
Pancreas	+	-	-	(Zhao *et al.*, 1996)
Urogenital tract	+	+	-	(Bals *et al.*, 1998; Valore *et al.*, 1998)
Respiratory tract	+	-	+	(Goldman *et al.*, 1997; McCray & Bentley, 1997; Harder *et al.*, 2001)
Foreskin	+	-	-	(Harder *et al.*, 1997)
Ocular surface	+	+	+	(Haynes *et al.*, 1999; McDermott *et al.*, 2003)
Intestine	+	+	+	(Bals *et al.*, 1998; O'Neil *et al.*, 1999; Harder *et al.*, 2001)
Haematopoietic system	-	+	-	(Bals *et al.*, 1998)
Brain tissues	+	-	+ / -	(Nakayama *et al.*, 1999; Hao *et al.*, 2001)
Skin	+	+	+	(Harder *et al.*, 1997; Fulton *et al.*, 1997; Harder *et al.*, 2001)

[1] – Constitutive expression [2] – Inducible expression

In vitro experiments indicate that hBD-1 and hBD-2 exhibit broad-spectrum antimicrobial activity predominantly against gram-negative bacteria, the fungal pathogen *Candida albicans* and some enveloped viruses (Table 1.5). However, hBD-1 and hBD-2 demonstrate only low, if any, microbiocidal activity against gram-positive bacteria, whereas hBD-3 has been shown to kill *Staphylococcus aureus* and *Streptococcus* pyogenes (Harder *et al.*, 2001). Although the three-dimensional structure of β-defensins is not available, the fact that they are cationic peptides with a predicted amphiphilic antiparallel β-sheet structure suggests a mode of action similar to that of α-defensins (Martin *et al.*, 1995). The killing event by α-defensins, for both gram-positive and gram-negative bacteria, is insertion into the cytoplasmic membrane, where they assemble into multimeric pores that destroy membrane integrity (White *et al.*, 1995; Hancock, 1997). The low membrane potentials and high levels of cholesterol characteristic of eukaryotic cells, which serve to discourage pore formation, explain the selectivity of these peptides for bacteria (Nakajima *et al.*, 1987; Schroder, 1999). However, recent studies have indicated that β-defensin bactericidal activity could also result from electrostatic charge-based mechanisms of membrane-permeabilisation, rather than the formation of bilayer-spanning pores (Hoover *et al.*, 2000).

Human β-defensins can also act in synergy with other antimicrobial proteins, such as lactoferrin and lysozyme, which are found in proximal human airways (Bals *et al.*, 1998). The antimicrobial activity of both hBD-1 and hBD-2 is not changed appreciably by low pH but is inhibited by high salt conditions (Smith *et al.*, 1996; Goldman *et al.*, 1997; Bals *et al.*, 1998; Valore *et al.*, 1998). This characteristic has been linked with the pathogenesis of cystic fibrosis (CF), where the inactivation of human β-defensin activity in patients with CF may contribute to recurrent airway infections (Guggino, 1999). By contrast, the bactericidal activity of hBD-3 is not salt-sensitive at physiological concentrations, making the contribution of this β-defensin of particular relevance in CF (Harder *et al.*, 2001).

Table 1.5: The antimicrobial activity of human β-defensins.

Organism	References:
Gram-negative bacteria:	
Escherichia coli	(Valore *et al.*, 1998; Hiratsuka *et al.*, 1998; Ganz, 2001)
Pseudomonas aeruginosa	(Smith *et al.*, 1996; Goldman *et al.*, 1997; Bals *et al.*, 1998)
Gram-positive bacteria:	
Staphylococcus aureus	(Harder *et al.*, 2001)
Streptococcus pyogenes	(Harder *et al.*, 2001)
Entrococcus faecalis	(Bals *et al.*, 1998)
Enveloped viruses:	
Human Immunodeficiency Virus (HIV)	(Nakashima *et al.*, 1993)
Herpes simplex virus (HSV)	(Yasin *et al.*, 2000)
Fungus:	
Candida albicans	(Harder *et al.*, 1997)

1.5 Aims of the project

Bacterial meningitis is characterised by an acute compartmentalised inflammatory response within the SAS, with little or no spread of the infection or inflammation to the underlying cerebral tissue (Brandtzaeg, 1995; Gray & Nordmann, 1997). Despite the pronounced influx of leukocytes into the CSF during inflammation, host defence mechanisms remain inefficient, due to the absence of functional opsonisation and bactericidal activity, which results in unchecked bacterial proliferation (Brown *et al.*, 1981). In addition, the identification of β-defensins in human choroid plexi (Nakayama *et al.*, 1999) and their up-regulation during bacterial infection (Stolzenberg *et al.*, 1997; O'Neil *et al.*, 1999), has suggested a role for these members of the innate immune response during bacterial meningitis. Recent studies have identified a pivotal role for the leptomeninges in the pathogenesis of bacterial meningitis, and they have described the molecular basis of interactions between meningococci and cells of the leptomeninges and the subsequent induction of pro-inflammatory cytokines and chemokines (Hardy *et al.*, 2000; Christodoulides *et al.*, 2002). However, comparable data on cellular interactions with other major meningeal pathogens and stimulation of the subsequent inflammatory response are scarce. In the current study, the following hypotheses will be tested: that,

1. *Neisseria meningitidis*, *Haemophilus influenzae*, *Escherichia coli K1* and *Streptococcus pneumoniae* demonstrate qualitative and quantitative differences in their interactions with leptomeningeal and epithelial cell lines.
2. leptomeningeal cells play a pivotal role in the acute inflammatory response through the production of pro-inflammatory cytokines and chemokines in response to the different bacterial pathogens.
3. the interactions of bacterial pathogens influence the mechanisms of innate immunity through the modulation of β-defensin mRNA expression in leptomeningeal and epithelial cell lines.

In the current project, the interactions of the meningeal pathogens *H.influenzae*, *E.coli K1* and *S.pneumoniae* with leptomeningeal cells will be investigated and compared with the interactions of *N.meningitidis*. In addition, the interactions of these bacterial pathogens with a representative epithelial cell line will be compared and contrasted. Next, the cellular immune responses induced by these meningeal pathogens will be investigated, with respect to the induction of pro-inflammatory cytokines and chemokines and the expression of β-defensin mRNA.

CHAPTER 2 MATERIALS AND METHODS

2.1 Water and general reagents

Distilled water (dH_2O) was produced by reverse osmosis on a euRO40 system (Triple Red Ltd, Thame, UK), whilst ultra high quality (UHQ) water was prepared by further purification to 18.0mΩ-cm on a NANOpure DIamond ultrapure system (Barnstead-Thermolyne, Iowa, USA). Phosphate buffered saline (PBS) was prepared by the dissolution of tablets (Oxoid, Basingstoke, UK) in UHQ, to produce a buffer of pH 7.3. Both UHQ water and PBS were sterilised by autoclaving at 1.05kg cm^{-2} for 15 min before use. Unless otherwise stated, all reagents were purchased from VWR International, Lutterworth, UK.

2.2 Bacterial strains and growth conditions

Materials:

Proteose peptone broth dH_20 containing 1% (w/v) proteose peptone (BD Biosciences, Oxford, UK) and 8% (v/v) glycerol. Sterilised by autoclaving at 1.05kg cm^{-2} for 15 min.

The bacteria used in this study are listed in Table 2.1. *Neisseria meningitidis* piliated variant MC58-7 ($Pil^+Opa^+Opc^+Cap^+LPS^+$) was isolated from a case that occurred during an outbreak of meningococcal infection in Stroud, Gloucestershire in the mid-1980's (McGuinness *et al.*, 1991). A non-piliated variant, MC58-9 ($Pil^-Opa^+Opc^+Cap^+LPS^+$), was subsequently obtained by colony selection using a stereomicroscope (Hardy *et al.*, 2000). *Escherichia coli* strain IH3080 (O18:K1:H7) isolated from a neonate presenting with meningitis, was obtained from the National Public Health Institute Helsinki, Finland (Nowicki *et al.*, 1986). *E.coli* DSM (O18:K1:H-) is a spontaneous nalidixic acid-resistant strain of *E.coli* RS228, which was originally isolated from a faecal specimen from a healthy individual (Achtman *et al.*, 1983). This strain has been shown to be pathogenic in the infant rat model of bacteraemia and meningitis (Pluschke *et al.*, 1983). *Haemophilus influenzae* type b strain Eagan (Cap^+) is a clinical isolate from the CSF of a patient with meningitis in Boston, USA (Anderson *et al.*, 1972). The *Haemophilus* strain RD (Cap^-) is an unencapsulated, spontaneous mutation of a type d bacterium derived from a CSF sample taken from an individual presenting with bacterial meningitis (Alexander & Leidy, 1952). *Streptococcus pneumoniae* D39 and *Neisseria lactamica* were both obtained from the National Collection of Type Cultures (NCTC), Colindale, UK.

Bacterial strains were stored in liquid nitrogen at –196°C as thick suspensions in proteose peptone (PP) broth. For growth of bacteria, the frozen stocks were used to inoculate appropriate agar medium plates, which were subsequently incubated at 37°C for 16-18 hours in either an

ambient atmosphere or in 5% (v/v) CO_2. The medium and growth conditions specific to each bacterium are listed in Table 2.1 and detailed in Appendix 1.

2.3 Preparation of bacterial suspensions

Materials:

B salts dH_20 containing 1mM magnesium chloride and 0.68μM calcium chloride. Sterilised by autoclaving at 1.05kg cm^{-2} for 15 min.

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

Absorbance solution UHQ containing 1% (w/v) SDS and 0.1% (w/v) sodium hydroxide.

N.meningitidis, *E.coli*, *H.influenzae* and *N.lactamica* were grown for 16-18 hours on appropriate agar medium plates and resuspended in 1ml of plating buffer. Each bacterial suspension was centrifuged at 300g for one minute to remove any clumps of bacteria and the retained supernatant was diluted by a factor of one in ten in plating buffer. *S.pneumoniae* was incubated at 37°C for 16-18 hours in liquid medium (10ml), centrifuged at 300g for ten minutes, the pellet was then resuspended in fresh medium (20ml) and incubated for a further two hours. The bacterial suspension was centrifuged again at 300g for ten minutes and the pellet was resuspended in 1ml of plating buffer and diluted by a factor of one in ten.

2.3.1 Quantification of bacterial suspensions

A sample of each bacterial suspension was further diluted by a factor of one in ten in absorbance solution and the absorbance was measured at a wavelength of 260nm (A_{260}). The bacterial suspensions were serially diluted in plating buffer and 15μl volumes were used to inoculate appropriate agar plates in triplicate for viable counting. The number of colony forming units (cfu) per ml of bacteria equivalent to an A_{260} value of 1.0 was determined for each bacterium so that bacterial inoculum could be adjusted to the desired concentration for subsequent experiments. Table 2.2 describes the bacterial cfu ml^{-1} values equivalent to an A_{260} reading of 1.0.

2.4 Detection of capsule

The presence or absence of a capsule was determined for all the bacteria by the use of either a slide agglutination assay or a negative staining protocol.

Table 2.1: Basic characteristics of bacteria used in this study.

Organism	Strain	Source	Capsule type	Pili / Fimbriae	Reference	Growth medium	CO_2 conc. (v/v)
Neisseria meningitidis	MC58-7	CSF	Group B	Class I pili	(McGuinness *et al.*, 1991)	PP	5%
	MC58-9	CSF	Group B	None	(Hardy *et al.*, 2000)	PP	5%
Escherichia coli	IH3080	CSF	K1	Type S and I fimbriae	(Nowicki *et al.*, 1986)	Luria-Bertani	Ambient
	DSM	Faecal	K1	Type S and I fimbriae	(Pluschke *et al.*, 1983)	Luria-Bertani	Ambient
Haemophilus influenzae	Eagan	CSF	Type b	None	(Anderson *et al.*, 1972)	HTM [1]	5%
	RD	Lab strain	Acapsular (originally type d)	None	(Alexander & Leidy, 1952)	HTM [1]	5%
Streptococcus pneumoniae	D39	NCTC	Type 2	None	(Avery *et al.*, 1944)	Todd Hewitt [2]	Ambient
Neisseria lactamica	-	NCTC	Acapsular	Class II pili	(Hollis *et al.*, 1969)	PP	5%

[1] – Haemophilus Test Medium (Oxoid) supplemented with 15μg ml^{-1} of both β-Nicotinamide adenine dinucleotide (β-NAD) and Hematin (Sigma, Poole, UK).

[2] – Grown in liquid culture.

Table 2.2: Bacterial cfu ml^{-1} values equivalent to an A_{260} reading of 1.0

Organism	Strain	cfu ml^{-1} equivalent to A_{260}=1.0
N.meningitidis	MC58-7	3.30×10^{10}
	MC58-9	3.30×10^{10}
E.coli	IH3080	3.80×10^{10}
	DSM	2.32×10^{11}
H.influenzae	Eagan	1.25×10^{10}
	RD	2.20×10^{9}
S.pneumoniae	D39	4.28×10^{8}
N.lactamica	-	2.5×10^{9}

2.4.1 The slide agglutination assay

The test was performed using the Wellcome Meningococcus Group B Monoclonal Antibody solution following the manufacturer's instructions. Briefly, *N.meningitidis*, *E.coli* and *N.lactamica* were grown for 16-18 hours on appropriate agar medium plates. A loop of culture was emulsified into a dense suspension in two separate drops of PBS on a glass microscope slide (Fisher, Loughborough, UK). One drop (approximately 50µl) of undiluted antibody was added to one bacterial suspension, while PBS was added to the other bacterial suspension as a negative control. The samples were mixed by gently rocking the slide for two minutes. A positive reaction was characterised by the rapid and strong agglutination of the test suspension, and the result was validated by the absence of agglutination in the negative control sample.

2.4.2 The negative stain for capsule expression

Materials:

Congo red solution UHQ containing 1% (w/v) Congo red (Sigma)

A single drop (approximately 50µl) of Congo red solution was added to a clean glass microscope slide, which had been previously passed through a bunsen flame to remove any grease. Next, one or two bacterial colonies were mixed with the Congo red solution to create an emulsion which was spread thinly over the surface of the slide and allowed to air-dry. The slide was flooded with crystal violet (Difco, Heidelberg, Germany) for two minutes and was then gently washed under running tap water. After air-drying, the sample was viewed with a Leitz Dialux light microscope (Leica Microsystems, Milton Keynes, UK) under immersion oil with a x100 objective lens.

2.5 Detection of pili or fimbriae

The presence, or absence, of pili or fimbriae on all bacteria was determined by transmission electron microscopy (TEM). In addition, an immunodot blot assay using specific antibodies, and a haemagglutination assay, were both used to characterise the type of pili or fimbriae.

2.5.1 Transmission Electron Microscopy

Materials:

Phosphotungstic acid (PTA) UHQ containing 1% (w/v) phosphotungstic acid, pH 7.4.

The presence, or absence, of pili or fimbriae in all strains of bacteria was investigated by electron microscopy following a negative staining protocol adapted from the method of Lin and colleagues (Lin *et al.*, 2002). A thin strip of agar (70mm x 200mm) was carefully cut from an overnight culture of each bacterial strain and transferred to a 1.5ml tube (Fisher) containing PBS, and kept for 30 min at 25°C. This bacterial suspension was decanted into a fresh tube and fixed by the addition of gluteraldehyde (Agar Scientific Ltd, Stanstead, UK) to a final concentration of 0.5% (v/v), with incubation at 25°C for 20 min. Following centrifugation at 2500g for three minutes, the pellet was gently washed in UHQ and centrifuged again. The resulting pellet was gently resuspended in a 100µl volume of UHQ. A sample (7µl) was then placed onto a formvar-coated copper grid and left to adsorb for two minutes. The grid was blotted dry and a 7µl volume of PTA added for 10 sec. The grid was blotted dry and viewed under a Hitachi H7000 transmission electron microscope at 75kV.

2.5.2 Immunodot blot assay for the detection of pilus

Materials:

Absorbance solution UHQ containing 1% (w/v) SDS and 0.1% (w/v) sodium hydroxide.

Tris buffered saline (TBS) 20mM Tris-HCL pH 7.5 containing 500mM sodium chloride (Fisher), pH 7.5

Tween-Tris buffered saline (TTBS) TBS containing 0.05% (v/v) Tween 20

Block buffer TBS containing 3% (w/v) bovine serum albumin (BSA) (Sigma)

Antibody diluent TTBS containing 1% (w/v) BSA

Substrate buffer 100nM Tris-HCL pH 9.5 containing 100mM sodium chloride and 2mM magnesium chloride

Nitro blue tetrazolium (NBT) 30mg ml^{-1} NBT (Sigma) in 70% (v/v) dimethylformamide (DMF) (Sigma)

5-Bromo-4-chloro-3-indolyl phosphate (BCIP) 15mg ml^{-1} BCIP (Sigma) in DMF (100%)

Bacteria were grown for 16-18 hours on appropriate agar medium plates, scraped into 1 ml of PBS and were centrifuged at 300g for one minute to remove any clumps. A sample from the retained supernatant was diluted by a factor of 1/10 in absorbance solution and the protein concentration was estimated by measuring the absorbance at 260nm. Previous studies have shown that an A_{260} of 12.0 is equivalent to 1mg ml^{-1} of protein (Hardy *et al.*, 2000) and this standard concentration was used as a guide to prepare samples containing 1mg ml^{-1} of protein in UHQ. A sample (3µl) was added to the middle of a strip of nitrocellulose paper (Protran® Schleicher & Schuell, Dasell, Germany) and allowed to air-dry for 30 min. Strips were then incubated with block buffer for one hour. All incubations were carried out at 25°C. The block buffer was removed and the samples were washed three times, each for five minutes, in TTBS. The nitrocellulose was then incubated for one hour with the appropriate primary antibody (Table 2.3) both used at a dilution of 1/10000 in antibody diluent. Unbound antibody was removed by washing in TTBS (3x5min) and the sample incubated for one hour with the secondary antibody, an alkaline phosphatase-conjugated goat anti-mouse IgM (Bio-Rad, Hemel Hempstead, UK) used at a dilution of 1/1000 in antibody diluent. The nitrocellulose was washed in TTBS (4x5min) followed by TBS (3x5min). The substrate solution was prepared immediately before use by the addition of 1ml each of NBT and BCIP solutions to 100ml of substrate buffer. Antibody bound to the nitrocellulose was detected by the addition of the substrate solution for 10-15min in the dark, after which the reaction was stopped with a series of washes with cold tap water.

Table 2.3: Murine monoclonal antibodies used for the detection of class I and class II pilus

Primary antibody	Target	Source	Reference
SM-1	Pilin (Class I pili)	In-house	(Virji & Heckels, 1983)
AG123	Class IIb pili	A gift from M.Achtman	(Personal communication)

2.5.3 Haemagglutination assay (HA)

Human erythrocytes were isolated from fresh O-negative whole blood by centrifugation according to the method of Connor and Loeb (Connor & Loeb, 1983). An equal volume of PBS was added to the sample of whole blood and this was then centrifuged at 1800g for five minutes. The pellet was washed in fresh PBS and centrifuged again at 1800g for five minutes. This step was repeated twice and the final pellet was resuspended in 2ml PBS and mixed thoroughly. The concentration of red blood cells was calculated by running an automated packed cell volume report on the sample, kindly undertaken by the Department of Haematology, Southampton General Hospital on

a XE2100 multitask analyser (SYSMEX, Milton Keynes, UK). A working stock of erythrocyte suspension was prepared by diluting the cells to a concentration of 1% (v/v) in PBS.

All HA reactions were undertaken in U-shaped microtitre plates (Fisher) at 25°C (Pichichero *et al.*, 1982). Briefly, the bacteria to be tested were resuspended in PBS (Section 2.3) and the concentration was adjusted to approximately $1x10^8$ cfu ml^{-1}. To 90µl of each bacterial suspension, 10µl of the 1% (v/v) erythrocyte solution was added and the sample gently mixed. The plates were incubated at 25°C on a non-vibrating surface for 1-2 hours. A positive reaction was characterised by the agglutination of the erythrocytes forming a network of cells spread evenly across the bottom of the microtitre well. In contrast, a negative result was visualised as a dense button of erythrocytes formed at the centre of the well bottom.

2.6 Culture of human cell lines

2.6.1 Isolation and culture of human meningioma cell lines

Meningioma tissue was obtained from patients undergoing surgical removal of meningeal tumours and was kindly provided by the Department of Neuropathology, Southampton General Hospital. These tumours were confirmed as meningiomas by the Neuropathology Department using histological methods, and classified into subtypes, which included meningothelial, transitional, anaplastic and atypical.

Materials:

Growth medium Dulbecco's modified Eagles medium with Glutamax-1 and sodium pyruvate (DMEM) (Invitrogen, Paisley, UK) supplemented with 10% (v/v) decomplemented FCS (Invitrogen), 100IUml^{-1} penicillin (Invitrogen), 10µg ml^{-1} streptomycin (Invitrogen) and 10µg ml^{-1} gentamicin (Invitrogen).

Storage medium Growth medium containing 10% (v/v) dimethyl sulfoxide (DMSO).

Trypsin-EDTA Hanks' Balanced Salt solution containing 0.5mg ml^{-1} and 0.2mg ml^{-1} EDTA•4NA (Invitrogen).

2.6.1.1 Coating of cell culture plastic surfaces with collagen

All cell culture flasks and plate wells (Greiner, Gloucestershire, UK) were coated with collagen, type I from rat-tail (BD Biosciences), according to the manufacturer's instructions. Briefly, collagen stocks were diluted to 50µg ml^{-1} in 0.02M acetic acid (Fisher) and culture vessels were coated with 100µl of collagen solution per cm^2 for one hour at 25°C. The excess collagen was discarded and the vessels were rinsed with PBS and allowed to air-dry before storage.

2.6.1.2 Primary culture of meningioma tissue

Necrotic tissue and blood clots were removed from fresh meningioma tissue with a sterile scalpel and the remaining tissue was washed several times in PBS. The tissue was cut into small pieces (<2mm^3), transferred into 10ml of trypsin-EDTA solution and incubated at 37°C for 30 min with gentle shaking. The tissue suspension was centrifuged at 300g for ten minutes, the supernatant was discarded and the tissue pellet was resuspended in 5ml of growth medium before being seeded into a collagen-coated 25cm^2 culture flask. All cultures were maintained in a humid environment at 37°C with 5% (v/v) CO_2 and growth medium was typically changed every three days.

2.6.1.3 Subculture of meningioma cells

Meningioma cells were passaged upon reaching confluence. Growth medium was removed and the monolayers were washed once with PBS to remove debris and traces of dFCS. Trypsin-EDTA solution was added to sufficiently cover the surface of the flask (1-2ml) for five to ten minutes under normal growth conditions. Enzymatic activity was inhibited by the addition of at least an equal volume of growth medium and the cells were centrifuged at 300g for ten minutes. The cells were resuspended in growth medium and were typically diluted by a factor of 1 in 3 for expansion into 75cm^2 collagen-coated culture flasks.

2.6.1.4 Frozen storage of meningioma cell lines

For long-term storage, the cells were isolated as described in Section 2.6.1.3 from a confluent monolayer grown in a 75cm^2 flask as described in Section 2.6.1.3. The pellet was resuspended in 3ml of storage medium and 1ml volumes were transferred to cryovial storage tubes (Greiner). The cryovials were placed in a polystyrene box packed with tissue paper and frozen overnight at –80°C before they were finally stored in liquid nitrogen at –196°C. Frozen cells were resuscitated by rapid thawing of the vial at 37°C in a water bath, followed by dilution of the contents with fresh growth medium. This was then centrifuged at 300g for 10 min and the pellet was resuspended in fresh growth medium and seeded into a 25cm^2 culture flask.

2.6.2 Culture of Chang cells

Chang conjunctival epithelial cells were obtained from the European Type Culture Collection (ETCC), Porton Down, UK. Chang cells were cultured and stored in the same manner as meningioma cells, as described above in Sections 2.6.1.3 and 2.6.1.4.

2.7 Immunocytochemistry of cellular markers in cultured human cell lines

Materials:

Block buffer PBS containing 10% (v/v) normal rabbit serum (Invitrogen).

Secondary antibody Fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse antibody (Dako, Ely, UK): Used at a dilution of 1/100 in PBS.

Meningioma cells were grown to approximately 60-80% confluence in Lab-TekII chamber slides (Invitrogen) coated with 5μg cm^{-2} collagen. Chang cells were grown in uncoated chamber slides. The cellular markers investigated are detailed in Table 2.4.

Table 2.4: Primary antibodies and fixatives used for the staining of cellular markers

Cellular marker	Source of primary antibody	Fixative	Working dilution of primary antibody
Cytokeratin AE1/AE3	Zymed	Acetone (absolute)	1/20
Desmoplakin I &II	Boehringer Mannheim	Ice-cold methanol (absolute)	1/20
Epithelial membrane antigen (EMA)	Dako	Ice-cold methanol	1/20
Vimentin	Sigma	Ice-cold methanol	1/200

2.7.1 Immunocytochemistry

The culture medium was removed and the cell monolayers were washed three times in PBS. The monolayers were fixed for ten minutes with the appropriate fixative solution, which was then removed. The monolayers were washed three times in PBS, and block buffer (200μl) was added for 30 min at 37°C to reduce non-specific binding of the primary antibody. The primary antibodies, as described in Table 2.3, were diluted in blocking buffer (100μl), and the cell monolayers were incubated for 16-18 hours with these antibodies at 4°C. The monolayers were washed three times in PBS before the addition of 100μl volumes of the secondary antibody for one hour at 37°C. Following a further three washes in PBS, the monolayers were incubated for 10 minutes at 25°C with 100μl per well of propidium iodide (Sigma) diluted 1/1000 in PBS. The monolayers were then washed six times in PBS before being mounted monolayer-down onto a drop of anti-fade fluorescent mounting medium (Dako) on a clean glass microscope slide. Cell staining was visualised under a Leitz Dialux fluorescent microscope (Leica Microsystems) using an epifluorescence system of illumination.

2.7.2 Confocal microscopy

Staining was also viewed under a Leica SP-2 laser-scanning confocal microscope system. Images were obtained by simultaneous 2-channel scanning at 488 and 568nm to excite FITC and propidium iodide respectively. Maximum projection images were subsequently prepared from 20 optical sections.

2.8 Quantification of the association of bacteria to cultured human cells

Materials:

Maintenance medium DMEM containing 0.1% (v/v) dFCS.

Wash buffer PBS containing 1% (v/v) dFCS.

Lysing buffer Wash buffer containing 1% (w/v) saponin (Sigma) and sterilised using a 0.2μm filter (Nalgene, supplied by Fisher).

Gentamicin solution Maintenance medium containing 200μg ml^{-1} gentamicin (Sigma).

Cytochalasin D (CD) Maintenance medium containing 2μg ml^{-1} CD (Sigma).

In all experiments in which bacterial association with host cells was quantified, meningioma cells of between passage numbers three and nine were grown to confluence on collagen-coated 96 well culture plates (Greiner). Chang cells were grown to confluence on uncoated 24 well culture plates. The growth of the cell monolayers was arrested by replacing the growth medium with maintenance medium, 48 hours prior to bacterial challenge. The infection experiments were based on the procedures of Virji and colleagues (Virji *et al.*, 1991a; Virji *et al.*, 1992c) with all solutions pre-warmed to 37°C.

2.8.1 Optimisation of bacterial growth conditions

Bacterial suspensions were prepared as described in Section 2.3 and adjusted to 1 x 10^6 cfu ml^{-1} in maintenance medium. Duplicate samples of bacterial suspensions were incubated under normal culture conditions in 24 well culture plates (1ml /well). At 1, 3, 6, 9 and 24 hours post-inoculation, the growth kinetics of each bacterium was investigated by serially diluting the challenge medium in plating buffer and inoculating the appropriate agar medium plates with 15μl volumes of each sample.

The concentration of dFCS in the maintenance medium was subsequently optimised for each bacterium so that their *in vitro* growth kinetics were similar to that of *N.meningitidis* strain MC58-7. The optimised maintenance medium will hereafter be referred to as "experimental medium" as described in Table 2.5. The experimental medium of *H.influenzae* required supplementation with β-NAD (Sigma) and hematin (Sigma), the concentrations of which were also optimised in conjunction with dFCS as described above.

Table 2.5: Experimental media for the culture of bacteria.

Bacteria	Experimental medium
N.meningitidis MC58-7 & MC58-9	DMEM + 0.1% (v/v) dFCS
E.coli DSM & IH3080	DMEM + 0.1% (v/v) dFCS
H.influenzae Eagan & RD	DMEM + 2% (v/v) dFCS + 2μg ml^{-1} β-NAD + 10μg ml^{-1} hematin
S.pneumoniae D39	DMEM + 2% (v/v) dFCS
N.lactamica	DMEM + 1% (v/v) dFCS

2.8.2 Quantification of bacterial adhesion

Bacterial suspensions were prepared as described in Section 2.3 and adjusted to the desired concentration (typically $1x10^6$ cfu ml^{-1}) in the appropriate experimental medium. The actual infecting dose was determined by serially diluting the neat bacterial suspension in plating buffer and inoculating appropriate agar plates for viable counting. The cell monolayers were gently washed once with wash buffer and the bacteria were added to the wells. The volume of bacterial suspension added depended on the type of well used; 1ml / well in 24 well plates and 200μl / well in 96 well plates. The inoculated monolayers were incubated under normal culture conditions. Triplicate wells were included for all samples at time points of 1, 3, 6, 9 and 24 hours post-challenge. At each designated time point, the challenge medium was removed from the wells and the monolayers washed four times in wash buffer. The concentration of non-adherent bacteria was measured by pooling the challenge medium and subsequent washes, followed by serial dilution in plating buffer then 15μl of each sample was used to inoculate appropriate plates. The washed cell monolayers were lysed with the addition of lysing buffer (150 or 250μl per well in 96 and 24 well plates respectively) and incubated at 37°C for 15 min. The lysate was mixed thoroughly, diluted and plated as described above.

2.8.3 Quantification of bacterial internalisation

To investigate bacterial internalisation, the bacteria were removed at each time point and a gentamicin solution (200μl or 1ml well in 96 and 24 well plates respectively) added for 90 min under normal culture conditions to eliminate all extracellular bacteria. The gentamicin solution was discarded and the monolayers were washed, then lysed and samples were plated as described in Section 2.8.2.

2.8.3.1 Incubation with cytochalasin D

To determine whether the apparent internalisation of bacteria was dependent on the microfilament activity of the cells, the monolayers were pre-treated with cytochalasin D (CD). Monolayers were incubated for 30 min with CD (100 or 500µl per well of CD stock solution in 96 and 24 well plates respectively) prior to the addition of bacteria. Equal volumes of bacterial suspension (diluted to $2x10^6$ cfu ml^{-1}) were then added to each well, to give final concentrations of 1µg ml^{-1} of CD and $1x10^6$ cfu ml^{-1} of bacteria. Monolayers were processed at appropriate time points for the determination of bacterial internalisation as described above.

2.8.4 Statistical analysis

The relative association of different bacteria was compared using one-way ANOVA analysis to compare the level of significance between the means. A P value of ≤0.05 was considered significant. All statistical analyses were done using Microsoft's EXEL package.

2.9 Visualisation of bacterial interactions with human cells

The association of each bacterium with meningioma cells was also visualised by staining infected monolayers with fluorescent dyes and bacteria with specific antibodies and viewing by confocal microscopy. The internalisation of bacteria by meningioma cells was determined by processing infected monolayers for TEM.

2.9.1 Fluorescence immunocytochemistry of infected cell monolayers

Meningioma cells were grown to confluence on size O (13mm diameter) uncoated glass coverslips (VWR International) in 24 well tissue culture plates as described in Section 2.6.1.

Materials:

Wash buffer PBS containing 1% (v/v) dFCS.

Experimental medium Supplemented DMEM (Section 2.8.1).

2.9.1.1 Immunocytochemistry

Bacterial suspensions were prepared as described in Section 2.3 and adjusted to either $1x10^6$ or $2.5\text{-}x10^8$ cfu ml^{-1} in experimental medium. Cell monolayers were washed once with wash buffer and the bacteria were added (1ml per well). Duplicate wells were included for all samples at time points of 3, 6 and 24 hours post-challenge. At each time point, the challenge medium was removed from the wells and the monolayers were washed three times in PBS before being fixed in a solution of 3% (w/v) paraformaldehyde in PBS for 30 min at 25°C. The paraformaldehyde solution was discarded and free aldehyde groups were neutralised by the addition of a 50mM ammonium carbonate solution for five minutes at 25°C. After washing three times in PBS, the

monolayers were incubated for 16-18 hours at 4°C with the appropriate primary antibody (Table 2.6). The monolayers were washed a further three times in PBS and incubated for one hour at 37°C with the secondary antibody, a FITC-conjugated rabbit anti-mouse antibody or FITC-conjugated goat anti-rabbit antibody at a dilution of 1/100. After washing three times in PBS, the cells were counter stained with 0.0025% (w/v) Evans Blue in PBS for 20 min at 25°C. Finally, the coverslips were washed six times in PBS and mounted monolayer-down onto a drop of anti-fade fluorescent mounting medium on a clean glass microscope slide.

Table 2.6: Primary polyclonal antibodies specific for bacterial pathogens.

Antibody	Target [1]	Working dilution	Source	Species raised in
M418-422	*N.meningitidis* OM	1/100	In-house	Mouse
Ab8064-1	*E.coli* K1 capsule	1/100	Abcam	Mouse
R699	*H.influenzae* Egan OM	1/100	Section 2.12	Rabbit
R701	*H.influenzae* RD OM	1/100	Section 2.12	Rabbit
Type Serum 2	*S.pneumoniae* type 2 capsule	1/100	Statens Serum Institute	Rabbit
R141	*N.meningitidis* Porin [2]	1/100	In-house	Rabbit

[1] – OM = Outer membranes. [2] – Cross-reacts with *N.lactamica.*

2.9.1.2 Confocal microscopy

Staining was also viewed under a Leica SP-2 laser-scanning confocal microscope system. Images were obtained by simultaneous 2-channel scanning at 488 and 568nm to excite FITC and propidium iodide respectively. Maximum projection images were subsequently prepared from between 14 and 25 optical sections.

2.9.2 Processing of cell monolayers for TEM

Meningioma cells were grown to confluence on collagen-coated microporous membrane (0.4μm pore size) culture plate inserts (Falcon) in 24 well tissue culture plates.

Materials:

Wash buffer PBS containing 1% (v/v) dFCS.

Experimental medium Supplemented DMEM (section 2.8.1).

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

Main fixative PIPES buffer containing 3% (v/v) gluteraldehyde and 4% (w/v) formaldehyde.

Post fixative PIPES buffer containing 1% (w/v) osmium tetroxide (Oxchem, Oxford, UK).

Graded alcohols 30, 50, 70, 95% (v/v) ethanol in UHQ; absolute ethanol.

Epoxy resin Spurr's resin (Agar Scientific)

Reynolds lead dH_2O containing 0.17M lead nitrate and 0.2M tri-sodium citrate

Bacterial suspensions were prepared as described in Section 2.3 and adjusted to $1x10^6$ cfu ml^{-1} in experimental medium. Cell monolayers were washed once with wash buffer, the bacteria were then added (1ml per well) and incubated under standard conditions. Bacteria were removed and the monolayers were washed four times in wash buffer before the addition of the main fixative for 15 min at 25°C followed by 50 min at 4°C. The cells were then washed in PIPES buffer (2x10 min) and post-fixed for 1 hour at 25°C. The samples were washed in PIPES buffer (2x10 min) and dehydrated through a series of graded ethanol solutions at 30, 50, 70 and 95% (1x10min each) and absolute ethanol (2x20 min). Following the 30% ethanol stage, the membrane was carefully cut away from the cell culture insert ensuring that the monolayers did not desiccate. As an intermediary between ethanol and resin, acetonitrile was added for ten minutes and then replaced with a 50:50 acetonitrile: resin mix for 16-18 hours. The samples were incubated with neat resin for six hours before the membranes were embedded in fresh resin, which was polymerised at 60°C for 20-24 hours.

Gold sections (80-120nm) were cut on a Reichert-Jung Ultracut E ultramicrotome (Leica Microsystems) with glass knives, and mounted onto 200 square mesh copper/palladium grids (Agar Scientific). The sections were then stained in saturated alcoholic uranyl acetate (Agar Scientific) for 15 min in the dark, followed by Reynolds lead stain for five minutes in the presence of sodium hydroxide pellets to absorb excess CO_2. Samples were viewed under a Hitachi H7000 transmission electron microscope at 75kV.

2.9.3 Determination of cell viability

Meningioma cells were grown to confluence on size O (13mm diameter) uncoated glass coverslips (VWR International) in 24 well tissue culture plates as described in Section 2.6.1.

Materials:

Wash buffer PBS containing 1% (v/v) dFCS.

Experimental medium Supplemented DMEM (section 2.8.1).

Bacterial suspensions were prepared as described in Section 2.3 and adjusted to $1x10^6$ cfu ml^{-1} in experimental medium. Cell monolayers were washed once with wash buffer, the bacteria were

then added (1ml per well) and incubated under standard conditions. Bacteria were removed and the monolayers were washed four times in wash buffer before the addition of the Molecular Probes LIVE/DEAD® Viability / Cytotoxicity kit reagents, according to the manufacturer's instructions. The coverslips were mounted monolayer-down onto clean glass microscope slides and viewed immediately.

2.9.3.1 Confocal microscopy

Staining was also viewed under a Leica SP-2 laser-scanning confocal microscope system. Images were obtained by simultaneous 2-channel scanning at 488 and 568nm to excite FITC and propidium iodide respectively. Maximum projection images were subsequently prepared from 18 optical sections.

2.10 Detection of cytokine expression by human cells following challenge with bacteria

The expression of a range of pro-inflammatory cytokines and chemokines from human cells challenged with various bacteria was investigated both at the mRNA level by reverse-transcriptase (RT)-PCR assays and at the protein level by sandwich immunoassay.

Meningioma cells of between passage number three and nine were grown to confluence on collagen-coated 96 well culture plates. Chang cells were grown to confluence on uncoated 24 well culture plates. The growth medium was replaced with maintenance medium 48 hours prior to bacterial challenge to arrest the growth of the cell monolayers.

2.10.1 Reverse-transcriptase (RT)-Polymerase chain reaction (PCR)

Bacterial suspensions were prepared as described in Section 2.3 and adjusted to $1x10^6$ cfu ml^{-1} in experimental medium. Cell monolayers were washed once with wash buffer and the bacterial suspensions were then added (1ml per well). Triplicate or quadruplicate wells were included for Chang or meningioma cell samples respectively and the challenged monolayers were incubated under normal culture conditions.

2.10.1.1 Extraction and isolation of total cellular RNA

At appropriate time points the experimental medium was discarded and the total RNA harvested with the addition of Trizol reagent (0.5ml / well) (Invitrogen) for five minutes at 25°C. Triplicate or quadruplicate wells were pooled and chloroform was added at a ratio of 1:5, followed by vigorous mixing and incubation at 25°C for three minutes. The samples were centrifuged at 15000g for ten minutes at 4°C. The upper aqueous phase containing RNA, was carefully transferred to fresh tubes and precipitated with the addition of isopropyl alcohol (Fisher) at a ratio

of 1:1. The samples were briefly mixed and kept at –20°C for one hour before centrifuging at 15000g at 4°C for ten minutes. The resulting pellet was washed in ice cold 75% (v/v) ethanol and centrifuged again at 15000g for ten minutes at 4°C. The ethanol was discarded and the RNA pellet was kept under vacuum for five to ten minutes until almost dry, redissolved in 10µl of UHQ and kept at –20°C for 30 min. After thawing, a 1/600 dilution was made for each sample and the concentration of total RNA was calculated from the A_{260} value, based on a reading of 1.0 being equal to 40µg ml^{-1} of single-stranded RNA (Anonymous, 1996). Any contaminating genomic DNA was eliminated by the addition of 1µl of DNase enzyme (1IU ml^{-1}) (Invitrogen) and 1µl of DNase buffer (Invitrogen). The samples were incubated at 25°C for 15 min, after which a further period of incubation at 65°C for 10 min inactivated the DNase enzyme.

2.10.1.2 Synthesis of complementary (c) DNA

Materials:

RT mastermix 20% (v/v) 5x superscript buffer (Invitrogen), 0.1M DTT (Promega, Southampton, UK), 10mM each of dATP, dCTP, dGTP & dTCP (Promega), 1mg ml^{-1} acetylated BSA (Promega), 40,000IU ml^{-1} RNase inhibitor (Promega), 200IU ml^{-1} Superscript RT enzyme (Invitrogen)

Duplicate tubes for each sample, one to serve as a negative RT control, were prepared by adjusting the sample to contain 1µg of total RNA in a final volume of 17.2µl of UHQ. cDNA synthesis was primed by the addition of 50ng of random hexamers (Promega) in a 2µl volume to each tube and incubating for five minutes at 65°C, followed by ten minutes at 25°C. Both tubes containing the annealed RNA / primer samples were made up to a final volume of 40µl with either UHQ (negative RT control) or RT mastermix. The samples were incubated at 42°C for one hour to enable polymerisation, after which heating to 90°C for five minutes inactivated the RT enzyme. The resulting cDNA samples were stored at –20°C.

2.10.1.3 PCR for the detection of cytokines and chemokines

Materials:

PCR mastermix	10% (v/v) $MgCl_2$-free buffer (Promega), 0.01M each dNTP, 1.0IU / reaction Taq DNA polymerase (Promega)
Agarose gel	Tris-borate-EDTA buffer (TBE) (Fisher) containing 2% (w/v) agarose (Oswel, Southampton, UK) and 1/10000 (v/v) VistraGreen (Amersham Pharmacia Biotech, Bucks, UK)
Loading buffer	30% (w/v) glycerol, 60% (v/v) TBE, 0.25% (w/v) orange G dye

Amplification of cytokine and chemokine cDNA was carried out by PCR with the primer pairs described in Table 2.7, with GAPDH primers used as an internal control in each reaction. cDNA samples were diluted 1/10 in UHQ of which 10µl volumes were added to 20µl of a reaction solution containing PCR mastermix, appropriate primer pairs (20-30 pmol μl^{-1}) and varying concentrations of $MgCl_2$ (Table 2.7). Sample cDNA was replaced with cDNA prepared from concanavalin A-stimulated peripheral blood mononuclear cells as a positive control, while UHQ replaced sample cDNA for negative controls. The reaction consisted of 30 cycles of denaturation at 94°C for 30 sec, followed by annealing for 30 sec at the temperatures described in Table 2.6 and extension at 72°C for one minute.

PCR products were visualised after agarose gel electrophoresis in the presence of VistraGreen. A 6µl volume of each PCR product was mixed with 2µl of loading buffer and subjected to electrophoresis at 100V on a 2% (w/v) agarose gel submerged in TBE buffer. The bands were visualised by scanning chemifluorescent signals with a Molecular Dynamics Storm 860 imager (Amersham Pharmacia Biotech).

2.10.2 Sandwich immunoassay

The cytokines and chemokines secreted into the experimental medium of human cells stimulated with bacteria were quantified using a sandwich immunosorbent assay technique. Briefly, the protein molecules are trapped in the solid phase between unlabelled capture antibodies and biotin-labelled detector antibodies. The detector antibodies are in turn detected by the use of a europium (Eu)-labelled streptavidin system. A chelating solution disassociates Eu^{3+} from this solid phase allowing the use of time-resolved fluorometry instead of the traditional enzymatic / colorimetric substrate system.

2.10.2.1 Details of challenge experiments

Bacterial suspensions were prepared as described in Section 2.3 and adjusted to $1x10^6$ cfu ml^{-1} in experimental medium. Cell monolayers were washed once with wash buffer and the bacterial suspensions added (1ml per well). Triplicate wells were included for all samples and the inoculated monolayers were incubated under normal culture conditions. At 3, 6, 9, 24, 36 and 48 hours post-challenge, the experimental medium was removed, centrifuged at 9000g for five minutes to pellet the bacteria and the supernatant samples dispensed into smaller aliquots before being stored at –80°C.

2.10.2.2 Immunoassay protocol

Unless otherwise stated, all incubations took place at 37°C in a humidified box and 100µl volumes of reagents were dispensed into each well. Paired capture and detection antibodies were supplied by R&D Systems, Oxon, UK, and the recombinant cytokine and chemokine protein standards were supplied by Peprotech, London, UK.

Materials:

Carbonate buffer	35mM sodium hydrogen carbonate and 15mM sodium carbonate, pH 9.6
Coating buffer	Carbonate buffer containing 0.05% (w/v) sodium azide.
Block buffer	PBS containing 1% (w/v) BSA and 5% (w/v) sucrose.
Wash buffer	25mM Tris-HCL pH 8.0 containing 100mM sodium chloride and 0.05% (v/v) Tween 20

Capture antibodies were diluted in coating buffer as described in Table 2.8 and used to coat the surface of 96-well FluoroNunc™ Maxisorp plates (Nunc, supplied by Greiner). The plates were covered and incubated for 16-18 hours at 37°C. The coating solution was removed and replaced with block buffer (100µl / well) for one hour, following which the wells were washed three times immediately before the addition of the samples and standards to the plates. Sample aliquots stored in 96 well polystyrene plates (Greiner) were thawed and diluted in DELFIA® assay buffer (PE Applied Biosystems, Warrington, UK) as described in Table 2.8. A 2.5-fold dilution series of the recombinant cytokine standard was then prepared in assay buffer, ranging from 12,500 to 8pg ml^{-1}. Negative control wells contained assay buffer only. Following a two-hour incubation, the plates were washed four times with wash buffer. Appropriate dilutions of biotinylated detector antibodies (Table 2.8) were prepared in assay buffer and added to the wells for a further two hours. The plates were washed four times and Eu-labelled streptavidin (PE Applied Biosystems) was added to the wells for one hour at a dilution of 100ng ml^{-1} in assay buffer. A final series of four washes in wash buffer was followed by the addition of DELFIA® Enhancement Solution (PE Applied Biosystems) to each well, after which the plate was incubated in the dark for ten minutes on a shaking platform at 37°C. Fluorescence was subsequently measured on a Wallac 1234 DELFIA®fluorometer.

Table 2.7: Oligonucteotide primer sequences, reaction conditions and product sizes for cytokine and chemokine RT-PCR.

Gene [1]	Direction	Sequence	Annealing temp (°C)	$MgCl_2$ conc. (mM)	Product size (bp)
IL-6	Sense	5'-ATGAACTCCTTCTCCACAAGCGC-3'	50	1.0	628
	Antisense	5'-GAAGAGCCCTCAGGCTGGACTG-3'			
IL-8	Sense	5'-ATGACTTCCAAGCTGGCCGTGGCT-3'	50	1.0	289
	Antisense	5'-TCTCAGCCCTCTTCAAAAACTTCTC-3'			
MCP-1	Sense	5'-TCTGTGCCTGCTGCTCATAGC-3'	50	1.0	510
	Antisense	5'-GGGTAGAACTGTGGTTCAAGAGG-3'			
RANTES	Sense	5'-ATGAAGGTCTCCGCGGCACGCCTCGCTGTC-3'	45	1.5	252
	Antisense	5'-CTAGCTCATCTCCAAAGAGTTGAT-3'			
GM-CSF	Sense	5'-CAGCACGCAGCCCTGGGAGCATGTG-3'	55	1.5	253
	Antisense	5'-CCGGGGTTGGAGGGCAGTGCTGCTT-3'			
GAPDH	Sense	5'-GGGAAGGTGAAGGTCGGACT-3'	60	2.0	229
	Antisense	5'-TGGAAGATGGTGATGGGATTTC-3'			

[1] – Primer sequences derived from the study of Jung and colleagues (Jung *et al.*, 1995).

Table 2.8: Concentrations of capture antibodies, detection antibodies and recombinant standards.

Cytokine / Chemokine [1]	Antibody concentration		Sample dilution
	Capture ($\mu g\ ml^{-1}$)	Detection ($ng\ ml^{-1}$)	
IL – 6	4	25	1/2 - 1/20
IL – 8	4	20	1/2 - 1/20
MCP-1	4	50	1/2 - 1/10
RANTES	4	50	Neat
GM – CSF	4	2000	Neat
IL – 1α	2	50	1/2 - 1/8
IL – 1β	4	100	1/2 - 1/8
TNF - α	4	200	1/2 - 1/8
TGF - β	4	100	1/2 - 1/8
MIP - 1α	4	40	1/2 - 1/8
MIP - 1β	4	20	1/2 - 1/8
IL – 10	4	500	1/2 - 1/8

[1] – See abbreviation list.

The relative fluorescence units were converted into cytokine concentrations through the application of linear regression analysis to the standard curve, taking into account the background levels and the dilution factor of the samples.

2.11 Detection of β-defensin mRNA produced by human cells challenged with *N.meningitidis*

The mRNA expression of both hBD-1 and hBD-2 from meningioma and Chang cells was investigated using semi-quantitative reverse-transcriptase (RT)-PCR assays.

Bacterial suspensions were prepared as described in Section 2.3 and adjusted to the desired concentration (typically $1x10^6$ cfu ml^{-1}) in experimental medium. Cell monolayers were washed once with wash buffer and the bacterial suspensions were then added (1ml per well). Triplicate or quadruplicate wells were included for Chang or meningioma cell samples respectively and the inoculated monolayers were incubated under normal culture conditions.

2.11.1 Isolation of RNA and synthesis of cDNA

Total cellular RNA was isolated and cDNA synthesised as described in sections 2.9.2.1 and 2.9.2.2 respectively.

2.11.2 Preparation of standards for quantification of β-defensin expression

The quantification of β-defensin gene expression relative to ribosomal RNA gene expression was carried out using a standard curve method (Anonymous, 1997). Serial dilutions of a cDNA stock, prepared from unchallenged Chang cells, were used to construct standard curves for the ribosomal 18S gene assay. Plasmid DNA was similarly diluted for the construction of both hBD-1 and hBD-2 standard curves.

2.11.2.1 Preparation and isolation of β-defensin plasmids

Plasmid constructs from *E.coli,* which contained the cloned sequences of both hBD1 and hBD2 mRNA, were kindly supplied by Dr. M. Pickett and Yu Deng, Molecular Microbiology and Infection. Briefly, hBD-1 and hBD-2 PCR products were purified using a Wizard kit (Promega), treated with kinase, re-purified and blunt end ligated into a pSPT18 vector (Roche Diagnostics Ltd, Lewes, UK) cut with Sma I restriction enzyme (Promega). Plasmid DNA for both hBD-1 and hBD-2 was isolated and purified using Midi prep techniques (Qiagen, Crawley, UK). The isolated plasmid DNA was diluted 1/100 in UHQ and the concentration of DNA was calculated from the A_{260} value, based on a reading of 1.0 equal to 50μg ml^{-1} of double-stranded DNA (Anonymous, 1996). The molarity of each sample was then calculated by dividing this concentration by the molecular weight of the plasmid and cloned insert. The molarity of each sample was converted into the number of plasmid copies μl^{-1} by multiplying by Avagadro's number, which states that one mole contains approximately 6.02×10^{23} molecules.

2.11.3 Real-time TaqMan® PCR

Materials:

TaqMan® mastermix 10% (v/v) buffer A, 1.25mM dNTP mix, 1.25IU / reaction AmpErase® uracil *N*-glycosylase (UNG), 0.5IU / reaction AmpliTaq Gold™ (all supplied in TaqMan® Core Reagent Kit (PE Applied Biosytems))

Amplification of β-defensin cDNA was carried out by semi-quantitative, real-time TaqMan® PCR. The hBD-1 and hBD-2 primer / probe combinations were designed using Primer Express software (PE Applied Biosystems) and they are detailed in Table 2.9. Sequence data was obtained from the EMBL sequence database, accession numbers Aac51728 (hBD-1) and AF040153 (hBD-2). A ribosomal 18S RNA primer / probe combination (PE Applied Biosystems) was used as a "housekeeping" gene to control for variations in reverse-transcriptase efficiency.

A 1/10 dilution of template cDNA (10µl volume) was added to a PCR mastermix to give a final volume of 25µl. The PCR mastermix was supplemented with $MgCl_2$ and the appropriate primer / probe combination, optimised for each gene (Table 2.10). All TaqMan® PCR reactions were subjected to identical parameters on an ABI PRISM™ 7700 Sequence Detector (PE Applied Biosystems). Briefly, and preceding amplification, a 50°C incubation for two minutes allowed AmpErase®UNG to digest any potential contaminants from previous runs. In addition, a ten minute hold step at 95°C served to inactivate AmpErase®UNG and was also required to activate AmpliTaq Gold™. All samples were run for 40 cycles of amplification, consisting of denaturation (95°C for 15 sec) and a combined annealing-extension step (60°C for 60 sec).

Table 2.9: β-defensin primer and probe sequences.

Gene	Primer / Probe	Nucleotide sequence
hBD-1	Forward	5' –CCTTCTGCTGTTTACTCTCTGCTTACT- 3'
	Reverse	5' –CCACTGCTGACGCAATTGTAA T- 3'
	Probe	5' –CTGAGATGGCCTCAGGTGGTAACTTTCTCAC- 3'
hBD-2	Forward	5' –CCCAGCCATCAGCCATGA- 3'
	Reverse	5' –TAGGGCAAAAGACTGGATGACA- 3'
	Probe	5' –CACCAAAAACACCTGGAAGAGGCATCAG- 3'

Table 2.10: Optimised TaqMan® PCR conditions.

Components	hBD-1	hBD-2	18S
$MgCl_2$ (mM)	4.0	3.5	5.5
Sense primer (nM)	300	300	300
Anti-sense primer (nM)	300	300	300
Probe (nM)	100	300	100

Serial dilutions (ten-fold) of β-defensin plasmid or cDNA stock were used to create a standard curve (*input target quantity against* C_T value) for β-defensins and ribosomal RNA respectively. The CT values for the experimental unknowns could then be extrapolated from the standard curve.

2.12 Preparation of *Haemophilus* anti-sera

Polyclonal antibodies were raised against separate outer membrane preparations isolated from *H.influenzae* strains Eagan and RD.

2.12.1 Isolation of *H.influenzae* outer membranes

Materials:

Peptone broth dH_20 containing 1% (w/v) proteose peptone (BD Biosciences) and 8% (v/v) glycerol. Sterilised by autoclaving at 1.05kg cm^{-2} for 15 min.

Detergent solution HEPES buffer (Sigma) containing 2% (w/v) sodium lauryl sarcosinate (Sigma).

2.12.1.1 Bulk growth of H.influenzae

From *H.influenzae* strains Eagan and RD, which had been grown for 16-18 hours on supplemented HTM agar, six colonies of each bacteria were subcultured separately onto supplemented HTM agar and incubated for a further 16-18 hours at 37°C and 5% (v/v) CO_2. The growth from one plate was resuspended in 1.5 ml of peptone broth and used to inoculate five large plates (145mm diameter) of supplemented HTM agar and incubated for 16-18 hours as described above.

2.12.1.2 Preparation of detergent-insoluble outer membranes

The method used for outer membrane isolation was adapted from the method of Carlone and colleagues (Carlone *et al.*, 1986). Briefly, the growth from approximately 30 large plates of each *H.influenzae* strain was resuspended in 10ml of 10mM HEPES buffer (pH 7.4) and disrupted by sonication at 20microns for 20 min on a MSE Soniprep 150 (Sanyo-Gallenkamp PLC, Loughborough, UK). Cellular debris and any intact bacteria were removed by centrifugation at 27000g (Beckman ultracentrifuge, Beckman Coulter, UK) for ten minutes at 4°C. The supernatant was removed and centrifuged at 115000g for 60 min at 4°C. The pellets containing cell membrane material were resuspended in 5ml of detergent solution for 30 min, followed by centrifugation at 115000g for 60 min at 4°C. The pellet was washed once, without resuspending, in 10mM HEPES buffer. Finally, the outer membrane pellet was resuspended in 1ml of 10mM HEPES buffer and the protein concentration determined using a BSA protein assay (Pierce, Illinois, USA) before being stored at –20°C.

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

Separation of *H.influenzae* Eagan and RD outer membrane preparations was carried out with SDS-PAGE using a 10-25% (w/v) acrylamide gradient as described by Heckels (Heckels, 1981), with the discontinuous buffer system of Laemmli (Laemmli, 1970).

Materials:

Acrylamide monomer solution	UHQ containing 40% (w/v) solution of Acrylogel 2.6 premix.
SDS solution	UHQ containing 2% (w/v) SDS.
Separating gel buffer	1.2M Tris-HCL, pH 8.8
Stacking gel buffer	0.25M Tris-HCL, pH 6.8 containing 0.08% (v/v) TEMED (Eastman Chemical Company, New York, USA).
Ammonium persulphate	(Eastman Chemical Company) 1% (w/v) in UHQ.
Running buffer	25mM Tris-HCL, pH 8.3 containing 192mM glycine and 0.1% (w/v) SDS.
Dissociation buffer	125mM Tris-HCL, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) mercaptoethanol and 0.002% (w/v) bromophenol blue (Sigma)
Staining solution	10% (v/v) glacial acetic acid (Fisher), 20% (v/v) isopropanol (Fisher) and 0.5mg ml^{-1} Coomassie brilliant blue R-250.
Destain solution	10% (v/v) glacial acetic acid and 10% (v/v) isopropanol

2.12.2.1 Construction of gradient gels

The glass gel casting plates were thoroughly cleaned with methanol, separated by a rubber gasket and held together with 'bulldog clips'. Solutions of 10% (v/v) and 25% (v/v) acrylamide were prepared according to Table 2.11 and any dissolved oxygen removed with a vacuum pump. The separating gel (1x110x170mm) was cast using a triple channel peristaltic pump (Pharmacia, P-3). Water-saturated butanol was carefully layered onto the surface of the gel, to exclude air, and the gel was left to polymerise at 25°C for one hour. The butanol was removed and the top of the gel rinsed with dH_2O. The stacking gel (1x40x170mm) was prepared as described in Table 2.12 and cast on top of the separating gel. A 20-well comb was inserted and the gel was left to polymerise for 30 min at 25°C, after which the comb was removed and the wells washed with dH_2O.

Table 2.11: Composition of SDS-PAGE separating gel.

Components	Acrylamide concentration	
	25%	**10%**
Acrylamide monomer solution	6.25 ml	2.5 ml
Separating gel buffer	3.1 ml	3.1 ml
SDS solution	0.62 ml	0.62 ml
Glycerol	1.25 ml	-
dH_2O	1.1 ml	6 ml
* Ammonium persulphate	0.125 ml	0.25 ml
* TEMED	6.2 µl	6.2 µl

* - Added after the removal of dissolved oxygen and immediately before gel casting.

Table 2.12: Composition of SDS-PAGE stacking gel.

Components	Acrylamide concentration
	4%
Acrylamide monomer solution	1.25 ml
Stacking gel buffer	7.8 ml
SDS solution	0.775 ml
UHQ	4.2 ml
* Ammonium persulphate	1.55 ml

* - Added after the removal of dissolved oxygen and immediately before gel casting.

2.12.2.2 Preparation of outer membrane samples

Each outer membrane preparation was adjusted to contain 20µg of protein in a final volume of 25µl in PBS. This was mixed in a ratio of 1:1 with dissociation buffer to a final volume of 50µl, of which 25µl was loaded into each well. Before loading, the membrane protein was denatured by heating to 100°C for five minutes. A Dalton mark VII-L molecular weight standard (molecular weight range of 14,000-70,000) (Sigma) was loaded into the first lane of each gel.

2.12.2.3 Gel running conditions

The wells were carefully overlaid with running buffer prior to filling the gel tank reservoirs with the same buffer. Electrophoresis was performed in a Biometra tank (Biometra, Göttingen, Germany) at 200V for 18-20 hours at 4°C.

2.12.2.4 Staining of polyacrylamide gels

After electrophoresis was completed the stacking gel was removed and the proteins were fixed and stained in staining buffer for one hour. The gel was destained with several washes of destain solution until the background staining was minimal.

2.12.3 Immunisation of animals with OM preparations

Materials:

Aluminium hydroxide gel adjuvant: Alhydrogel®2.0% (Superfos Biosector, Vedbaek, Denmark)

New Zealand White half-lop rabbits were housed under standard conditions of temperature and humidity with a 12-hour lighting cycle, and with water and food *ad libitum*. A pre-immunisation sample of whole blood was taken from the ear vein, and processed by incubating at 37°C for one hour followed by storage at 4°C overnight in order to allow clot formation and expression of serum. The rabbits were each immunised sub-cutaneously with 20μg of *H.influenzae* OM preparation, which had been adsorbed to an equal volume of aluminium hydroxide to a final volume of 1.0 ml. For each preparation of *H.influenzae* OM, two rabbits were immunised three times in total and then terminally bled by cardiac puncture 14 days after the final dose. Serum was stored at –20°C until used. Rabbits R0699 and R0700 were immunised with OM from *H.influenzae* Eagan and rabbits R0701 and R0702 with OM from *H.influenzae* RD.

2.12.4 Specificity of anti-*Haemophilus* anti-sera

The specificity of anti-sera raised against *Haemophilus* outer membrane preparations was investigated by the labelling of samples of whole bacteria. The cross-reactivity of these anti-sera with human cells was also investigated.

Materials:

Block buffer PBS containing 10% (v/v) dFCS.

Secondary antibody FITC-conjugated goat anti-rabbit antibody.

2.12.4.1 Labelling of whole Haemophilus bacteria

H.influenzae strains Eagan and Rd were grown for 16-18 hours on supplemented HTM agar medium plates. Several colonies were mixed with a single drop (approximately 50μl) of PBS to

create an emulsion, which was spread thinly over the surface of a clean microscope slide and allowed to air-dry for 30 min. The samples were fixed for ten minutes in absolute methanol, which was then removed and the slide was washed three times in PBS. Block buffer was added for 30 min at 37°C to reduce non-specific binding of the primary antibody. The primary antibodies, R699 and R701, as described in Table 2.5, were diluted in blocking buffer, and the fixed bacteria were incubated for one hour with these antibodies at 37°C. The monolayers were washed three times in PBS before the addition of the secondary FITC antibody used at a dilution of 1/100 and incubated for one hour at 37°C. Following a further four washes in PBS, the smears of bacteria were mounted sample-down onto a drop of an anti-fade fluorescent mounting medium (Dako) on a clean glass microscope slide. Staining of bacteria was visualised under a Leica SP-2 laser-scanning confocal microscope system, with images being obtained by scanning at 488 to excite FITC. Maximum projection images were subsequently prepared from 10 optical sections.

2.12.4.2 Investigating cross-reactivity of Haemophilus anti-sera with human cells

Human cells were grown to confluence on size O (13mm diameter) uncoated glass coverslips in 24 well culture plates as described in Section 2.8.1. Cell monolayers were washed three times in PBS and fixed for ten min in absolute methanol, which was then removed. The monolayers were washed three times in PBS and block buffer added for 30 min at 37°C to reduce non-specific binding of the primary antibody. The primary antibodies, R699 and R701, as described in Table 2.5, were diluted in blocking buffer, and the fixed bacteria were incubated for one hour with these antibodies at 37°C. The monolayers were washed three times in PBS before the addition of the secondary FITC antibody used at a dilution of 1/100 and incubated for one hour at 37°C. Following a further three washes in PBS, the monolayers were counter-stained for 15 min at 37°C with 0.0025% (w/v) Evan's Blue solution. The monolayers were then washed six times in PBS before being mounted monolayer-down onto a drop of a anti-fade fluorescent mounting medium (DAKO) on a clean glass microscope slide. Cell staining was visualised under a Leitz Dialux fluorescent microscope (Leica Microsystems) using an epifluorescence system of illumination.

CHAPTER 3 CHARACTERISATION OF BACTERIAL STRAINS AND HUMAN CELLS

Prior to any *in vitro* investigation of bacterial interactions with human host cells, panels of meningeal pathogens and human cells were established and characterised. The panel of bacteria included the main causative agents of pyogenic meningitis as well as a representative commensal organism. Cell lines were established from human meningioma tissue and characterised to determine their suitability for use in an *in vitro* culture model of the leptomeninges.

3.1 Selection and characterisation of bacterial strains

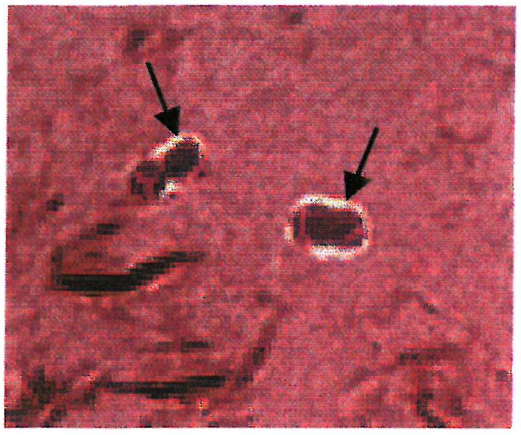
The pathogens *N.meningitidis*, *E.coli*, *H.influenzae* and *S.pneumoniae* included clinical isolates that were isolated from the CSF of patients with meningitis and are detailed in Table 2.1. Initially, the bacteria were characterised with respect to their expression of capsule and pili or fimbriae, since these surface components are important virulence factors during infection (Saukkonen *et al.*, 1988; Virji *et al.*, 1991a; Virji *et al.*, 1993b; Gilsdorf *et al.*, 1997; Hoffman *et al.*, 1999; Mitchell, 2000).

3.1.1 Characterisation of capsular state

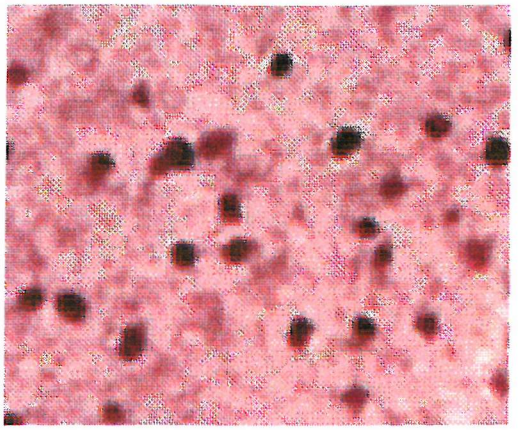
The expression of capsule on *N.meningitidis* MC58, variants -7 and -9 were investigated using the agglutinating properties of group B specific monoclonal antibodies. Both meningococcal variants exhibited positive agglutination with these antibodies. In addition, *E.coli* strains IH3080 and DSM also exhibited strong agglutination. In contrast, the commensal organism *N.lactamica* was not agglutinated.

A negative capsule stain was used to investigate the expression of capsule by *H.influenzae* and *S.pneumoniae* (Figure 3.1). The acidic Congo red solution stains both the bacteria and the background, but cannot penetrate the dense capsule, which can be seen as a clear halo around encapsulated bacteria. Such halos of encapsulation were clearly visible around *S.pneumoniae* and to lesser extent *H.influenzae* strain Eagan. In contrast, no capsule was seen with *H.influenzae* strain RD bacteria. *N.meningitidis* MC58-7 and *N.lactamica*, previously characterised as encapsulated and non-encapsulated with specific monoclonal antibodies, were included as positive and negative controls respectively.

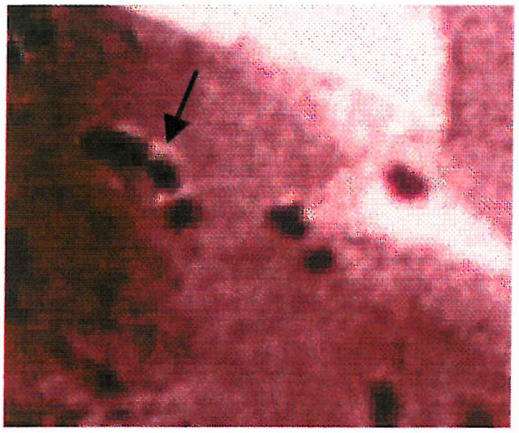
(A) *N.meningitidis* MC58-7



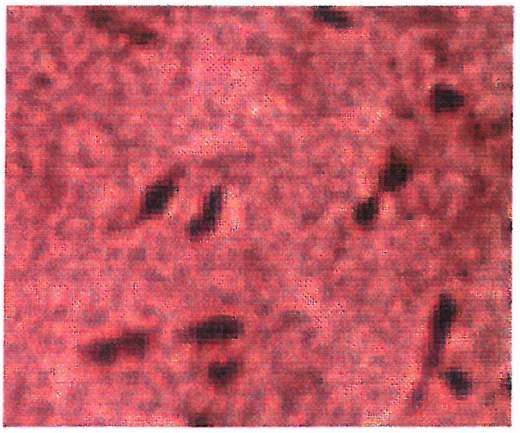
(B) *N.lactamica*



(C) *H.influenzae* Eagan



(D) *H.influenzae* RD



(E) *S.pneumoniae* D39

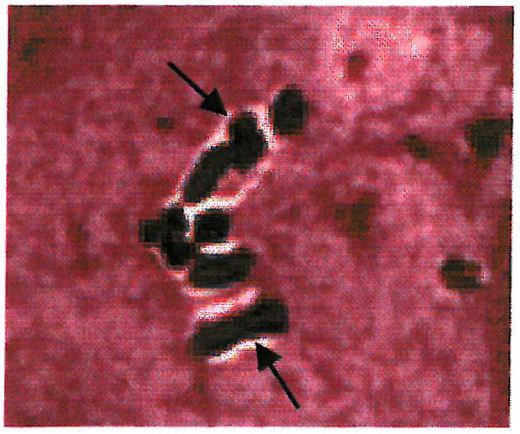


Figure 3.1: Negative capsule stain of *N.meningitidis* MC58-7 (A), *N.lactamica* (B), *H.influenzae* Eagan (C), *H.influenzae* RD (D) and *S.pneumoniae* (E). The bacteria are stained a darker purple to the background with the capsule (arrow) visualised as a clear halo around the cells. Magnification x7500.

3.1.2 Identification and characterisation of pili and fimbriae

3.1.2.1 TEM analysis

The expression of pili and fimbriae was investigated by transmission electron microscopy (TEM) following negative staining with phosphotungstic acid (Figure 3.2). The piliation of *N.meningitidis* strain MC58-7 was confirmed by TEM, with the pili visualised as hair-like structures that projected several microns from the surface of the bacteria. Although, meningococci normally possess numerous pili, the majority of negatively stained meningococci possessed only one pilus (Figure 3.2A), although bundles of five or more pili were occasionally seen (data not shown). Individual pili-like structures were also seen on the commensal bacterium *N.lactamica* (Figure 3.2H), but were absent from the *N.meningitidis* variant MC58-9 (Figure 3.2B).

Filamentous surface structures were also visible on the surface of both *E.coli* strains IH3080 and DSM. In contrast to the *N.meningitidis* MC58-7 pili, the *E.coli* fimbriae were significantly shorter (<1μm) and covered the whole surface of the bacteria (Figures 3.2C and 3.2D). In addition, *E.coli* IH3080 bacteria were shown to possess peritrichous flagella, which are structures distinctly different from fimbriae (Figure 3.2C). The flagella were approximately three-fold greater in thickness than the fimbriae and when fully intact extended well in excess of two microns from the cell surface. However, no filamentous structures were visualised on negatively stained *H.influenzae* Eagan (Figure 3.2E), *H.influenzae* RD (Figure 3.2F) or *S.pneumoniae* (Figure 3.2G) bacteria.

3.1.2.1 Immunoblot analysis

The pili of *N.meningitidis* MC58-7 and *N.lactamica* were characterised further by immunoblotting with the monoclonal antibodies SM1, which recognises class I pilin and AG123, which recognises class II pili. *N.meningitidis* MC58-7 reacted strongly with SM1 antibodies (Figure 3.3A) confirming the presence of class I pili. Non-piliated *N.meningitidis* MC58-9 was included as a negative control (Figure 3.3B). In contrast, the *N.lactamica* pili identified by TEM were characterised as class II pili by virtue of their strong reactivity with the antibody AG123 (Figure 3.3C).

Figure 3.3: The reactivity of *N.meningitidis* MC58-7 (A) but not MC58-9 (B) with SM1 antibody specific for class I pili, and reactivity of *N.lactamica* (C) with AG123 antibody specific for the expression of class II pili. Blots are representative of experiments carried out at least twice.

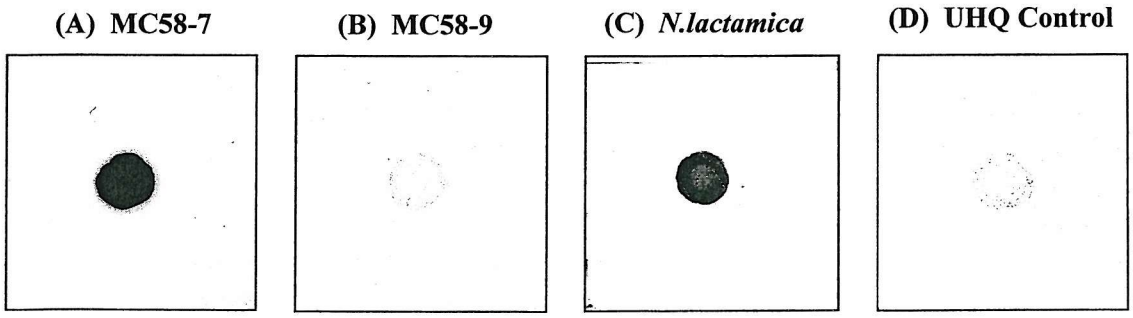
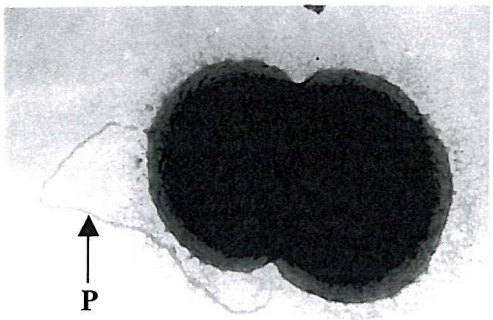
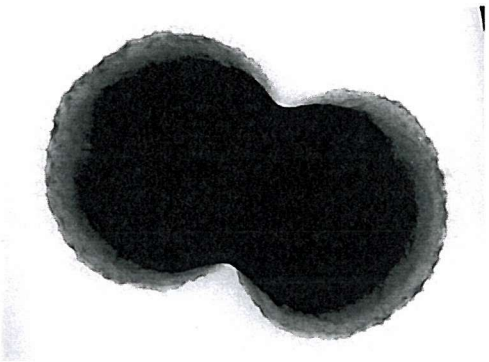


Figure 3.2: Transmission electron micrographs of negatively stained bacteria. Arrows indicate the bacterial surface structures pili (P), fimbriae (F) and flagellae (Fg). Individual magnification is shown in brackets.

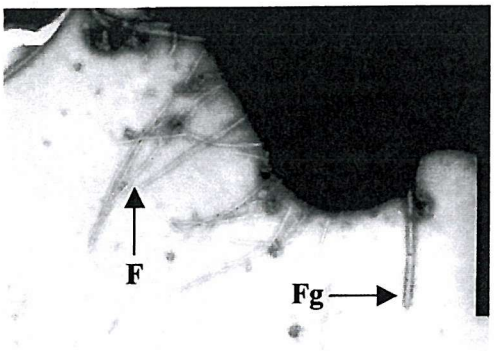
(A) *N.meningitidis* #7 (x30,000)



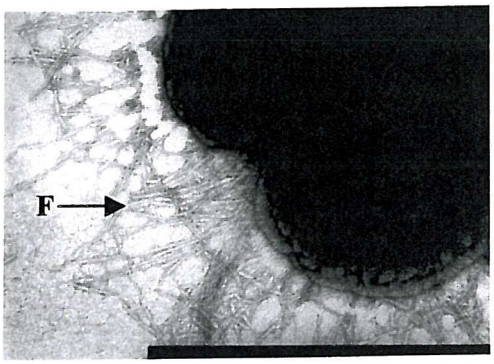
(B) *N.meningitidis* #9 (x40,000)



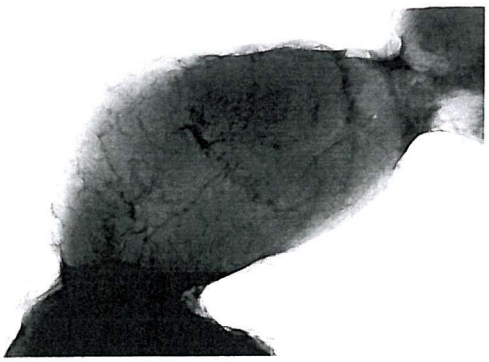
(C) *E.coli* IH3080 (x40,000)



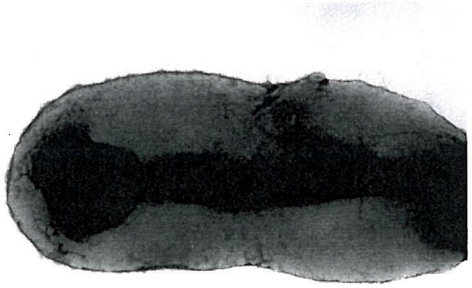
(D) *E.coli* DSM (x50,000)



(E) *H.influenzae* Eagan (x40,000)

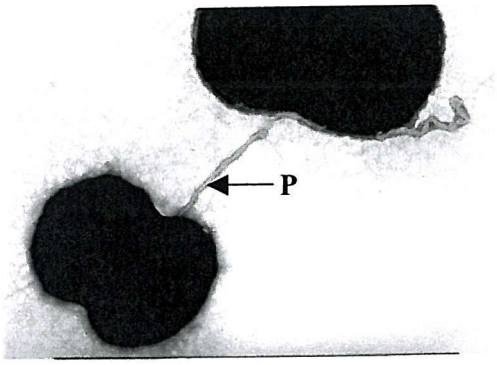


(F) *H.influenzae* RD (x40,000)



(G) *S.pneumoniae* D39 (x40,000)

(H) *N.lactamica* (x40,000)



3.1.2.3 *Haemagglutination analysis*

The fimbriae of *E.coli* were characterised further by their ability to agglutinate human erythrocytes in a haemagglutination assay. The *E.coli* strain DSM, and to a lesser extent strain IH3080, induced a slight agglutination of erythrocytes, which confirmed the presence of fimbriae, previously shown by TEM. In contrast, no agglutination was seen with either *N.meningitidis* or *H.influenzae* strains.

Table 3.1: Summary of the expression of capsule and pili or fimbriae by meningeal pathogens.

Organism	Strain	Capsule	Pili	Fimbriae
N.meningitidis	MC58-7	+	+ Class I	-
	MC58-9	+	-	-
E.coli	IH3080	+	-	+ Haemagglutinating
	DSM	+	-	+ Haemagglutinating
H.influenzae	Eagan	+	-	-[1]
	RD	-	-	-
S.pneumoniae	D39	+	-	-
N.lactamica		-	+ Class II	-

[1] – The expression of fimbriae on *H.influenzae* Eagan is lost during *in vitro* culture (Apicella *et al.*, 1984; Gilsdorf & Ferrieri, 1984; Pichichero, 1984; Mason *et al.*, 1985).

3.2 Characterisation of human meningioma cells

Cells derived from fresh human meningiomas were grown *in vitro* and characterised to determine their suitability for use in an established *in vitro* model of the human leptomeninges (Hardy *et al.*, 2000).

3.2.1 Growth and selection of meningioma cells

Fresh samples of meningioma tissue were processed as described in Section 2.5.1 and labelled numerically, with the prefix 'M' (Table 3.2). A number of meningioma cell lines had been previously established in this laboratory, but due to limited stocks only newly established and fully characterised cell lines were used for this study. The potential contamination of cell cultures with macrophages present in the original tissue samples was overcome by using only meningioma cells of passage number of three or greater. Since the macrophages are terminally differentiated cells it

was unlikely that they would survive longer than the first passage in tissue culture (Hardy *et al.*, 2000).

The tumours were confirmed as meningiomas by the Department of Neuropathology using histological methods, and were classified into the respective subtypes (Table 3.2). The meningioma subtypes of 52 fresh meningiomas included transitional (38%), meningothelial (25%), atypical (15%) and fibroblastic (4%). However, only meningiomas of the meningothelial subtype were considered for use in the *in vitro* model of the human leptomeninges. Consequently, only the cell lines M26, M27, M28, M29, M35, M37, M42, M45, M61 and M66 were characterised further, having been confirmed histologically as being of meningothelial origin and showing good growth characteristics.

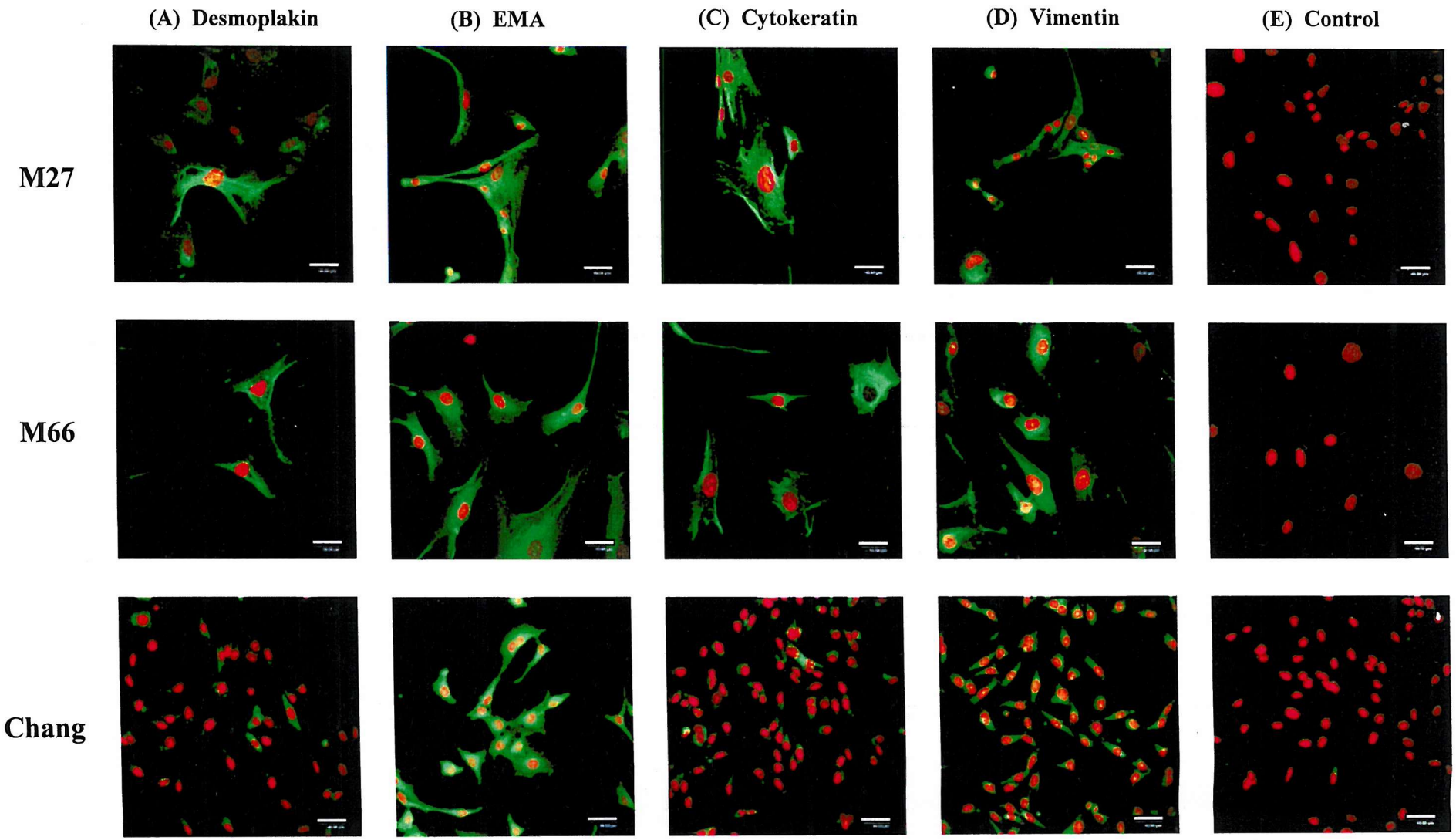
3.2.2 Immunocytochemistry for the identification of specific cellular markers

The meningothelial meningioma cell lines were characterised with a panel of antibodies directed against the specific cellular markers desmosomal desmoplakin (I & II), epithelial membrane antigen (EMA), cytokeratin and vimentin. In conjunction with the growth and histological classification of meningioma cultures, these cellular markers were used as selection criteria for cell lines suitable for subsequent infection experiments, which have been described previously (Hardy *et al.*, 2000). Chang conjunctival cells were included for comparison, as they have been widely used as a model epithelial cell line in studies of meningococcal cell interactions (Virji *et al.*, 1993b; Virji *et al.*, 1995a; de Vries *et al.*, 1998). The cell lines were incubated with specific antibodies to these cellular markers (detailed in Table 2.4). Table 3.4 outlines the staining characteristics of all the meningioma cell lines established during this study and the Chang epithelial cell line.

Although the tumours M29, M42, M45 and M61 were confirmed histologically as meningothelial meningiomas, they were not used further due to their inconclusive staining for the expression of desmoplakin. Other suitable meningothelial meningiomas, including M26, M28, M35 and M37 were not available in sufficient quantities for large scale studies. Only the cell lines M27 and M66 appeared to fulfil all the stringent requirements for use as an *in vitro* model of the leptomeninges.

Desmosomes are cytoskeletal-associated structures found in a variety of cells, including epithelial cells and cells derived from the arachnoid or pia mater (Parrish *et al.*, 1986). The presence within a culture of desmosome-associated proteins, desmoplakin I and II, confirmed the leptomeningeal origin of the cells (Hardy *et al.*, 2000). Both meningioma cell lines M27 and M66 tested strongly positive for the expression of desmoplakin, which was shown as a punctate staining throughout the cells (Figure 3.4A). Desmosomes were also observed in Chang epithelial cells, although the intensity of immunoreactivity was weaker compared with meningioma cells.

Figure 3.4: Immunocytochemistry for the detection of cellular markers on both meningioma and Chang epithelial cell lines. The scale bar represents 40 microns.



EMA is widely expressed in both epithelial and meningeal cells (Theaker *et al.*, 1986; NG & Wong, 1993). Meningioma cell lines M27 and M66 were both positive for the expression of EMA, which was observed as a punctate staining throughout the cells with distinct cell borders (Figure 3.4B). In addition, Chang epithelial cells stained uniformly in a similar manner.

Cytokeratin is an intermediate filament frequently expressed in meningiomas and commonly co-expressed with another intermediate filament, vimentin (Kartenbeck *et al.*, 1984; Theaker *et al.*, 1986). Both meningioma cell lines stained strongly positive for the expression of cytokeratin and vimentin, although the staining of the latter was weaker compared to the former (Figure 3.4C). In contrast, the expression of cytokeratin in Chang cells was equivocal, since the staining was very weak or absent. Chang cells also exhibited equivocal staining for vimentin and was scored as a weakly positive result when analysed by confocal microscopy (Figure 3.4D).

The two meningothelial meningioma cell lines, M27 and M66, were therefore selected for future infection experiments, since their growth and overall cell staining patterns were consistent with previously reported meningioma cell lines (Hardy *et al.*, 2000).

3.3 Chapter discussion

3.3.1 Establishment of a panel of meningeal pathogens

The expression of capsule and pili or fimbriae by the meningeal pathogens used in this study are summarised in Table 3.1 on page 65. *N.meningitidis* MC58-7 is representative of the meningococcal phenotype commonly recovered from the CSF of patients with meningitis, being both encapsulated and piliated (DeVoe & Gilchrist, 1975; Stephens *et al.*, 1982). Since the primary ligand for mediating adhesion of meningococci to epithelial, endothelial and meningeal cells has been shown to be the pilus (Virji *et al.*, 1991a; Virji *et al.*, 1992a; Hardy *et al.*, 2000), the initial interactions between host cells and meningococci were investigated in this project by comparing the piliated clinical isolate to its non-piliated variant. The other meningeal pathogens investigated in this study, namely *E.coli* IH3080, *H.influenzae* Eagan and *S.pneumoniae* D39, were also selected primarily on their phenotypic resemblance to bacteria recovered from the CSF of individuals with meningitis. In addition to the clinical isolates, two other contrasting bacteria were included; *E.coli* DSM and *H.influenzae* RD. Although *E.coli* DSM was isolated from a faecal sample provided by a healthy individual, it is similar to the clinical isolate since it possesses a K1 capsule. However, care must be taken when comparing with the clinical isolate since *E.coli* DSM has been shown to cause bacteraemia, but not meningitis, in the infant rat model (Pluschke *et al.*, 1983). The non-capsulate *H.influenzae* strain RD provided a contrast to the type b clinical isolate since the polysaccharide capsule has been shown to influence the association of *H.influenzae* to epithelial and endothelial cells (St.Geme & Falkow, 1991; Virji *et al.*, 1991b). However, *H.influenzae* RD is an avirulent strain, incapable of causing invasive disease, and consequently may be deficient in other adhesins and invasins associated with the clinical isolate in addition to

Table 3.2: The characterisation of meningioma and Chang cell lines based on the detection of specific cell markers.

Cell line	Histological subtype	Growth in culture	Cellular markers [1]			
			Desmoplakin	EMA	Cytokeratin	Vimentin
Chang	-	Yes	+/-	+++	+/-	+/-
M26	Meningothelial	Yes	++	++	++	++
M27	Meningothelial	Yes	++	++	+++	++
M28	Meningothelial	Yes	++	+	++	+++
M29	Meningothelial	Yes	+	+	+++	+
M30	Atypical	Yes	nd	nd	nd	nd
M31	Transitional	Yes	++	++	++	++
M32	Transitional	Yes	+++	+++	+++	+++
M33	Transitional	Yes	nd	nd	nd	nd
M34	Atypical	No	nd	nd	nd	nd
M35	Meningothelial	Yes	++	+	++	+++
M36	Meningothelial	No	nd	nd	nd	nd
M37	Meningothelial	Yes	++	+	++	++
M38	Fibroblastic	Yes	nd	nd	nd	nd
M39	Paraganglioma	Yes	nd	nd	nd	nd
M40	Transitional	Yes	nd	nd	nd	nd
M41	Transitional	Yes	nd	nd	nd	nd
M42	Meningothelial	Yes	+	+++	+++	+
M43	Transitional	Yes	nd	nd	nd	nd
M44	Anaplastic	Yes	nd	nd	nd	nd
M45	Meningothelial	Yes	+/-	+/-	+/-	++
M46	Transitional	Yes	nd	nd	nd	nd
M47	Atypical	Yes	nd	nd	nd	nd
M48	Prostate metastases	Yes	nd	nd	nd	nd
M49	Transitional	Yes	nd	nd	nd	nd
M50	Atypical	Yes	nd	nd	nd	nd
M51	Transitional	Yes	nd	nd	nd	nd
M52	Haemangioperic -ytoma	No	nd	nd	nd	nd

Table 3.2: (Continued)

Cell line	Histological subtype	Growth in culture	Cellular markers [1]			
			Desmoplakin	EMA	Cytokeratin	Vimentin
M53	Transitional	Yes	nd	nd	nd	nd
M54	Psammomatous	Yes	nd	nd	nd	nd
M55	Transitional	Yes	nd	nd	nd	nd
M56	Transitional	Yes	nd	nd	nd	nd
M57	Meningothelial	No	nd	nd	nd	nd
M58	Atypical	Yes	nd	nd	nd	nd
M59	Fibroblastic	Yes	nd	nd	nd	nd
M60	Transitional	Yes	nd	nd	nd	nd
M61	Meningothelial	Yes	+	++	+++	+
M62	Angiomatous	Yes	nd	nd	nd	nd
M63	Atypical	Yes	nd	nd	nd	nd
M64	Transitional	Yes	nd	nd	nd	nd
M65	Transitional	Yes	nd	nd	nd	nd
M66	Meningothelial	Yes	++	++	+++	+
M67	Transitional	Yes	nd	nd	nd	nd
M68	Transitional	Yes	nd	nd	nd	nd
M69	Meningothelial	No	nd	nd	nd	nd
M70	Transitional	Yes	nd	nd	nd	nd
M71	Transitional	Yes	nd	nd	nd	nd
M72	Psammomatous	Yes	nd	nd	nd	nd
M73	Atypical	Yes	nd	nd	nd	nd
M74	Transitional	Yes	nd	nd	nd	nd
M75	Unknown	No	nd	nd	nd	nd
M76	Benign	Yes	nd	nd	nd	nd
M77	Atypical	Yes	nd	nd	nd	nd

[1] - Staining scored '+' positive, '++' strongly positive, '+++' very strongly positive, '+/-' equivocal , 'nd' not done.

The meningioma cell lines M5-M25 were previously established and characterised by Hardy and colleagues (Hardy *et al.*, 2000).

the polyribosyl phosphate capsule. Finally, the interactions of these meningeal pathogens with human cell lines were compared with the interactions of *N.lactamica*, which is a non-pathogenic commensal bacterium of the human nasopharynx.

3.3.2 Characterisation of meningioma cell lines

The primary culture of meningioma cells from clinical samples was very successful, with 46 out of a total of 52 (~92%) growing *in vitro*. The histological classification of these meningeal tumours identified a large variety of subtypes (Lantos *et al.*, 1997), with transitional (38%) and meningothelial (25%) subtypes being the most common in the current study. However, only cells from benign meningothelial meningiomas were considered for use in the *in vitro* model, since they most closely resemble typical leptomeningeal cells (Feurer & Weller, 1991). In conjunction with the growth and histological classification of meningioma cultures, various cellular markers were used as selection criteria to ensure the suitability of cell lines for subsequent infection experiments (Hardy *et al.*, 2000). Meningioma cell lines were only considered to be of meningeal origin if they strongly expressed desmosomal desmoplakin and EMA, although presence of the latter is not as reliable an indicator as presence of the former (Feurer & Weller, 1991). The co-expression of cytokeratin and vimentin intermediate filaments was also identified in most cell lines, although the staining was not homologous for either marker. This co-expression is thought to reflect the dual embryological origins of the leptomeninges from both neuroectodermal and mesenchymal elements (O'Rahilly & Muller, 1986; Al-Rodhan & Laws, 1991).

Although meningioma cell lines were not immortal, they could be expanded sufficiently to perform a large number of experiments, thus eliminating inter-sample variation and allowing experimental replication to test for statistical significance. However, by passage number nine signs of senescence were evident, which included an increased cytoplasmic:nuclear ratio and the presence of giant, often multinucleated, cells (Rutka *et al.*, 1986). The purity of the meningioma cultures was ensured by only using cells sub-cultured a minimum of three times, since any contaminating differentiated cells, such as macrophages, endothelial cells or smooth muscle cells, would be unlikely to survive longer than the first passage in tissue culture (Hardy *et al.*, 2000). However, in the current study, only two meningothelial meningioma cell lines, M27 and M66, were selected for the infection experiments, since their growth and overall cell staining patterns were consistent with previously reported meningioma cell lines (Hardy *et al.*, 2000).

Overall, the panel of meningeal pathogens chosen for this study includes clinical isolates from patients with meningococcal (strain MC58-7), *H.influenzae* type b (strain Eagan), *E.coli* (strain IH3080), and pneumococcal (stain D39) meningitis. In addition, a non-piliated meningococcus (strain MC58-9), an *E.coli* variant isolated from a healthy individual (strain DSM), an acapsulate laboratory *H.influenzae* variant (strain RD), and a commensal of the nasopharynx, *N.lactamica*, were included to provide a comparison to the clinical isolate phenotypes. The interactions of these bacteria with meningioma cells that closely resemble normal human leptomeningeal cells, both in morphology and the expression of cellular markers, will be investigated in the next chapter.

CHAPTER 4 BACTERIAL INTERACTIONS WITH HUMAN CELLS

Bacterial meningitis is believed to occur as a result of pathogens invading the subarachnoid space and initiating an acute compartmentalised inflammatory response. In meningitis, both the bacterial infiltrate and the inflammatory exudates are largely contained within the SAS. The inability of bacterial pathogens causing meningitis to gain access to the underlying brain has been accredited to the barrier properties of the leptomeninges (Gray & Nordmann, 1997). Recently, Hardy and colleagues (Hardy *et al.*, 2000) have established an *in vitro* model of the leptomeninges using cultured meningioma cells, which could be used to investigate the interactions of bacterial pathogens. However, it is also important to compare the interactions between meningeal pathogens and meningioma cells and their interactions with cultured cells that are representative of another potential barrier to microbial penetration. In the current study, the interactions of the meningeal pathogens were also investigated with Chang conjunctival epithelial cells, an established and representative epithelial cell line that has been extensively used in various studies of the pathogenesis of *N.meningitidis* and *H.influenzae* (Virji *et al.*, 1993a; Virji *et al.*, 1995a; de Vries *et al.*, 1998; Prasadarao *et al.*, 1999a; van Schilfgaarde *et al.*, 2000).

4.1 Establishment of challenge experiment protocols

The methods used to investigate the interactions of meningococci with both meningioma and Chang cells have been established by both Virji and colleagues (Virji & Everson, 1981; Virji *et al.*, 1992a; Virji *et al.*, 1992c) and Hardy *et al.* (Hardy *et al.*, 2000). However, in the context of challenge experiments in this study, it was necessary initially to optimise the growth bacterial characteristics and challenge conditions prior to infection experiments.

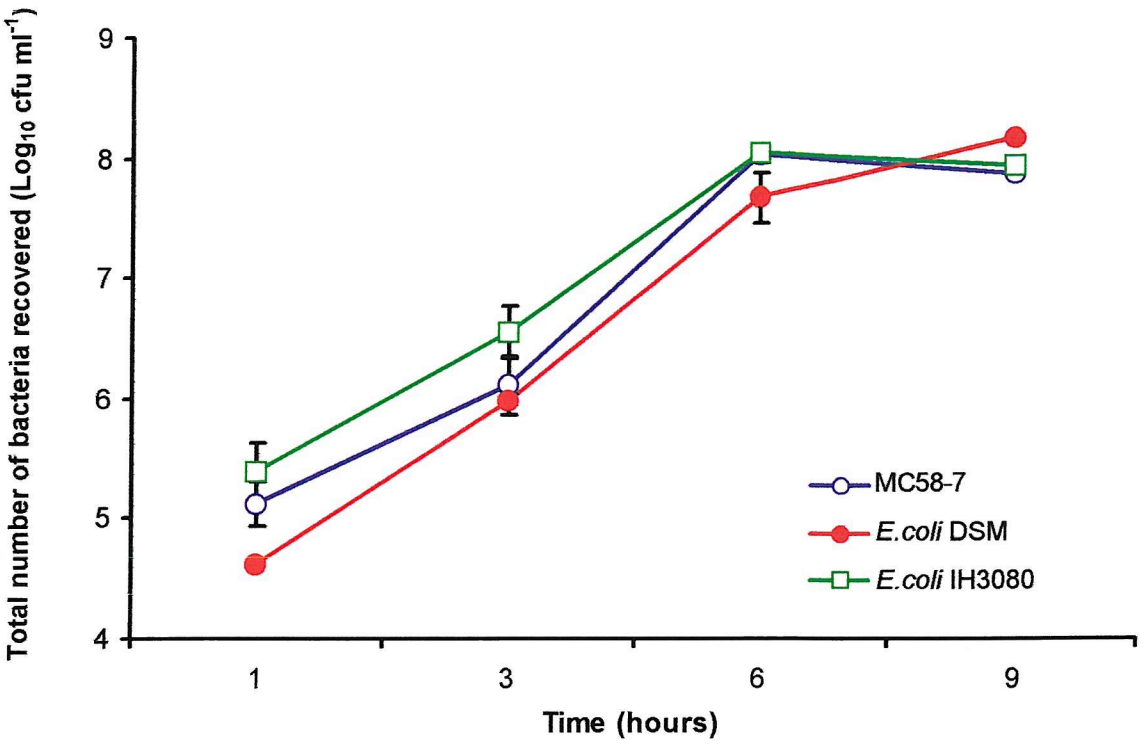
4.1.1 Optimisation of bacterial growth conditions

Initial growth experiments demonstrated that meningococci grew rapidly in experimental medium containing only 0.1% (v/v) dFCS (data not shown). The concentration of dFCS in experimental medium was subsequently optimised for each bacterium so that their *in vitro* growth characteristics were similar to, or approached those of *N.meningitidis* strain MC58-7.

4.1.1.1 Optimisation of the growth conditions of E.coli

The *in vitro* growth kinetics of *E.coli* strains DSM and IH3080 in experimental medium during challenge of meningioma cell lines are shown in Figure 4.1, alongside the growth dynamics of *N.meningitidis* MC58-7. The time course of these experiments was stopped at nine hours due to the rapid fall in pH of the experimental medium and the severe damage to the monolayer observed following prolonged incubation with either *E.coli* strain. Although the growth rate of *E.coli* IH3080 was approximately two fold higher than that of *N.meningitidis* MC58-7 for the first three

Figure 4.1: *E.coli* strains IH3080 and DSM exhibit similar growth characteristics to piliated meningococci in experimental medium.



Meningioma cell monolayers were challenged with approximately $2x10^5$ cfu per monolayer of bacteria in experimental medium containing 0.1% (v/v) dFCS. The symbols denote the mean and the error bars denote the standard deviation of triplicate samples.

hours ($P \leq 0.05$), there was no significant difference between the relative growth rates at either six or nine hours ($P \geq 0.1$). In addition, *E.coli* strain DSM exhibited similar growth rates to both piliated meningococci and *E.coli* IH3080 over the nine hour time course (Figure 4.1).

4.1.1.2 Optimisation of the growth conditions of H.influenzae

Standard experimental medium containing 0.1% (v/v) dFCS proved to be inadequate for the growth of either *H.influenzae* strain RD or Eagan, with no bacteria being recovered from culture supernatants (data not shown). The active growth of *H.influenzae in vitro* required the addition of both β-NAD and haem (supplied as hematin) to the experimental medium, although, by themselves, even high concentrations of these essential components did not allow the growth of *H.influenzae* in the presence of 0.1% (v/v) dFCS. However, increasing the concentration of dFCS from 0.1% (v/v) to 1% (v/v), in conjunction with β-NAD and haem, permitted successful growth of *H.influenzae*.

The growth kinetics of *H.influenzae* strain RD in experimental medium containing 1% (v/v) and 2% (v/v) are shown in Figure 4.2. Initially, up to three hours, there was no significant difference between the growth rates of *H.influenzae* RD in any of the supplemented experimental media ($P \geq 0.1$). However, increasing the concentration of dFCS from 1% (v/v) to 2% (v/v), regardless of the concentration of β-NAD and haem, resulted in the significantly increased growth rate of *H.influenzae* strain RD by approximately five- and ten-fold at six and nine hours respectively ($P \leq 0.01$). In addition, the concentrations of β-NAD and hematin were optimised at 2mg ml^{-1} and 10mg ml^{-1} respectively, since higher concentrations of both (15mg ml^{-1}) resulted in a two-fold reduction in the number of cfu of *H.influenzae* RD recovered after six and nine hours growth ($P \leq 0.05$).

The growth kinetics of *H.influenzae* RD in experimental medium containing 1% (v/v) and 2% (v/v) dFCS was then compared with *N.meningitidis* MC58-7 (Figure 4.3). There was no significant difference in the growth rate of *N.meningitidis* MC58-7 in either concentration of dFCS ($P \geq 0.1$) with the number of bacteria increasing over time. Initially, there was no significant difference in the growth rates of *H.influenzae* RD grown in 1% (v/v) or 2% (v/v) dFCS ($P \geq 0.05$) after three hours and the numbers of bacteria recovered were approximately two-fold higher than *N.meningitidis* MC58-7. However, by nine hours, the growth of *H.influenzae* strain RD in experimental medium containing 1% (v/v) dFCS was approximately twelve-fold lower compared with *N.meningitidis* MC58-7. Increasing the concentration of dFCS to 2% (v/v) resulted in growth kinetics of *H.influenzae* RD that was similar to meningococci ($P \geq 0.3$). In addition, essential the same results were seen with *H.influenzae* strain Eagan (data not shown). Therefore, for all subsequent challenge experiments, both *H.influenzae* strains RD and Eagan were cultured in experimental medium containing 2% (v/v) dFCS and 2mg ml^{-1} β-NAD and 10mg ml^{-1} hematin.

Figure 4.2: Comparison of the growth of *H.influenzae* RD in experimental medium supplemented with various concentrations of dFCS, β-NAD and hematin.

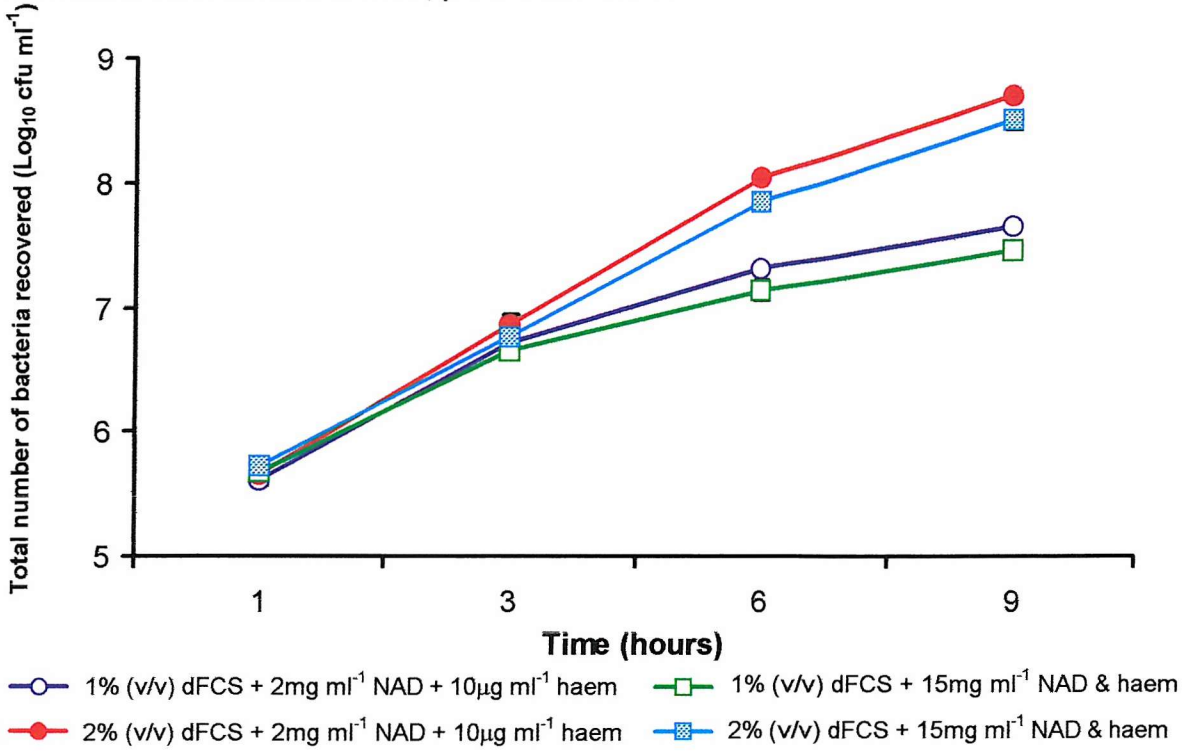
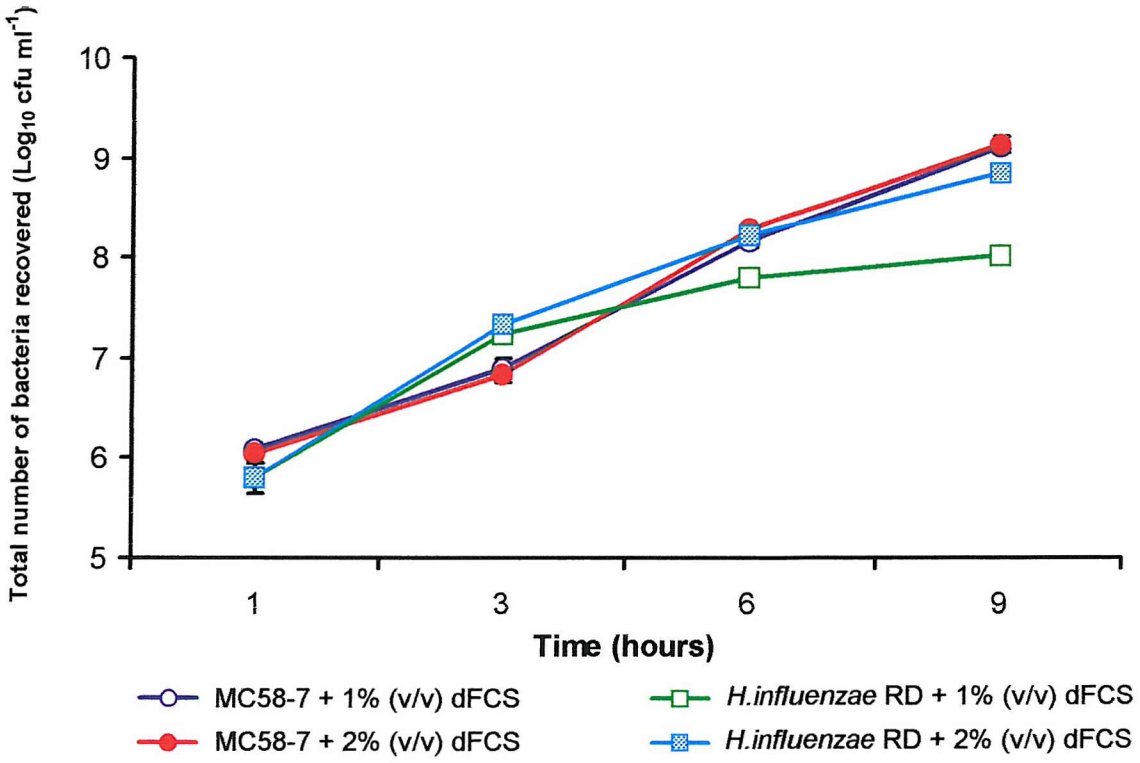


Figure 4.3: Comparison of the growth kinetics of *H.influenzae* RD alongside piliated meningococci in experimental medium containing either 1% (v/v) or 2% (v/v) dFCS.



In both experiments (Figures 4.2 and 4.3), the concentrations of bacteria were adjusted to approximately $1x10^6$ cfu ml^{-1} in experimental medium. The symbols denote the mean and the error bars denote the standard deviation of triplicate samples.

4.1.1.3 *Optimisation of the growth conditions of S.pneumoniae*

S.pneumoniae demonstrated a slow rate of growth in experimental medium containing 0.1% (v/v) dFCS and bacteria were recovered in significant numbers only after 24 hours (Figure 4.4). However, increasing the concentration of dFCS to 1% (v/v) resulted in the exponential growth of *S.pneumoniae* as early as 3 hours, although there was no significant difference (P=0.65) in the total number of bacteria recovered after 24 hours, compared to bacteria grown in 0.1% (v/v) dFCS. The growth dynamics of *S.pneumoniae* in experimental medium containing 1% (v/v) and 2% (v/v) dFCS were subsequently investigated and compared to with the growth kinetics of *N.meningitidis* MC58-7 (Figure 4.5). There was no significant difference in the growth rate of *N.meningitidis* MC58-7 in either 1% (v/v) or 2% (v/v) dFCS ($P \geq 0.09$) with the number of bacteria increasing over time. The numbers of *S.pneumoniae* recovered also increased over time in experimental medium containing either concentration of dFCS, but these levels were consistently between 50 and 500 fold lower than those of *N.meningitidis* MC58-7 at each time point ($P \leq 0.001$). In addition, the growth rate of *S.pneumoniae* in 2% (v/v) dFCS was significantly higher (two to three fold) ($P \leq 0.01$) than that observed in 1% (v/v) dFCS. Although increasing the concentration of dFCS in the experimental medium increased the growth rates of *S.pneumoniae*, the concentration of dFCS was capped at 2% (v/v) in order to prevent the stimulation of human cells by components of the dFCS that may mask the effects of bacteria and bacterial components on the induction of inflammatory mediators.

4.1.1.4 *Optimisation of the growth conditions of N.lactamica*

In preliminary experiments the growth kinetics of *N.lactamica* in experimental medium supplemented with 0.1% (v/v) and 1% (v/v) dFCS was investigated. However there was no significant difference in the growth rates of *N.lactamica* with either concentration of dFCS ($P \geq 0.4$) (data not shown). Therefore, the growth dynamics of *N.meningitidis* an *N.lactamica* were subsequently investigated in more detail using experimental medium containing 1% (v/v) and 2% (v/v) dFCS (Figure 4.6). The growth rate of *N.meningitidis* MC58-7 in experimental medium containing either 1% (v/v) or 2% (v/v) dFCS was similar over time ($P \geq 0.2$). In addition, there was no significant difference in the growth rate of *N.lactamica* with either 1% (v/v) or 2% (v/v) dFCS-supplemented media ($P \geq 0.1$), although the numbers of bacteria recovered were approximately 10-100 fold lower than *N.meningitidis* MC58-7 ($P \leq 0.05$) at all time points. Therefore, the concentration of dFCS was maintained at 1% (v/v) for all future experiments.

Figure 4.4: The *in vitro* growth of *S.pneumoniae* in experimental medium is dependent on the concentration of dFCS.

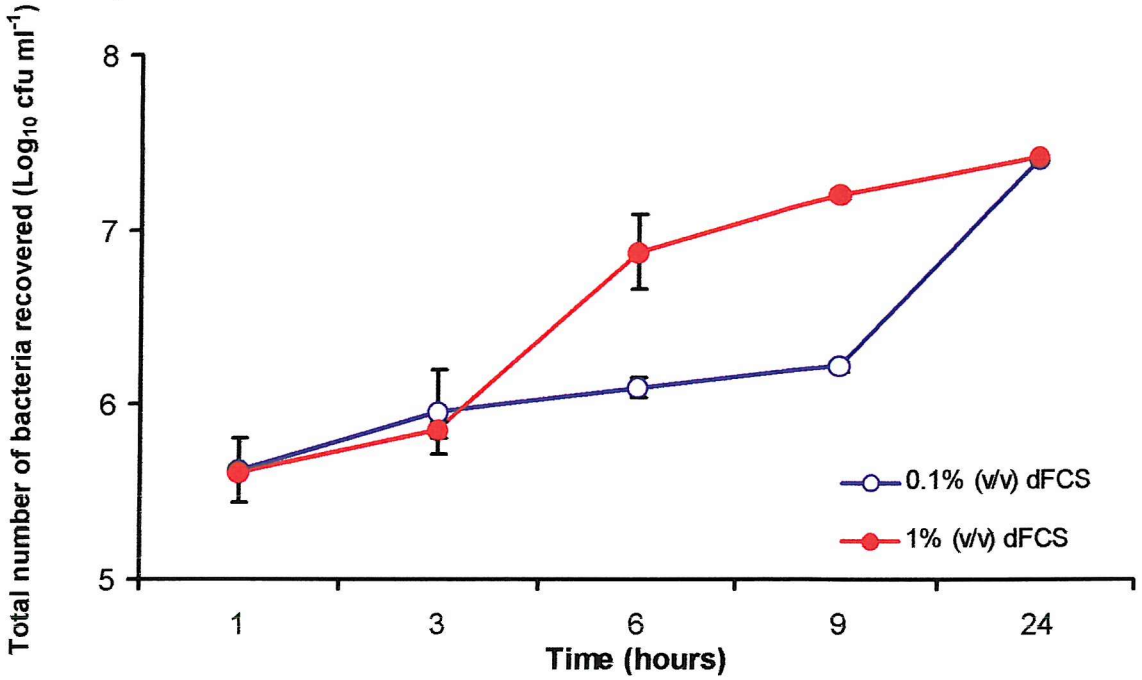
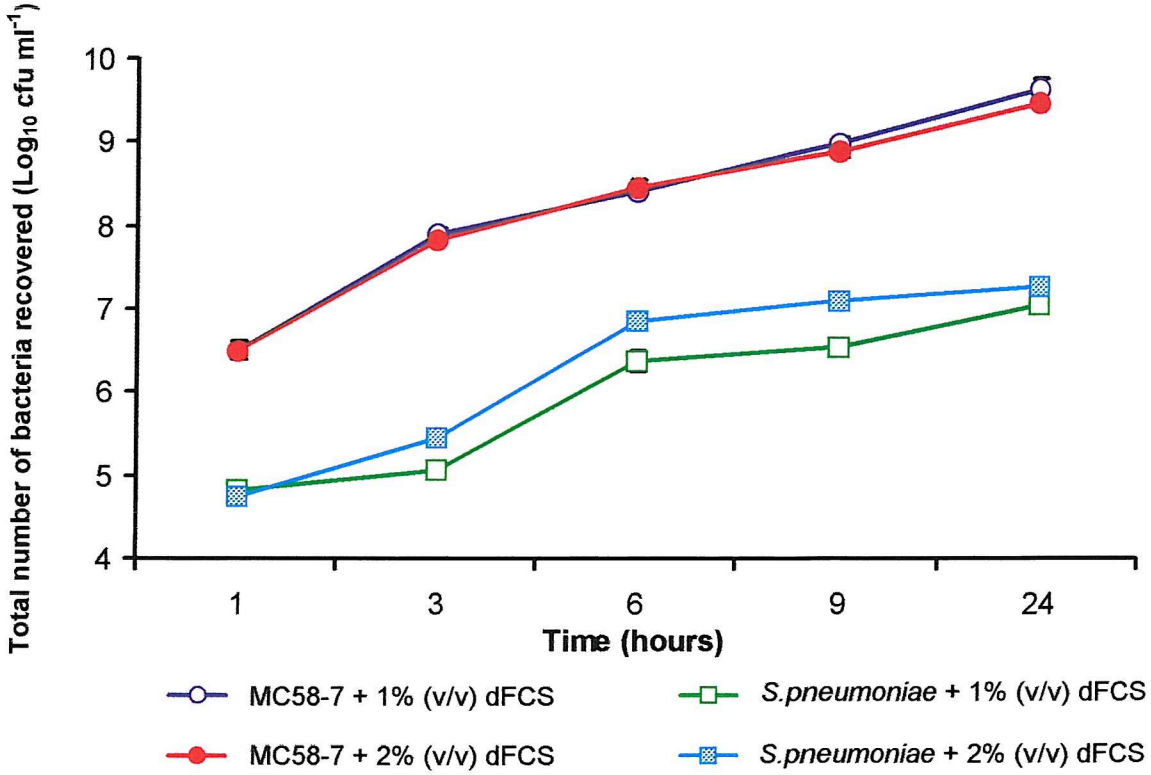
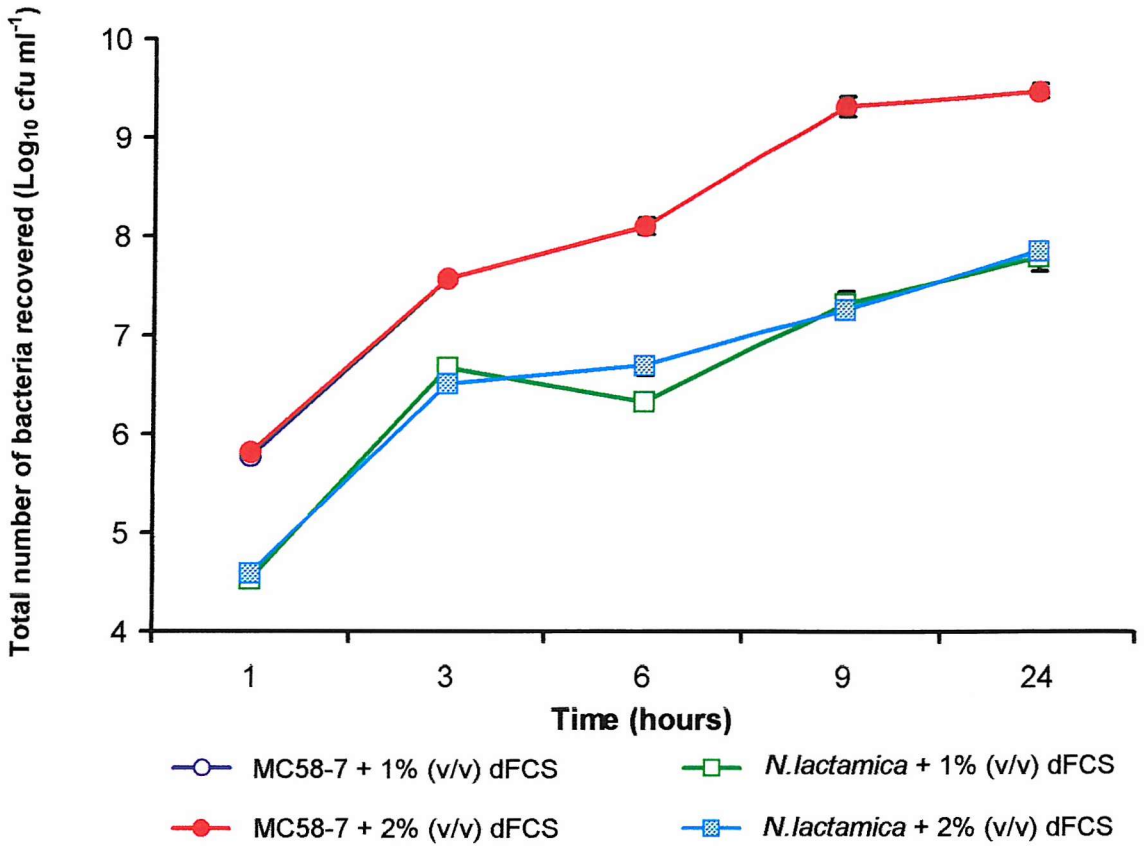


Figure 4.5: The effects of increasing the concentration of dFCS in experimental medium from 1% (v/v) to 2% (v/v) on the growth kinetics of *S.pneumoniae* compared to *N.meningitidis* MC58-7.



In both experiments (Figures 4.4 and 4.5), the concentrations of bacteria were adjusted to approximately $1x10^6$ cfu ml^{-1} in experimental medium. The symbols denote the mean and the error bars denote the standard deviation of triplicate samples.

Figure 4.6: Comparison of the growth kinetics of N.lactamica and piliated meningococci in experimental medium supplemented either 1% (v/v) or 2% (v/v) dFCS.



The concentrations of bacteria were adjusted to approximately $1x10^6$ cfu ml^{-1} in experimental medium. The symbols denote the mean and the error bars denote the standard deviation of triplicate samples.

4.2 The interactions of bacterial pathogens with human meningioma cells

In the current study, the following experiments were undertaken to test the hypothesis that the specific interaction between bacterial pathogen(s) and cells of the leptomeninges is a pivotal event in meningitis

4.2.1 Quantification of bacterial association with meningioma cell lines

The interaction of bacterial pathogens with meningioma cell lines was initially investigated using quantitative viable counting methods. All experiments were carried out using the meningothelial meningioma cell lines M27 and M66. The interactions of each bacterial pathogen were investigated in at least three independent experiments, in which the similar levels of association of piliated meningococci were used to validate any comparisons between experiments.

4.2.1.1 Association of N.meningitidis with meningioma cell lines

Previous studies by Hardy and colleagues (Hardy *et al.,* 2000) have demonstrated that piliated meningococci adhered to meningothelial meningioma cell lines in significantly higher numbers than the corresponding non-piliated variant ($P \leq 0.05$). Initially, the meningioma cell lines M27 and M66 used in this current study were characterised in a similar manner by challenge with *N.meningitidis* MC58-7 and MC58-9 (~$2x10^5$ cfu / monolayer). The association dynamics of meningococci with meningioma cell lines M27 and M66 are shown in Figures 4.7A and 4.7B respectively. The association of piliated and non-piliated meningococci with both meningioma cell lines showed similar trends, with bacterial numbers increasing consistently over time. However, at all time points the number of piliated meningococci associated with both cell lines was approximately 10-30 fold higher than the non-piliated variant ($P \leq 0.005$). Significantly, the association dynamics of meningococci with both meningioma cell lines were similar to those of meningothelial meningioma cell lines used previously (Hardy *et al.,* 2000).

The investigation into the effect of different concentrations of bacterial inocula on the association of meningococci to meningioma cell lines has been touched upon in a previous study (Hardy *et al.,* 2000). Hardy observed that an increase of 1000-fold in the infecting concentration of bacteria only resulted in a two-fold increase in the number of associated bacteria after six hours. However, the current study extended these initial observations by investigating the levels of association of a range of bacterial concentrations ($1x10^2$ to $1x10^8$ cfu) over an extended time course, in order to mimic the events that may occur in the SAS when initial low levels of entering bacteria are followed by unchecked proliferation. Regardless of the concentration of the bacterial inoculum, piliated meningococci always associated with M27 cell monolayers at significantly higher levels than the non-piliated variant ($P \leq 0.05$) (Figure 4.8). Challenge with the highest concentrations ($1x10^6$ to $1x10^8$ cfu) resulted in an early and rapid saturation of the monolayer with both piliated and non-piliated meningococci. However, by 24 hours the levels of association of piliated meningococci were similar ($P \geq 0.1$), regardless of the initial challenge dose. This was also

Figure 4.7: Comparison of meningothelial meningioma cell lines M27 (A) and M66 (B) for the association of piliated and non-piliated meningococci.

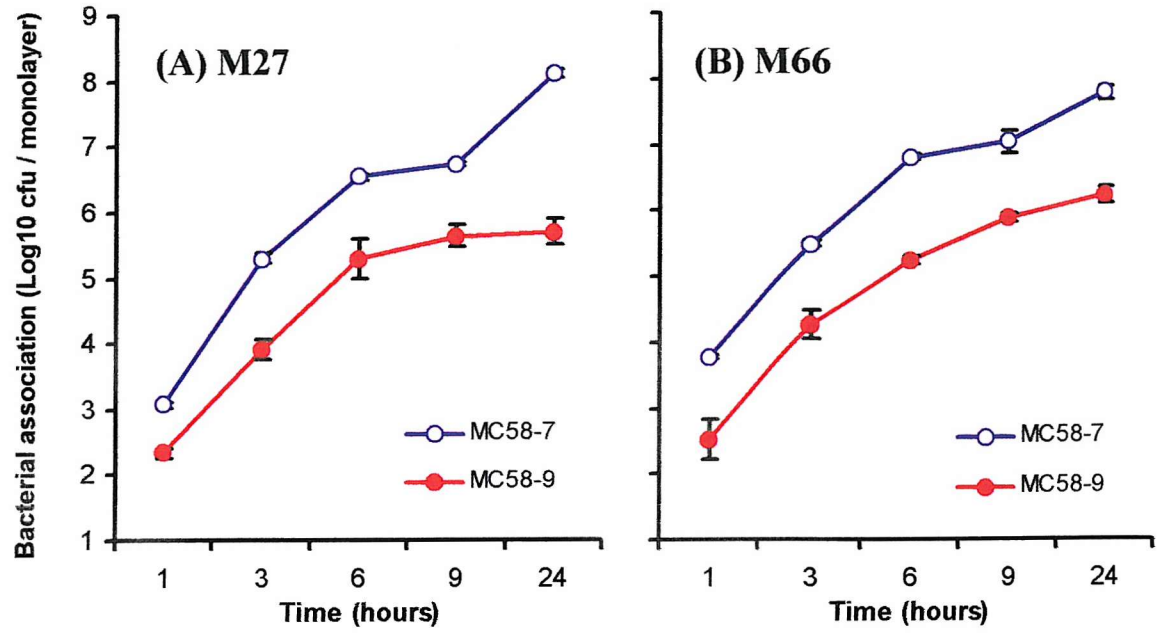
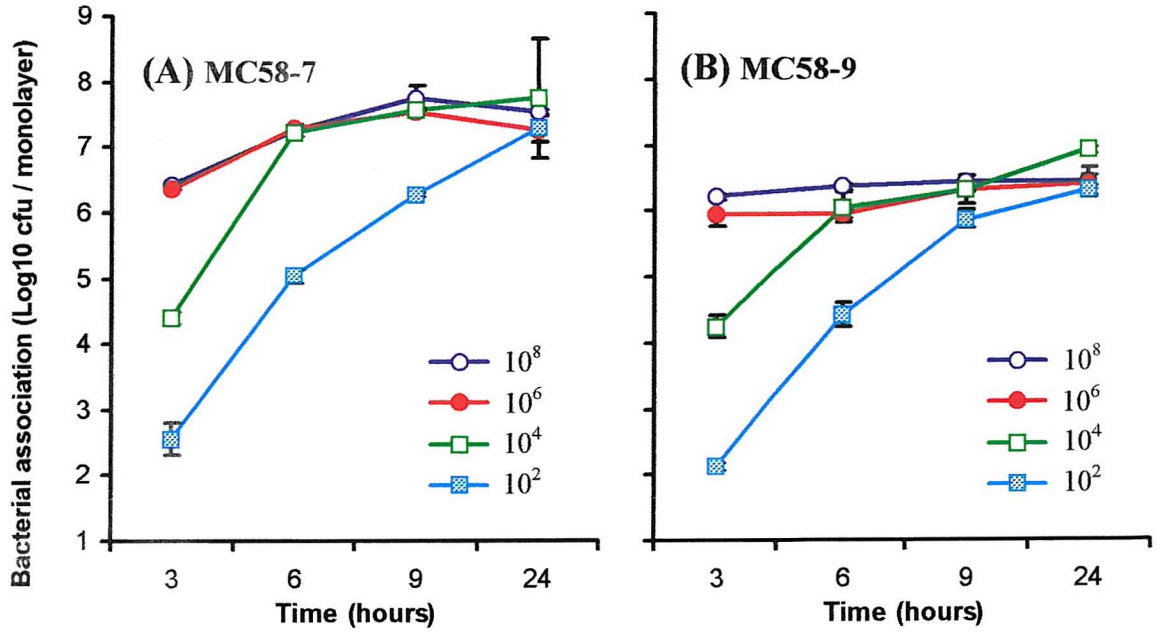


Figure 4.8: The effect of increasing the concentrations of *N.meningitidis* MC58-7 (A) and MC58-9 (B) on the association of bacteria to the meningioma cell line M27.



The symbols denote the mean and the error bars denote the standard deviation of triplicate samples.

observed with the non-piliated variant, although the overall levels of association were still statistically lower compared with piliated meningococci. A similar pattern of results was also seen with the meningioma cell line M66 challenged with bacteria (data not shown).

4.2.1.2 Association of E.coli with meningioma cell lines

Initially, monolayers of the meningioma cell line M27 were challenged with approximately $2x10^5$ cfu of *E.coli* strains DSM and IH3080 to investigate bacterial association. The time course of these experiments was stopped at nine hour post-challenge based on preliminary observations, which showed that the meningioma cell monolayers did not survive prolonged incubation with either *E.coli* strain (data not shown). The levels of association of both *E.coli* strains (DSM and IH3080) with meningioma cell monolayers increased over time (Figure 4.9), although at the earliest time points, up to three hours, the association of *E.coli* IH3080 was approximately ten-fold higher than *E.coli* DSM ($P \leq 0.05$). However, there was no significant difference between the association dynamics of either *E.coli* strain after six hours post-challenge ($P \geq 0.0.9$). In contrast, the numbers of *N.meningitidis* MC58-7 associating with meningioma cell monolayers over the first six hours were approximately 5-10 and 10-100 fold greater than *E.coli* strains IH3080 ($P \leq 0.01$) and DSM ($P \leq 0.01$) respectively. However, by nine hours post-challenge, there was no significant difference ($P \geq 0.1$) between the levels of association of either *E.coli* strain and MC58-7. Similar results were also seen with the other meningioma cell line, M66, following challenge with *E.coli* strains DSM and IH3080 (data not shown).

Due to the extensive damage to the monolayer induced by as few as $2x10^5$ cfu of *E. coli* IH3080 or DSM, the maximum challenge concentration of $1x10^8$ cfu was not tested, but instead the concentration range was downshifted to include a very low concentration (10 cfu). Increasing the concentration of bacteria (from 10 cfu up to $1x10^6$) resulted in incremental increases in association of both *E.coli* DSM (Figure 4.10A) and IH3080 (Figure 4.10B) to monolayers of M66 meningioma cells by nine hours. However, although the meningioma monolayers had been destroyed by nine hours, the numbers of *E.coli* IH3080 and DSM recovered from the cell culture wells continued to increase and peaked at 24 hours, at which time the apparent levels of association were similar ($P \geq 0.05$), regardless of the initial challenge concentration. The data collected for both *E.coli* strains at 24 hours were an artefact, caused by the bacteria associating with cellular debris and the collagen matrix and not with live cells.

4.2.1.3 Association of H.influenzae to meningioma cells

Initially, monolayers of meningioma cell line M27 were challenged with approximately $2x10^5$ cfu of *H.influenzae* strains Eagan and RD to investigate bacterial association. The levels of association of both *H.influenzae* strains with meningioma cell monolayers increased over time (Figure 4.11), with the numbers of adherent encapsulated *H.influenzae* Eagan approximately 50-150 fold lower than the acapsulate *H.influenzae* RD during the first nine hours ($P \leq 0.01$).

Figure 4.9: Comparison of the levels of association of *E.coli* strains IH3080 and DSM and *N.meningitidis* MC58-7 with meningioma cell line M27.

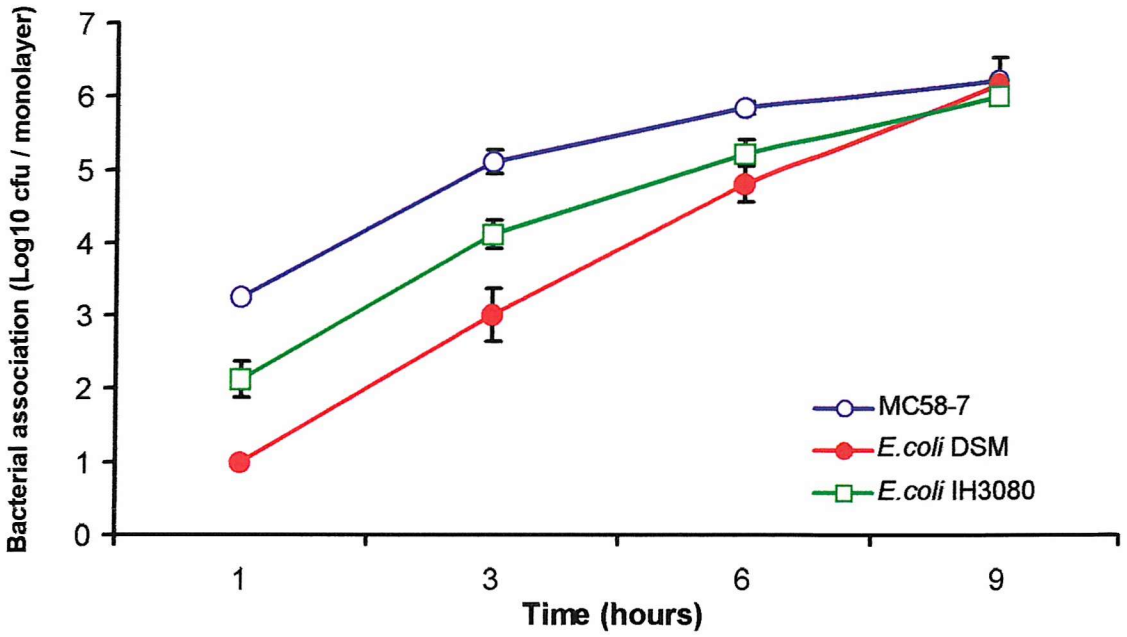
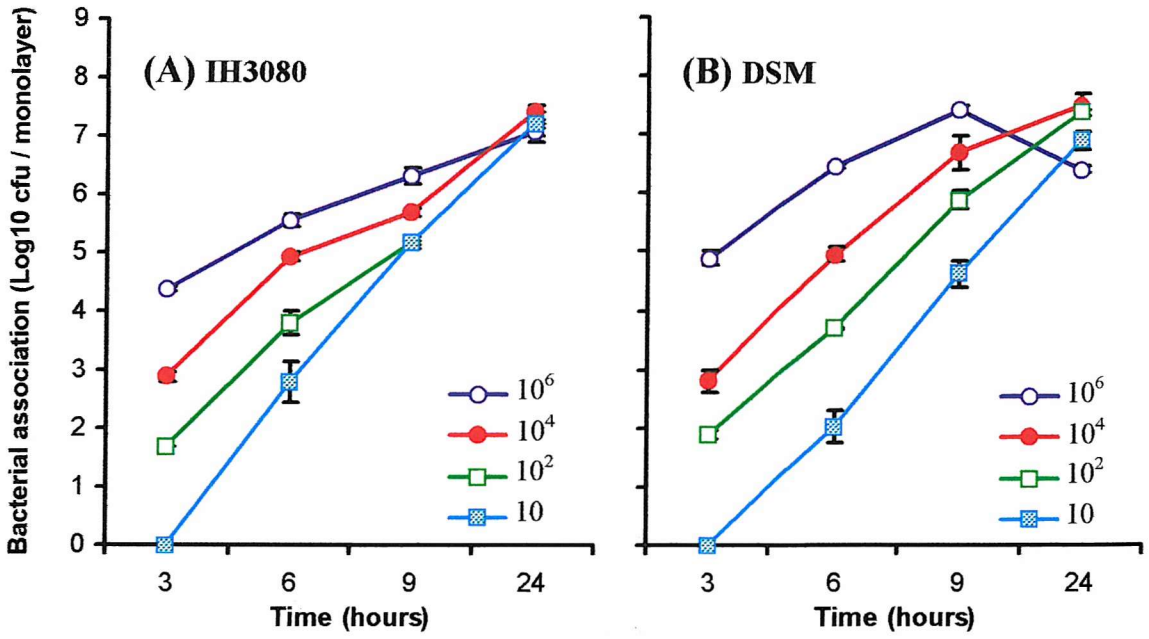


Figure 4.10: The effect of increasing the concentrations of *E.coli* strains IH3080 (A) and DSM (B) on the association of bacteria to the meningioma cell line M27.



The symbols denote the mean and the error bars denote the standard deviation of triplicate samples.

Figure 4.11: Comparison of the levels of association of *H.influenzae* strains Eagan and RD alongside *Neisseria meningitidis* MC58-7 with the meningioma cell line M27.

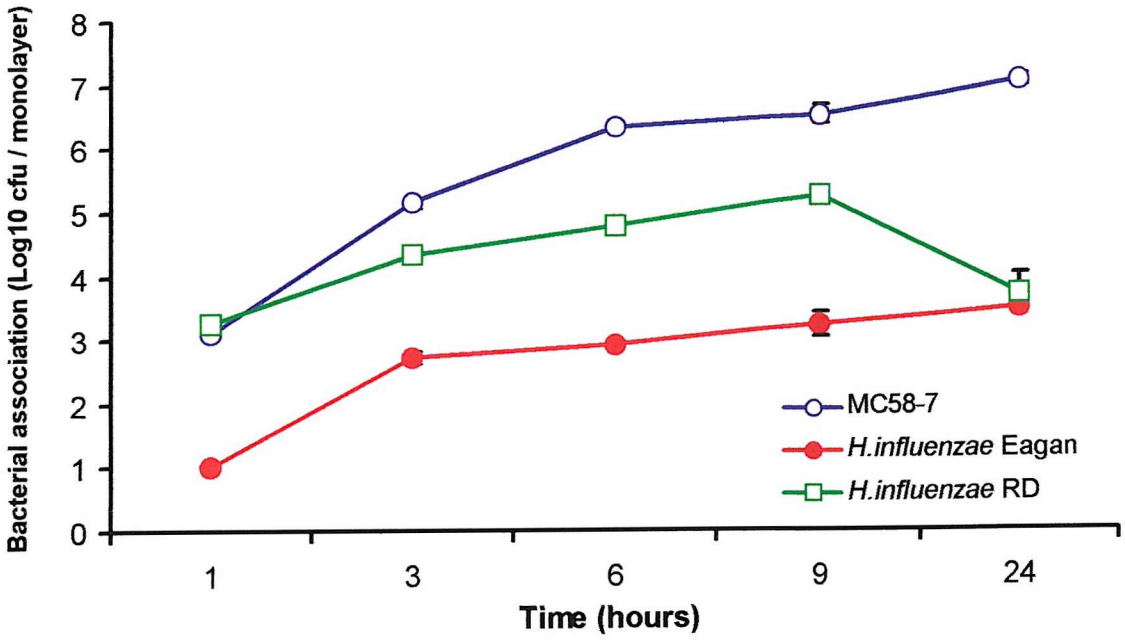
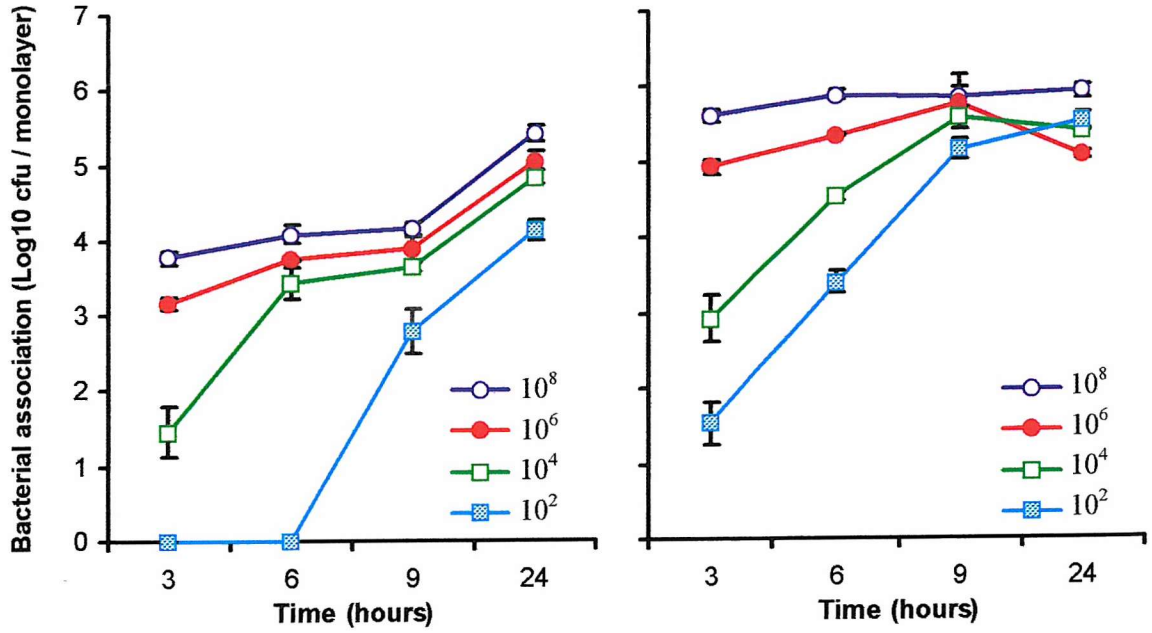


Figure 4.12: The effect of increasing the concentrations of *H.influenzae* strains Eagan (A) and RD (B) on the association of bacteria to the meningioma cell line M27.



The symbols denote the mean and the error bars denote the standard deviation of triplicate samples.

However, by 24 hours post-challenge there was no significant difference between the levels of association of either *H.influenzae* strain (P≥0.3), given the observation of a 30-fold reduction in the numbers of *H.influenzae* strain RD associated with meningioma cells, compared with nine hours. In contrast, the levels of association of *N.meningitidis* MC58-7 with meningioma cell monolayers, at all time points, was approximately 30 and 300-3000 fold greater than *H.influenzae* strains Eagan (P≤0.005) and RD (P≤0.005) respectively. Furthermore, neither *H.influenzae* strain reached the levels of association observed with MC58-7 after 24 hours challenge.

Monolayers of meningioma cells were then challenged with various concentrations of both strains of *H.influenzae* (Fig.4.12). Challenge with the highest concentrations (10^6 –10^8 cfu) of either strain resulted in an early and rapid saturation of the monolayer. By 24 hours post-challenge with *H.influenzae* RD, although statistically significant (P≤0.05), there was only a four-fold difference in the levels of association between all concentrations of bacteria tested. In contrast, the levels of association of *H.influenzae* Eagan continued to rise by 24 hours post-challenge, and there was a 30-fold difference in the numbers of bacterial cfu between the highest and lowest concentrations tested (P≤0.005). Furthermore, a significant lag in bacterial association was observed up to six hours with the lowest dose of *H.influenzae* Eagan, and the levels quantified at nine and 24 hours remained consistently lower than the other concentrations tested (Figure 4.12).

4.2.1.4 Association of S.pneumoniae to meningioma cells

Initially, monolayers of meningioma cell line M27 were challenged with approximately $2x10^5$ cfu of *S.pneumoniae* to investigate bacterial association in comparison to *Neisseria meningitidis* MC58-7. The association dynamics of *S.pneumoniae* with meningioma cells are shown in Figure 4.13. Although the numbers of *S.pneumoniae* associating with the cells increased over time, they were approximately 50-100 fold lower compared with piliated meningococci (P≤0.005) at all time points sampled.

Monolayers of meningioma cells were then challenged with various concentrations of *S.pneumoniae* (Figure 4.14). Challenge with the highest concentrations ($1x10^6$ to $1x10^8$ cfu) resulted in an early and rapid saturation of the monolayer. In contrast, the intermediate concentration ($1x10^4$ cfu) associated more slowly, and a significant time lag was also observed with the lowest dose tested ($1x10^2$ cfu). However, by 24 hours post-challenge the differences in association between all the concentrations tested were not significant (P≥0.1).

Figure 4.13: Comparison of the levels of association of *S.pneumoniae* and *N.meningitidis* MC58-7 with the meningioma cell line M27.

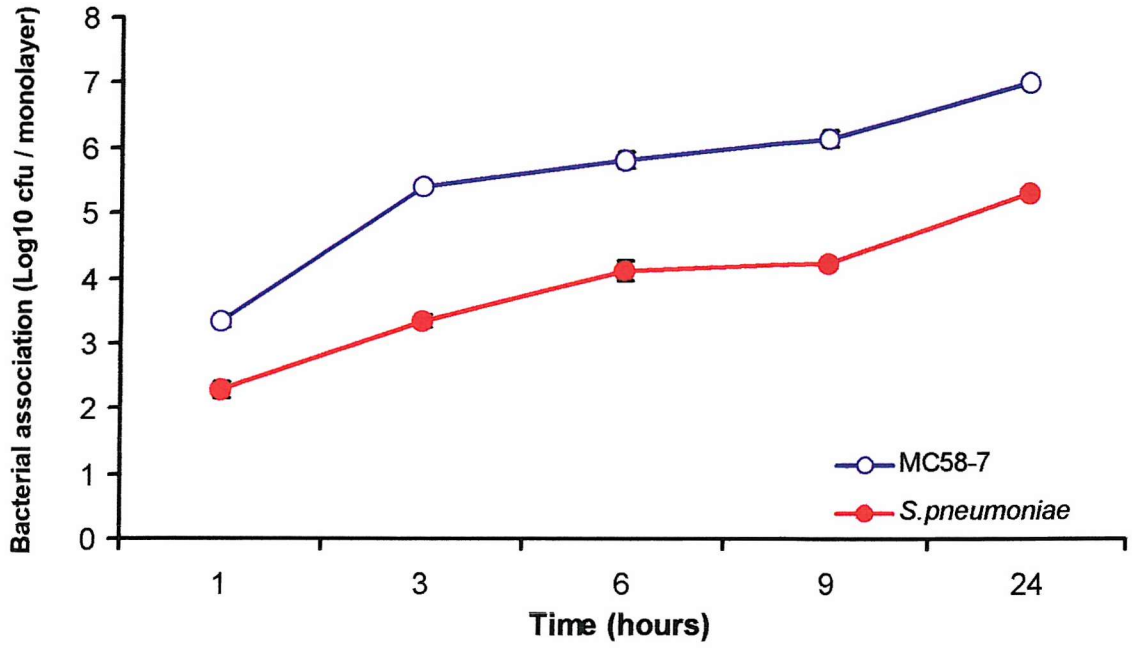
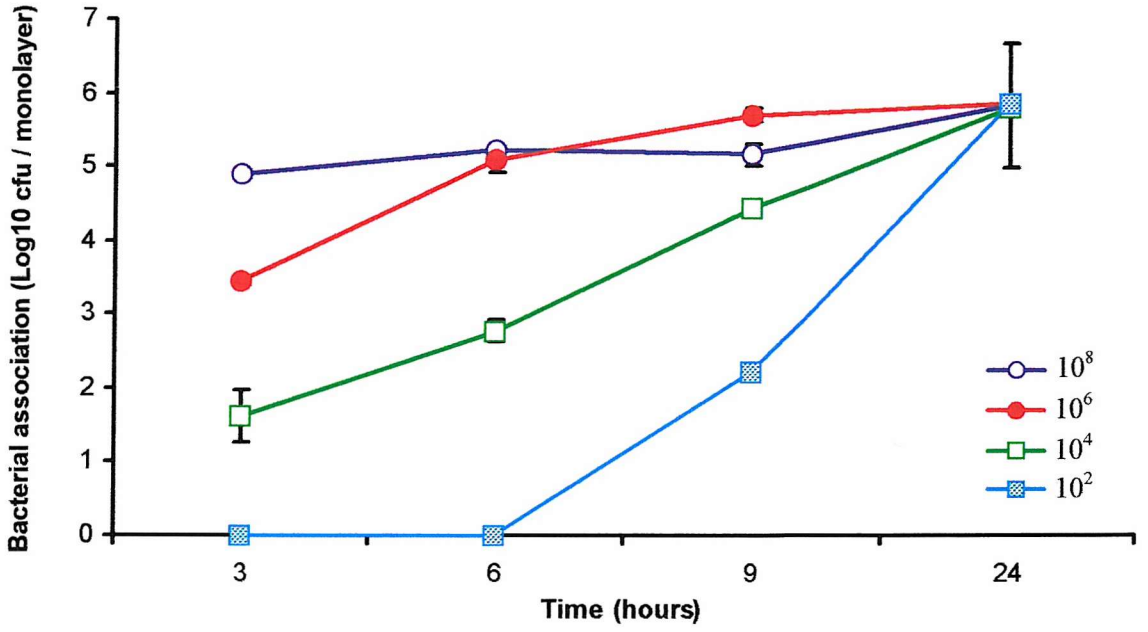


Figure 4.14: The effect of increasing the concentrations of *S.pneumoniae* on the association of bacteria to the meningioma cell line M27.



The symbols denote the mean and the error bars denote the standard deviation of triplicate samples.

4.2.1.5 *Association of N.lactamica to meningioma cells*

Initially, monolayers of meningioma cell line M27 were challenged with approximately $2x10^5$ cfu of *N.lactamica* to investigate bacterial association in comparison to *Neisseria meningitidis* MC58-7. The association dynamics of *N.lactamica* with meningioma cells are shown in Figure 4.15. Although the numbers of *N.lactamica* associating with the cells increased over time, they were approximately 400 fold lower than piliated meningococci ($P \leq 0.005$) at all time points sampled.

Monolayers of meningioma cells were then challenged with various concentrations of *N.lactamica* (Figure 4.16). Challenge with the highest concentrations ($1x10^6$ to $1x10^8$ cfu) resulted in an early and rapid saturation of the monolayer. In contrast, the intermediate concentration ($1x10^4$ cfu) associated more slowly, and a significant time lag was also observed with the lowest dose tested ($1x10^2$ cfu). However, by 24 hours post-challenge the differences in association between all the concentrations tested were not significant ($P \geq 0.3$).

4.2.2 Visualisation of bacterial association with human meningeal cells

The association of meningeal pathogens with human meningioma cells, which was quantified in Section 4.2.1 by viable counting, was also visualised by confocal microscopy following staining of infected monolayers with fluorescent dyes and bacteria with specific antibodies.

4.2.2.1 *Analysis of anti-sera raised against H.influenzae outer membrane (OM) preparations*

Preliminary immunocytochemistry experiments demonstrated the suitability of the primary antibodies specific to *N.meningitidis*, *N.lactamica*, *E.coli* and *S.pneumoniae* (detailed in Table 2.6) for confocal microscopy studies (data not shown). However, an antibody (Mab3B9) that recognises a surface-exposed epitope of the *H.influenzae* outer membrane P6 (Bogdan & Apicella, 1995), and which was kindly supplied by Dr. M.Apicella (Department of Microbiology, University of Iowa, USA), demonstrated specific but low-level reactivity with *H.influenzae* strains Eagan and RD (data not shown). Since Mab3B9 was unsuitable for confocal studies of the interactions of *H.influenzae* with human cells, anti-sera were raised in the current study against outer membrane (OM) preparations isolated from *H.influenzae* strains Eagan and RD, as described in Section 2.12.

The OM preparations from both *H.influenzae* strains were subjected to SDS-PAGE on gradient gels. Preliminary experiments, in which approximately 10μg of protein was loaded per well, both OM preparations displayed a single weak band on the gradient gel, equivalent to approximately 40kDa (data not shown). However, the experiments were repeated with a very high concentration of each OM preparation in order to check for the presence of other bands (Figure 4.17). In addition to the one prominent band, equivalent to approximately 40kDa, many minor bands were observed with both OM preparations.

Figure 4.15: Comparison of the levels of association of *N.lactamica* and *N.meningitidis* MC58-7 with the meningioma cell line M27.

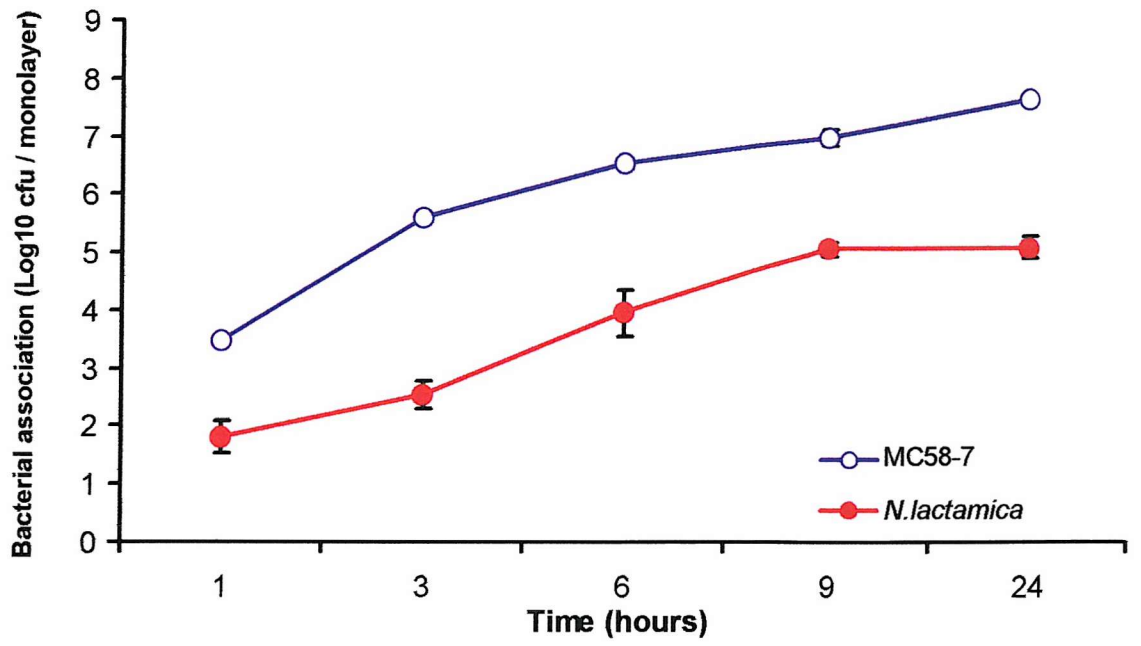
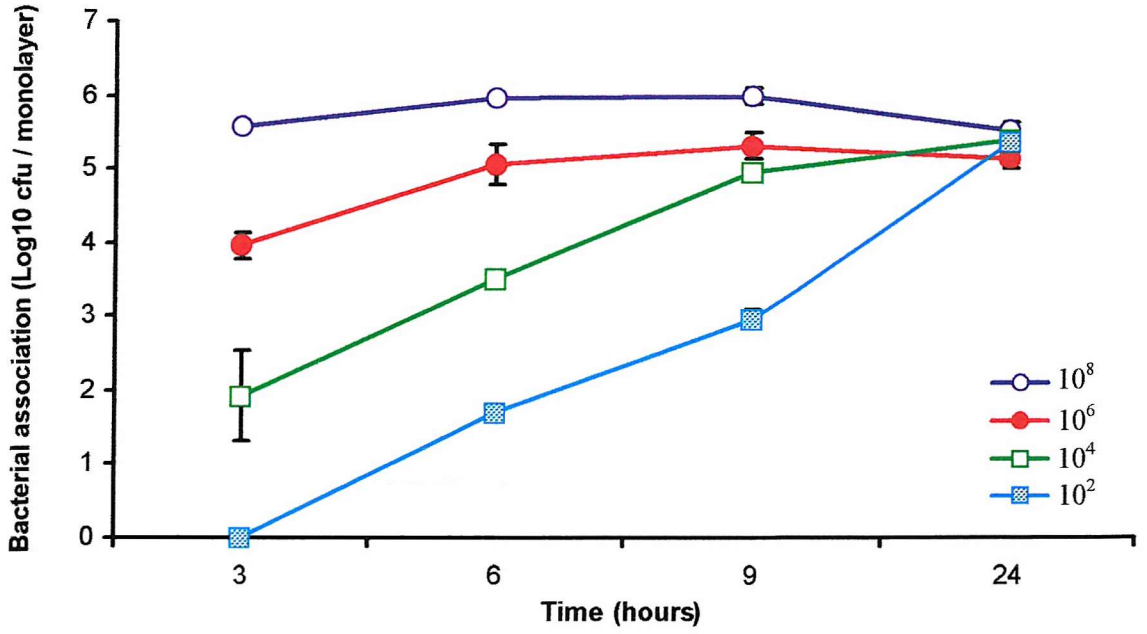


Figure 4.16: The effect of increasing the concentrations of *N.lactamica* on the association of bacteria to the meningioma cell line M27.



The symbols denote the mean and the error bars denote the standard deviation of triplicate samples.

Anti-sera were raised in rabbits to OM preparations isolated from *H.influenzae* strains Eagan and RD and they were designated R699 and R701 respectively. The ability of these anti-sera to label the respective *H.influenzae* strain is shown in Figure 4.18. Significantly, no cross reactivity was observed following incubation of human meningothelial meningioma cell lines or Chang epithelial cells with either antibody (data not shown). Therefore, anti-serum R699 and R701 could be used as primary antibodies in subsequent experiments for the visualisation of *H.influenzae* association with human meningeal cells.

4.2.2.2 Fluorescent staining and confocal microscopy analysis

In the negative control samples, the uninfected meningioma cells stained a deep red colour against a black background (Figure 4.19A-C). The simulated fluorescence projections of the confocal microscope images provided excellent detail of the individual cell surfaces and cellular processes, which could be seen to mediate contact between cells. Unless otherwise stated, the monolayers of meningioma cell line M27 were challenged with approximately $2.5x10^8$ cfu of each bacterium.

Meningococci were visible as green cocci associating only with the surface of meningioma cells and not the glass coverslip underneath. Analysis of the meningioma cell monolayers challenged with piliated meningococci showed that as early as three hours meningococci had associated with most, but not all, of the meningioma cells in the monolayer (Figure 4.19D). The bacteria were present as discrete diplococci and were spread evenly over the cell surfaces. Considerably more meningococci had associated with the meningioma cells by six hours, with some discrete clusters of bacteria beginning to form on the cell surfaces (Figure 4.19E). By 24 hours post-challenge (Figure 4.19F), there were even greater numbers of associated meningococci, the majority of which were present in large clusters of bacteria. In contrast, meningioma cell monolayers challenged with non-piliated meningococci (Figure 4.19G-I) showed far lower levels of association of bacteria at all time points, compared with the piliated variant. The numbers of non-piliated meningococci associating with meningioma cells increased marginally between three (Figure 4.19G) to six hours (Figure 4.19H) and by 24 hours there were significantly greater numbers of bacteria associated with the cell monolayer, although they were present as diplococci and had not formed the clusters apparent with piliated meningococci (Figure 4.19I). It was also interesting to note that bacteria did not associate with some of the cells within the monolayer.

Figure 4.17: SDS-PAGE analysis of the protein compositions of outer membrane preparations from *H.influenzae* strains Eagan and RD.

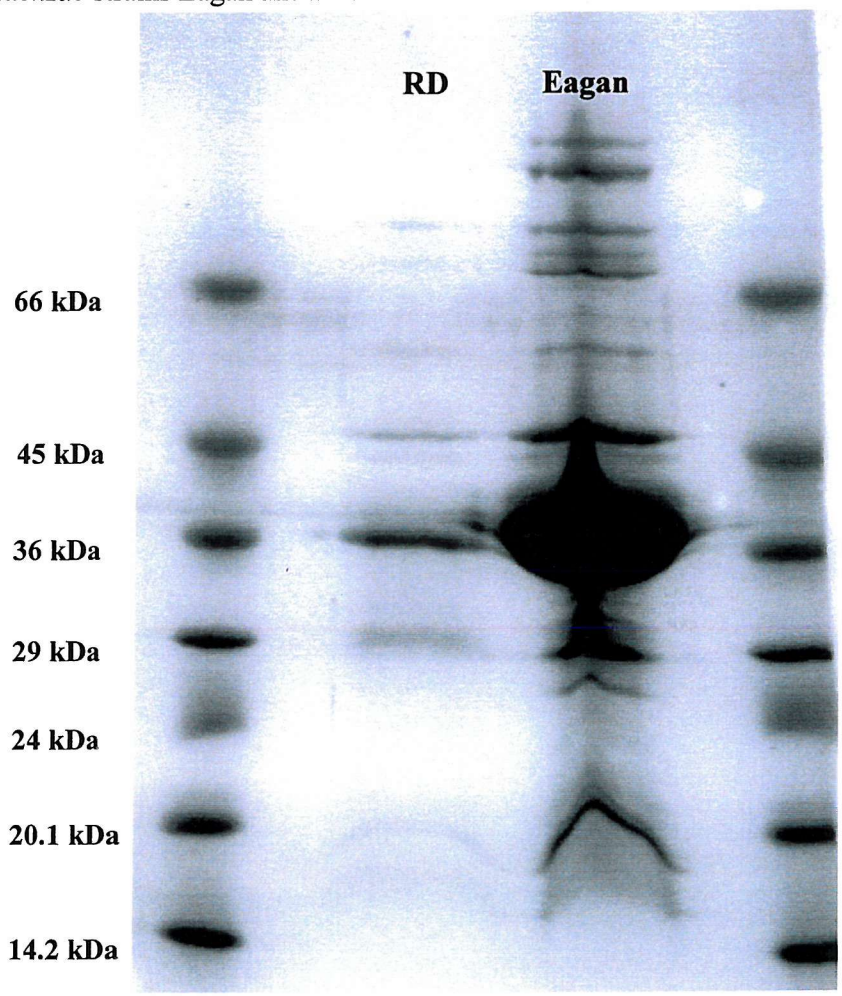
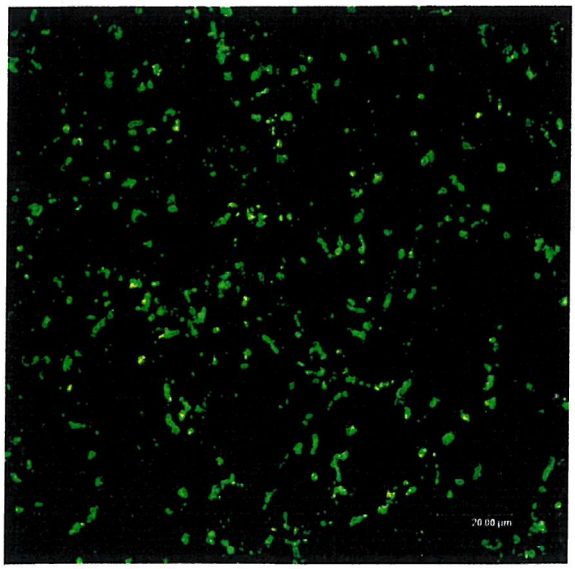
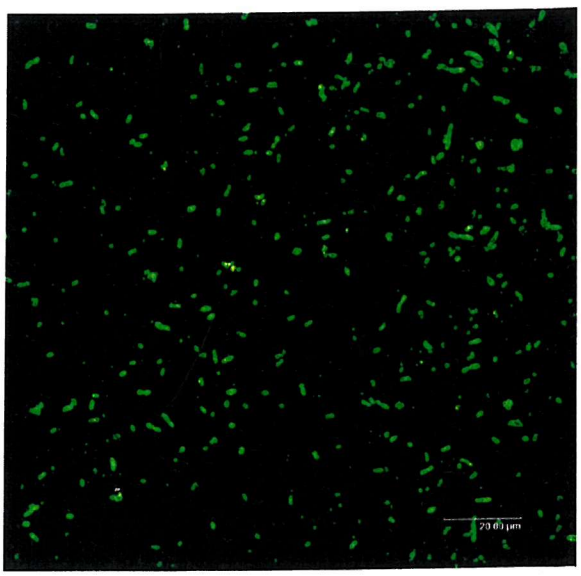


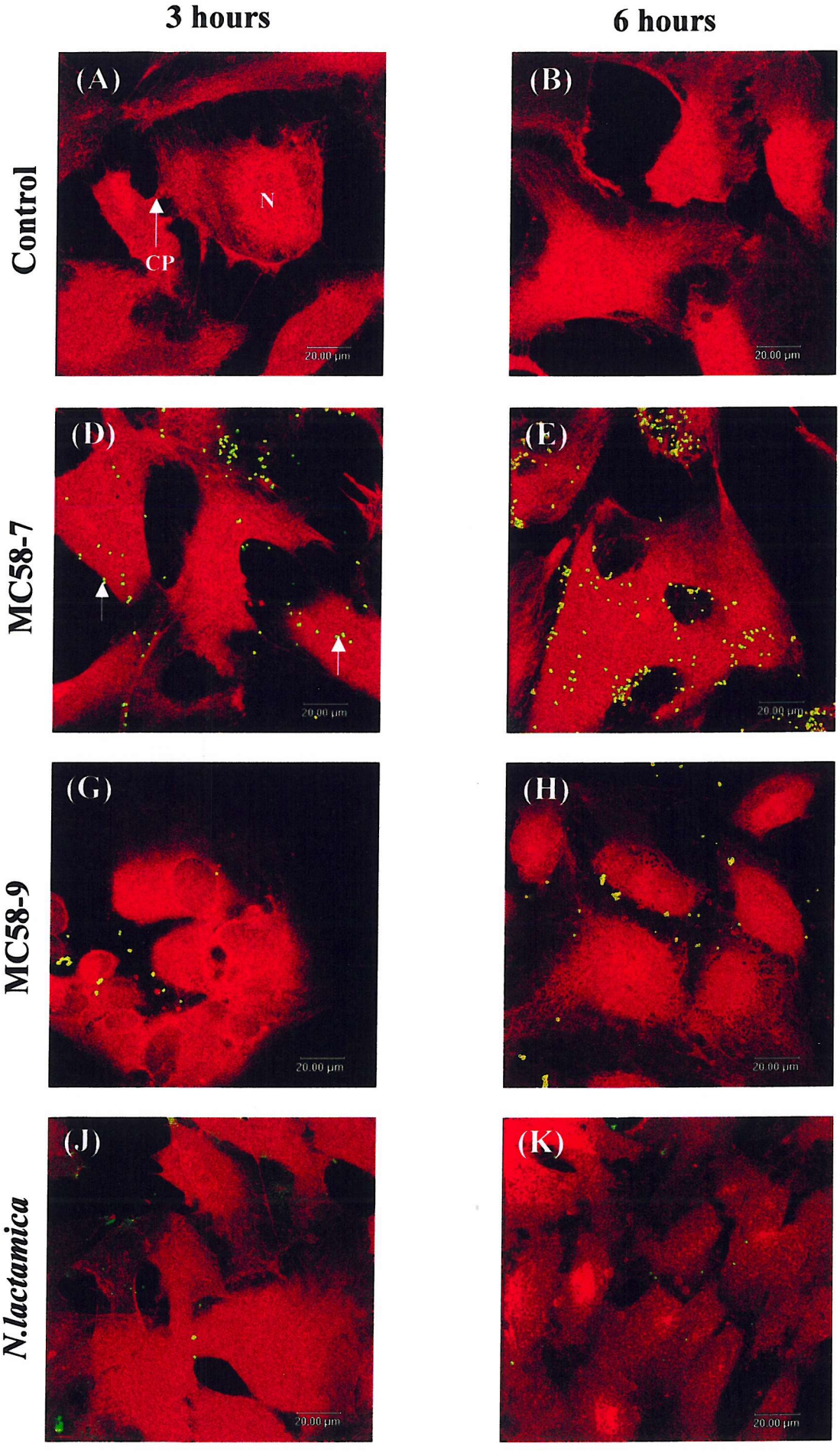
Figure 4.18: Confocal images of *H.influenzae* strains Eagan (A) and RD (B) incubated with the antibodies R699 and R701 respectively.

(A) *H.influenzae* Eagan

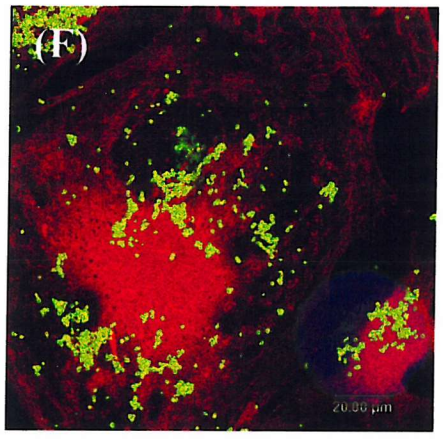


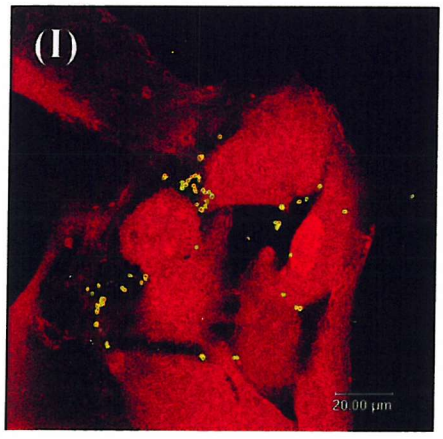
(B) *H.influenzae* RD




3 hours
6 hours
Control
MC58-7
N.lactamica
(A)
(B)
(D)
(E)
(G)
(H)
(J)
(K)
N
CP
20.00 µm

24 hours





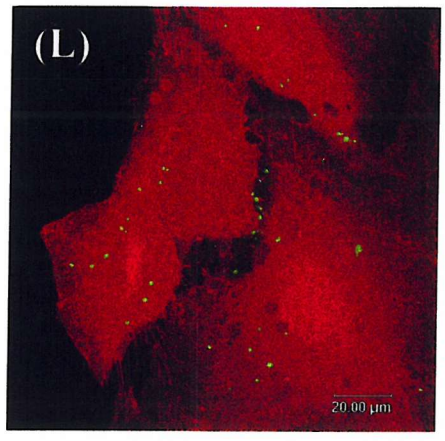


Figure 4.19:

Simulated fluorescence projection confocal images of uninfected meningothelial meningioma cell line M27 monolayers (Figure 4.19A-C). The meningioma cells stained a deep red colour with cellular processes (CP) clearly visible against the background. In addition the nuclei of some cells are visible as raised circular areas (N)

The association of piliated meningococci (green) with M27 cell monolayers (red) increased over time. The meningococci were present on the cell surface as discrete diplococci (arrows) at three hours (Figure 4.19D) but had formed large clusters of bacteria at six (Figure 4.19E) and 24 hours (Figure 4.19F) post-challenge.

The association of non-piliated meningococci with M27 cell monolayers at 3 (Figure 4.19G), 6 (Figure 4.19H) and 24h (Figure 4.19I).

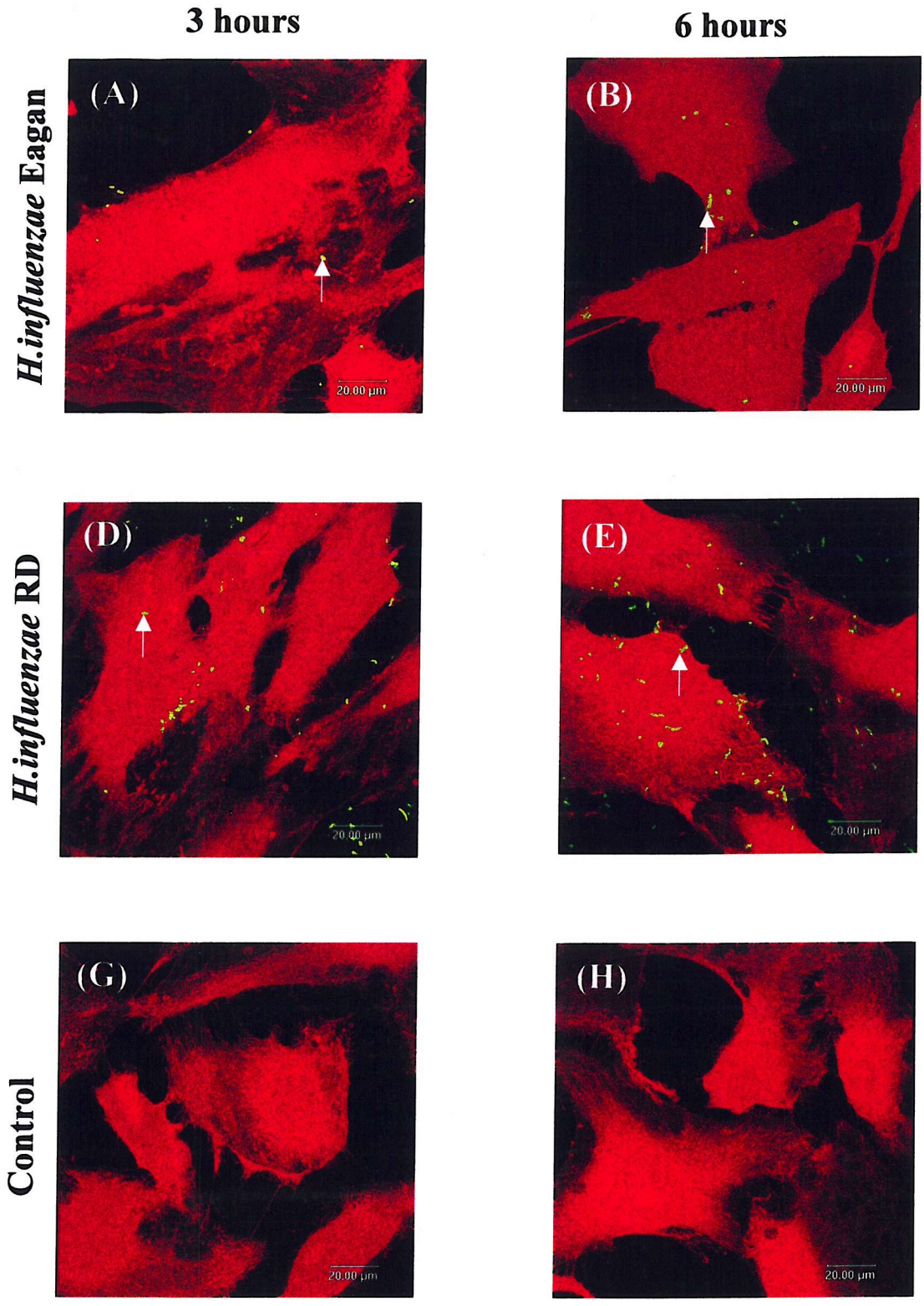
The association of *N.lactamica* with M27 cell monolayers at 3 (Figure 4.19J), 6 (Figure 4.19K) and 24h (Figure 4.19L).

Monolayers were challenged with approximately $2x10^8$ cfu of each bacterium. The scale bars represent 20µm.

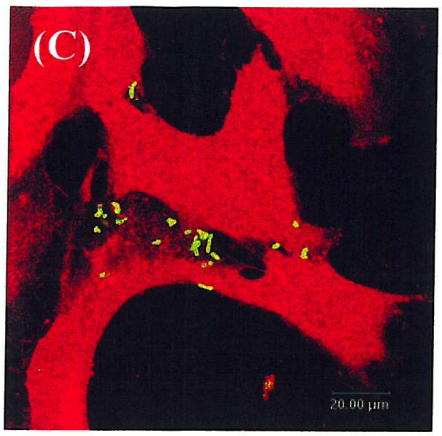
The association of the commensal *N.lactamica* with the meningioma cell line M27 was also investigated and compared with meningococci. The association of *N.lactamica* with meningioma cells at three (Figure 4.19J) and six hours (Figure 4.19K) was significantly lower than either piliated or non-piliated meningococci, with on average only two bacteria observed in association with each cell. However, by 24 hours the numbers of *N.lactamica* associated with the M27 cell monolayer had increased approximately five-fold, to levels similar to non-piliated meningococci, and the bacteria were observed as discrete diplococci spread evenly over the cell surfaces (Figure 4.19K).

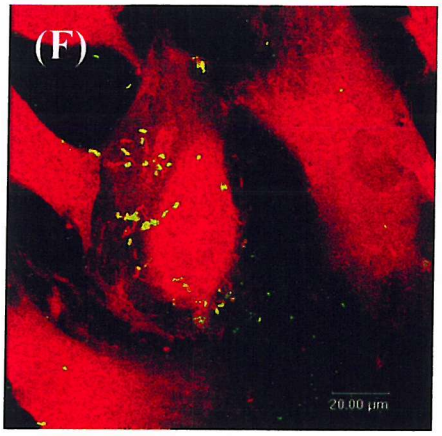
The association of *H.influenzae* strains Eagan and RD with meningioma cells is shown in Figure 4.20. The numbers of *H.influenzae* Eagan associating with the cells increased marginally from three (Figure 4.20A) to six hours (Figure 4.20B), although the bacteria did not associate with some cells in the monolayer. By 24 hours, the numbers of *H.influenzae* Eagan associating with the monolayer had increased by approximately two-fold and small clusters of bacteria could be seen on the cell surface (Figure 4.20C). In contrast, the acapsulated *H.influenzae* strain RD associated with meningioma cells in greater numbers than the capsulated *H.influenzae* strain Eagan. The numbers of adherent *H.influenzae* strain RD, which were observed as individual bacteria in association with meningioma cells, increased approximately two-fold from three (Figure 4.20D) to six hours (Figure 4.20E). By 24 hours (Figure 4.20F), there were greater numbers of adherent bacteria, which had begun to form small clusters on the cell surface.

In contrast to *N meningitidis*, *N.lactamica* and *H.influenzae*, challenge with *E.coli* resulted in severe damage to the monolayers of the meningioma cell line M27. Challenge of meningioma cells with *E.coli* strain DSM resulted in approximately 50% of the monolayer being destroyed after only three hours, leaving only small patches of cells. Bacteria were seen associating in clusters with the remaining cells, which exhibited extensive contraction of their cytoplasm (Figure 4.21D). At six (Figure 4.21E) and 24 hours (Figure 4.21F) the meningioma cell monolayers had been completely destroyed, with clusters of *E.coli* DSM now adhering to cellular debris. Experiments using a lower inoculum concentration of bacteria (~$2.5x10^6$ cfu per monolayer) yielded similar results (data not shown). In contrast, meningioma cell monolayers challenged with *E.coli* strain IH3080 remained intact after three hours (Figure 4.21A) with individual bacteria adhering randomly to the cell surface. After six hours (Figure 4.21B), considerably more *E.coli* had associated with the undamaged meningioma cells, with some bacteria forming discrete clusters over the cell surface. However, by 24 hours (Figure 4.21C) only approximately 50% of the meningioma cell monolayer was still intact, with the remaining cells showing signs of cytoplasmic shrinkage. In addition, large clusters of *E.coli* IH3080 were seen associated with cellular debris.


3 hours
6 hours
H.influenzae Eagan
H.influenzae RD
Control
(A)
(B)
(D)
(E)
(G)
(H)
20.00 µm

24 hours





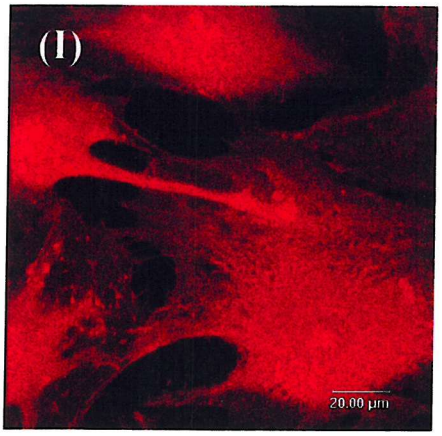


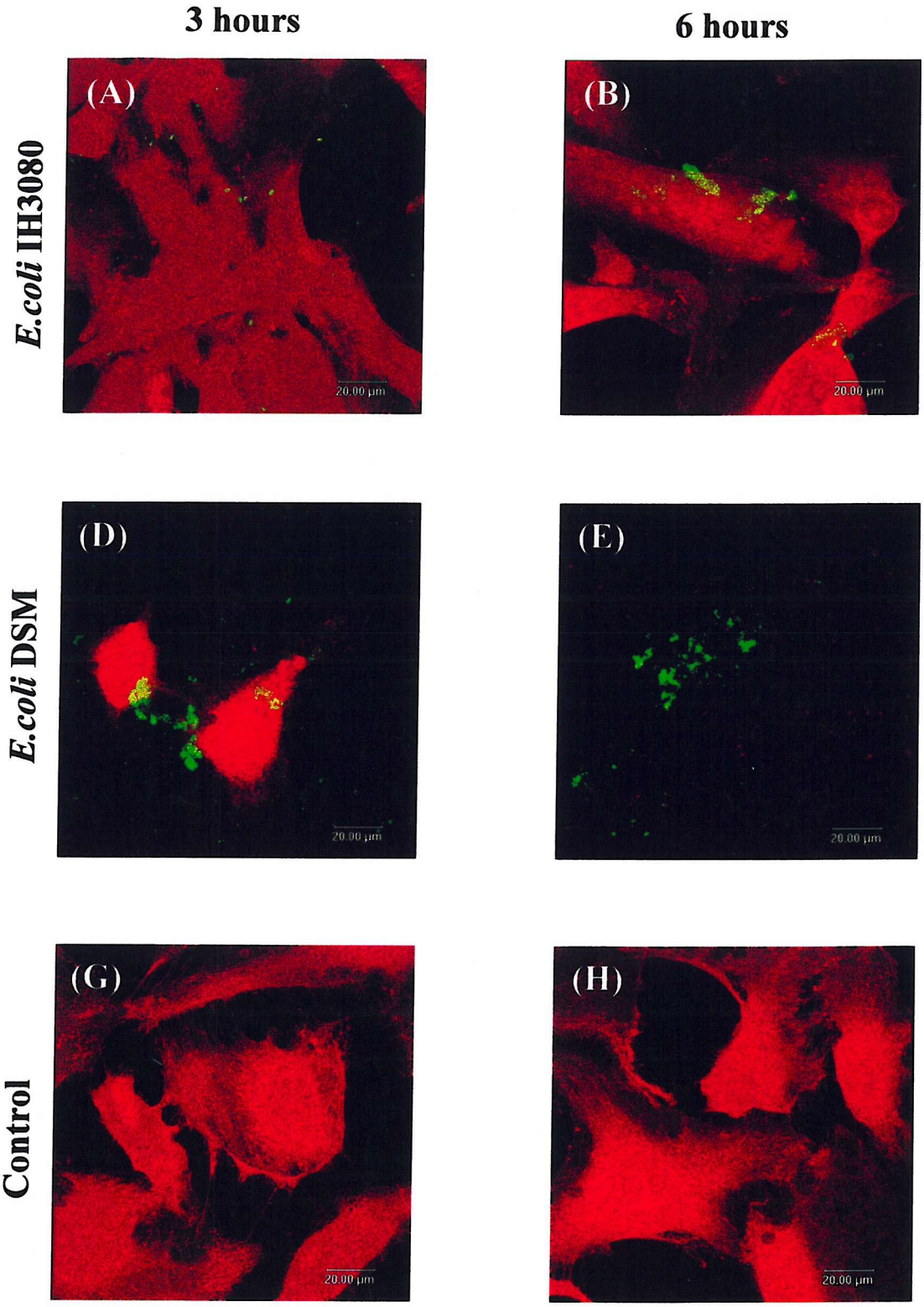
Figure 4.20:

The association of *H.influenzae* Eagan with M27 cell monolayers at 3 (Figure 4.20A), 6 (Figure 4.20B) and 24 hours (Figure 4.20C). The bacteria were present on the cell surface as discrete rods (arrows) at 3 (Figure 4.20A) and 6 hours (Figure 4.20B) but had formed large clusters of bacteria at 24 hours (Figure 4.20C) post-challenge.

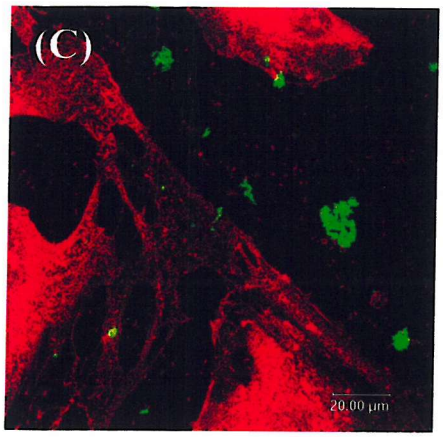
The association of *H.influenzae* RD with M27 cell monolayers at 3 (Figure 4.20D), 6 (Figure 4.20E) and 24 hours (Figure 4.20F). The bacteria were present on the cell surface as discrete rods (arrows) at three (Figure 4.20D) and six hours (Figure 4.20E) but had formed large clusters of bacteria at 24 hours (Figure 4.20F) post-challenge.

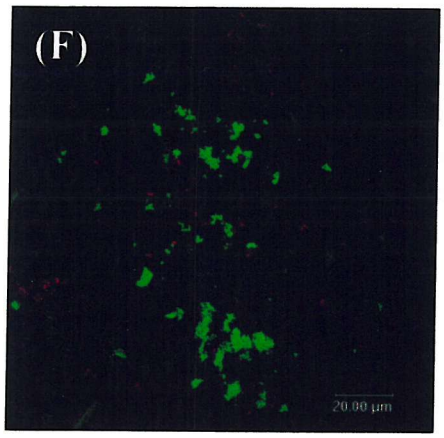
Simulated fluorescence projection confocal images of uninfected meningothelial meningioma cell line M27 monolayers (Figure 4.22G-I).

Monolayers were challenged with approximately $2x10^8$ cfu of each bacterium. The scale bars represent 20µm.


3 hours
6 hours
E.coli IH3080
E.coli DSM
Control
(A)
(B)
(D)
(E)
(G)
(H)
20.00 µm

24 hours





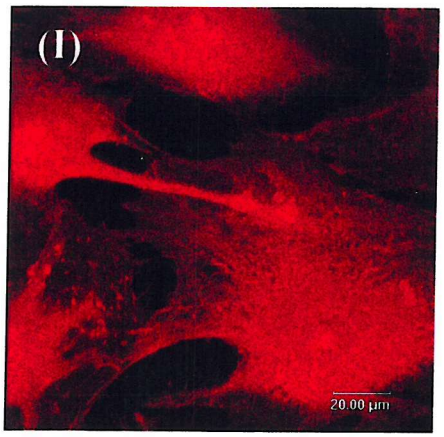


Figure 4.21:

The association of *E.coli* IH3080 with M27 meningioma cell monolayers at 3 (Figure 4.21A), 6 (Figure 4.21B) and 24 hours (Figure 4.21C). Bacteria formed clusters on the cell surface after 6 hours (Figure 4.21B) and by 24 hours (Figure 4.21C), only 40% of the monolayer was still intact. The surviving cells were contracted and rounded with *E.coli* associating with cellular debris.

After three hours incubation with *E.coli* DSM only patches of the monolayer were still intact (Figure 4.21D). Clusters of *E.coli* were seen associating with the remaining cells, which were contracted and rounded. At six and 24 hours the monolayer had been completely destroyed with bacteria seen associating with cellular debris (Figure 4.21E and 4.21F).

Simulated fluorescence projection confocal images of uninfected meningothelial meningioma cell line M27 monolayers (Figure 4.21G-I). Cell monolayers remained intact throughout the experimental time course.

Monolayers were challenged with approximately $2x10^8$ cfu of each bacterium. The scale bars represent 20µm.

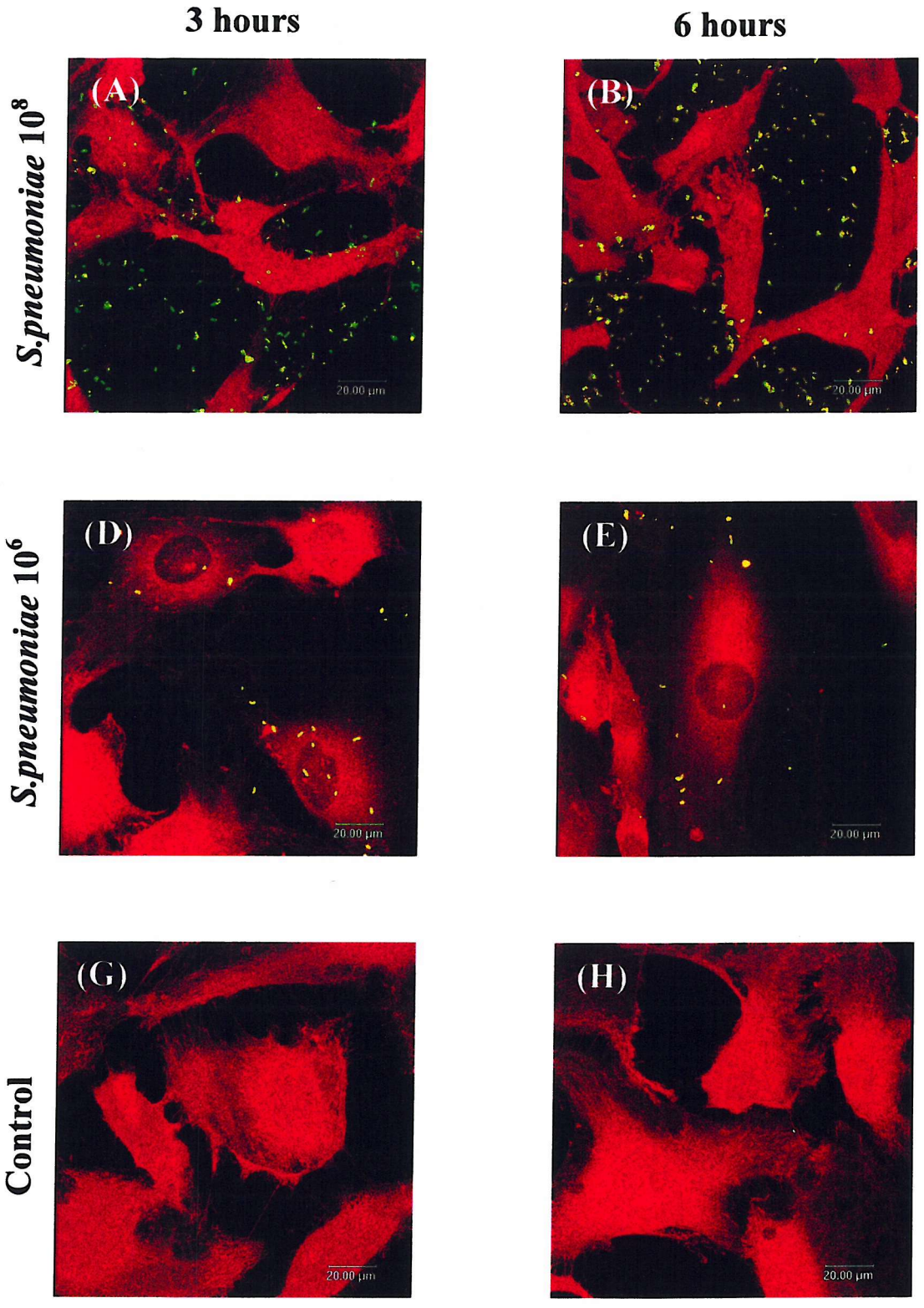
Analysis of the meningioma cell monolayers challenged with $2.5x10^8$ cfu per monolayer of *S.pneumoniae* showed that at three hours (Figure 4.22A) many small clusters of bacteria were observed in association with the meningioma cells and also non-specifically to the collagen-coated glass slide. The meningioma cell monolayer was still intact, although the cells were showing signs of cytoplasmic shrinkage. By six hours (Figure 4.22B), >95% of the monolayer had been destroyed, and clusters of *S.pneumoniae* were observed in association with the remaining small patches of damaged cells and the substratum.. At 24 hours post-challenge (Figure 4.22C) the few remaining meningioma cells of the monolayer were also rounded and displayed significant cytoplasmic shrinkage. In contrast, meningioma cell monolayers challenged with $1x10^6$ cfu per monolayer of *S.pneumoniae* only began to show signs of cellular damage after 24 hours (Figure 4.22F). The numbers of adherent bacteria, which were observed as individual bacteria, increased marginally from three (Figure 4.22D) to six hours (Figure 4.22E), and by 24 hours, increasing numbers of adherent pneumococci formed small clusters on the cell surfaces (Figure 4.22F).

4.2.3 The invasion of meningioma cells by bacterial pathogens

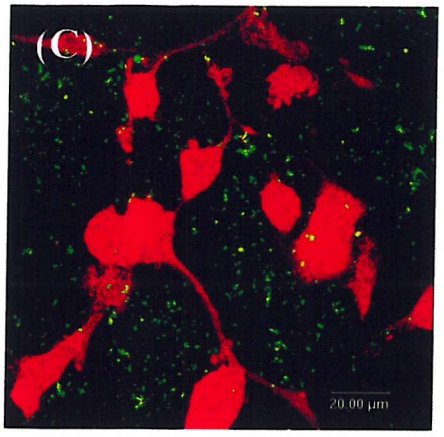
A previous study by Hardy and colleagues (Hardy *et al.*, 2000) demonstrated that *Neisseria meningitidis* did not invade meningothelial meningioma cells *in vitro*. In the current study, the hypothesis that the leptomeninges also provides a barrier to the penetration by other meningeal pathogens was tested. Initially, the ability of bacterial pathogens to invade meningothelial meningioma cells was investigated using quantitative viable counting methods following gentamicin treatment and pre-incubation with cytochalasin D. Any potential internalisation of bacteria by meningioma cells was subsequently investigated with TEM. Monolayers of the meningioma cell line M27 were challenged with approximately $2x10^5$ cfu of each pathogen in at least two independent experiments.

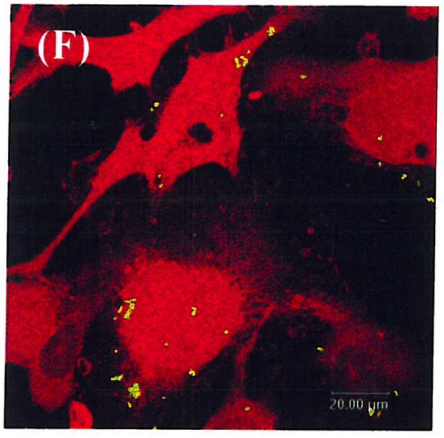
4.2.3.1 Quantification of bacterial invasion of meningioma cells

The ability of meningeal pathogens to invade meningioma cell monolayers after nine hours post-challenge is shown in Figure 4.23. No viable *H.influenzae*, *S.pneumoniae*, *N.lactamica* or *E.coli* strain DSM were recovered from challenged meningioma cells following gentamicin treatment. However, significant numbers of *N.meningitidis* variants MC58-7 and MC58-9 and *E.coli* IH3080 were isolated from gentamicin treated meningioma cells. The numbers of piliated meningococci that were recovered from meningioma cells following antibiotic treatment was approximately three and nine fold greater than *E.coli* IH3080 and *N.meningitidis* MC58-9 respectively. However, there was no significant difference between the numbers of *N.meningitidis* MC58-7 (P=0.901) or MC58-9 (P=0.586) recovered following gentamicin treatment alone or pre-incubation with cytochalasin D., which inhibits cellular microfilament activity.


3 hours
6 hours
S.pneumoniae 10^8
S.pneumoniae 10^6
Control
(A)
(B)
(D)
(E)
(G)
(H)
20.00 µm

24 hours





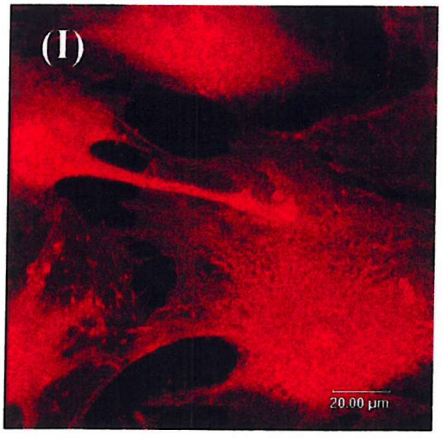


Figure 4.22:

Monolayers of meningioma cell line M27 were challenged with approximately $1x10^8$ cfu of *S.pneumoniae*. By 3 hours, large numbers of bacteria associated with the cell monolayer, within which many of the cells of were contracted (Figure 4.22A). By 6 hours, 95% of the monolayer had been destroyed with the surviving cells severely contracted and rounded (Figure 4.22B). At 24 hours clusters of bacteria were associated with cellular debris and not the remaining contracted cells (Figure 4.22C).

Monolayers of meningioma cell line M27 were challenged with approximately $1x10^6$ cfu of *S.pneumoniae* at 3 (Figure 4.22D), 6 (Figure 4.22E) and 24 hours (Figure 4.22F). The monolayer remained intact up to 24 hours, although some cells showed signs of shrinkage. The association of *S.pneumoniae* with M27 monolayers increased over time, forming clusters of bacteria by 24 hours (Figure 4.22F).

Simulated fluorescence projection confocal images of uninfected meningothelial meningioma cell line M27 monolayers (Figure 4.22G-I). Cell monolayers remained intact throughout the experimental time course.

The scale bars represent 20µm.

Figure 4.23: A comparison of the ability of bacterial pathogens to invade meningioma cells following treatment with the antibiotic gentamicin and the inhibitor of microfilament activity, cytochalasin D.

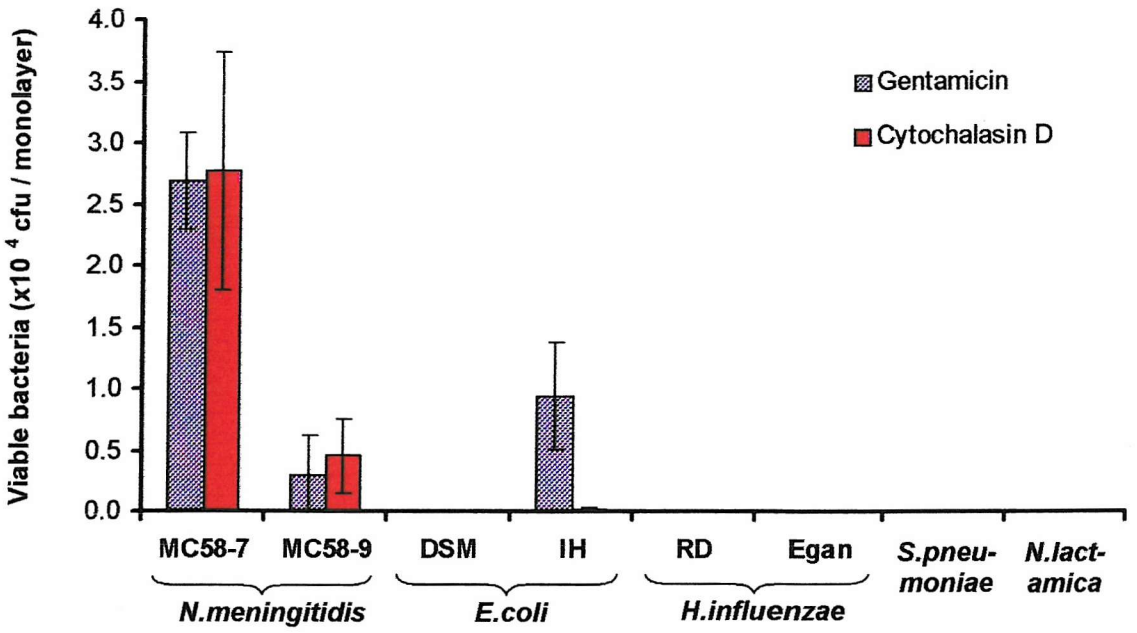
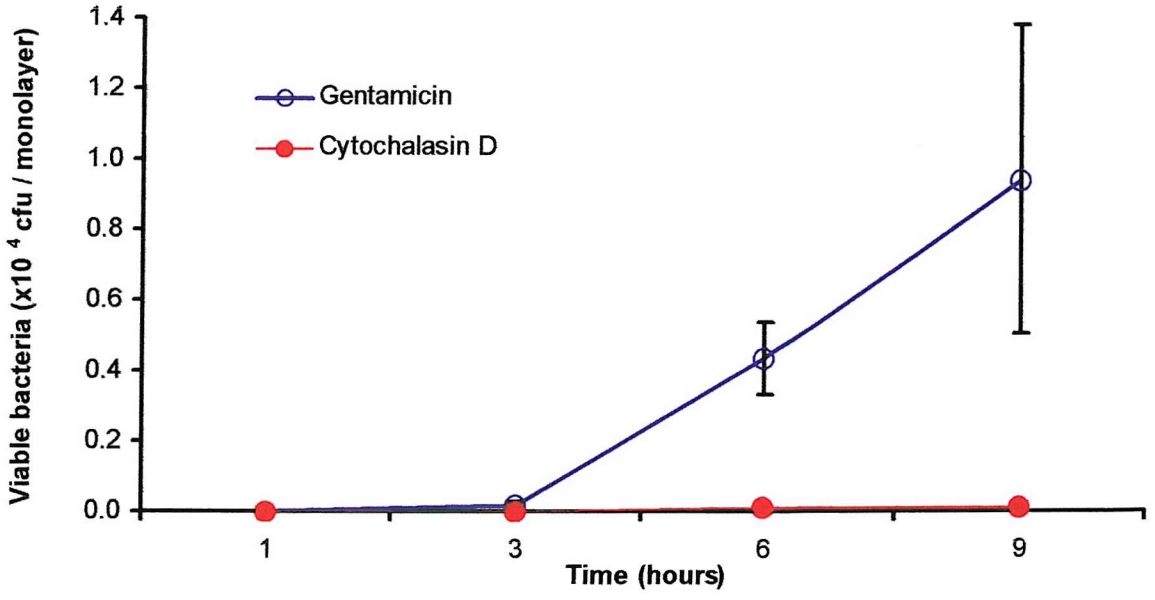


Figure 4.24: The significant reduction in the numbers of viable *E.coli* IH3080 recovered at all time points ($P \leq 0.05$) from meningioma cell monolayers treated with gentamicin as compared to those pre-incubated with cytochalasin D.



The symbols denote the mean and error bars denote the standard deviation of triplicate samples.

In contrast to meningococci, the pre-incubation of meningioma cell monolayers with cytochalasin D resulted in an approximate seven-fold reduction in the numbers of *E.coli* strain IH3080 recovered following gentamicin treatment at nine hours (P=0.022). In addition, the significant reduction in the numbers of viable *E.coli* IH3080 as a result of pre-incubation with cytochalasin D was evident over time, as shown in Figure 4.24 ($P \leq 0.05$). The numbers of *E.coli* strain IH3080 surviving antibiotic treatment at nine hours represented approximately two percent of the total number of bacteria associating to the surface of the meningioma cell monolayer.

4.2.3.2 Investigation of E.coli IH3080 invasion of meningioma cells with TEM

The potential invasion of meningioma cells by *E.coli* strain IH3080 was investigated further using TEM to detect the presence of any intracellular bacteria. Figure 4.25 is representative of a control, uninfected meningioma cell, with the nucleus, mitochondria and rough endoplasmic reticulum clearly visible. The apical surfaces of the control cells were smooth and cellular processes were generally absent. Examination of more than 50 grids (each containing at least one section) of monolayers challenged with *E.coli* IH3080 showed bacteria in close association with the apical surface of meningioma cells after six hours (Figure 4.26).

The apical surfaces of challenged meningioma cells generally exhibited more cellular processes than those of control cells. The depth of the cell monolayer was generally very thin, and often the bacteria had greater depth than the distance between the apical and basolateral surfaces of the meningioma cells, especially in regions of the cell processes. Occasionally, *E.coli* could be seen sitting in cup-shaped depressions of the apical cell membrane, partially surrounded by folds of the cell cytoplasm (Figure 4.27). The ability of *E.coli* IH3080 to invade meningioma cells was confirmed by the presence of intracellular bacteria within membrane-bound vacuoles (Figure 4.28). The membranes of both the cell vacuole and the bacteria can be clearly seen in Figure 4.29, with the depth of one bacterium causing a protrusion in the cell apical surface. In addition, bacteria were commonly seen associated to meningioma cells between the processes of overlapping cells (Figure 4.30). In these sections (Figures 4.25-4.30), fimbriae-like structures on the bacteria were not evident, although they were present when observed in different planes of view (data not shown).

Figure 4.25: TEM micrograph of a representative unchallenged meningioma (M66) cell. The nucleus (N), mitochondria (M) and rough endoplasmic reticulum (rER) are clearly visible. The apical cell surface is smooth and cellular processes are not evident. Magnification x17,000.

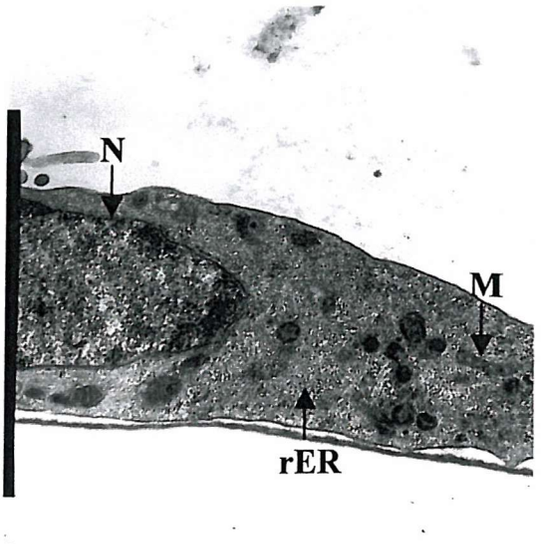


Figure 4.26: TEM micrograph of a representative meningioma (M66) cell challenged with *E.coli* IH3080 for six hours. A single bacterium (arrow) is seen in close association with the apical surface of the meningioma cell at a slight depression of the plasmalemma. In contrast to the negative control, the apical surface of the challenged cell has many cellular processes. Magnification x20,000.

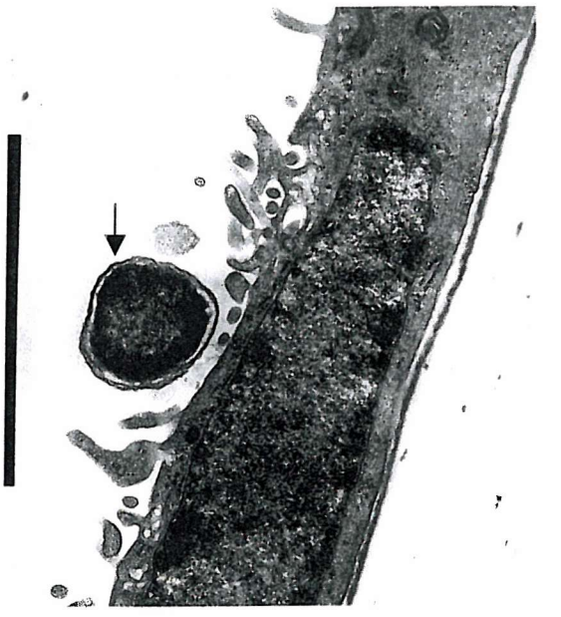


Figure 4.27: TEM micrograph of a representative meningioma (M66) cell challenged with *E.coli* IH3080 for six hours. Bacteria (arrow) are shown sitting in a cup-shaped depression in the apical cell membrane, partially surrounded by folds of the cell cytoplasm. Magnification x15,000.

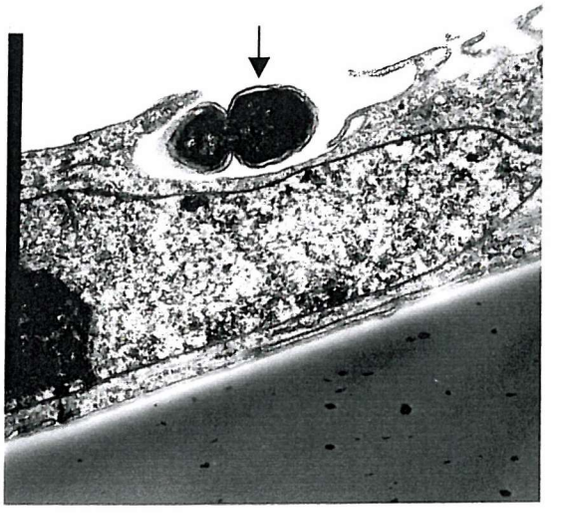


Figure 4.28: TEM micrograph of a representative meningioma (M66) cell challenged with *E.coli* IH3080 for six hours. Invasive bacteria (arrows) are shown within vacuoles in the meningioma cell cytoplasm. All the micrographs are orientated with the apical surface upwards. Magnification x12,000.

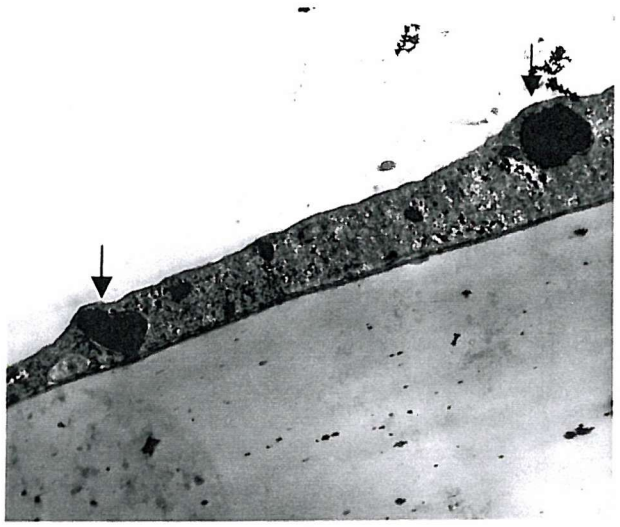


Figure 4.29: Close-up of the micrograph shown in Figure 4.36. A bacterium (B) is shown within a vacuole (V) in the meningioma cell cytoplasm. Magnification x12,000.

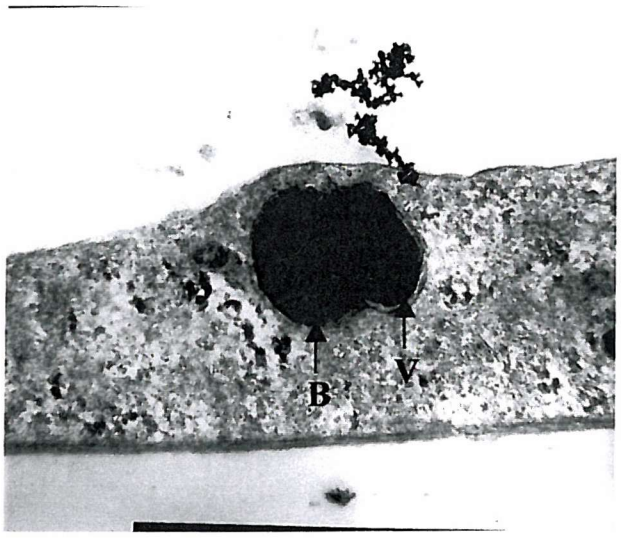
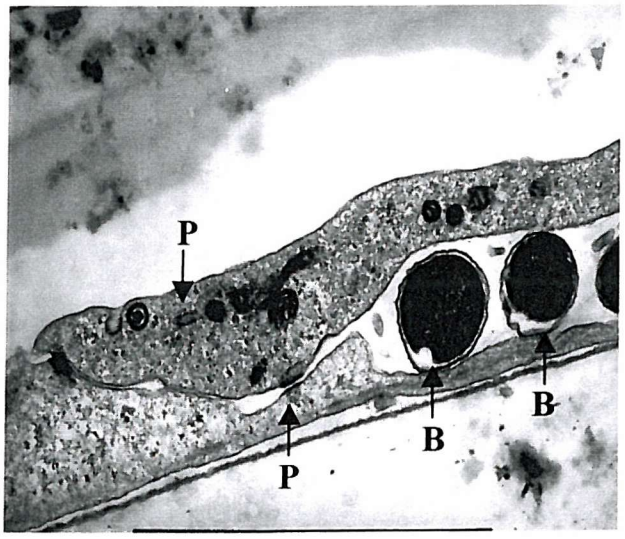


Figure 4.30: TEM micrograph of a representative meningioma (M66) cell challenged with *E.coli* IH3080 for six hours. Bacteria (B) are shown in-between the overlapping cytoplasmic processes of the meningioma cells (P). Magnification x15,000.



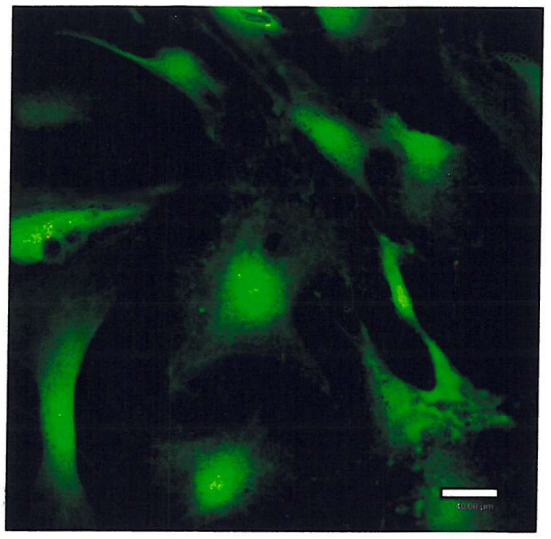
4.2.4 Viability of meningioma cell monolayers challenged with bacteria

The damage to cell monolayers (Section 4.2.2) as a result of challenge with *E.coli* strains DSM and IH3080, and *S.pneumoniae* was investigated further with the use of Molecular Probes Live/Dead® kit. The assay is based on the use of two dyes that measure recognised parameters of cell viability. The conversion of calcein Am into the intensely fluorescent calcein (green) by intracellular esterase activity is predictive of live cells. In addition, ethidium homodimer (ethD-1) undergoes a 40x enhancement of fluorescence (red) upon binding to nucleic acids in cells with damaged membranes, but is excluded by the intact plasma membrane of live cells.

The viability of uninfected meningioma cell monolayers remained at 100% over 24 hours as shown by their intense green fluorescence (Figure 4.31A), a property also shared by monolayers challenged with *N.meningitidis* MC58-7 over the same time course (Figure 4.31B). At six and 24 hours post-challenge with *S.pneumoniae*, the majority of meningioma cells remained viable, with their intact plasma membranes excluding the red dye (Figure 4.32). In contrast, the cytopathic effects of *E.coli* DSM on the monolayer described in Section 4.2.2.2 were confirmed, with the few meningioma cells remaining after six and 24 hours staining red (Figure 4.34). The majority of meningioma cells challenged with *E.coli* IH3080 remained viable at six hours post-challenge (Figure 4.33A), although some cells showed slight red fluorescence. However, by 24 hours only small patches of the monolayer remained, the cells of which were non-viable. In addition, challenge experiments with both *E.coli* strains also resulted in a decrease in the pH of the experimental medium, which was characterised by an acidic yellow discolouration.

Figure 4.31: Determination of cell viability using the Molecular Probes Live/Dead® kit of control, unchallenged meningioma cells (A) and meningioma cells challenged with *N.meningitidis* MC58-7 (B) after six hours. Both meningioma cell monolayers exhibited 100% viability, shown by the intense green fluorescence. Similar results were seen after 24 hours post-challenge. The scale bar represents 40 microns.

(A) Control cells



(B) Cells challenged with *N.meningitidis* MC58-7

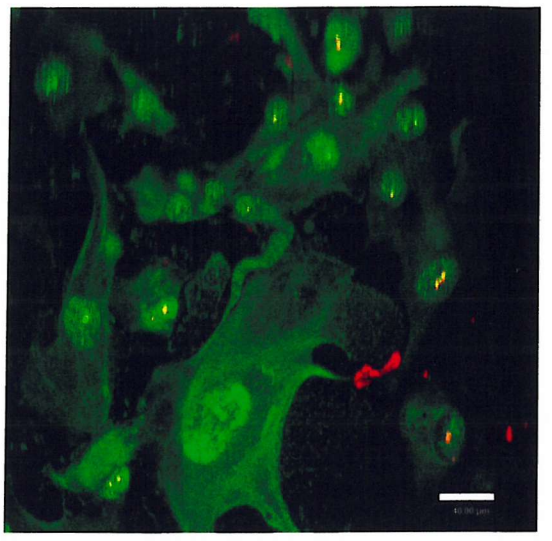
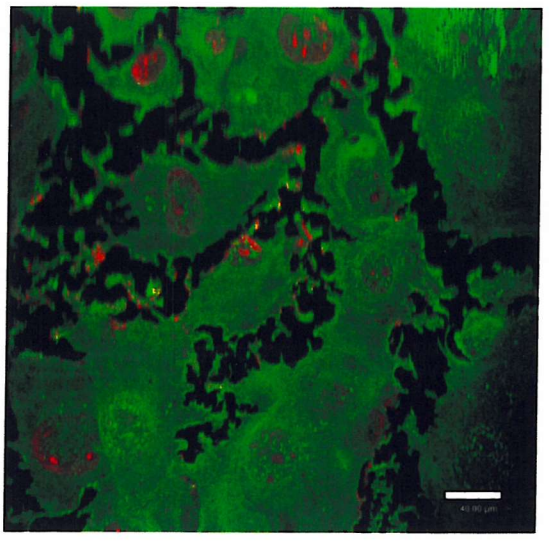


Figure 4.32: Determination of cell viability of meningioma cell monolayers challenged with *S.pneumoniae* after six (A) and 24 (B) hours. At both six and 24 hours post-challenge with *S.pneumoniae*, the majority of meningioma cells remained viable, as demonstrated by the intense green fluorescence and the exclusion of the red dye by intact plasma membranes. Controls as above.

(A) *S.pneumoniae* 6hrs post-challenge



(B) *S.pneumoniae* 24hrs post-challenge

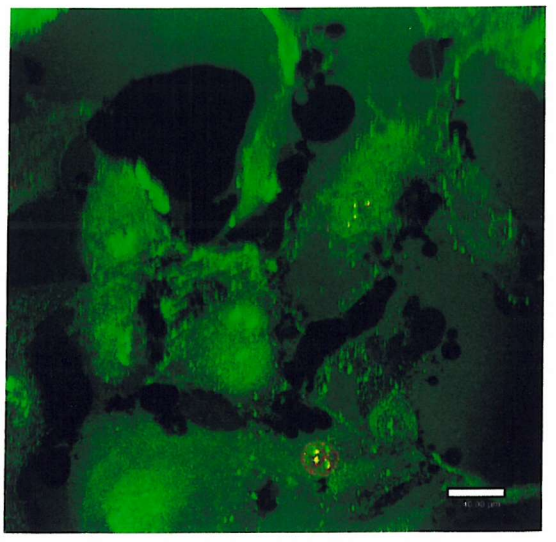
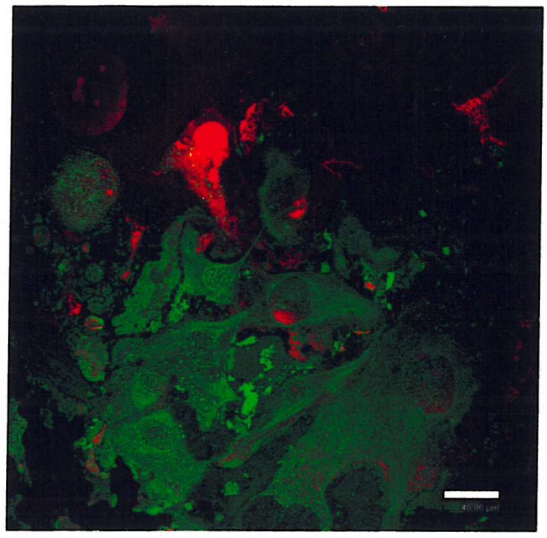


Figure 4.33: Determination of cell viability of meningioma cell monolayers challenged with *E.coli* IH3080 after six (A) and 24 (B) hours. The majority of meningioma cells challenged with *E.coli* IH3080 remained viable at six hours exhibiting green fluorescence. However, by 24 hours only small patches of the monolayer remained, the cells of which had damaged membranes resulting in the uptake of red fluorescence. Controls as above. The scale bar represents 40 microns.

(A) *E.coli* IH3080 6hrs post-challenge



(B) *E.coli* IH3080 24hrs post-challenge

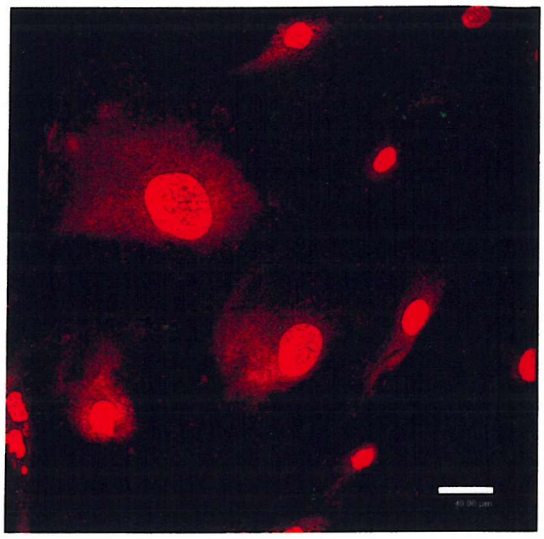
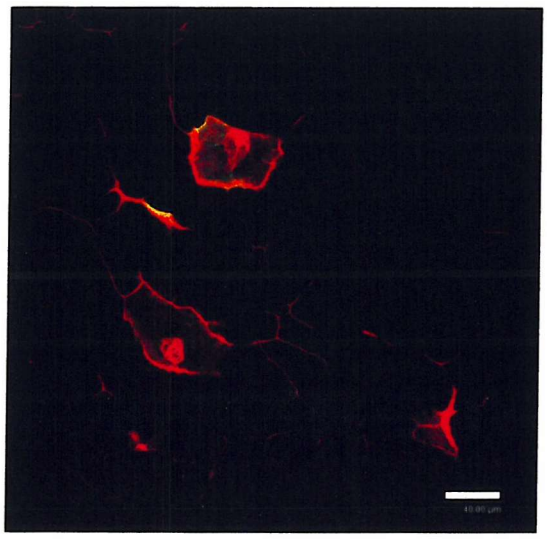
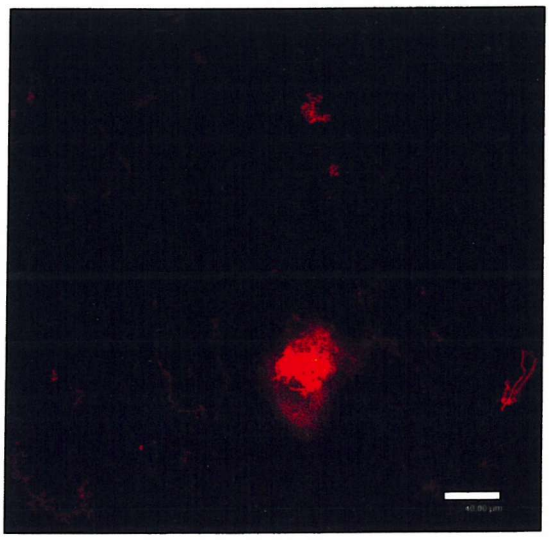


Figure 4.34: Determination of cell viability of meningioma cell monolayers challenged with *E.coli* DSM after six (A) and 24 (B) hours. Over 90% of the monolayer had been destroyed by six hours post-challenge, with the remaining cells staining red due to the uptake of the dye through their damaged membranes. By 24 hours post-challenge, only a few dead cells were observed.

(A) *E.coli* DSM 6hrs post-challenge



(B) *E.coli* DSM 24hrs post-challenge



4.3 The interactions of bacterial pathogens with Chang epithelial cells

To determine whether the interactions of bacterial pathogens with meningioma cell monolayers, as described in Section 4.2, were representative of all human cell types the experiments were repeated with Chang epithelial cells. Chang cells are an established representative epithelial cell line that has been extensively used in various studies of bacterial pathogenesis (Virji *et al.,* 1993a; Virji *et al.,* 1995a; de Vries *et al.,* 1998; Prasadarao *et al.,* 1999a; van Schilfgaarde *et al.,* 2000)

4.3.1 Quantification of bacterial association with Chang cells

The interaction of bacterial pathogens with epithelial cells was initially investigated using quantitative viable counting methods. These interactions were investigated for each bacterial pathogen in at least two independent experiments, with *N.meningitidis* strain MC58-7 included in all challenge studies, in which the similar levels of association of piliated meningococci were used to validate any comparisons between experiments. Unless otherwise stated, Chang cell monolayers were challenged with approximately $1x10^6$ cfu per monolayer.

4.3.1.1 Association of N.meningitidis to Chang cells

In preliminary experiments, monolayers of Chang epithelial cells were challenged with piliated and non-piliated meningococci. The association of both variants with Chang cell monolayers increased over time (Figure 4.35). However, from three to nine hours the numbers of piliated meningococci associating with the epithelial cell monolayer were approximately six fold greater than those of the non-piliated variant ($P \leq 0.005$). Furthermore, by 24 hours, the levels of piliated meningococci associating with Chang cell monolayers had increased by approximately 14 fold, compared with non-piliated meningococci ($P=0.03$).

Monolayers of Chang epithelial cells were then challenged with various concentrations of both *Neisseria meningitidis* MC58-7 and MC58-9 (Fig.4.36). Regardless of the challenge concentration, the levels of association of piliated meningococci with Chang cells were approximately ten fold greater than those of the non-piliated variant at all time points sampled ($P \leq 0.005$). Challenge with the highest concentrations ($1x10^6$ to $1x10^8$ cfu) of either variant resulted in an early and rapid saturation of the monolayer (Figure 4.36). However, with the exception of the lowest concentration of bacteria tested ($1x10^2$ cfu), by nine hours there was no significant difference in the levels of piliated meningococci in cultures of Chang cells infected with 10^4-10^8 cfu per monolayer ($P \geq 0.05$) (Figure 4.36A). This pattern was also apparent with the association of non-piliated variant (Figure 4.36B). Although the levels of the association of piliated and non-piliated meningococci also increased over time for the lowest concentration tested ($1x10^2$ cfu), they were approximately three to five fold lower than any of the other concentrations tested, at both nine and 24 hours ($P \leq 0.05$).

Figure 4.35: Comparison of the levels of association of *N.meningitidis* strain MC58 variants -7 and –9 with Chang cell monolayers. Chang cell monolayers were challenged with approximately $1x10^6$ cfu per monolayer and data is representative of experiments carried out at least three times.

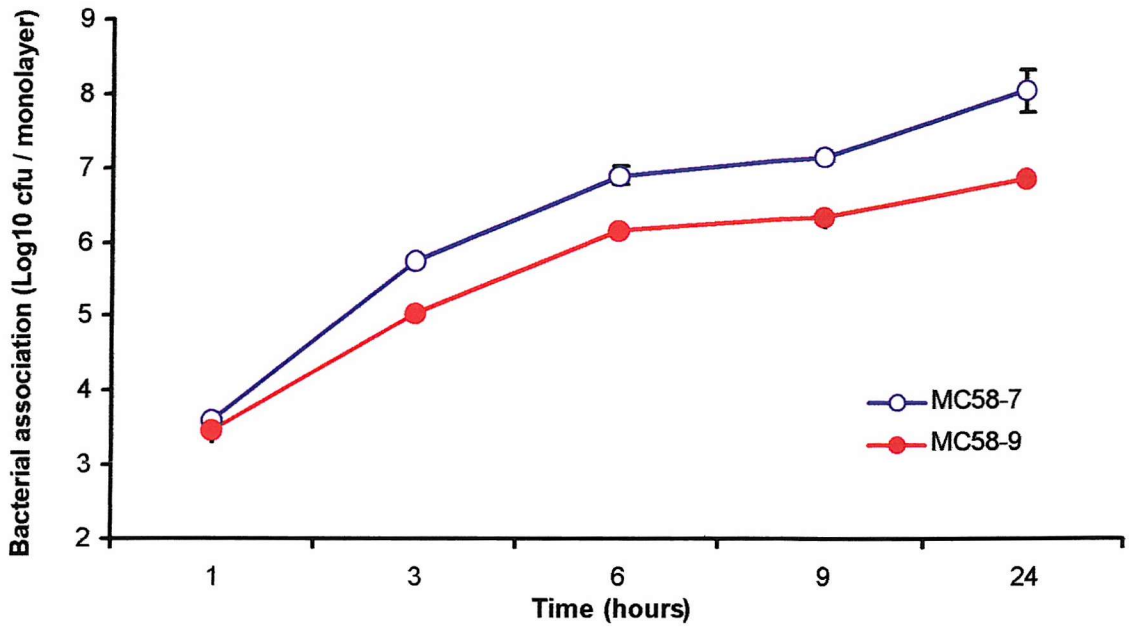
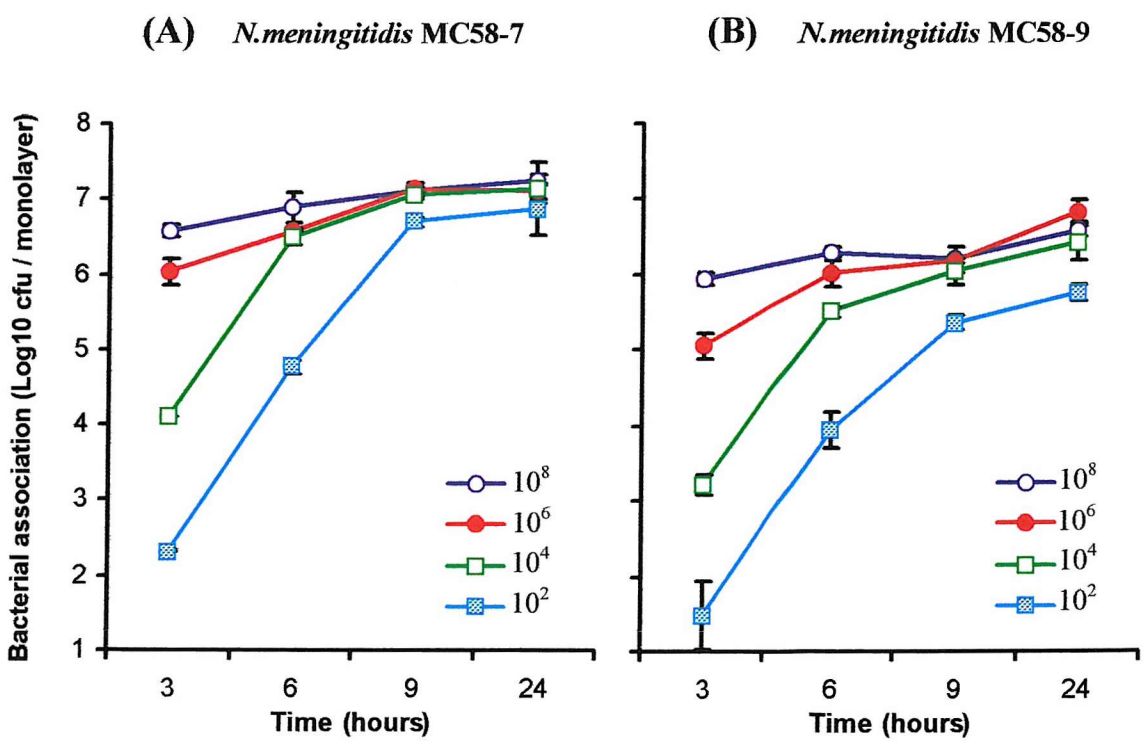


Figure 4.36: The effect of increasing the concentrations of *N.meningitidis* MC58-7 (A) and MC58-9 (B) on the association of bacteria to Chang epithelial cells. Chang cell monolayers were challenged with between approximately $1x10^2$ and $1x10^8$ cfu per monolayer.



The symbols denote the mean and the error bars denote the standard deviation of triplicate samples.

4.3.1.2 *Association of E.coli to Chang cells*

Monolayers of Chang epithelial cells were challenged *E.coli* strains IH3080 and DSM and the levels of association compared with *Neisseria meningitidis* MC58-7 (Figure 4.37). Both *E.coli* strains IH3080 and DSM were found in association with monolayers of Chang cells, and the levels increased over time. In addition, there were no significant differences in the levels of association of either strain at all time points sampled ($P \geq 0.05$). However, the time course was limited to nine hours due to a cytopathic effect to the cell monolayers following prolonged incubation with either *E. coli* strain. By contrast, the levels of piliated meningococci associating with Chang cell monolayers was approximately six and three fold greater than either strain of *E.coli* at three ($P \leq 0.01$) and six ($P \leq 0.001$) hours respectively. However, by nine hours post-challenge there were no significant differences ($P \geq 0.05$) in the levels of association of *E.coli* strain IH3080, DSM and piliated meningococci.

4.3.1.3 *Association of H.influenzae to Chang cells*

Monolayers of Chang epithelial cells were challenged with the acapsulate *H.influenzae* strain RD, the capsulated strain Eagan, and the levels of association of each strain compared with *Neisseria meningitidis* MC58-7 (Figure 4.38). The numbers of acapsulate *H.influenzae* strain RD associating with Chang cell monolayers increased over time, and after three hours there was no significant difference between the levels of association of this strain compared with piliated meningococci ($P \geq 0.05$) (Figure 4.38). Although the association of *H.influenzae* strain Eagan to Chang cells also increased over time, the numbers of bacteria recovered were approximately 300 fold lower than either *H.influenzae* RD or MC58-7 at three and six hours ($P \leq 0.005$). However, by nine hours, there was only a 60-fold difference between the levels of association of the Eagan strain and strain RD and meningococci ($P \leq 0.001$); and by 24 hours post-challenge the levels of association of all three bacteria were similar ($P \geq 0.05$).

4.3.1.4 *Association of S.pneumoniae to Chang cells*

Monolayers of Chang epithelial cells were challenged with the *S.pneumoniae* and the levels of association compared with *Neisseria meningitidis* MC58-7 (Figure 4.39). Although the association of *S.pneumoniae* increased over time (Figure 4.39), the levels of associated bacteria were approximately two to three fold lower than those of piliated meningococci at six hours ($P=0.01$). At subsequent time points, the levels of associated *S.pneumoniae* were consistently less compared with piliated meningococci, being approximately seven and fifteen fold lower at nine ($P=0.002$) and 24 ($P=0.03$) hours respectively.

Figure 4.37: Comparison of the levels of association of *E.coli* strains IH3080 and DSM and *N.meningitidis* MC58-7 with monolayers of Chang epithelial cells.

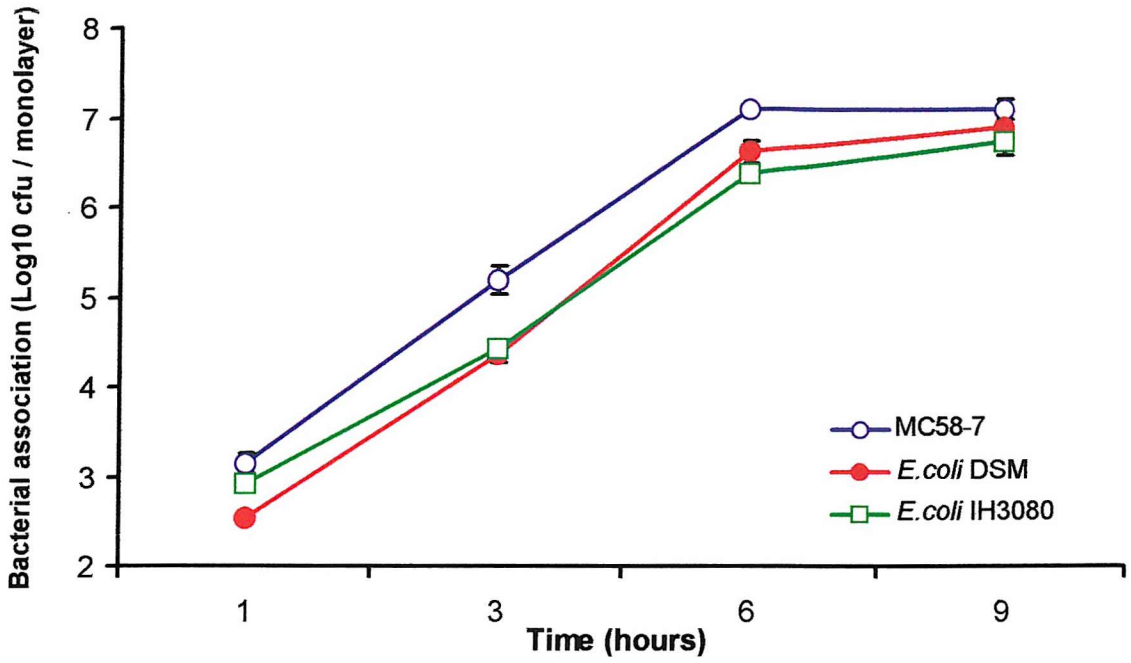
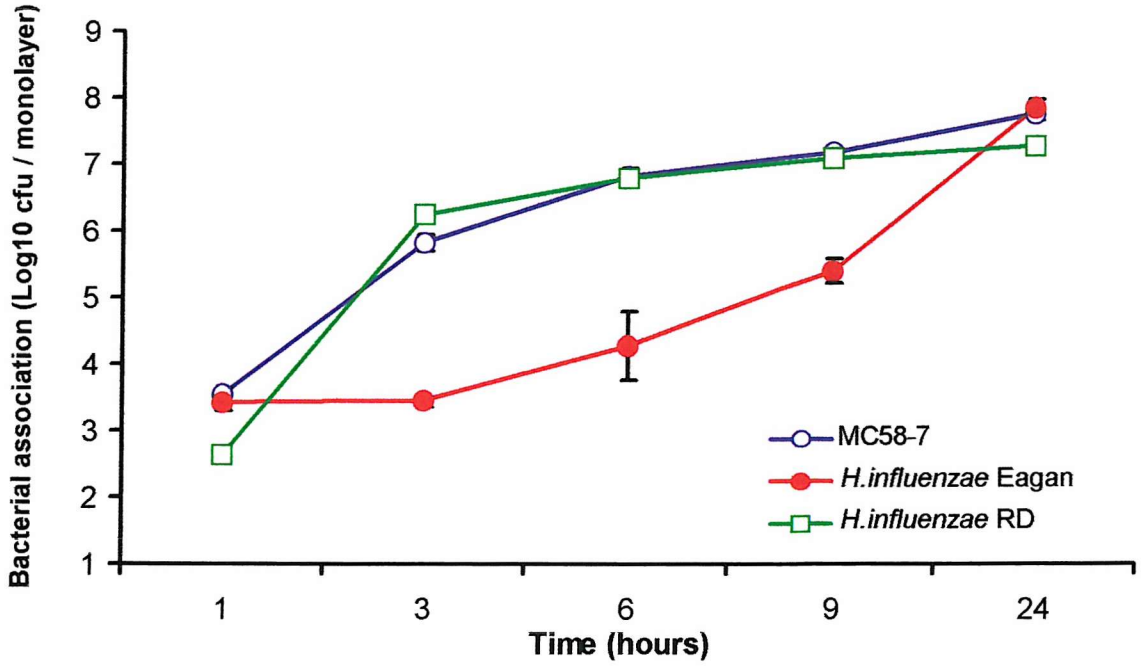


Figure 4.38: Comparison of the association of *H.influenzae* strains Eagan and RD and *Neisseria meningitidis* MC58-7 with monolayers of Chang epithelial cells.



Data is representative of experiments carried out at least three times; the symbols denote the mean and the error bars denote the standard deviation of triplicate samples.

Figure 4.39: Comparison of the association of *S.pneumoniae* and *N.meningitidis* MC58-7 with monolayers of Chang epithelial cells.

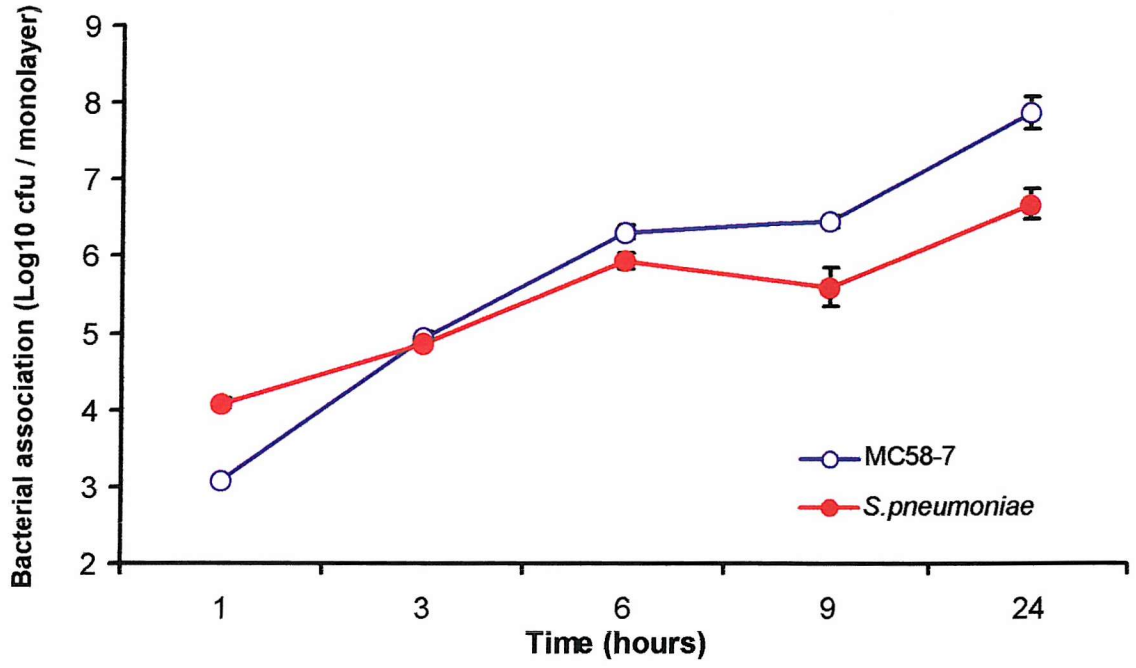
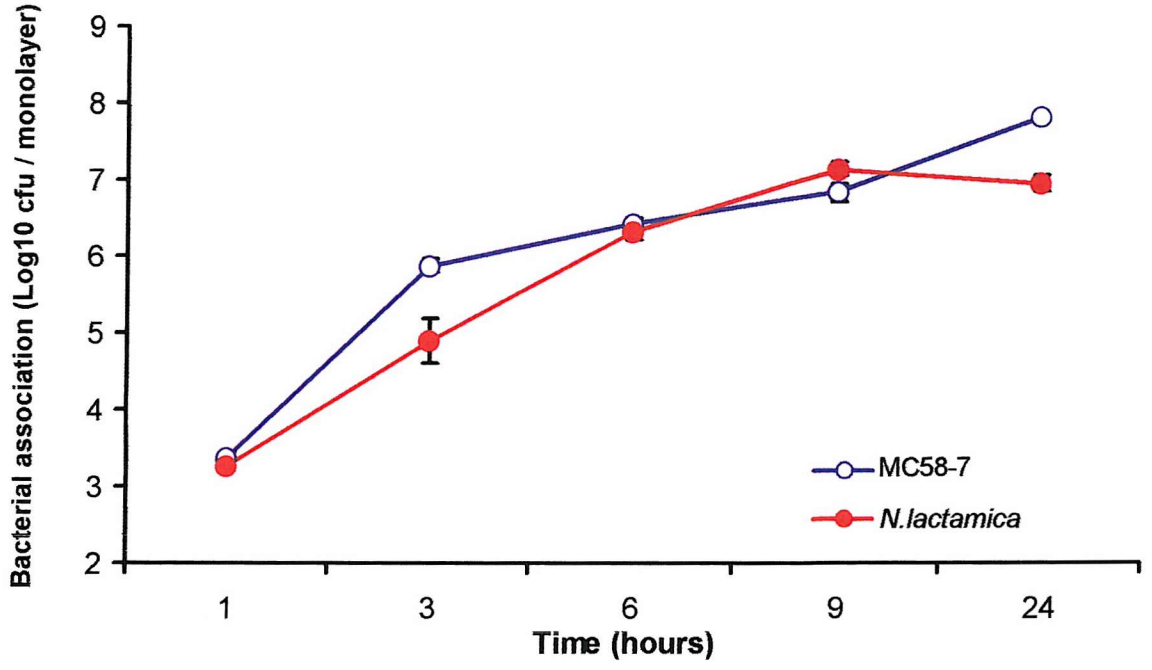


Figure 4.40: Comparison of the association of the commensal organism *Neisseria lactamica* and the meningeal pathogen *Neisseria meningitidis* MC58-7 with monolayers of Chang epithelial cells.



Data is representative of experiments carried out at least three times; the symbols denote the mean and the error bars denote the standard deviation of triplicate samples.

4.3.1.5 Association of N.lactamica to Chang cells

Monolayers of Chang epithelial cells were challenged with *Neisseria lactamica* and the levels of association of this commensal organism compared with that of the meningeal pathogen *Neisseria meningitidis* MC58-7 (Figure 4.40). The levels of association of *N.lactamica* with Chang cells increased over time (Figure 4.40). Although there were significant differences between the numbers of *N.lactamica* and piliated meningococci recovered from the cell monolayers at three, nine and 24 hours ($P \leq 0.05$) the overall dynamics of association of both bacteria were similar.

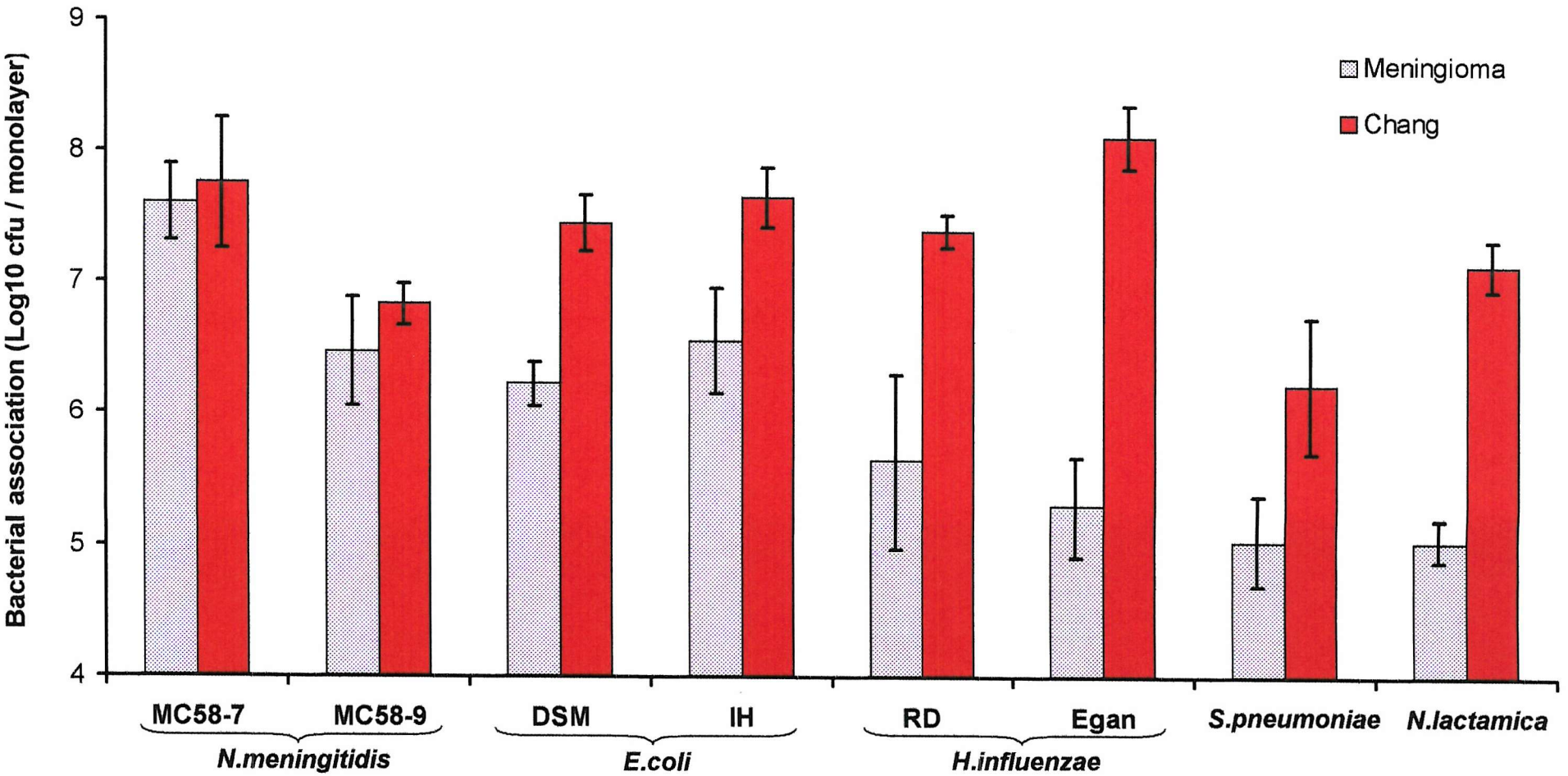
4.3.2 Comparison between the bacterial association with meningioma and Chang cell monolayers

During the course of this study, given the number of independent experiments carried out to investigate bacterial-host cell interactions, it was possible to compare relatively the association of all the different bacteria with both Chang epithelial cells and meningothelial meningioma cells. The mean numbers and standard deviations of bacteria associated with monolayers of either meningioma or Chang cells taken from three independent experiments are shown in Figure 4.41. Data are presented for 24 hours, expect for *E.coli* strains IH3080 and DSM, which were taken at nine hours due to the extensive damage to the monolayers following prolonged incubation with these bacteria.

There was no significant difference in levels association of piliated meningococci with either meningioma or Chang cell monolayers ($P=0.12$). In addition, non-piliated meningococci showed adhered equally to both cell types ($P=0.52$), but at levels approximately 10-fold lower compared with piliated meningococci. However, significant differences were observed between meningioma and Chang cell monolayers for the association of *E.coli*, *H.influenzae*, *S.pneumoniae* and *N.lactamica*. The association of *E.coli* strains IH3080 and DSM with Chang epithelial cells was approximately nine and eighteen fold greater than with meningioma cells respectively ($P \leq 0.00005$).

There was a 23 fold increase in the numbers of *H.influenzae* RD bacteria associating with Chang cells compared to meningioma cells ($P \leq 0.00001$); furthermore, the differences were more pronounced with *H.influenzae* Eagan, which was found associated with Chang cells at levels approximately 500 fold greater than those measured with meningioma cells ($P \leq 0.00005$). Similar trends were observed with *S.pneumoniae* and *N.lactamica*, which associated with Chang cells at levels of eight ($P \leq 0.05$) and 128 ($P \leq 0.00005$) fold higher compared with association to meningioma cell monolayers, respectively.

Figure 4.41: Comparison between meningioma cells and Chang cells for the association of bacteria. The data represent the average numbers and standard deviations from three individual experiments of bacteria associated with human cell monolayers.



4.3.3 Investigation of bacterial invasion of Chang epithelial cells

An important event in the host-pathogen interaction is the initial contact between bacteria and mucosal epithelial cells of the nasopharynx and upper respiratory tract. The barrier properties of Chang epithelial cells against potentially invasive meningeal pathogens were investigated using quantitative viable counting methods following treatment with gentamicin and cytochalasin D. The interactions of each bacterium was investigated in at least two independent experiments, with *N.meningitidis* strain MC58-7 included in all challenge studies, in which the similar levels of association of piliated meningococci were used to validate any comparisons between experiments. Unless otherwise stated, Chang cell monolayers were challenged with approximately $1x10^6$ cfu of bacteria per monolayer

4.3.3.1 Quantification of bacterial invasion of Chang cells

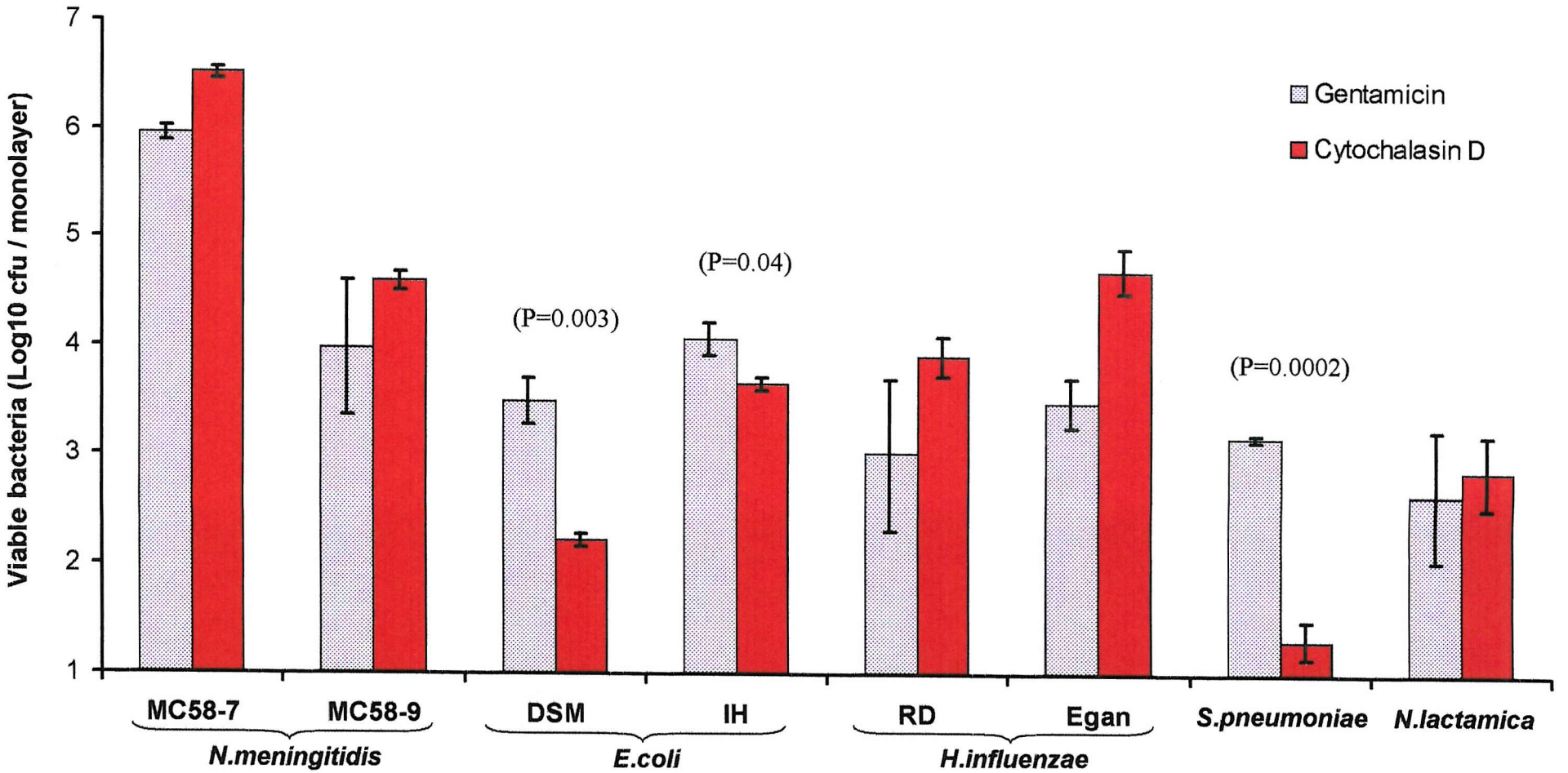
Following treatment with gentamicin, viable bacteria were isolated from Chang cell monolayers challenged with the various organisms (Figure 4.42). However, at 24 hours post-challenge, there was no reduction in the numbers of *N.meningitidis*, *H.influenzae* and *N.lactamica* bacteria recovered from Chang cells pre-incubated with cytochalasin D. Indeed, for *Neisseria meningitidis* and *H.influenzae*, the numbers of viable bacteria recovered actually increased. By contrast, pre-incubation of Chang cell monolayers with cytochalasin D resulted in a 70-fold reduction in the numbers of *S.pneumoniae* (P=0.0002) recovered following gentamicin treatment at nine hours. In addition, pre-incubation with cytochalasin D also resulted in a three and twenty fold reduction in the numbers of *E.coli* IH3080 (P=0.04) and *E.coli* DSM (P=0.003) recovered at nine hours, respectively. No invasion data were available at 24 hours for Chang cells challenged with both *E.coli* strains and *S.pneumoniae* due to extensive cell monolayer disruption. Challenge experiments involving *E.coli* resulted in the acidification of the experimental media after nine hours, as described in Section 4.2.4 for meningioma cells, and the destruction of the Chang cell monolayer between nine and 24 hours. In contrast to meningioma cells, Chang cell monolayers challenged with *S.pneumoniae* exhibited extensive damage at 24 hours, with few remaining cells intact (data not shown).

4.4 Chapter discussion

4.4.1 Summary of results

The association with meningioma cell monolayers of the meningeal pathogens *N.meningitidis*, *E.coli*, *H.influenzae*, *S.pneumoniae* and the commensal organism *N.lactamica* all increased consistently over time. However, there were significant differences between the pathogens in the dynamics of these interactions, with the overall numbers of adherent bacteria used to rank the organisms. *N.meningitidis* MC58-7 and *E.coli* strains DSM and IH3080 adhered to meningioma cell monolayers in the greatest numbers, closely followed by *H.influenzae* strain RD.

Figure 4.42: Comparison of the ability of different bacteria to invade monolayers of Chang epithelial cells. The bars denote the mean and the errors the standard deviation of triplicate samples.



N.meningitidis MC58-9 and *S.pneumoniae* associated with meningioma cells at levels approximately ten-fold lower than those observed with MC58-7 and both *E.coli* strains. In addition, the corresponding adherence of *N.lactamica* and *H.influenzae* strain Eagan were one hundred-fold lower than those observed with MC58-7 and both *E.coli* strains.

The association dynamics of these meningeal pathogens were also investigated with Chang epithelial cell lines. The numbers of *N.meningitidis* MC58-7 associating with Chang cell monolayers were consistent with those observed with meningioma cells, and being approximately ten-fold higher than *N.meningitidis* MC58-9. However, the levels of association of *H.influenzae*, *E.coli*, *S.pneumoniae* and *N.lactamica* to Chang cells were approximately ten to twenty fold higher than the corresponding values with meningioma cells.

With respect to internalisation, only the *E.coli* strain IH3080 was capable of invading meningioma cell monolayers *in vitro*, identified through gentamicin assays and electron microscopy. By contrast, both *E.coli* strains DSM and IH3080 and *S.pneumoniae* were internalised by Chang epithelial cell lines in an actin-dependent manner.

4.4.2 The association of meningeal pathogens with human cell lines

In the current study, established viable count assays comparing the total association of the panel of bacteria with meningioma cells and Chang epithelial cells, demonstrated that the dynamics of adherence were specific to each individual organism. Preliminary experiments demonstrated the significantly increased adherence of piliated meningococci to meningioma cell lines compared to the non-piliated variant, which confirms the observations of Hardy and colleagues (Hardy *et al.*, 2000). Almost identical dynamics of association were seen following the challenge of Chang epithelial cells with piliated and non-piliated meningococci, which is also consistent with previous *in vitro* studies (Virji *et al.*, 1992a; Virji *et al.*, 1995). Although the pilus protein is thought to be the primary bacterial ligand to mediate these interactions, the association of non-piliated meningococci to human cell lines suggests either a spontaneous switch to the piliated phenotype or the involvement of other non-pilus ligands. The dynamic on-off nature of meningococcal pilus production could result in pilus up-regulation from a non-piliated stock (Tinsley & Heckels, 1986; Achtman *et al.*, 1992). However other studies have demonstrated that over 95% of the non-piliated bacteria recovered after infecting either meningioma or human umbilical vein endothelial (HUVEC) cell lines remained non-piliated (Virji *et al.*, 1991a; Hardy *et al.*, 2000). Although increases in the concentration of bacterial inoculum resulted in the earlier association of meningococci to meningioma and Chang cell lines, very high doses resulted in the monolayers rapidly becoming saturated. However, even with high doses of bacteria significant differences in the levels of cellular association between the piliated and non-piliated variants were still evident.

Although the data from this study demonstrates a significant difference in the levels of adhesion between piliated and non-piliated meningococci to both meningioma and Chang cells, it is important to identify the limitations of these experiments. Since the challenge medium is not

replenished through the experimental time course, the cultures will become stagnant once bacterial growth has exhausted the available nutrients. Consequently, the bacteria reaching a stationary growth phase may overshadow the role of the pilus in the adhesion of meningococci to meningioma and Chang cells. Indeed, it may be appropriate to conduct future experiments at lower temperatures to limit the effect of microbial growth on the overall adherence of bacteria to cultured human cells *in vitro*. Although the data from this study suggests that the initial adherence of meningococci to human cells is mediated by pili, other adhesins including Opa and Opc may contribute to the increase in adherence demonstrated at the later time points. Indeed, the sequencing of the meningococcal genome has identified over 500 open reading frames that potentially encode novel surface-exposed or secreted proteins, including homologs of the *H.influenzae* outer membrane proteins Hsf, Hap and Hia (Pizza *et al.*, 2000; van Ulsen *et al.*, 2001). Additionally, the phenotype of the meningioma and Chang cells may also have changed during the experimental time course possibly to reflect these changes in the predominant surface adhesin, *i.e.* up- or down-regulation of specific surface receptor expression.

In contrast to the class I pilus expressed by N.meningitidis MC58-7, the commensal *N.lactamica* expresses class II pili, which are of a distinctively different antigenic type (Diaz *et al.*, 1984; Aho *et al.*, 1997). Nevertheless, *N.lactamica* also exhibited high levels of association with Chang epithelial cells, which is consistent with a commensal bacterium of the human nasopharynx. In contrast to meningococci, *N.lactamica* adhered to meningioma cell lines at very low levels, suggesting that the class II pili may require distinct receptors that may not be present on some cell types. However, Hardy and colleagues demonstrated that *N.meningitidis* stain C114, which expresses class II pili, showed high levels of adherence to cultured meningioma cells, although it was less effective than those with class I pili (Hardy *et al.*, 2000). These data suggest that other bacterial surface structures, possibly including various outer membrane proteins and capsule expression, may contribute to the interactions of pathogenic *Neisseriae* with meningeal cells, and their absence in *N.lactamica* may explain the inability of this organism to cause invasive disease. Indeed, porin and Opa outer membrane protein sequences have been identified in *N.lactamica*, although the absence of corresponding protein production suggests only the potential of this commensal to express these virulence factors (Stern & Meyer, 1987; Ward *et al.*, 1992; Wolff & Stern, 1995). However, *N.lactamica* lacks a number of major virulence factors associated with pathogenic *Neisseriae* including a polysaccharide capsule (Petering *et al.*, 1996), Opc genes (Zhu *et al.*, 2003), IgA1 protease (Muller, 1983) and PilC1 (Pron *et al.*, 1997).

Although class I and class II pili are specific to *Neisseria*, the majority of the other meningeal pathogen panel also expressed some form of surface adhesin. Since *E.coli*, *H.influenzae* and *S.pneumoniae* are all capable of causing pyogenic meningitis in humans, it was hypothesised that these diverse surface adhesins may possibly mediate the adhesion of these pathogens to meningioma and Chang cell lines at similar levels to each other and meningococci. Although the initial association of both *E.coli* strains to meningioma cells was less rapid than the piliated meningococci, the differences were negligible by nine hours post-challenge. This lag

phase in adherence may possibly be a result of a reduced surface expression on leptomeningeal cells of the sialic acid containing glycoconjugate receptors for S-fimbriae compared to the putative pilus receptor CD46. However, high levels of sialic acid expression have been reported on human meningiomas and other cancerous tissues (Sonmez *et al.*, 1995). This suggests that the lag phase in adherence may be due to *E.coli* S-fimbriae having less avidity for their glycoconjugate receptors compared to meningococcal type IV pili and CD46. By contrast, there was no significant difference in the levels of adherence to Chang epithelial cells between *E.coli* K1 and piliated meningococci, suggesting the involvement of additional non-fimbrial adhesions in the association of *E.coli* with epithelial cell lines, including the outer membrane protein Hek (Smith, 2003). A role for S-fimbriae has been identified for the adhesion of *E.coli* K1 with the epithelial lining of the choroid plexi and vascular endothelium, potentially critical stages in the penetration of the B-CSFB (Parkkinen *et al.*, 1988). Indeed, the observation that S-fimbriae are typically only found on *E.coli* strains associated with neonatal meningitis confirms their importance in the pathogenesis of *E.coli* infection (Saukkonen *et al.*, 1988). The age-specific predisposition to *E.coli* K1 meningitis may be associated with the age-dependent expression of binding sites for S-fimbriae within the human CNS. However, the association of *E.coli* K1 to meningioma cells suggests that the absence of meningitis in neonates older than one month may not be due solely to the loss of potential receptors on the meningeal cell surface. This is supported by experiments using the infant rat model of meningitis, which have identified the resistance to high grade bacteraemia in rats of over thirty days as the pivotal point in the protection against *E.coli* K1 meningitis (Parkkinen *et al.*, 1988). Indeed, the deficiencies in humoral and cellular immunity, such as the lack of specific antibodies and immature complement functions (Berger, 1990; Schelonka & Infante, 1998), may contribute to the susceptibility of human neonates to high-grade bacteraemia and consequent *E.coli* K1 meningitis.

Compared with piliated meningococci and *E.coli* K1, both *H.influenzae* strains associated poorly with meningioma cell lines *in vitro*. In addition, there was also a significant difference in the adhesion dynamics between the two *H.influenzae* strains, with the acapsulate *H.influenzae* RD adhering rapidly to meningioma cells, while the levels of the capsulated *H.influenzae* Eagan remained very low throughout the experiments. Similarly, the association of *H.influenzae* Eagan with Chang cells was initially low compared to strain RD, although these differences progressively decreased over time. The reduced association of *H.influenzae* with meningioma cells may be due in part to the low concentration of the phosphorylcholine receptor PAF reported in the CSF of children with bacterial meningitis (Townsend & Scheld, 1994). Despite the fact that the clinical isolates of *H.influenzae* type b obtained from individuals with meningitis have fimbriae, their expression has been reported to be lost during prolonged *in vitro* cultivation of *H.influenzae* strain Eagan (Apicella *et al.*, 1984; Gilsdorf & Ferrieri, 1984; Pichichero, 1984; Mason *et al.*, 1985). By contrast, *H.influenzae* RD is non-pathogenic and lacks the genes for expression of fimbriae (Mhlanga-Mutangadura *et al.*, 1998).

While there have been no previous reports on the interactions of *H.influenzae* with meningeal cells, the current data are consistent with other observations that acapsulate strains associate with both epithelial and endothelial cells more rapidly than capsulated variants (St.Geme & Falkow, 1991; Virji *et al.*, 1991b; Virji *et al.*, 1992b). Indeed, our data suggest that the polysaccharide capsule of *H.influenzae* Eagan may be impeding the interactions of non-fimbrial adhesins, such as phosphorylcholine and surface fibrils, with host cell receptors (St.Geme & Cutter, 1995). However, the data from this study cannot define a precise role for the polysaccharide capsule in the adherence of *H.influenzae* to human cells due to the additional differences between the clinical isolate Eagan and the avirulent strain RD. Indeed, a more appropriate future experiment would be to introduce a mutation in the capsular genes of *H.influenzae* Eagan and compare this acapsular phenotype to the clinical isolate with respect to levels of adherence to meningioma and Chang cells.

By contrast, the low association rates of both *H.influenzae* strains with meningioma cell lines, compared to piliated meningococci, may in part be due to their poor growth in the experimental medium during these challenge experiments, an observation not seen in corresponding experiments with Chang cells. The reason for this reduced growth rate is unclear; it is possible that compounds secreted by meningioma cell lines may reduce the availability of the essential supplements β-NAD and haem to the bacteria, either by binding directly to these proteins or indirectly to their receptors. An alternative explanation may be that meningioma cells secrete antimicrobial compound(s); however, the growth of neither meningococci nor *E.coli* K1 was affected in a similar way. It is possible that meningococci and *E.coli* K1 are more resistant to the antimicrobial activity of meningioma cells than *H.influenzae*, or these pathogens may down-regulate the expression or secretion of these compounds.

S.pneumoniae associated with Chang cells at levels equivalent to piliated meningococci, an observation that is consistent with previous studies demonstrating the high association of *S.pneumoniae* (type 2 capsulated) with Chang cells (Barthelson *et al.*, 1998), A549 respiratory epithelial cells (Rubins *et al.*, 1998) and human bronchial epithelial cells (Adamou *et al.*, 1998). However, the corresponding levels of pneumococcal association with meningioma cell lines were significantly lower than those with Chang epithelial cells, and were similar to the levels of association of non-piliated meningococci and acapsulate *H.influenzae*. This may be a result of a low expression of the polymeric immunoglobulin receptor (pIgR) and / or PAF on leptomeningeal cells, hence reducing the availability of receptors for the pneumococcal CbpA adhesin. Indeed, a previous study has reported the low concentration of PAF in the CSF of children with bacterial meningitis (Townsend & Scheld, 1994), suggesting that the expression of PAF is restricted to endothelial cells, PMNLs and macrophages (Camussi *et al.*, 1987; Bussolino *et al.*, 1988; Bussolino *et al.*, 1992).

In summary, all of the pathogenic bacteria adhere to meningioma cell lines *in vitro,* although there were significant quantitative differences between the pathogens. These differences are probably due to the expression of various surface adhesins and capsule by the individual pathogens (Table 1.1), and the expression of corresponding receptors on the host cells. Indeed, it is important to recognise that due to changes in the expression of bacterial ligands during infection, the bacterial strains selected for this study represent only a snapshot of all the possible phenotypes in the disease process. In addition, the entire panel of meningeal pathogens adhered in high levels to a representative epithelial cell line, an ability associated with colonisation of the nasopharynx.

4.4.3 Do bacterial pathogens invade human meningeal cell lines?

Meningitis is characterised by the presence of bacteria within the SAS, with little or no spread to the underlying brain. One hypothesis is that the leptomeninges, and in particular the pia mater, provides a barrier to prevent the spread of meningeal pathogens from the CSF to the underlying cerebral tissue. In this study, the meningioma model was used to investigate this potential barrier function *in vitro*.

Despite high levels of adherence, piliated meningococci did not invade meningothelial meningioma cells, confirming the results from a previous study (Hardy *et al.*, 2000). Although viable bacteria were recovered from meningioma cell monolayers following gentamicin treatment, pre-incubation with cytochalasin D (CD) did not significantly reduce these numbers. These data suggest the absence of true invasion, since the cytoskeletal activity of the cells, in particular the polymerisation of actin filaments, which has been shown to be necessary for the invasion of epithelial and endothelial cells is inhibited by CD (Virji *et al.*, 1991b). It is also likely that the efficiency of the gentamicin assay was hampered by the large numbers of adhering bacteria associated in clumps on the monolayer or protected in crypts between cells. Similar observations have been reported with gonococci and HeLa cells (Bessen & Gotschlich, 1986) and with meningococci with HUVECs (Virji *et al.*, 1991a). Significantly, the uptake of inert latex beads by meningioma cells has been demonstrated *in vitro*, suggesting that the lack of meningococcal internalisation was not due to the inability of the cells to phagocytose foreign material (Feurer & Weller, 1991). No invasion of meningioma cells was observed for *E.coli* DSM, *H.influenzae* Eagan and RD or *S.pneumoniae,* demonstrating that the leptomeninges is also likely to provide a barrier *in vivo* to these pathogens. Furthermore, as expected the commensal organism *N.lactamica* was also unable to penetrate monolayers of meningioma cells. However, the presence of bacteria in the subpial space *in vivo* during infection has not been conclusively shown, but it is possible that it occurs over time as a result of the inflammatory response and cell death within the pia mater during the later stages of disease.

By contrast, the encapsulated *E.coli* K1 strain IH3080 was internalised by meningioma cell lines, which was demonstrated by the significant reduction in the numbers of bacteria recovered following pre-incubation with CD and gentamicin treatment. Internalisation of bacteria

was subsequently confirmed by TEM. However, no invasion was observed with *E.coli* strain DSM, a faecal isolate obtained from a healthy individual. Various strains of *E.coli* associated with meningitis can be isolated from the faeces of a large proportion of healthy individuals (Sarff *et al.*, 1975). The fact that *E.coli* DSM bacteria possess a K1 capsule and have been shown to cause bacteraemia in the infant rat (Pluschke *et al.*, 1983) suggest that it is capable of invasive disease. However, a recent study by Johnson and colleagues has identified that while a number of faecal *E.coli* K1 strains express a similar repertoire of virulence factors to those expressed by strains isolated from the CSF, including S-fimbriae and haemolysin, other isolates are deficient in one or more of them (Johnson *et al.*, 2001). Indeed, the lack of invasion of meningeal cells by *E.coli* DSM may be the due to the absence of specific invasin(s). For example, the prevalence of the invasin ibeA has been found to be significantly increased in *E.coli* K1 strains from the CSF compared to faecal and blood isolates (Huang *et al.*, 1995; Bingen *et al.*, 1997). Alternatively, it is possible that *E.coli* DSM could invade meningioma cell lines, but this event may be masked by the rapid and severe cytotoxicity to host cells associated with this strain. Significantly, the invasion of meningioma cells, and hence traversal of the leptomeningeal barrier *in vivo*, may be an important stage in the pathogenesis of *E.coli* K1 meningitis. The possible rapid spread of pathogenic bacteria into the underlying cerebral tissue may be a contributory factor to the high mortality and morbidity associated with neonatal *E.coli* meningitis.

To compare and contrast the data obtained from meningothelial meningioma cell lines with another site important in the pathogenesis of infection, the ability of these meningeal pathogens to invade Chang epithelial cell monolayers was also investigated. The invasion of Chang cells by *E.coli* IH3080, *E.coli* DSM, and *S.pneumoniae* was consistent with previous reports in the literature, which suggested that these bacteria used a transcellular mechanism for traversing the nasopharyngeal mucosa (Geelen *et al.*, 1993; Cundell *et al.*, 1995; Smith, 2003). Although, no meningococci were observed invading Chang epithelial cells in the current study, previous studies have demonstrated that piliated meningococci traverse nasopharyngeal epithelial cells via a transcellular pathway (de Vries *et al.*, 1996; Merz & So, 2000). However, the invasion of epithelial cell lines has only been demonstrated for acapsulate meningococci (Virji *et al.*, 1992c), suggesting that the down-regulation of polysaccharide capsule expression is essential for internalisation. Indeed, the majority of meningococcal strains isolated from the nasopharyngeal mucosa have been shown to express a non-capsulate phenotype (Craven, 1980). The presence of capsular polysaccharide on the *N.meningitidis* strains MC58-7 and MC58-9 used in the current study may be masking the surface adhesins Opa and Opc, which are involved in intimate adhesion to and invasion of epithelial cells through interactions with CD66 and heparan sulfate proteoglycan receptors respectively (Virji *et al.*, 1996; de Vries *et al.*, 1998). The absence of Chang cell invasion by both *H.influenzae* strains is consistent with previous studies that have demonstrated that the traversal of the nasopharyngeal mucosal epithelium by *H.influenzae* is via a paracellular pathway (Stephens & Farley, 1991; Quagliarello & Scheld, 1992).

4.4.4 Cytopathic effects of meningeal pathogens on human cells

There are two major forms of cell death: apoptosis and necrosis. Apoptosis involves an inherent cellular programme of cell death, and at the tissue level produces little or no inflammation due to the engulfment of apoptotic cells by neighbouring cells, especially macrophages. By contrast, necrosis results entirely from circumstances outside of the cell and causes local inflammation through the release of cellular contents into the extracellular fluid (Studzinski, 1999). In addition, apoptosis cell death differs from necrosis on a morphological basis. The key morphological features of apoptosis include the shrinkage of cells away from their neighbours, the blebbing of the plasma membrane and cytoplasmic and nuclear condensation (Wilson & Potten, 1999). By contrast, necrotic cells exhibit an increased cellular water content and thus volume, which is subsequently followed by membrane rupture and cell lysis (Studzinski, 1999).

The challenge of both meningioma and Chang cell lines with *E.coli* K1 strains DSM and IH3080 resulted in rapid and severe damage to the cell monolayers. The few surviving cells were rounded with severely contracted cytoplasm, which suggests that the mechanism of cell death was bacterial-induced apoptosis and not necrosis. However, in addition to these morphological characteristics further direct evidence of apoptosis, such as chromosomal DNA fragmentation and increased intracellular caspase activity, is required to confirm this method of cell death. In addition to the classical virulence factors of S-fimbriae and K1 capsule, the serotype of *E.coli* associated with meningitis of neonates (O18:K1:H7) also expresses the toxin haemolysin, which may be responsible for the severe damage to the cell monolayers observed in this study. Indeed, haemolysin has been shown to exhibit a wide spectrum of cytocidal activity, damaging granulocytes (Bhakdi *et al.*, 1989), monocytes (Bhakdi *et al.*, 1990), endothelial cells (Suttorp *et al.*, 1990) and epithelial cells (Mobley *et al.*, 1990). The action of the toxin is through insertion into the lipid bilayer forming discrete hydrophilic transmembrane pores (Menestrina & Ropele, 1989), which alter the permeability of the target cell membrane and eventually leading to cell lysis (Stanley *et al.*, 1998). In addition, the physical damage caused to the plasma membrane by haemolysin may indirectly induce apoptosis in the host cell (Gao & Kwaik, 2000). Alternatively, the rapid multiplication of these *E.coli* K1 strains *in vitro* may cause human cell cytotoxicity through an indirect mechanism, such as the rapid acidification of the culture medium.

The challenge of meningioma cell lines with $1x10^8$ cfu of *S.pneumoniae* also resulted in damage to the cell monolayers. Although the severely contracted cytoplasm of all the cells is a characteristic of apoptosis, again further direct evidence of apoptosis is required to confirm this method of cell death. However, the majority of cells challenged with $1x10^6$ cfu remained viable and only began showing signs of cytotoxicity after 24 hours. These data are in agreement with a previous study in which the viability of National Cancer Institute (NCI) respiratory tract epithelial cells fell from 88% to 0% following 24 hours challenge with $1x10^6$ and $1x10^8$ cfu pneumococci respectively (Hakansson *et al.*, 1996). This rapid loss of cell viability was also observed in bovine

brain microvascular endothelial cells (Zysk *et al.*, 2001) and bovine pulmonary artery endothelial cells (Rubins *et al.*, 1992) challenged with high doses ($1x10^7$-$1x10^8$ cfu) of *S.pneumoniae*. In these cells, the shrinkage of cell cytoplasm was observed after six hours and apoptotic endothelial cells detected by 24 hours.

Pneumococci produce a wide range of toxic compounds that could potentially contribute to this cell death, including cell wall components and various toxins (Paton *et al.*, 1993). Indeed, two virulence factors unique to *S.pneumoniae* are the exotoxins pneumolysin and hydrogen peroxide (H_2O_2). Although the role of these two major toxins in interactions with the leptomeninges are unknown, both have been identified as inducing the rapid apoptosis of primary tissue from the rat hippocampus and human microglial and neuronal cell lines (Braun *et al.*, 2001). Indeed, the permanent loss of neurons in the hippocampus is likely to contribute to a high percentage of survivors of pneumococcal meningitis suffering from neurological sequelae (Zysk *et al.*, 1996; Tauber *et al.*, 1997). Hydrogen peroxide and pneumolysin have been shown to trigger apoptosis through rapid mitochondrial damage and the subsequent release of the apoptosis-inducing factor (AIF), which mediates large-scale DNA fragmentation and hypodiploidy (Cochrane, 1991; Braun *et al.*, 2001; Braun *et al.*, 2002). The haemolytic toxin pneumolysin may also contribute to cell cytotoxicity through the recognition of cholesterol in the eukaryotic cell membrane and subsequent formation of high molecular weight pores in the membrane (Mitchell *et al.*, 1997). In future studies the cytotoxic effects of the toxins hydrogen peroxide and pneumolysin on cultured meningioma cell lines could be investigated.

However, a central issue for the *in vivo* significance of this bacterial-induced host cell death is the ability of the toxins to invade the site of damage. Indeed, pneumococci do not directly penetrate the hippocampus during meningitis but accumulate in high numbers in the SAS. However, due to the close proximity of the CSF-containing lateral ventricles to the hippocampus, neurons could be directly exposed to high levels of locally secreted bacterial toxins such as H_2O_2 (Daum et al., 1978). By contrast, pneumolysin is located in the cytoplasm and is not actively secreted from the bacteria (Johnson, 1979). The observations of the current study suggest that at high concentrations of *S.pneumoniae* the normally inert cell wall-degrading enzyme, autolysin, may become activated and cause autolysis of bacterial cells. In addition to the degradation products of the cell wall that are toxic to neurons (Kim et al., 1995), the action of autolysin will also release cytoplasmic contents from the pneumococcus, including the potent pneumolysin toxin. Indeed the significance of autolysin-induced bacterial cell lysis could be investigated in future studies though the use of a specific autolysin inhibitor such as N-bromosuccinimide (Carvalho *et al.*, 1987).

In summary, all of the panel of pathogenic bacteria adhered to human leptomeningeal cells, although they differed significantly in their individual kinetics of adhesion to meningioma cell lines, which is possibly due to their differing expression of surface adhesins and / or capsular polysaccharide. The entire panel of meningeal pathogens also adhered in high levels to a representative epithelial cell line, which demonstrated an ability clearly associated with the early colonisation of the nasopharynx. In addition, there were significant differences between the panel of bacteria in their ability to invade leptomeningeal and Chang epithelial cells and their cytotoxicity towards human cell lines *in vitro*. The potential consequences of these differences will be investigated further in the next chapter, focusing on the stimulation of inflammatory mediators from challenged human cells.

CHAPTER 5 THE INDUCTION OF INFLAMMATORY MEDIATORS FROM HUMAN CELLS

Bacterial meningitis is characterised by an acute compartmentalised inflammatory response, initiated by the presence and proliferation of pathogenic bacteria within the SAS. The mediators of this immune response are pro-inflammatory cytokines and chemoattractant chemokines, although the exact molecular mechanisms are still unclear. While many resident and infiltrating cell types are capable of producing cytokines and chemokines within the SAS, the role of the leptomeninges in this inflammatory response is unknown. However, the meningioma cell model of the human leptomeninges allowed the investigation into the nature of the proinflammatory cytokines and chemokines induced and the contribution of the leptomeninges to the inflammatory response.

5.1 The induction of pro-inflammatory cytokines and chemokines from human meningioma cells following challenge with bacterial pathogens

In order to investigate the role played by cells of the leptomeninges in the inflammatory response, the induction of cytokines and chemokines from meningioma cells challenged with a variety of bacterial pathogens was investigated.

5.1.1 Profile of cytokines and chemokines produced by meningioma cells following challenge with *N.meningitidis*.

Recently, Christodoulides and colleagues (Christodoulides *et al.*, 2002) identified the significant up-regulation of mRNA from meningioma cells challenged with meningococci for the proinflammatory cytokine IL-6 and the chemokines IL-8 and MCP-1. In addition, lower levels of mRNA up-regulation were seen for the chemokine RANTES and the cytokine growth factor GM-CSF. The secretion and accumulation of individual cytokine and chemokine proteins from meningioma cells was investigated in this study, focusing upon those that exhibited an up-regulation in mRNA levels following bacterial challenge.

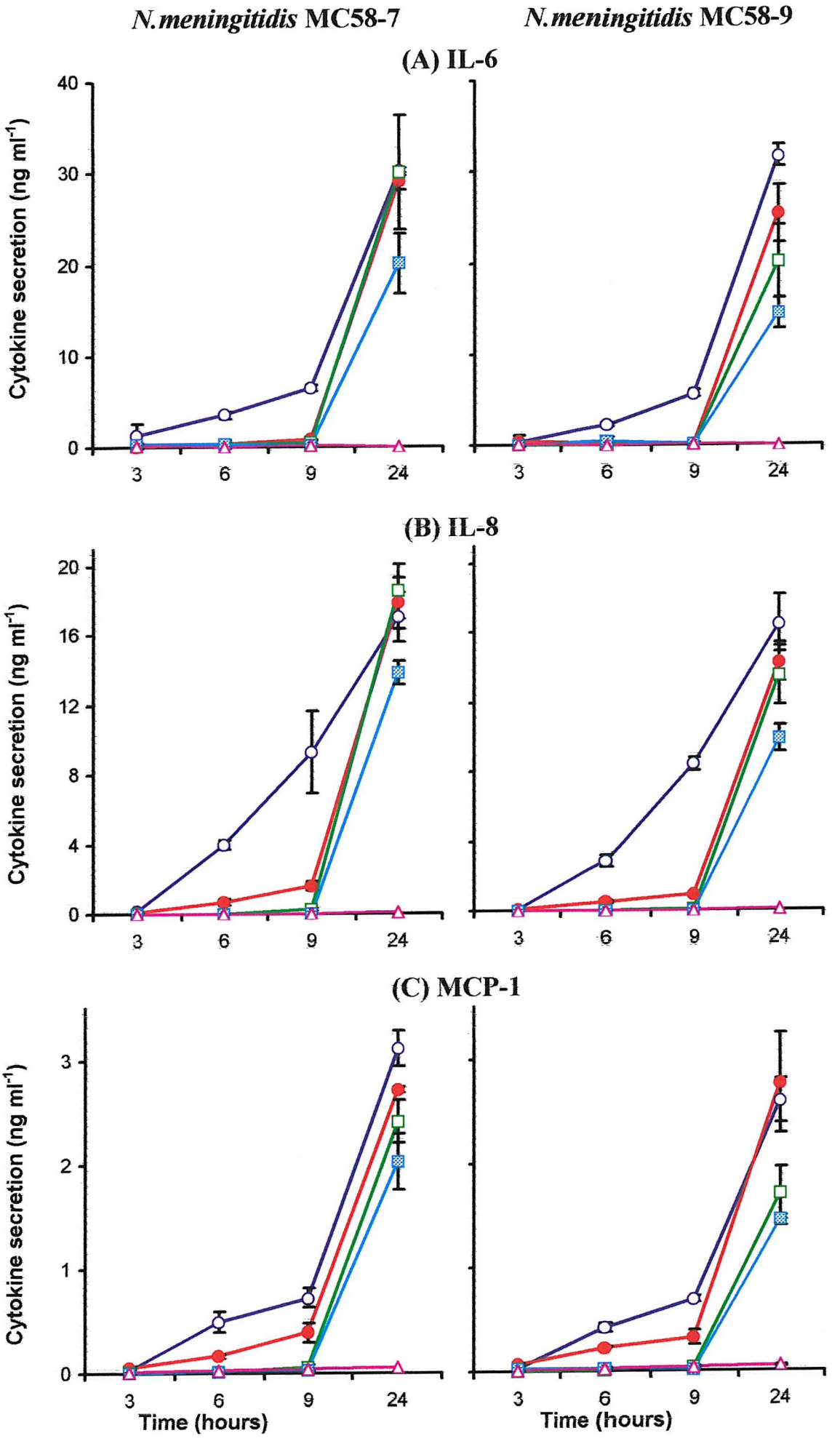
Initially, the role of bacterial association in modulating the secretion of cytokine and chemokine proteins from meningioma cells was investigated. The meningioma cell line M66 was challenged with a range of concentrations of meningococcal strains MC58-7 ($Pil^+Cap^+Opa^+Opc^+$) and MC58-9 ($Pil^-Cap^+Opa^+Opc^+$) over an extended time course. This is in an attempt to mimic the events that occur in the SAS, whereby the initial low concentration of bacteria entering the SAS is followed by unchecked bacterial proliferation. The dose-dependent kinetics of cytokine and chemokine protein secretion from meningioma cell monolayers following challenge with piliated and non-piliated meningococci are shown in Figure 5.1. The increased adherence of bacteria to cell monolayers, associated with the expression of pili, had no effect on the kinetics or

quantitative secretion of either IL-6 or IL-8 from meningioma cells challenged with meningococci ($P \geq 0.05$) (Figure 5.1A and 5.1B). The highest concentration ($1x10^8$ cfu ml^{-1}) of bacteria induced early increases in the levels of IL-6 and IL-8, which at six and nine hours were approximately ten-fold higher than those induced by any other concentration (Figure 5.1A and 5.1B). The intermediate ($1x10^6$ cfu ml^{-1}) and lower ($1x10^4$ and $1x10^2$ cfu ml^{-1}) concentrations exhibited a significant time lag in the secretion of IL-6 and IL-8, with protein secretion occurring between nine and 24 hours. However, by 24 hours there was no significant difference ($P \geq 0.05$) between the levels of protein accumulation induced by the various concentrations of bacteria except for the lowest concentration, which was approximately two-fold less. Non-challenged, control cell monolayers exhibited no significant levels of either IL-6 or IL-8 protein secretion.

The induction of MCP-1 from challenged meningioma cells was similar to both IL-6 and IL-8 in that there were no significant differences in the kinetics or levels of secretion induced by either piliated or non-piliated meningococci (Figure 5.1C). Although smaller amounts of MCP-1 protein were secreted from challenged meningioma monolayers compared to IL-6 and IL-8, these levels were approximately 60 fold greater than the amounts in the control cells. The highest concentration of meningococci induced a ten-fold increase in MCP-1 at the early time points compared with the levels induced by the lower doses, although it was only two-fold higher than those induced by the intermediate concentration. In contrast to IL-6 and IL-8 there were still significant differences ($P \leq 0.05$) in the levels of MCP-1 accumulation between the various concentrations although these differences were only approximately two-fold.

In contrast to IL-6, IL-8 and MCP-1 a time lag in the secretion of both RANTES and GM-CSF was observed with all of the challenge concentrations, with significant protein secretion occurring between nine and 24 hours (Figure 5.1D and 5.1E). Increasing the concentration of piliated meningococci from $1x10^2$ to $1x10^6$ cfu per monolayer resulted in an approximately two to three-fold and three to seven-fold increase in the secretion of RANTES and GM-CSF respectively at 24 hours post-challenge. However, challenge with the highest concentration of piliated meningococci resulted in the significantly lower levels of RANTES and GMCSF secretion compared to those of the intermediate concentration ($P \leq 0.005$). In addition, although non-piliated meningococci increased RANTES secretion above control levels, the amount of accumulated protein was approximately two to four-fold lower compared to piliated meningococci with no significant difference between the various concentrations ($P \geq 0.05$) (Figure 5.1D). In contrast, regardless of the concentration of bacteria, non-piliated meningococci did not induce the secretion of GM-CSF from meningioma cells above control levels (Figure 5.1E).

Furthermore, neither the challenge of meningioma cell monolayers with piliated nor non-piliated meningococci induced the secretion of IL-1α, IL-1β, TNF-α, MIP-1α, MIP-1β, TGF-β or IL-10 (data not shown).


N. meningitidis MC58-7
N. meningitidis MC58-9
(A) IL-6
(B) IL-8
(C) MCP-1
Cytokine secretion (ng ml-1)
Time (hours)
0
10
20
30
40
4
8
12
16
1
2
3
6
9
24

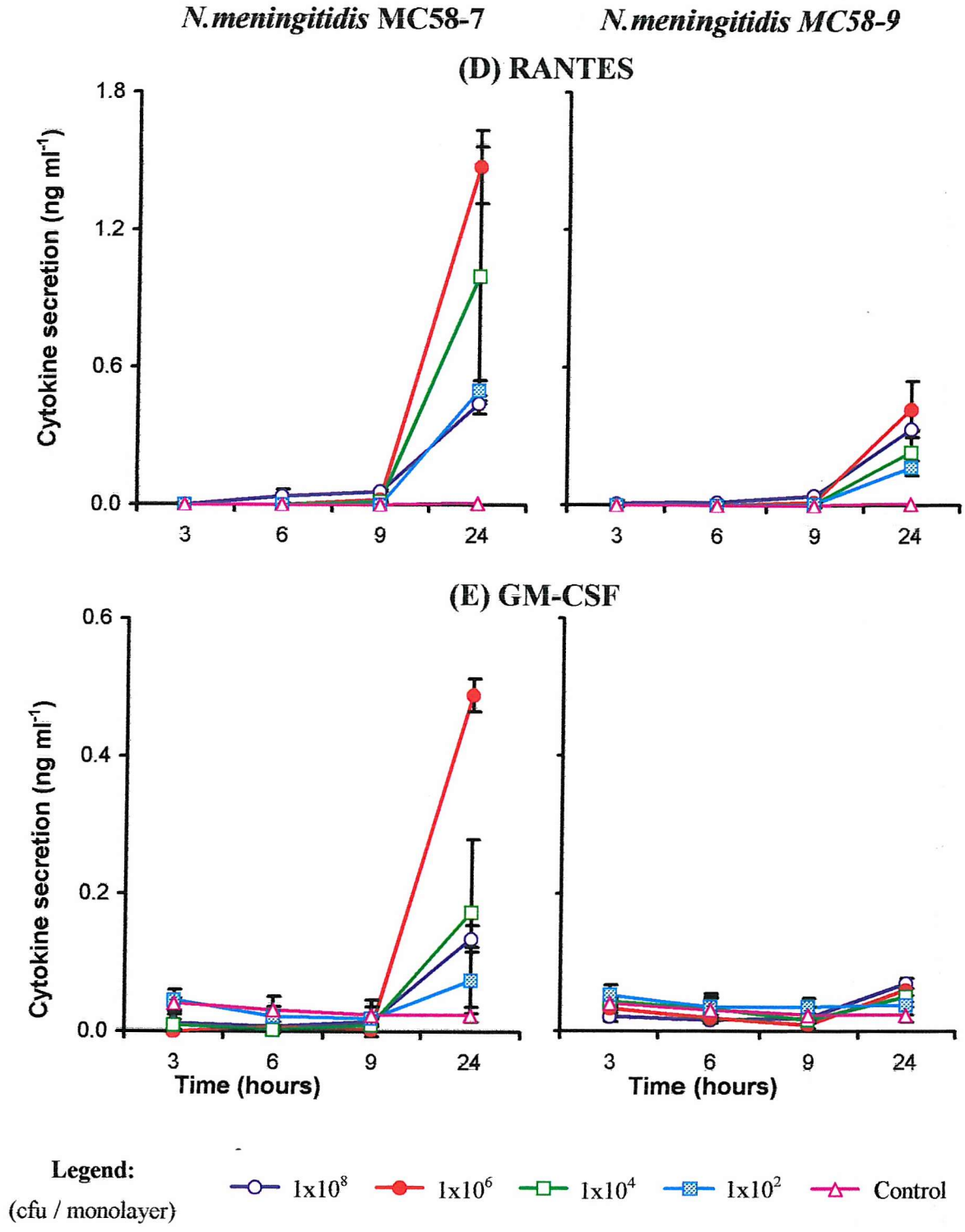


Figure 5.1: The challenge of the meningioma cell line M66 monolayers with *N.meningitidis* MC58-7 and MC58-9 induced the secretion of the pro-inflammatory cytokine IL-6 (A), the chemokines IL-8 (B), MCP-1 (C) and RANTES (D) and the cytokine growth factor GM-CSF (E).

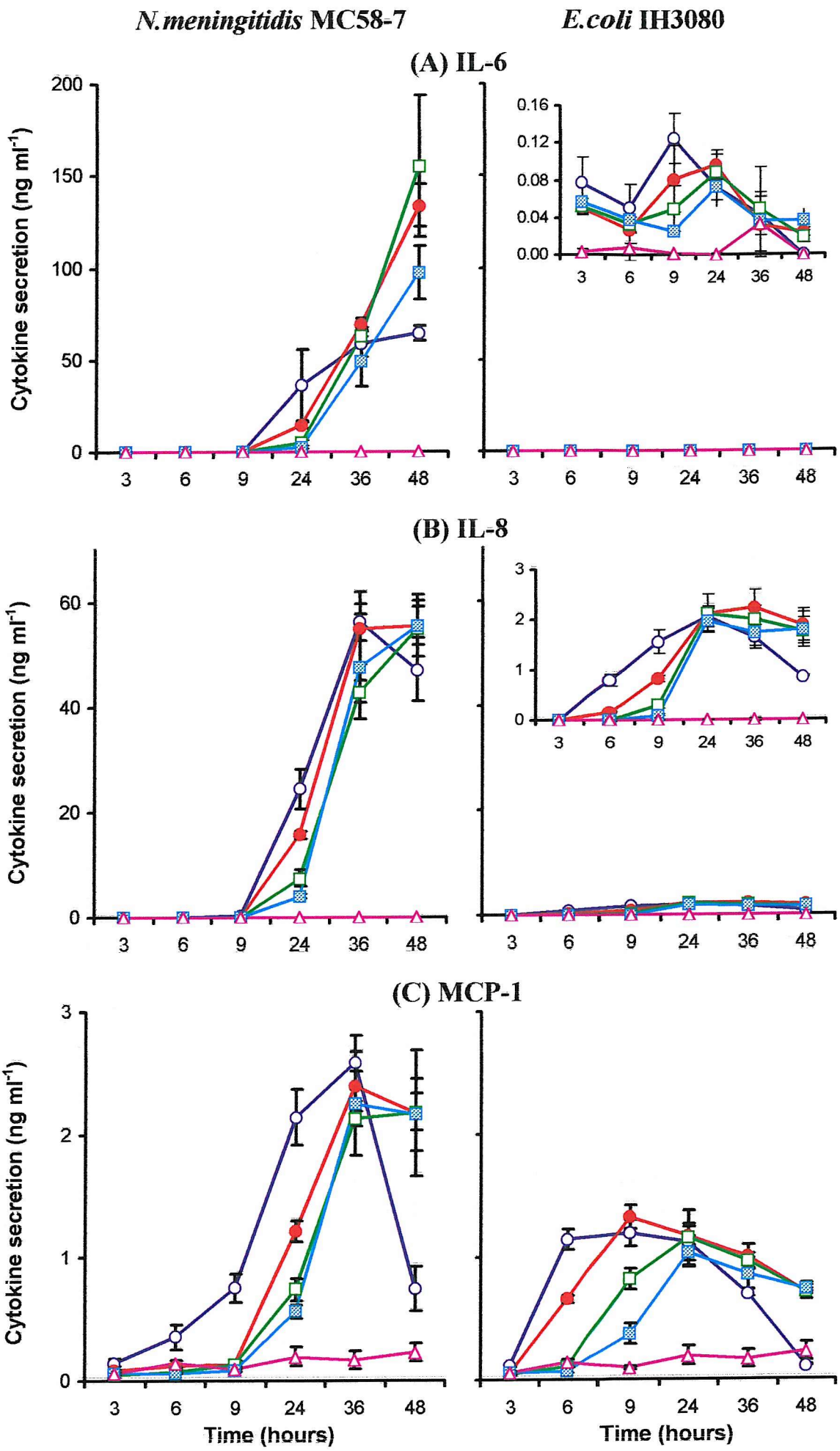
5.1.2 Profile of pro-inflammatory cytokines and chemokines produced by meningioma cells following challenge with other meningeal pathogens.

Having investigated the pro-inflammatory cytokines and chemokines induced from meningioma cell lines following challenge with meningococci, the study was extended to include the other meningeal pathogens *E.coli*, *H.influenzae*, *S.pneumoniae* and the non-pathogenic *N.lactamica*. Where possible the time course was extended to include 36 and 48 hour time points to investigate further the accumulation of pro-inflammatory cytokines and chemokines.

5.1.2.1 Profile of pro-inflammatory cytokines and chemokines produced by meningioma cells following challenge with E.coli IH3080.

In contrast to the experiments described in Section 5.1.1, the maximum challenge concentration of $1x10^8$ cfu per monolayer was replaced with a very low concentration (10 cfu), due to the extensive damage caused to the monolayer observed with high doses of *E.coli* strains IH3080 and DSM (Section 4.2.4). The dose-dependent kinetics of cytokine and chemokine protein secretion from meningioma cell monolayers following challenge with the *E.coli* IH3080, an encapsulated clinical isolate from the CSF of a neonate presenting with meningitis, compared to piliated meningococci are shown in Figure 5.2. Although the secretion of IL-6 was induced by challenge of meningioma cell monolayers with *E.coli* IH3080, the levels were very low ($\leq$0.2ng ml^{-1}) and significantly lower than the $\geq$100ng ml^{-1} induced by piliated meningococci ($P\leq0.005$) (Figure 5.2A). However, a dose dependent effect was observed, with 100-fold increases in the inoculum concentration of *E.coli* IH3080 resulting in the approximate two-fold increase in the secretion of IL-6 at nine hours ($P\leq0.01$), although by 24 hours these differences were no longer significant ($P=0.3$). In addition, the extended time course showed that the accumulation of IL-6 induced by the challenge with piliated meningococci continued to increase after 24 hours, although the highest concentration of bacteria did begin to plateau at 36 hours.

The challenge of meningioma cell monolayers with *E.coli* IH3080 induced the secretion of IL-8, the levels of which at 24 hours were approximately 500 fold higher than that of the control ($P\leq0.005$) (Figure 5.2B). However, these levels of approximately 2ng ml^{-1} were significantly lower than the $\geq$50ng ml^{-1} induced by piliated meningococci at 24 hours ($P\leq0.005$). Although, the highest concentration of *E.coli* IH3080 induced approximately five to ten-fold higher levels of IL-8 secretion at six and nine hours than any other concentration, by 24 hours these differences were no longer significant ($P=0.9$). In addition, the extended time course shows that the accumulation of IL-6 induced by the challenge with piliated meningococci continues to increase after 24 hours, peaking at 36 hours after which it begins to plateau.


N.meningitidis MC58-7
E.coli IH3080
(A) IL-6
(B) IL-8
(C) MCP-1
Cytokine secretion (ng ml-1)
Time (hours)
200
150
100
50
0
60
40
20
3
2
1
0.16
0.12
0.08
0.04
0.00
3
6
9
24
36
48

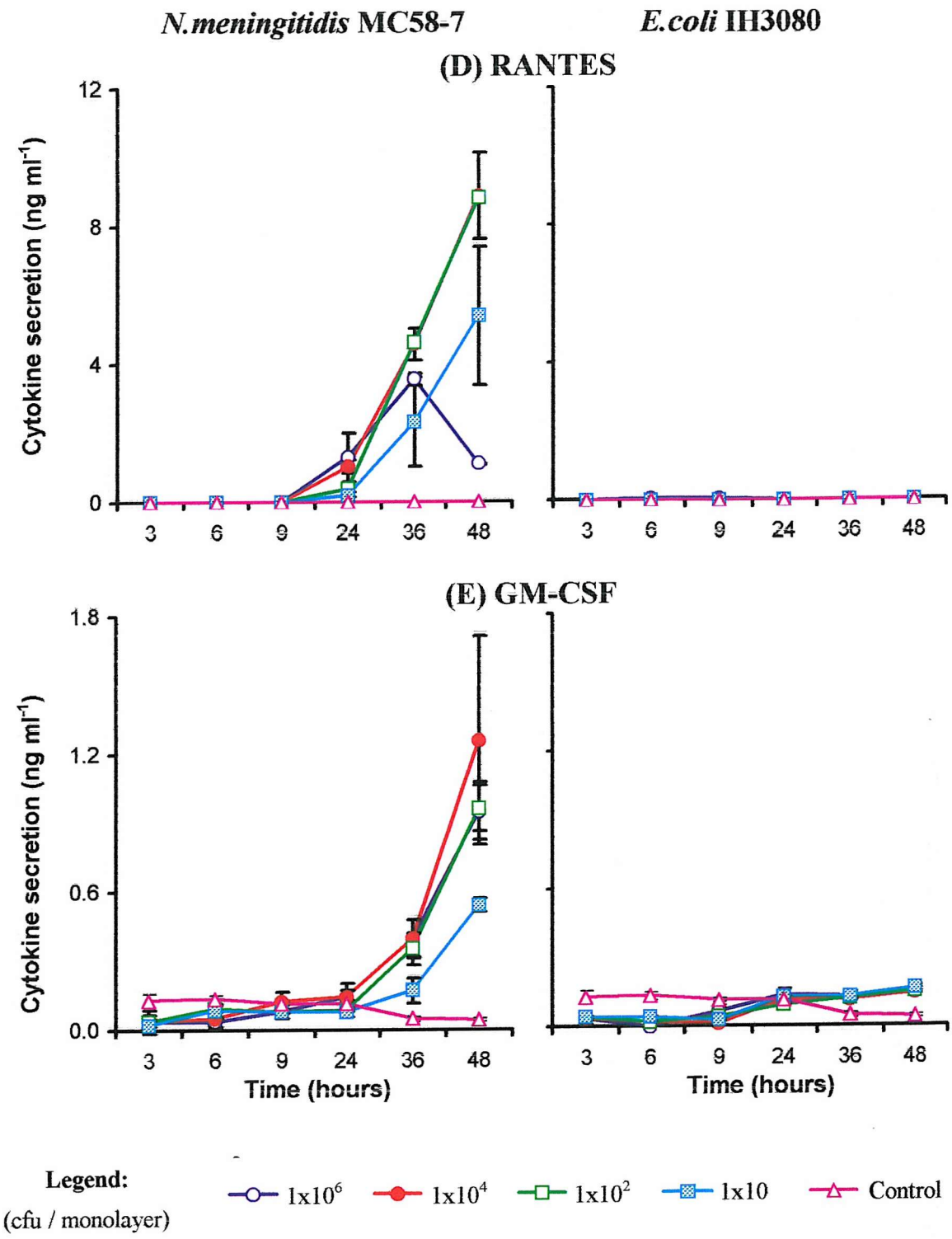


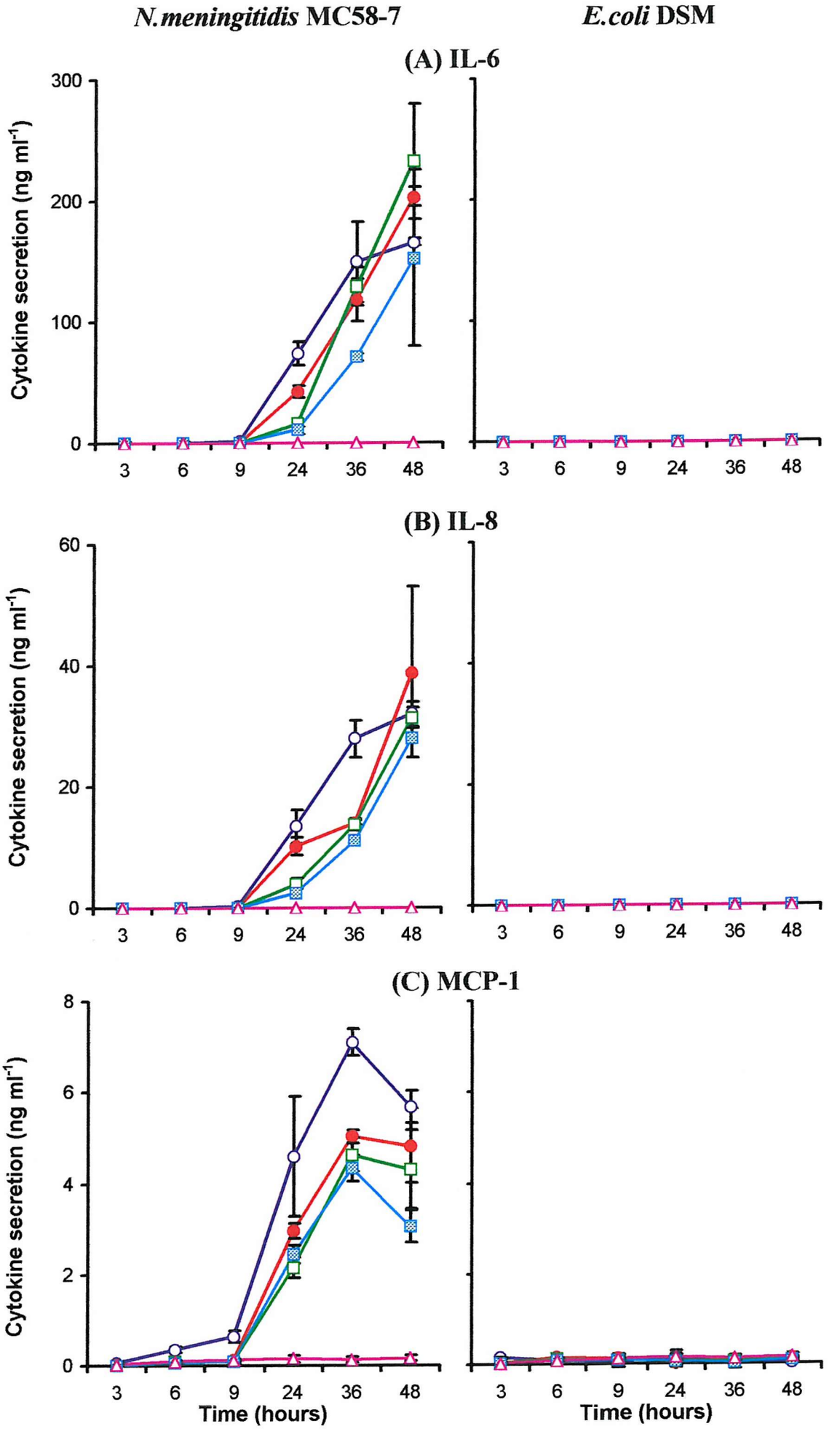
Figure 5.2: Comparison of the secretion of pro-inflammatory cytokines and chemokines from the meningioma cell line M66 induced by *E.coli* IH3080 and *N.meningitidis* MC58-7.

Challenge of meningioma cell monolayers with the highest and intermediate concentrations of *E.coli* IH3080 resulted in the secretion of MCP-1 protein after only six hours and reaching maximal secretion after nine hours (Figure 5.2C). Although there was a two-fold difference in the levels of MCP-1 secretion between the highest and intermediate concentrations of *E.coli* IH3080, the levels were both six-fold greater than those of piliated meningococci at six hours ($P \leq 0.05$). The lower concentrations of *E.coli* IH3080 exhibited a time lag in the secretion of MCP-1, with protein secretion occurring at nine hours, at which time point there was a two-fold difference between the concentrations but the levels were six-fold greater than those of piliated meningococci. However, by 24 hours there was no significant difference ($P=0.7$) between the levels of protein accumulation induced by the various concentrations of *E.coli* IH3080, after which the levels progressively reduced over the extended time course. In addition, the extended time course shows that the accumulation of MCP-1 induced by the challenge with piliated meningococci continued to increase after 24 hours, with there being no significant difference between the concentrations at 36 hours ($P=0.2$) after which the levels begin to fall.

Regardless of the concentration of bacteria the challenge of meningioma cell monolayers with *E.coli* IH3080 did not induce the secretion of either RANTES or GM-CSF (Figures 5.2D and 5.2E). In addition, the extended time course shows that the accumulation of RANTES and GMCSF induced by the challenge with piliated meningococci continued to increase from 24 to 48 hours.

5.1.2.2 Profile of cytokines and chemokines produced by meningioma cells challenged with E.coli DSM.

The *E.coli K1* strain DSM, which was originally isolated from a faecal specimen from a healthy individual, but has been shown to be pathogenic in the infant rat model of bacteraemia, was investigated as a comparison to the clinical isolate *E.coli* IH3080. The dose-dependent kinetics of cytokine and chemokine protein secretion from meningioma cell monolayers following challenge with *E.coli* DSM compared to piliated meningococci are shown in Figure 5.3. In contrast to *E.coli* IH3080, the challenge of meningioma cell monolayers with *E.coli* DSM did not result in the induction of IL-6, IL-8, MCP-1 or RANTES secretion. Although low-level secretion of GM-CSF was measured from meningioma cells challenged with *E.coli* DSM, the levels were not significantly different from those from uninfected control cells ($P \geq 0.05$). In addition, no up-regulation of IL-6, IL-8, MCP-1, RANTES or GM-CSF mRNA was observed from meningioma cells challenged with *E.coli* DSM at either six or nine hours (data not shown).


N.meningitidis MC58-7
E.coli DSM
(A) IL-6
(B) IL-8
(C) MCP-1
Cytokine secretion (ng ml-1)
Time (hours)
300
200
100
0
60
40
20
8
6
4
2
3
6
9
24
36
48

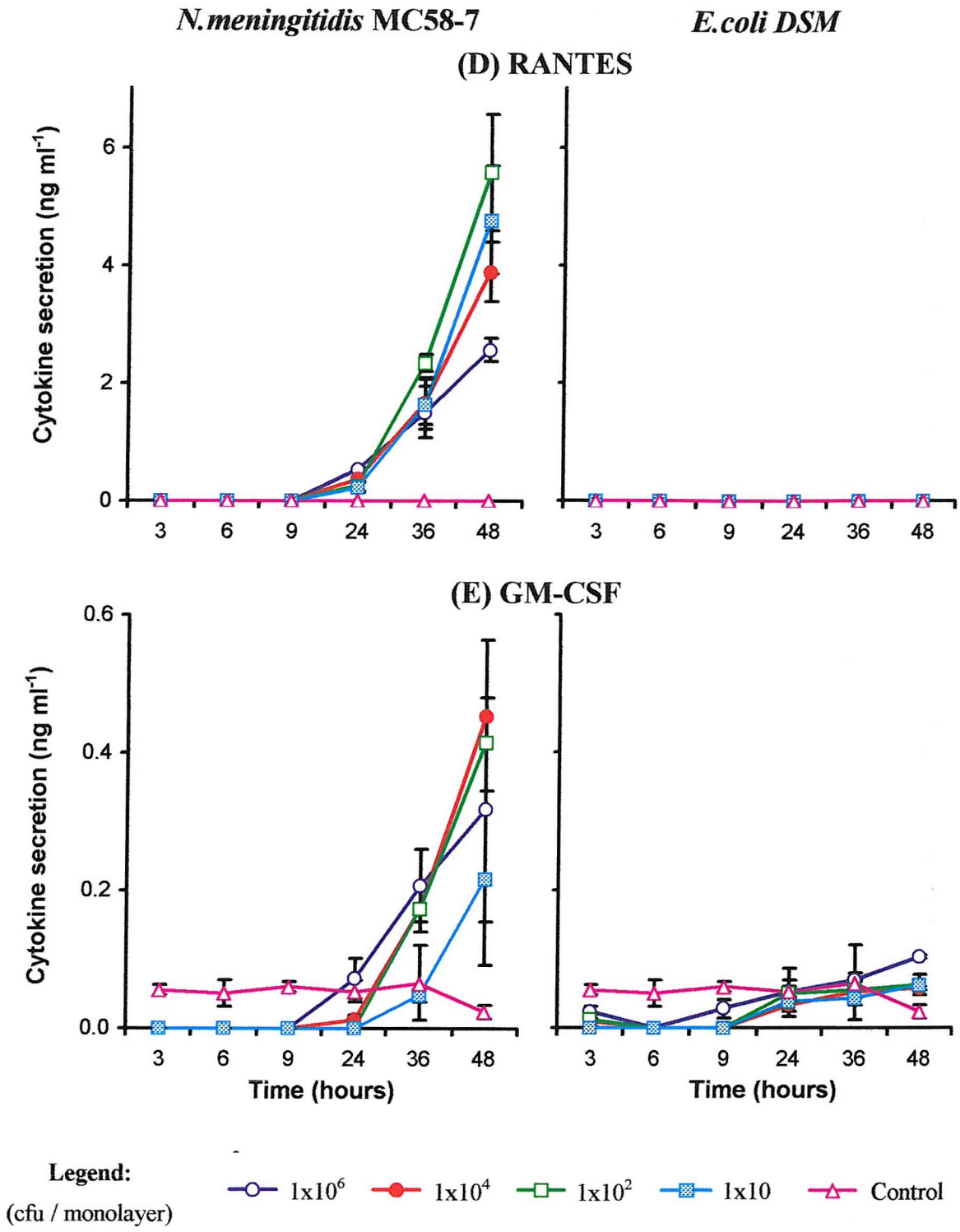


Figure 5.3: Comparison of the secretion of pro-inflammatory cytokines and chemokines from the meningioma cell line M66 induced by *E.coli* DSM and *N.meningitidis* MC58-7.

5.1.2.3 *Profile of pro-inflammatory cytokines and chemokines produced by meningioma cells following challenge with H.influenzae Eagan.*

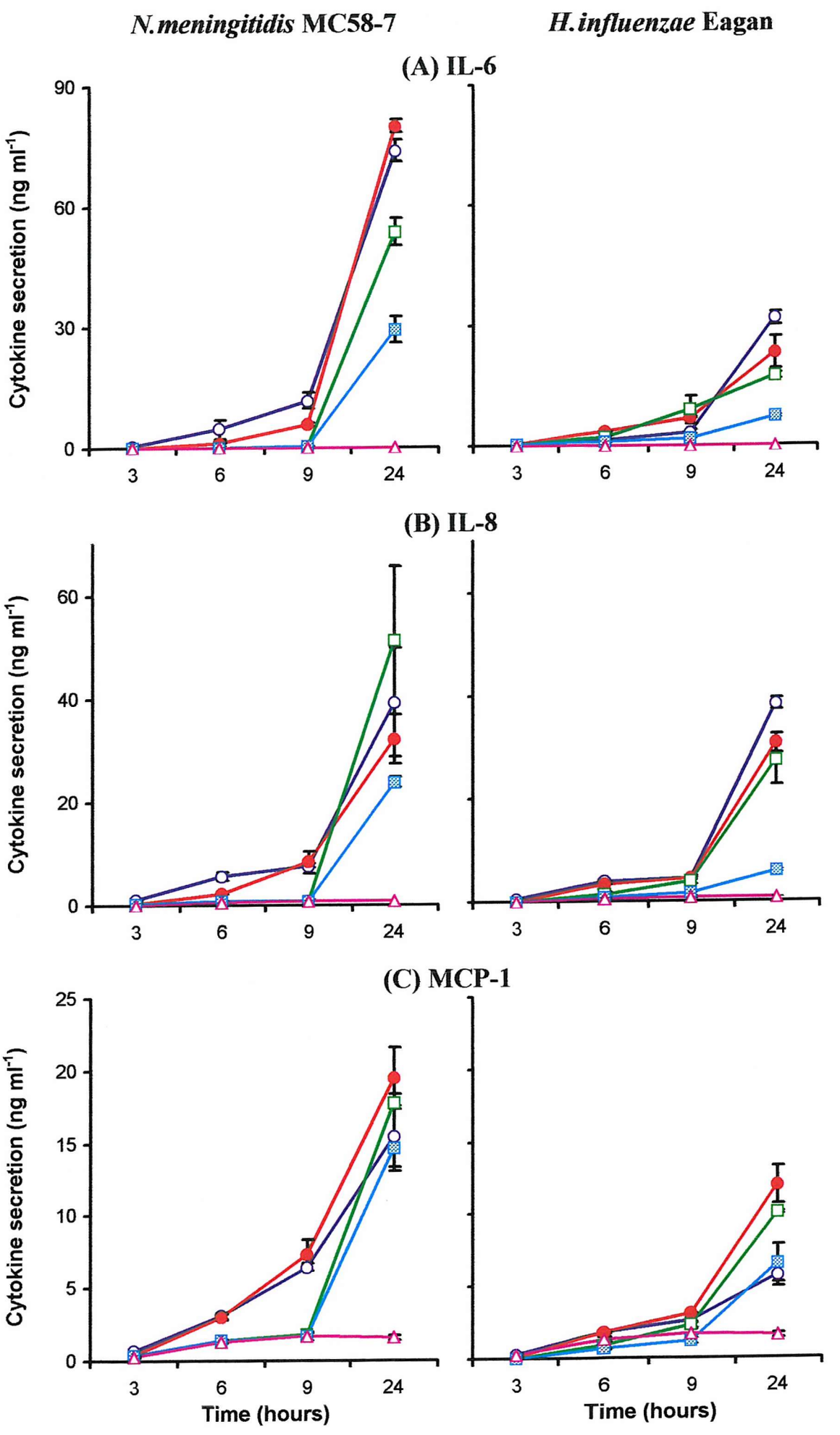
The dose-dependent kinetics of cytokine and chemokine protein secretion from meningioma cell monolayers following challenge with between $1x10^2$ and $1x10^8$ cfu per monolayer of *H.influenzae* Eagan, an encapsulated type b clinical isolate from the CSF of a patient with meningitis, and piliated meningococci are shown in Figure 5.4. The challenge of meningioma cells with between $1x10^4$ and $1x10^6$ cfu per monolayer resulted in the early induction of IL-6 secretion, the levels of which at nine hours were approximately two-fold and five-fold greater than the highest and lowest concentration respectively (Figure 5.4A). Although the highest concentration of bacteria exhibited a slight lag at nine hours in the secretion of IL-6, by 24 hours the levels of protein accumulated were approximately two to four-fold higher than any other concentration. Overall, the levels of IL-6 secretion induced from meningioma cells by *H.influenzae* Eagan were approximately three-fold lower than the levels induced by piliated meningococci at 24 hours.

The highest and intermediate concentrations of *H.influenzae* Eagan induced the early secretion of IL-8 from meningioma cells, the levels of which by six hours were approximately three-fold higher than those observed with the lower concentrations of bacteria (Figure 5.4B). By 24 hours, with the exception of the lowest concentration, there was no significant difference between the levels of protein accumulation induced by *H.influenzae* Eagan and piliated meningococci ($P \geq 0.05$). Although the level of IL-8 secretion induced by the lowest concentration of *H.influenzae* Eagan increased over time, by 24 hours it was approximately five-fold lower than any other concentration tested ($P \leq 0.05$).

Only the highest and intermediate concentrations of *H.influenzae* Eagan induced the secretion of MCP-1 from meningioma cells above the control levels at six hours (Figure 5.4C). By nine hours all but the lowest concentration of bacteria had induced the secretion of MCP-1, although there was no significant difference in the levels between the concentrations ($P \geq 0.05$). Maximal protein secretion was measured at 24 hours, although the highest concentration only induced levels of MCP-1 similar to the lowest concentration ($P=0.4$). Overall, the levels of MCP-1 secretion induced from meningioma cells by *H.influenzae* Eagan were approximately two-fold lower than the levels induced by piliated meningococci at 24 hours.

Although the challenge of meningioma cell monolayers with *H.influenzae* Eagan induced the secretion of RANTES at 24 hours, the levels were significantly lower than those seen with IL-6, IL-8 and MCP-1 and approximately six-fold lower than those induced by piliated meningococci (Figure5.4D). However, challenge with the highest concentration of *H.influenzae* Eagan resulted in the significantly lower level of RANTES secretion compared to that of the intermediate concentration ($P \leq 0.001$) but was similar to the lowest concentration ($P=0.7$).

Although the secretion of GM-CSF was induced from meningioma cell monolayers by all but the lowest concentration of *H.influenzae* Eagan, the levels were very low (≤ 0.1ng ml^{-1}) and not significantly above that of the control ($P \geq 0.05$) (Figure 5.4E).


N. meningitidis MC58-7
H. influenzae Eagan
(A) IL-6
(B) IL-8
(C) MCP-1
Cytokine secretion (ng ml-1)
Time (hours)
0
30
60
90
20
40
5
10
15
25
3
6
9
24

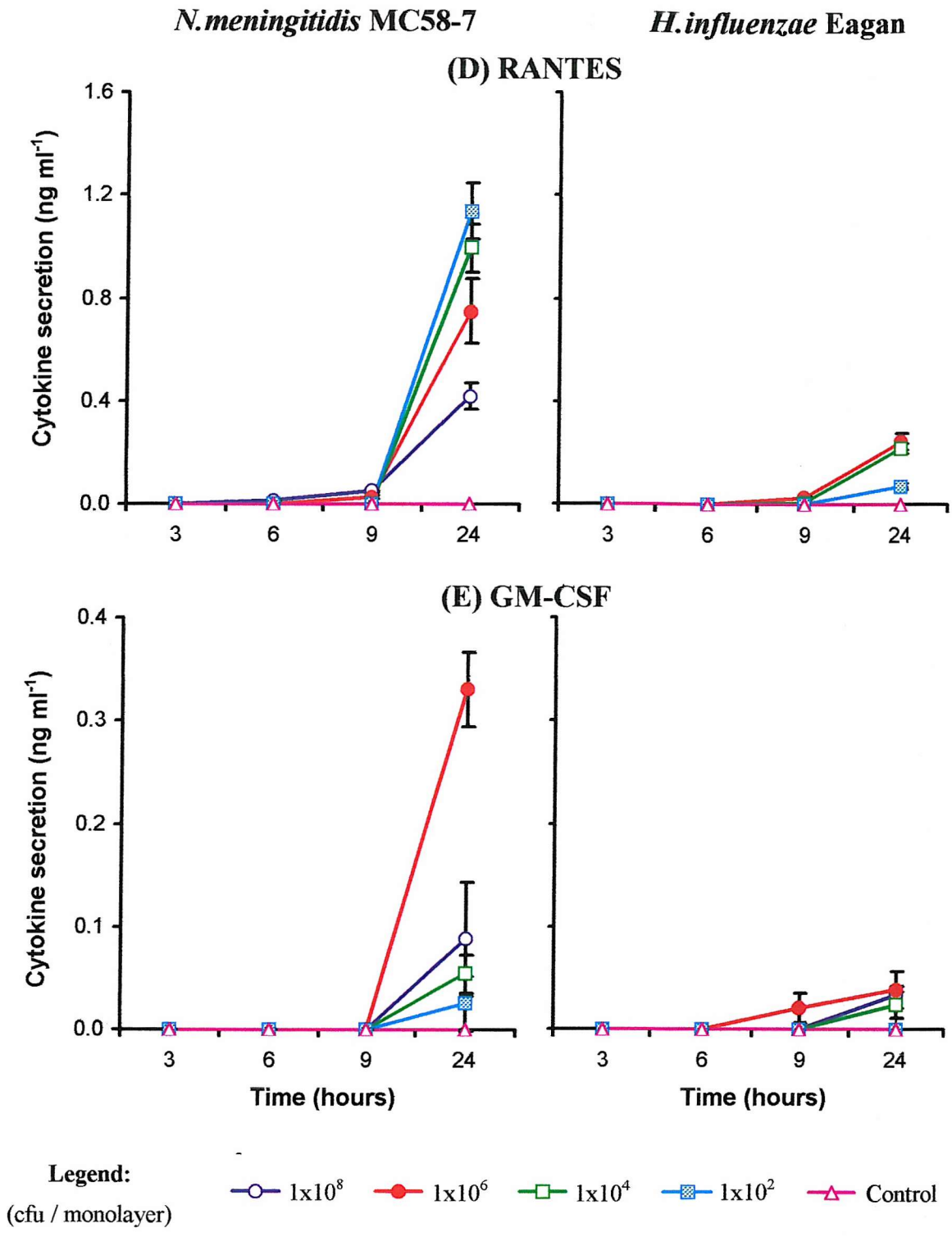


Figure 5.4: Comparison of the secretion of pro-inflammatory cytokines and chemokines from the meningioma cell line M66 induced by *H.influenzae* Eagan and *N.meningitidis* MC58-7.

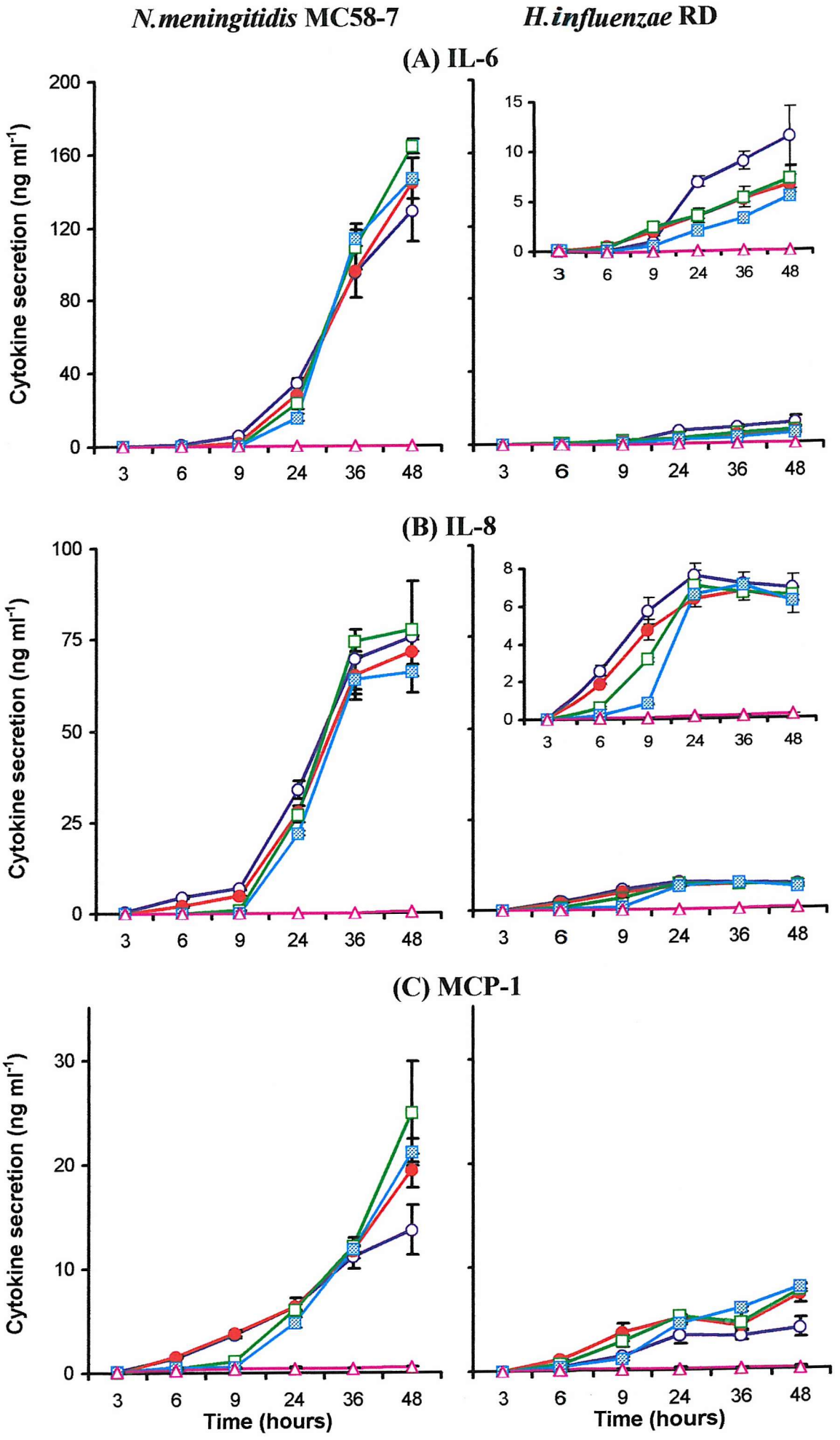
5.1.2.4 *Profile of cytokines and chemokines produced by meningioma cells following challenge with H.influenzae RD.*

The *H.influenzae* strain RD, which is an acapsulate, spontaneous mutation of a type d bacterium derived from a CSF sample taken from an individual presenting with bacterial meningitis, was investigated as a comparison to the encapsulated clinical isolate *H.influenzae* Eagan. The dose-dependent kinetics of cytokine and chemokine protein secretion from meningioma cell monolayers following challenge with between $1x10^2$ and $1x10^8$ cfu per monolayer of *H.influenzae* RD and piliated meningococci are shown in Figure 5.5. Although the secretion of IL-6 was induced by the challenge of meningioma cell monolayers with *H.influenzae* RD, the levels were low ($\leq$10ng ml^{-1}) and not significant when compared to over 120ng ml^{-1} induced by piliated meningococci ($P\leq0.005$). However, the highest concentration of *H.influenzae* RD induced levels of IL-6 secretion approximately three fold greater than those induced by the lowest concentration (Figure 5.5A). There was no significant difference in the levels of IL-6 protein secreted from meningioma cells challenged with either $1x10^4$ or $1x10^6$ cfu per monolayer at 24 hours onwards ($P=0.5$) at which points the levels were approximately two-fold lower than the highest concentration.

Although the challenge of meningioma cell monolayers with *H.influenzae* RD induced the early secretion of IL-8, the levels were low ($\leq$8ng ml^{-1}) and not significant when compared to over 70ng ml^{-1} induced by piliated meningococci ($P\leq0.005$) (Figure 5.5B). However, differences were seen between the various concentrations of bacteria, with 100-fold increases in the inoculum concentration of *H.influenzae* RD resulting in the approximate two-fold increase in the secretion of IL-8 at six and nine hours ($P\leq0.05$), although from 24 hours onwards these differences were no longer significant ($P=0.2$).

The challenge of meningioma cell monolayers with *H.influenzae* RD induced the early secretion of MCP-1, and although there were significant differences between the concentrations of bacteria ($P\leq0.01$) the magnitude of these differences were less than two-fold (Figure 5.5C). The accumulation of protein induced by all the various concentrations of *H.influenzae* RD increased over the extended time course, although they were approximately three fold lower than the levels induced by piliated meningococci ($P\leq0.005$).

Although the challenge of meningioma cell monolayers with *H.influenzae* RD induced the secretion of RANTES, the levels were very low ($\leq$0.6ng ml^{-1}) and not significant when compared to approximately 10ng ml^{-1} induced by piliated meningococci ($P\leq0.005$) (Figure 5.5D). The three fold differences seen in the induction of RANTES secretion between the lowest and the intermediate concentrations of *H.influenzae* RD progressively diminished over time and by 48 hours the differences were no longer significant ($P\geq0.9$). However, challenge with the highest concentration of *H.influenzae* RD resulted in approximately four-fold lower levels of RANTES secretion compared to those of the intermediate concentration ($P\leq0.005$) from 24 hours onwards.


N.meningitidis MC58-7
H.influenzae RD
(A) IL-6
(B) IL-8
(C) MCP-1
Cytokine secretion (ng ml-1)
Time (hours)
3
6
9
24
36
48

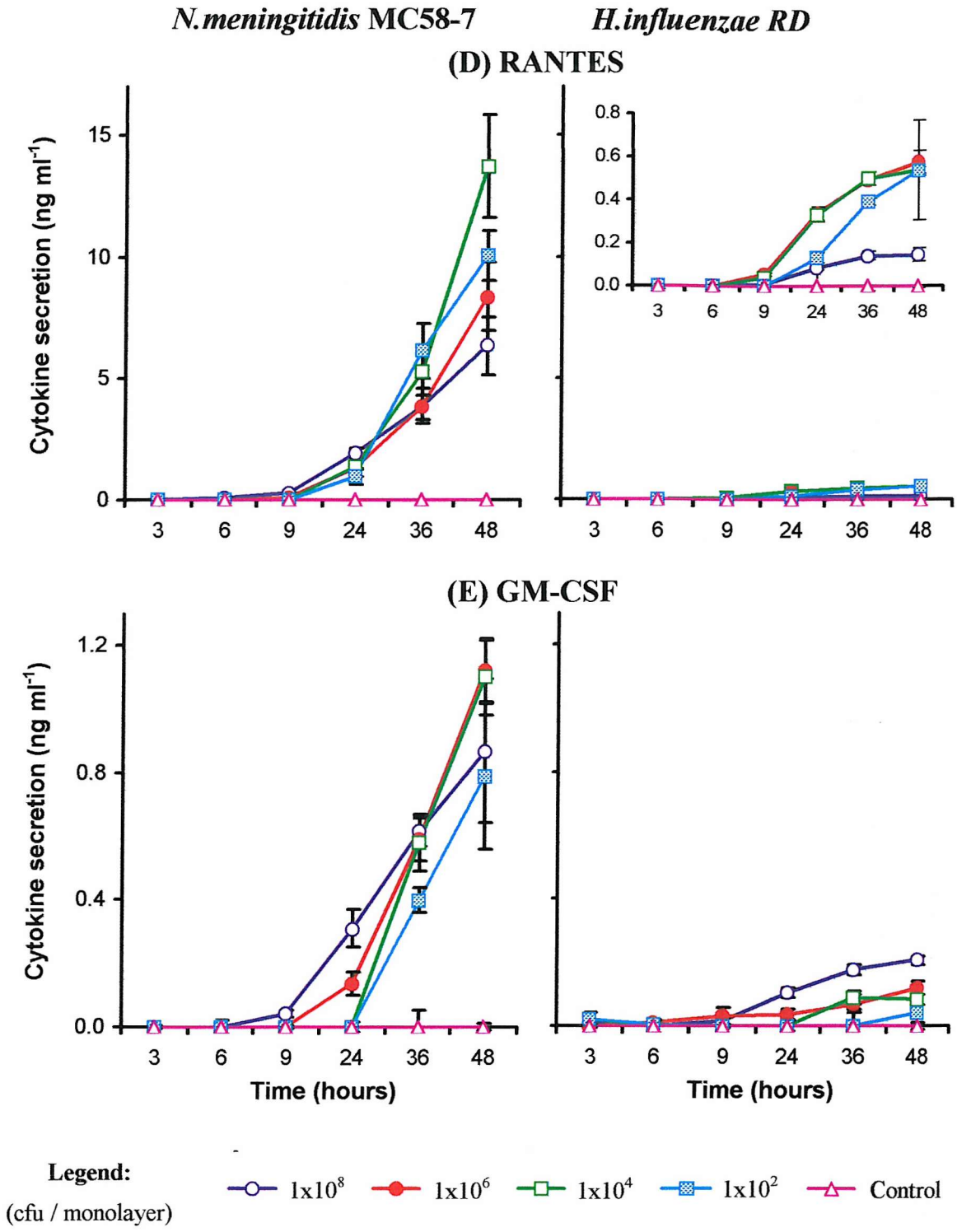


Figure 5.5: Comparison of the secretion of pro-inflammatory cytokines and chemokines from the meningioma cell line M66 induced by *H.influenzae* RD and *N.meningitidis* MC58-7.

The highest concentration of *H.influenzae* RD induced the secretion of GM-CSF from meningioma cells, the levels of which from 24 hours onwards were approximately three fold higher than those induced by any other concentration ($P \leq 0.05$) (Figure 5.5E). However, the levels of GM-CSF secretion from meningioma cells induced by *H.influenzae* RD were low, being approximately five to ten fold lower than the levels induced by piliated meningococci ($P \leq 0.05$).

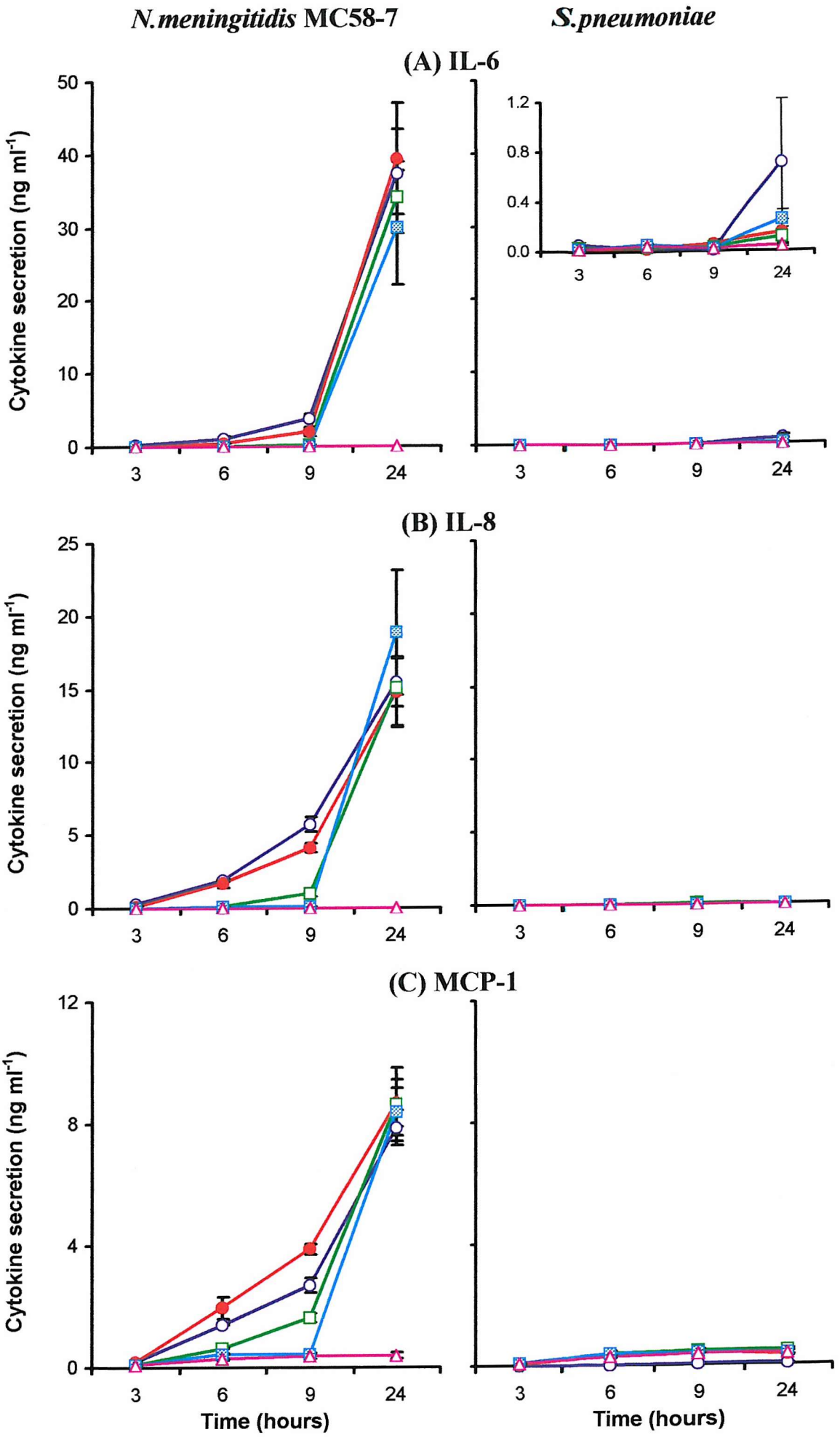
5.1.2.5 Profile of pro-inflammatory cytokines and chemokines produced by meningioma cells following challenge with S.pneumoniae.

The dose-dependent kinetics of cytokine and chemokine protein secretion from meningioma cell monolayers following challenge with between $1x10^2$ and $1x10^8$ cfu per monolayer of *S.pneumoniae* and piliated meningococci are shown in Figure 5.6. Although the challenge of meningioma cell monolayers with *S.pneumoniae* induced the secretion of IL-6 at 24 hours, the levels were very low (≤ 0.8ng ml^{-1}) and not significant when compared to over 40ng ml^{-1} induced by piliated meningococci ($P \leq 0.005$) (Figure 5.6A). There was no significant difference in the levels of IL-6 secretion at 24 hours between the various concentrations of *S.pneumoniae* ($P=0.07$).

In contrast to piliated meningococci, the challenge of meningioma cell monolayers with *S.pneumoniae* did not induce the secretion of IL-8, MCP-1 or RANTES protein (Figures 5.6B-D). However, the highest concentration of *S.pneumoniae* did induce the secretion of small amounts of GM-CSF from meningioma cells at 24 hours, and this level was approximately four fold lower than the equivalent concentration of piliated meningococci (Figure 5.6E).

5.1.2.6 Profile of pro-inflammatory cytokines and chemokines produced by meningioma cells following challenge with N.lactamica.

The dose-dependent kinetics of cytokine and chemokine protein secretion from meningioma cell monolayers following challenge with between $1x10^2$ and $1x10^8$ cfu per monolayer of *N.lactamica* and piliated meningococci are shown in Figure 5.7. The highest and intermediate concentrations of *N.lactamica* induced the secretion of IL-6 from meningioma cells between nine and 24 hours, the levels of which were approximately six fold greater than those induced by the lower concentrations ($P \leq 0.005$) (Figure 5.7A). The lower concentrations of *N.lactamica* exhibited a time lag in the secretion of IL-6, with protein secretion not occurring between 24 and 36 hours. The accumulation of IL-6 protein continued to increase over the extended time course for all concentrations of bacteria, with the highest and intermediate concentrations achieving approximately three fold greater levels than the lower concentrations ($P \leq 0.005$). However, the levels of IL-6 secretion from meningioma cells induced by *N.lactamica* were approximately five to ten fold lower than the levels induced by piliated meningococci ($P \leq 0.005$).


N.meningitidis MC58-7
S.pneumoniae
(A) IL-6
(B) IL-8
(C) MCP-1
Cytokine secretion (ng ml-1)
Time (hours)
3
6
9
24
0
10
20
30
40
50
0.0
0.4
0.8
1.2
5
15
25
4
8
12

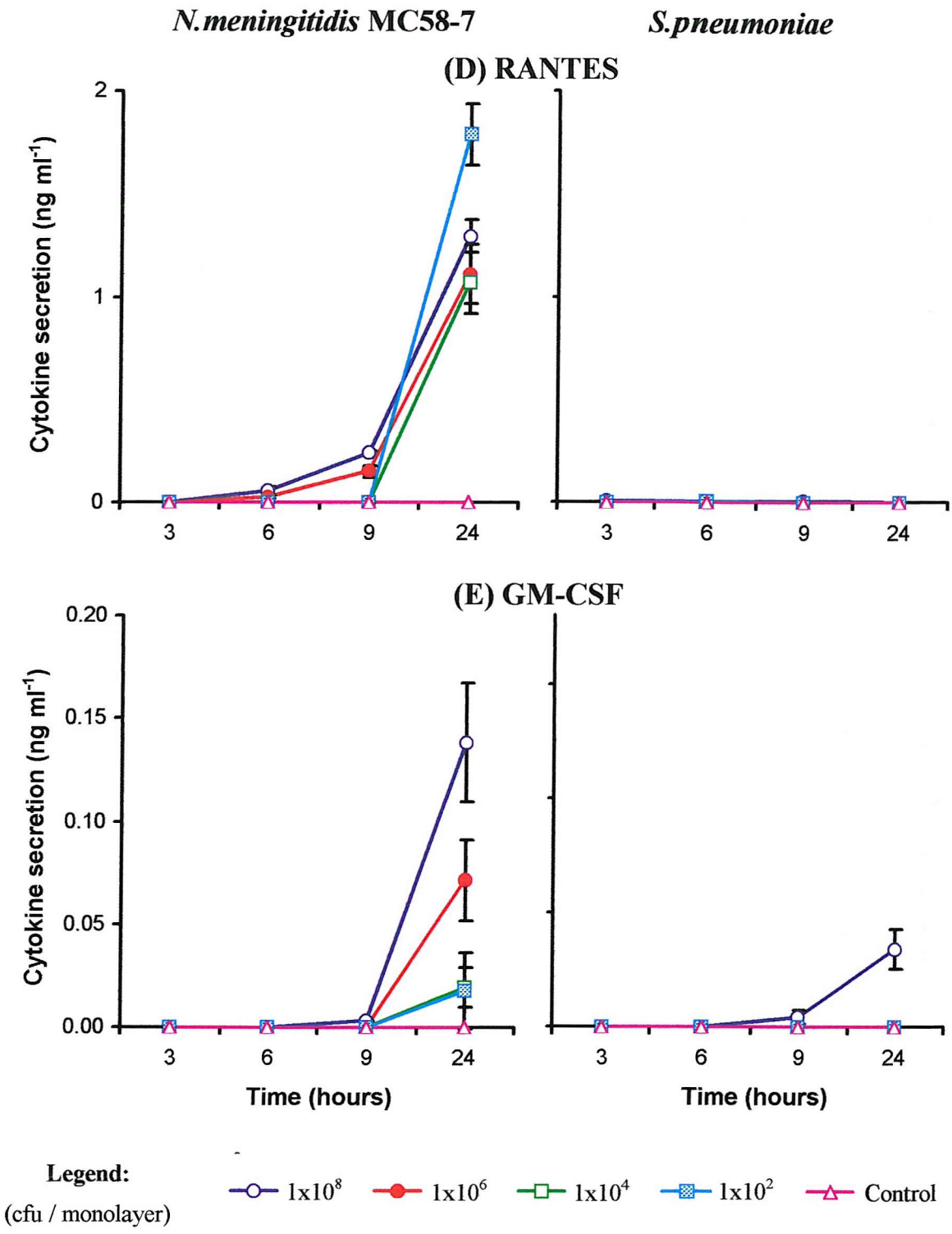


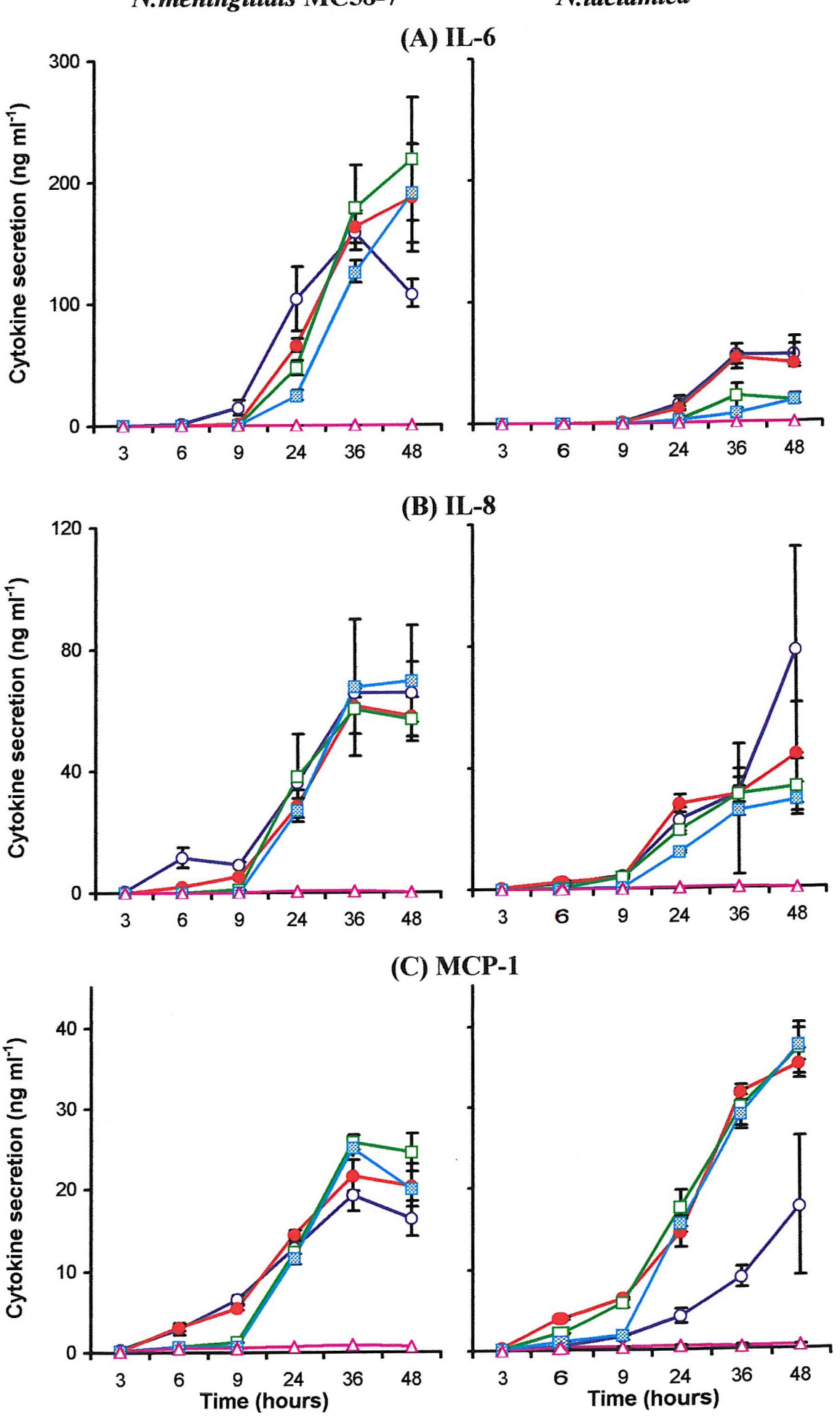
Figure 5.6: Comparison of the secretion of pro-inflammatory cytokines and chemokines from the meningioma cell line M66 induced by *S.pneumoniae* and *N.meningitidis* MC58-7.

The challenge of meningioma cell monolayers with *N.lactamica* resulted in the induction of IL-8 secretion between nine and 24 hours, with only a two fold difference between the highest and lowest concentration (Figure 5.7B). Over the extended time course the level of accumulated protein increased with no significant differences between the various concentrations of *N.lactamica* at 36 ($P \geq 0.9$) and 48 hours ($P \geq 0.05$). However, the levels of IL-8 secretion from meningioma cells induced by *N.lactamica* were approximately only two fold lower than the levels induced by piliated meningococci ($P \leq 0.05$).

The challenge of meningioma cell monolayers with either $1x10^4$ and $1x10^6$ cfu per monolayer of *N.lactamica* resulted in the secretion of MCP-1 as early as six hours (Figure 5.7C). There was no significant difference in the levels of MCP-1 secreted from meningioma cell monolayers challenged with either $1x10^4$ or $1x10^6$ cfu at nine hours ($P=0.18$), the values of which were approximately three fold greater than the lowest concentration ($P \leq 0.005$). Over the extended time course the level of accumulated protein increased with no significant differences between these concentrations of *N.lactamica* from 24 to 48 hours ($P \geq 0.1$). However, challenge with the highest concentration of *N.lactamica* resulted in the lower secretion of MCP-1 from meningioma cells, the levels being consistently three fold lower than the other concentrations of bacteria ($P \leq 0.005$). Significantly, the levels of MCP-1 secretion induced from meningioma cells by *N.lactamica* were approximately two fold greater than the levels induced by piliated meningococci ($P \leq 0.05$).

The challenge of meningioma cell monolayers with *N.lactamica* resulted in the induction of RANTES secretion at 24 hours, and there was no significant difference between the various concentrations of bacteria (Figure 5.7D). The accumulation of RANTES protein continued to increase over the next 24 hours, although the levels induced by the intermediate concentration of *N.lactamica* were approximately two to three fold greater than the other concentrations ($P \leq 0.05$). In addition, the challenge of meningioma cells with the highest concentration of *N.lactamica* again resulted in the lowest levels of RANTES secretion for the first 36 hours, after which the levels were similar to the lowest concentration of bacteria ($P \leq 0.05$). Overall, the levels of RANTES secretion induced from meningioma cells by *N.lactamica* were only approximately two fold lower than the levels induced by piliated meningococci ($P \leq 0.05$).

The highest concentration of *N.lactamica* induced the secretion of GM-CSF from meningioma cells at 24 hours, the level of which was approximately two fold greater than that induced by the intermediate concentration ($P=0.01$) (Figure 5.7E). The lower concentrations of *N.lactamica* exhibited a time lag in the secretion of GM-CSF, with protein secretion not occurring until between 24 and 36 hours, after which the levels remained very low. Despite the wide error bars at 48 hours, the accumulation of protein from meningioma cells challenged with the highest and intermediate concentrations of *N.lactamica* reached levels approximately ten fold and five fold respectively greater than those of the lower concentrations ($P \leq 0.005$). Overall, there were only one to two fold differences in the levels of secretion of GM-CSF induced by *N.lactamica* and piliated meningococci.


N.meningitidis MC58-7
N.lactamica
(A) IL-6
(B) IL-8
(C) MCP-1
Cytokine secretion (ng ml-1)
Time (hours)
0
100
200
300
40
80
120
10
20
30
3
6
9
24
36
48

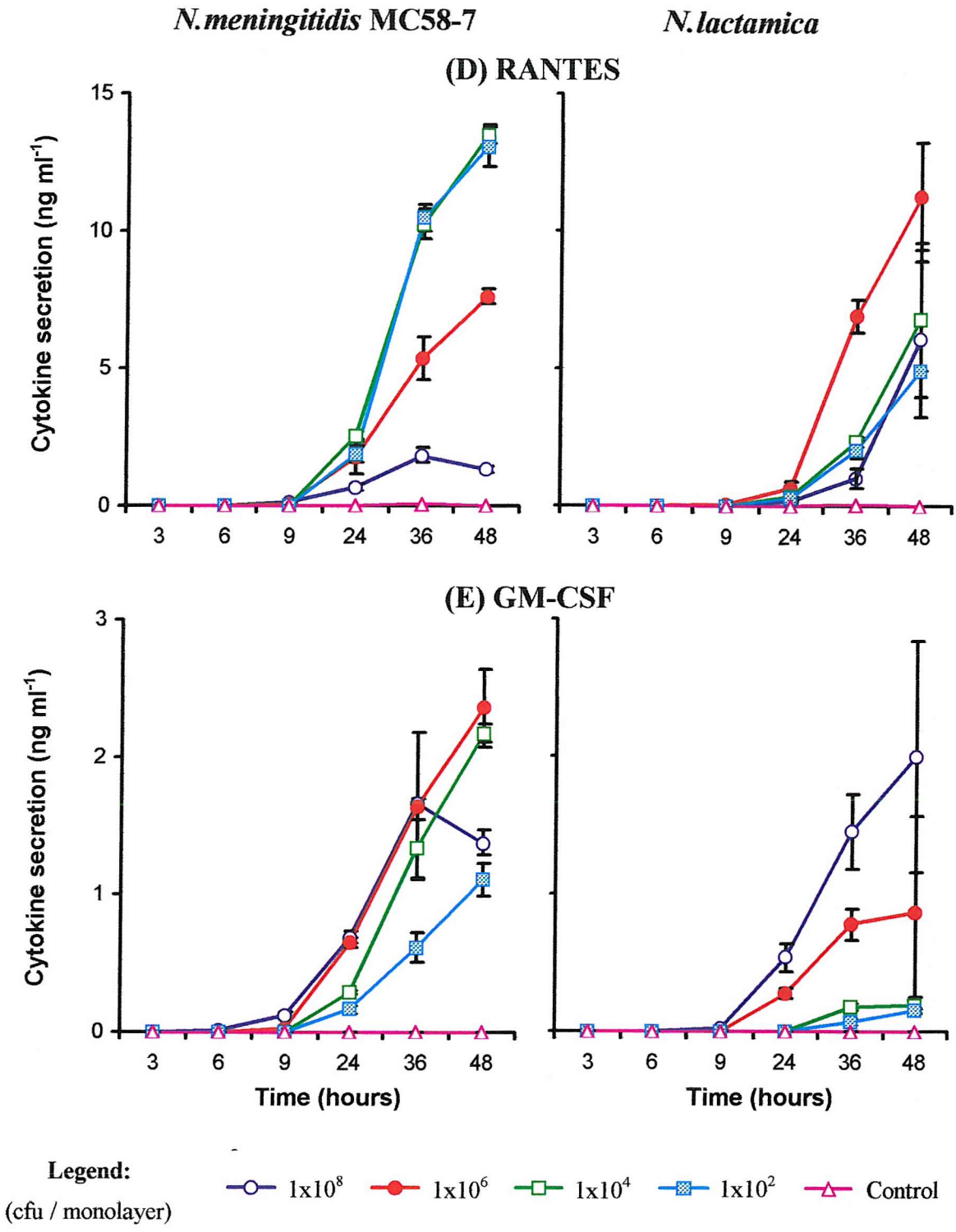


Figure 5.7: Comparison of the secretion of pro-inflammatory cytokines and chemokines from the meningioma cell line M66 induced by *N.lactamica* and *N.meningitidis* MC58-7.

Furthermore, the challenge of meningioma cell monolayers with *E.coli*, *H.influenzae*, *S.pneumoniae* and *N.lactamica* did not induce the secretion of IL-1α, IL-1β, TNF-α, MIP-1α, MIP-1β, TGF-β or IL-10 (data not shown).

5.2 The induction of pro-inflammatory cytokines and chemokines from Chang epithelial cells following challenge with bacterial pathogens

The pattern of pro-inflammatory cytokine and chemokine secretion from meningioma cell lines in response to meningeal pathogens has been characterised in Section 5.1. However, in order to determine whether this response is shared by other human cell types or is individual to meningioma cell lines, the study was extended to include Chang epithelial cells. Chang cell monolayers were challenged with approximately $1x10^6$ cfu per monolayer of *E.coli*, *H.influenzae*, *S.pneumoniae* and *N.lactamica* for 48 hours, with *N.meningitidis* strain MC58-7 included in all challenge studies to correct for any inter-experimental variations.

5.2.1 Profile of pro-inflammatory cytokines and chemokines produced by Chang cells following challenge with *Neisseria* species

The patterns of cytokine and chemokine proteins secreted from Chang cell monolayers following challenge with *N.meningitidis* MC58-7, *N.meningitidis* MC58-9 and *N.lactamica* are shown in Figure 5.8. No significant production of IL-6 was observed until 24 hours post-challenge, at which point there was less than a two fold difference between piliated and non-piliated meningococci ($P \leq 0.005$) (Figure 5.8A). The levels of IL-6 accumulation increased over time with the differences between piliated and non-piliated meningococci progressively diminishing until at 48 hours there was no significant difference between the levels ($P=0.13$), peaking at approximately 20ng ml^{-1}. Although the level of IL-6 secretion induced by *N.lactamica* at 24 hours was not significantly different from that of piliated meningococci ($P=0.06$), by 36 and 48 hours the levels of protein accumulation were approximately three and five fold greater than those induced by meningococci ($P \leq 0.005$).

The challenge of Chang cells with meningococci resulted in the secretion of IL-8 at levels approximately six fold greater than those observed with the unchallenged control cell monolayers (Figure 5.8B). There were less than two fold differences in the levels of IL-8 induction between piliated and non-piliated meningococci ($P \leq 0.01$) and at 48 hours these differences were no longer significant ($P=0.12$). However, the level of IL-8 protein secretion induced by *N.lactamica* at 24 hours was approximately two fold greater than that of meningococci ($P \geq 0.0005$). Furthermore, the accumulation of protein induced by challenge with *N.lactamica* continued to increase at 36 and 48 hours, the levels of which were five and eight fold higher, respectively, than either piliated or non-piliated meningococci ($P \leq 0.0005$).

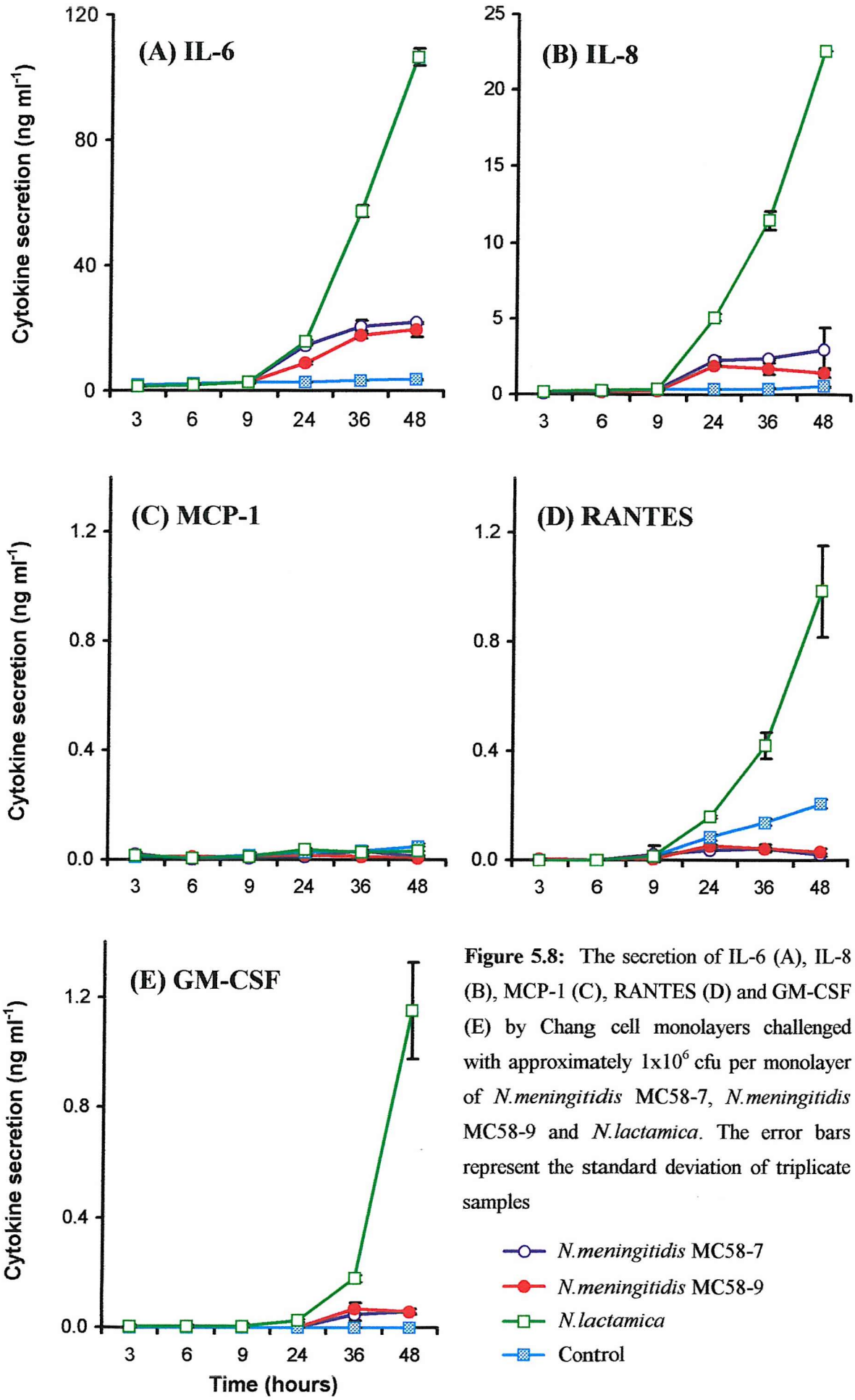


Figure 5.8: The secretion of IL-6 (A), IL-8 (B), MCP-1 (C), RANTES (D) and GM-CSF (E) by Chang cell monolayers challenged with approximately $1x10^6$ cfu per monolayer of *N.meningitidis* MC58-7, *N.meningitidis* MC58-9 and *N.lactamica*. The error bars represent the standard deviation of triplicate samples

No significant production of either MCP-1 or RANTES was observed from Chang cell monolayers challenged with either piliated or non-piliated meningococci (Figures 5.8C and 5.8D). However, the challenge of Chang cells with *N.lactamica* did result in the secretion of RANTES from 24 hours onwards, although the levels were significantly lower than either IL-6 or IL-8 (Figure 5.8D). The accumulation of RANTES secretion continued to increase reaching levels approximately three and five fold higher than the control at 36 and 48 hours respectively ($P \leq 0.001$).

There was no significant difference in the levels of GM-CSF secreted from Chang cells challenged with piliated or non-piliated meningococci ($P \geq 0.3$), with low levels of protein secretion only observed between 36 and 48 hours (Figure 5.8E). In contrast, the challenge of Chang cell monolayers with *N.lactamica* induced the secretion of GM-CSF from 24 hours onwards, although the levels were significantly lower than either IL-6 or IL-8. The accumulation of GM-CSF secretion continued to increase reaching levels of approximately three and 19 fold greater than both meningococcal variants at 36 and 48 hours respectively ($P \leq 0.005$).

5.2.2 Profile of pro-inflammatory cytokines and chemokines produced by Chang cells following challenge with *E.coli*

The patterns of cytokine and chemokine proteins secreted from Chang cell monolayers following challenge with *E.coli* strains IH3080 and DSM are shown in Figure 5.9. Although the challenge of Chang cell monolayers with piliated meningococci induced the secretion of IL-6 and IL-8 at levels similar to Section 5.2.1, the challenge with *E.coli* strains IH3080 and DSM did not result in the secretion of these proteins above the unchallenged cell levels ($P \geq 0.05$) (Figure 5.9A and 5.9B). In addition, no significant production above the control levels of either MCP-1 or RANTES was observed from Chang cell monolayers challenged with *E.coli* IH3080 and DSM or piliated meningococci (Figures 5.9C and 5.9D).

In contrast to IL-6, IL-8, MCP-1 and RANTES, the challenge of Chang cell monolayers with *E.coli* resulted in the secretion of very low levels (≤ 0.05ng ml^{-1}) of GM-CSF from 24 hours onwards (Figure 5.9E). Although the levels of GM-CSF were approximately two fold greater than those of the control, unchallenged cells ($P \leq 0.005$), they were not significant when compared to the levels induced by piliated meningococci.

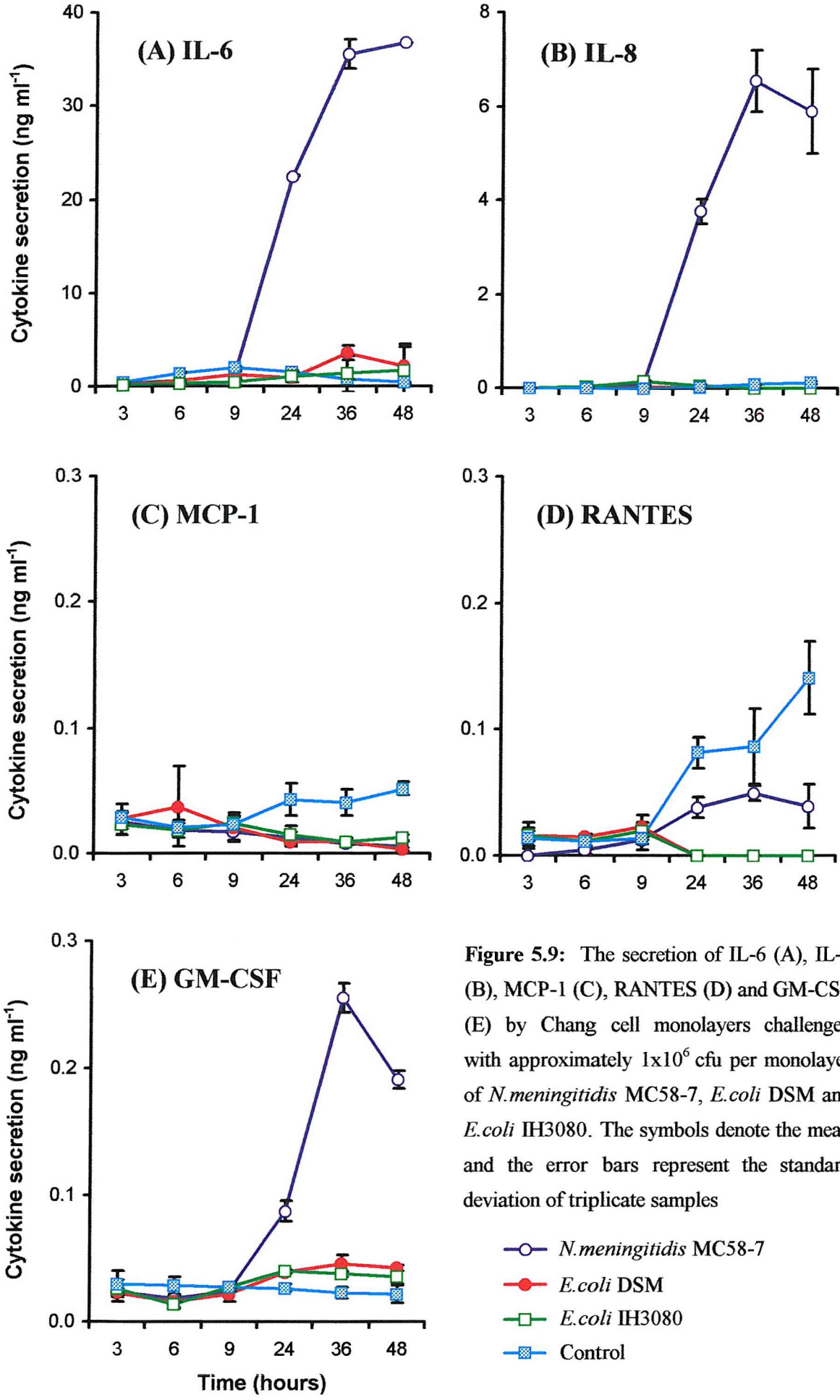


Figure 5.9: The secretion of IL-6 (A), IL-8 (B), MCP-1 (C), RANTES (D) and GM-CSF (E) by Chang cell monolayers challenged with approximately $1x10^6$ cfu per monolayer of *N.meningitidis* MC58-7, *E.coli* DSM and *E.coli* IH3080. The symbols denote the mean and the error bars represent the standard deviation of triplicate samples

5.2.3 Profile of pro-inflammatory cytokines and chemokines produced by Chang cells following challenge with *H.influenzae*

The patterns of cytokine and chemokine proteins secreted from Chang cell monolayers following challenge with *H.influenzae* strains Eagan and RD are shown in Figure 5.10. There was no significant difference between the quantity of IL-6 secreted from Chang cells challenged with *H.influenzae* strains Eagan or RD ($P \geq 0.4$), the levels of which were approximately two to three fold higher than those of the control cells from 24 hours onwards ($P \leq 0.001$) (Figure 5.10A). However, the levels of IL-6 secretion induced by *H.influenzae* were consistently two to three fold lower than those of piliated meningococci from 24 hours onwards ($P \leq 0.001$).

There was no significant difference between the quantities of IL-8 secreted from Chang cells challenged with *H.influenzae* strains Eagan or RD ($P \geq 0.1$). Although there were differences in the levels of IL-8 secreted between *H.influenzae* and piliated meningococci up to 36 hours ($P \leq 0.05$), these differences were less than two fold in magnitude (Figure 5.10B). However, by 48 hours post-challenge these differences were no longer significant ($P=0.07$) with the protein accumulation approximately three to four fold greater than that of the control cell monolayers.

No significant production above the control levels of either MCP-1 or RANTES was observed from Chang cell monolayers challenged with *H.influenzae* Eagan and RD or piliated meningococci (Figures 5.10C and 5.10D).

The challenge of Chang cells with *H.influenzae* Eagan and RD did not result in the secretion of GM-CSF at levels significantly above those of the control, unchallenged cells up to 36 hours ($P \geq 0.05$) (Figure 5.10E). At 48 hours post-challenge there was no significant difference in the quantities of GM-CSF protein secretion between *H.influenzae* strains Eagan and RD ($P=0.2$), the levels of which were two fold greater than those observed with the unchallenged control cell monolayers ($P=0.002$). However, the levels of GM-CSF secretion induced by piliated meningococci were consistently two fold higher than those of *H.influenzae* strains Eagan and RD from 24 hours onwards ($P \leq 0.05$).

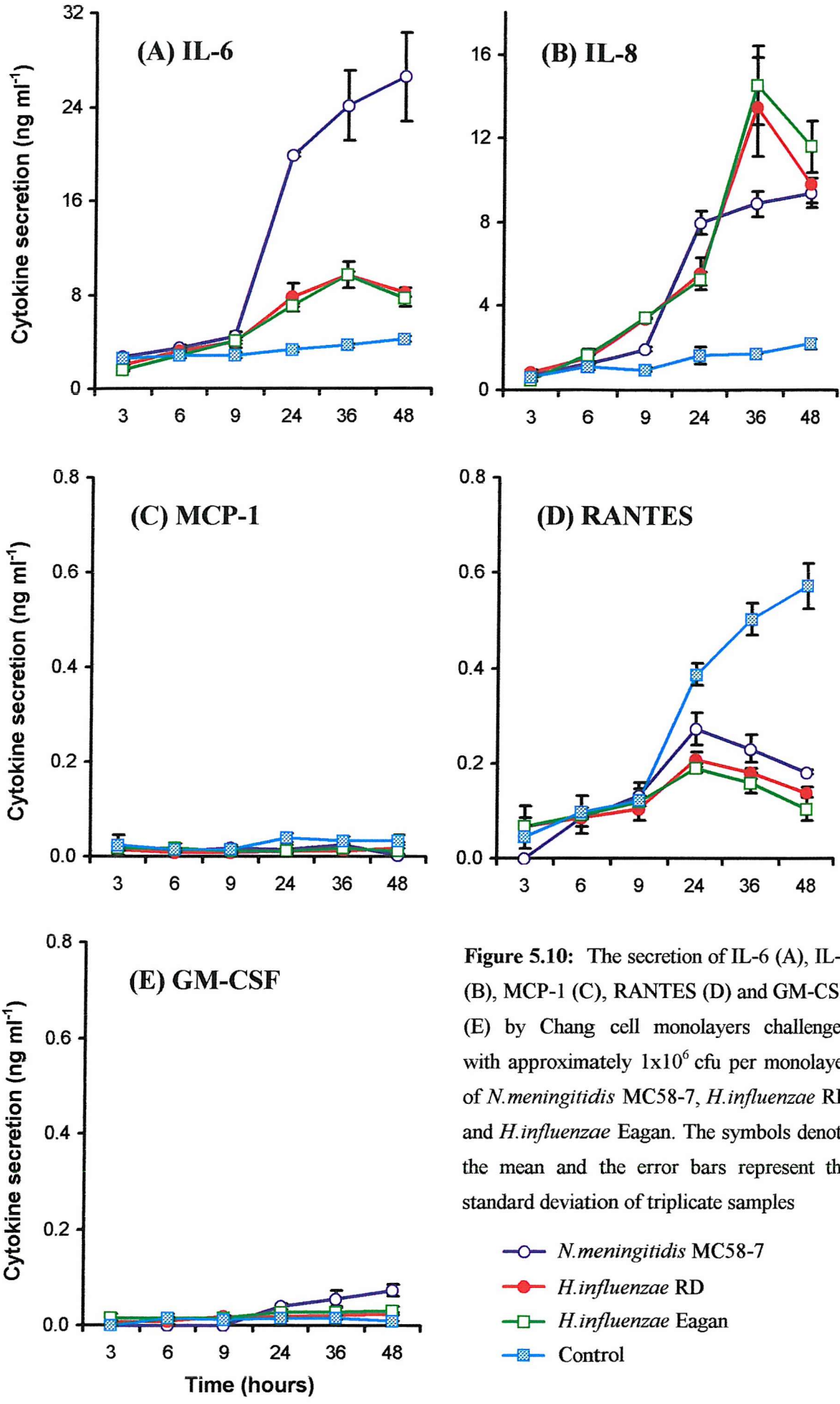


Figure 5.10: The secretion of IL-6 (A), IL-8 (B), MCP-1 (C), RANTES (D) and GM-CSF (E) by Chang cell monolayers challenged with approximately $1x10^6$ cfu per monolayer of *N.meningitidis* MC58-7, *H.influenzae* RD and *H.influenzae* Eagan. The symbols denote the mean and the error bars represent the standard deviation of triplicate samples

5.2.4 Profile of pro-inflammatory cytokines and chemokines produced by Chang cells following challenge with *S.pneumoniae*

The patterns of cytokine and chemokine proteins secreted from Chang cell monolayers following challenge with *S.pneumoniae* are shown in Figure 5.11. The challenge of Chang cell monolayers with *S.pneumoniae* induced the secretion of IL-6 after 24 hours, the levels of which were approximately three fold greater than those of the control ($P \leq 0.001$) (Figure 5.11A). However, the levels of IL-6 secretion induced by *S.pneumoniae* were consistently four to nine fold lower than those of piliated meningococci from 24 hours onwards ($P \leq 0.0001$). A similar pattern was observed for the secretion of IL-8, with the levels induced from 24 hours onwards by *S.pneumoniae* being eight fold higher than the control values ($P \leq 0.0001$), but two to five fold lower than those induced by piliated meningococci ($P \leq 0.0001$) (Figure 5.11B).

No significant production above the control levels of either MCP-1 or RANTES was observed from Chang cell monolayers challenged with *S.pneumoniae* or piliated meningococci (Figures 5.11C and 5.11D). In addition, although the challenge of Chang cell monolayers with piliated meningococci induced the secretion of GM-CSF at 36 hours, the challenge with *S.pneumoniae* did not result in the secretion of this protein above the unchallenged cell levels ($P \geq 0.9$) (Figure 5.11E).

Furthermore, the challenge of Chang cells with *N.meningitidis*, *E.coli*, *H.influenzae*, *S.pneumoniae* and *N.lactamica* did not induce the secretion of IL-1α, IL-1β, TNF-α, MIP-1α, MIP-1β, TGF-β or IL-10 (data not shown).

5.3 Chapter discussion

5.3.1 Summary of results

The induction of pro-inflammatory cytokines and chemokines from meningioma and Chang cell lines are summarised in Table 5.1. Meningioma cells challenged with *N.meningitidis* were found to secrete large amounts of the pro-inflammatory cytokine IL-6, the CXC chemokine IL-8, the CC chemokines MCP-1 and RANTES, and the cytokine growth factor GM-CSF. Although the challenge of meningioma cells with other meningeal pathogens also elicited the secretion of these cytokines and chemokines, the type and concentration of these inflammatory mediators induced was dependent on the individual bacteria. *H.influenzae* induced significant cytokine and chemokine production but at levels lower than those observed with meningococci. By contrast, meningioma cells challenged with either *E.coli* strain or *S.pneumoniae* only secreted very low levels, or none at all, of any of the inflammatory mediators. The secretion of cytokines and chemokines from Chang epithelial cells challenged with meningococci or *H.influenzae* was significantly lower than the levels observed with meningioma cells.

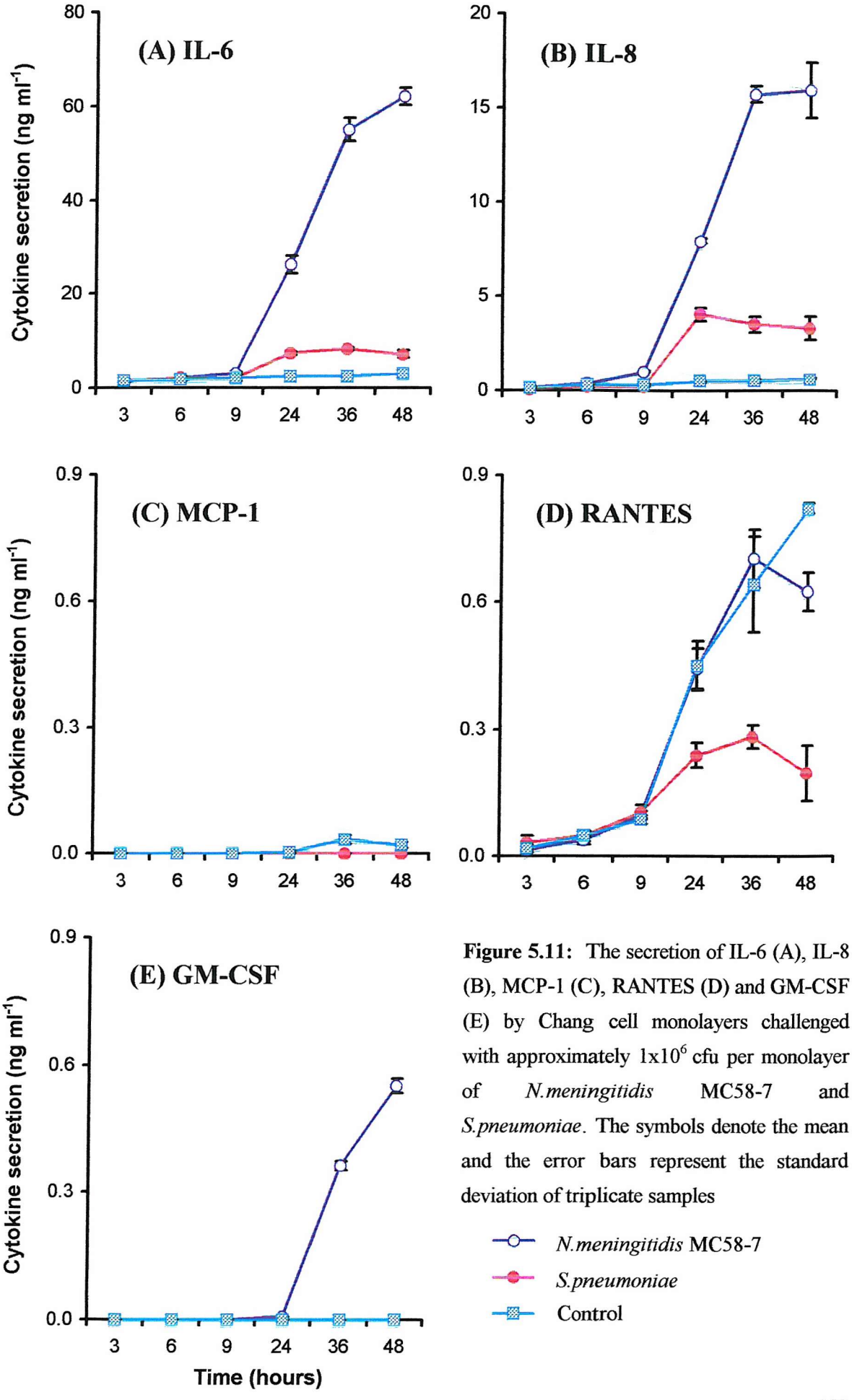


Figure 5.11: The secretion of IL-6 (A), IL-8 (B), MCP-1 (C), RANTES (D) and GM-CSF (E) by Chang cell monolayers challenged with approximately $1x10^6$ cfu per monolayer of *N.meningitidis* MC58-7 and *S.pneumoniae*. The symbols denote the mean and the error bars represent the standard deviation of triplicate samples

- *N.meningitidis* MC58-7
- *S.pneumoniae*
- Control

Table 5.1: Summary of the generic (Blue) and specific (Red) pro-inflammatory cytokines and chemokines secreted from (A) meningioma and (B) Chang cell lines following bacterial challenge.

(A) Meningioma cells

Bacteria	Cytokine levels (ng ml^{-1})				
	IL-6	IL-8	MCP-1	RANTES	GM-CSF
N.meningitidis MC58-7	128 (54)	37 (17)	15 (7)	1.2 (0.8)	1.0 (0.8)
E.coli DSM	nd	nd	nd	nd	nd
E.coli IH3080	nd	nd	nd	nd	0.15 (0.01)
H.influenzae RD	6.6 (0.9)	6.2 (0.1)	6.8 (0.7)	0.58 (0.1)	0.11 (0.02)
H.influenzae Eagan	23 (4)	30 (2)	12 (1)	0.24 (0.03)	0.02 (0.01)
S.pneumoniae	nd	nd	nd	nd	nd
N.lactamica	49 (21)	44 (17)	35 (2)	11.3 (1.9)	0.9 (0.7)

(B) Chang cells

Bacteria	Cytokine levels (ng ml^{-1})				
	IL-6	IL-8	MCP-1	RANTES	GM-CSF
N.meningitidis MC58-7	25 (7)	5.7 (2.9)	nd	nd	0.09 (0.06)
E.coli DSM	nd	nd	nd	nd	0.03 (0.01)
E.coli IH3080	nd	nd	nd	nd	0.03 (0.01)
H.influenzae RD	4.9 (0.4)	9.5 (0.7)	nd	nd	0.01 (0.01)
H.influenzae Eagan	4.5 (0.8)	11.3 (0.9)	nd	nd	0.02 (0.01)
S.pneumoniae	4.1 (0.8)	3.0 (0.3)	nd	nd	nd
N.lactamica	103 (2)	22.7 (0.1)	nd	0.6 (0.1)	1.2 (0.2)

Values represent the mean and (standard deviation) of triplicate samples above control values. *N.meningitidis* MC58-7 values represent the mean and (standard deviation) of four and six separate experiments for Chang and meningioma cell lines respectively.

nd = Not detected

Both *E.coli* strains failed to stimulate any cytokine or chemokine secretion, whereas *S.pneumoniae* induced the secretion of low levels of IL-6 and IL-8 from Chang cells. The commensal organism *N.lactamica* induced significant cytokine and chemokine production from meningioma cell lines, but at levels lower than meningococci. However, the challenge of Chang epithelial cells with *N.lactamica* resulted in the secretion of large amounts of IL-6, IL-8 and GM-CSF, which were approximately five to ten fold greater than the levels induced by meningococci.

5.3.2 The stimulation of inflammatory mediators from meningioma cells

Bacterial meningitis is characterised by an acute compartmentalised inflammatory response, with little or no spread of the infection to the underlying cerebral tissue. Elevated levels of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and the chemokine IL-8 have been detected early in the CSF of individuals with bacterial meningitis (Leist *et al.*, 1988; Waage *et al.*, 1989; Brandtzaeg *et al.*, 1992; Matsuzono *et al.*, 1995; van Furth *et al.*, 1996). Many cell types within the CNS have been shown to be capable of producing various cytokines and chemokines. These include PMNLs (Ossege *et al.*, 1996), mononuclear cells (Benveniste, 1992; Lapinet *et al.*, 2000) and astrocytes and microglia (Freyer *et al.*, 1996; Nakamura *et al.*, 1999). However, the ability of the leptomeninges to produce these molecules is not known. The current study has identified a specific subset of pro-inflammatory cytokines and chemokine proteins secreted by meningeal cells in response to bacterial challenge.

The challenge of meningioma cell lines with piliated and non-piliated meningococci stimulated the secretion of large amounts of the pro-inflammatory cytokine IL-6, but no TNF-α or IL-1β. The production of IL-6 is consistent with previous in vitro studies that have identified elevated levels of IL-6 in the CSF, prior to the influx of leukocytes from the blood (Waage et al., 1989; Matsuzono et al., 1995). The absence of TNF-α and IL-1β secretion from meningioma cells suggests a role for other cell types, such as infiltrating monocytes and vascular endothelial cells, as a source of these two cytokines (Lapinet *et al.*, 2000). By contrast, a recent study by Wells and colleagues demonstrated the up-regulation of TNF-α mRNA in meningioma cells challenged with meningococci (Wells *et al.*, 2001). However, in the current study, no bioactive TNF-α protein was detected suggesting that the TNF-α mRNA identified by Wells and colleagues is not actively translated. In addition to the pro-inflammatory cytokine IL-6, meningioma cell lines challenged with meningococci secreted early and large amounts of the CXC chemokine IL-8 and the CC chemokines MCP-1, followed by the later secretion of RANTES and the cytokine growth factor GM-CSF. These observations are consistent with the presence of elevated concentrations of IL-8, MCP-1, RANTES and GM-CSF in the CSF of individuals with bacterial meningitis (Frei *et al.*, 1992; Lopez-Cortes *et al.*, 1993; Spanaus *et al.*, 1997; Lahrtz *et al.*, 1998). The reported activity of IL-8 and RANTES in up-regulating adhesion molecules on the vascular endothelium (Inaba *et al.*, 1997; Adams & Lloyd, 1997) suggests that the leptomeninges plays a significant role in the recruitment of PMNL into the SAS. In addition, the gradual change of infiltrating inflammatory

cells from PMNL to monocytes (Lahrtz *et al.*, 1998) may be effected by MCP-1 with the late production of GM-CSF responsible for their maturation (Geissler *et al.*, 1989). Despite the correlation between pilus expression and increased bacterial association with meningioma cell lines, the secretion of IL-6, IL-8 and MCP-1 from meningioma cells was induced equally by the interactions of piliated and non-piliated meningococcal variants, suggesting the involvement of other bacterial components.

The mammalian innate immune system has evolved several mechanisms to detect conserved pathogen-associated molecular patterns (PAMPs) that cannot be found in higher eukaryotes (Medzhitov & Janeway, 1997). Although the details of these mechanisms are unclear, recent studies have demonstrated the involvement of the Toll-like receptor (TLR) family in the recognition of PAMPs (Aderem & Ulevitch, 2000). Indeed, the activation of the innate immune response by Gram-positive and Gram-negative bacteria is believed to commonly occur through the recognition of peptidoglycan by TLR2 molecules and LPS by TLR4 molecules respectively (Poltorak *et al.*, 1998; Takeuchi *et al.*, 1999; Aliprantis *et al.*, 1999). The innate immune recognition of bacterial LPS involves the interaction of LPS with the lipopolysaccharide-binding protein (LBP), which rapidly catalyses the transfer of LPS to membrane bound CD14 molecules (Wright *et al.*, 1990). Although CD14 has been identified as an LPS receptor, it is a glycosylphosphatidylinositol (GPI)-anchored protein and thus, lacks the transmembrane and intracellular domains necessary for signal transduction (Perera *et al.*, 2001). However, it is believed that CD14 forms a complex with the transmembrane TLR4, and signalling occurs through the sequential recruitment of the adapter molecule MyD88 and the IL-1 receptor-associated kinase (IRAK). Alternatively, LPS can bind directly to MD-2 and form a complex with TLR4 in the presence of CD14 (Heumann & Roger, 2002). Following dissociation from the receptor complex, IRAK interacts with the tumour necrosis factor receptor-associated factor 6 (TRAF6), which results in cell activation through NF-κB (Muzio *et al.*, 1998). The subsequent nuclear translocation of the transcription factor NF-κB results in the rapid induction of pro-inflammatory cytokines and chemokines (Medzhitov & Janeway, 1997; Zhang *et al.*, 1999).

The concentration of intracranial LPS during bacterial meningitis has been positively correlated to the clinical severity of the disease and the subsequent neurological sequelae (Mertsola *et al.*, 1991; Brandtzaeg *et al.*, 1992; Brandtzaeg *et al.*, 2001). However, a recent study has demonstrated that purified meningococcal LPS is a poor stimulator of IL-6 secretion from meningioma cells (Christodoulides *et al.*, 2002). These data suggest that other bacterial components are responsible for the induction of IL-6 from meningioma cell lines. Although these potential modulins remain to be identified, recent studies have demonstrated the upregulation of IL-6 production by peripheral blood mononuclear cells following challenge with purified IgA1 protease and peptidoglycan fragments, both of which are secreted by meningococci (Dokter *et al.*, 1994; Lorenzen *et al.*, 1999). By contrast, Christodoulides and colleagues also demonstrated the abilities of purified meningococcal LPS and outer membranes (OM) to induced MCP-1 secretion from meningioma cell lines, at levels comparable to those induced by viable bacteria. In addition,

the significant reduction in MCP-1 secretion observed with LPS-depleted OM suggests that LPS is an important stimulator of this chemokine from leptomeningeal cells. Purified meningococcal LPS also induced the production of IL-8 from meningioma cells, although the reduced levels compared to viable bacteria suggest a contributory role for other bacterial components (Christodoulides *et al.*, 2002). These observations indicate that the induction of IL-6, and MCP-1 and IL-8 from the leptomeninges may be due to the presence of secreted bacterial components in the CSF, and not dependent on bacterial adherence.

The challenge of meningioma cell lines with *H.influenzae* also resulted in the secretion of IL-6, IL-8, MCP-1, RANTES and GM-CSF. However, the levels were significantly lower than the corresponding values induced by challenge with meningococci. The reduced stimulation of pro-inflammatory cytokines and chemokines from meningioma cells by *H.influenzae* strains may be a result of their corresponding lower levels of association. However, the non-pathogenic *H.influenzae* strain RD induced a ten fold increase in IL-6 production compared to the clinical isolate strain Eagan. This suggests that the pathogenic *H.influenzae* type b is either able to down-regulate the inflammatory response or its polysaccharide capsule is masking stimulatory surface antigens, such as surface fibrils and phosphorylcholine (St.Geme & Cutter, 1995; Schweda *et al.*, 2000). The hypothesis that meningococcal LPS is associated with the induction of MCP-1 and IL-8 from meningioma cells, may also hold true for *H.influenzae*. The reduced levels of protein accumulation may be attributed to a slower release of outer membrane blebs by *H.influenzae* compared to the levels associated with meningococci. Alternatively, the *H.influenzae* LOS may be less immunostimulatory than meningococcal LPS (Gibson *et al.*, 1993).

The overall challenge of meningioma cells with *E.coli* K1 or *S.pneumoniae* did not induce the secretion of significant levels of pro-inflammatory cytokines or chemokines. The only exception was the very early secretion of MCP-1 stimulated by *E.coli* IH3080. Since there was insufficient time for the *de novo* production of protein, the observed secretion was probably due to the release of pre-synthesised intracellular stores of MCP-1. This is analogous to the rapid IL-1 secretion from peritoneal macrophages challenged with *Shigella flexneri* (Zychlinsky *et al.*, 1994). In the case of *E.coli* K1 DSM and IH3080, the absence of inflammatory mediators was most likely due to the severe cytotoxicity associated with these strains, with the viability of the cell monolayers rapidly declining over time. The ability of meningococcal LPS to stimulate IL-8 and MCP-1 from meningioma cell lines suggests that the absence of *E.coli* K1 LPS-induced chemokines was also due to loss of cellular viability. The absence of pro-inflammatory cytokine or chemokine mRNA up-regulation in meningioma cell lines challenged with *E.coli* DSM, suggests that this strain was unable to stimulate meningioma cells before they exhibited signs of cytotoxicity. However, *E.coli* strain DSM may have already irreversibly damaged the meningioma cells metabolic and protein synthesis machinery before the classical signs of cell death were apparent. In addition, as an isolate from a healthy individual, this bacterial strain may lack the cell surface antigens required for stimulating human cells. The absence of pro-inflammatory cytokine

or chemokine secretion from meningioma cell lines challenged with *E.coli* K1 could also suggest that other cell types within the CNS play a more significant role in the intracranial inflammatory response to *E.coli* than the leptomeninges, such as infiltrating leukocytes or endothelial cells.

By contrast to *E.coli* K1, the lack of pro-inflammatory cytokine and chemokine secretion from meningioma cell lines challenged with *S.pneumoniae* was not due to cytotoxicity since the monolayers remained viable throughout the experiment. These data are consistent with a previous study, in which *S.pneumoniae* did not induce the secretion of significant levels of IL-6 or IL-8 from NCI respiratory tract epithelial cells *in vitro* (Hakansson *et al.*, 1996). Taken together these observations suggest that the absence of inflammatory mediators maybe due to *S.pneumoniae* down-regulating secretion from meningioma cells. Conversely, the presence of pro-inflammatory cytokines and chemokines in the CSF during disease suggests that *S.pneumoniae* may preferentially stimulate other cell types in the CNS. Alternatively, pneumococci may be inhibiting the transcription of cytokine and chemokine genes through the suppression of NF-κB, a transcription factor that plays a key role in the expression of genes involved in inflammation and immune responses (Siebenlist *et al.*, 1994; Hatada *et al.*, 2000). However, this is unlikely since NF-κB regulates the majority of inflammatory mediators associated with pneumococcal meningitis and the NF-κB activity in mouse lung lavage cells was reported to significantly increase after challenge with *S.pneumoniae* (Amory-Rivier *et al.*, 2000). The potential modulation of NF-κB levels in meningioma cell lines challenged with *S.pneumoniae* could be investigated in future studies.

Toll-like receptors are also crucial in the recognition and activation of the innate immune response against Gram-positive bacterial pathogens. Indeed, TLR2 recognises the PAMP of bacterial peptidoglycan and lipoteichoic acids (Pugin *et al.*, 1994; Takeuchi *et al.*, 1999; Aliprantis *et al.*, 1999). The constitutive expression of TLR2 in the murine brain, and especially in the choroid plexi (Laflamme *et al.*, 2001), suggest that this molecule may act as a sensor for pneumococcal infection of the CSF. Indeed, meningioma cells have been shown to express TLR2 and TLR4 mRNA, although at low levels (M.Christodoulides, personal communication). However, the lack of pro-inflammatory cytokine or chemokine secretion from meningioma cell lines suggests that pneumococci may not stimulate these receptors on meningioma cells *in vitro.* Alternatively, *S.pneumoniae* may be capable of producing compounds analogous to the Vaccinia virus protein A52R, which can block the activation of the transcription factor NF-κB by multiple TLRs, including TLR2 (Harte *et al.*, 2003). In addition, TLR2-independent mechanisms also seem to play a central role in the induction of the host immune response during pneumococcal meningitis, which is in part mediated by the pneumococcal toxin pneumolysin (Koedel *et al.*, 2002). This fundamentally different mechanism of immune activation by *S.pneumoniae* compared to Gram-negative bacteria may in part be responsible for the non-stimulation of meningioma cell lines. The observation that pneumolysin can stimulate the secretion of inflammatory mediators,

including TNFα, IL-1β and IL-6, from monocytes and macrophages *in vitro* (Houldsworth *et al.*, 1994; Braun *et al.*, 1999), suggests the role of these cell types and not the leptomeninges as a major source of cytokines and chemokines during pneumococcal meningitis.

This study has demonstrated that cells of the leptomeninges are not inert but are active participants in the inflammatory response characteristic of bacterial leptomeningitis. However, this model of the human leptomeninges does not incorporate the potential role of other cell types present in the meninges, such as resident meningeal macrophages. Indeed, the resident macrophages of the leptomeninges are thought to be a major cellular source of the pro-inflammatory cytokine IL-1β, the anti-inflammatory cytokines IL-10 and TGF-β, and the cytokines MCP-1 and MIP-1α in the CSF during bacterial meningitis (Yoshimura *et al.*, 1989; Koch *et al.*, 1994; Lapinet *et al.*, 2000). A recent study by Koay and colleagues demonstrated that a generalised chemical depletion of macrophages in mice reduced NF-κB activation, generation of pro-inflammatory cytokines and chemokines, and neutrophilic lung inflammation in response to Gram-negative endotoxin (Koay *et al.*, 2002). Similarly the presence of macrophages in the human leptomeninges may be essential for maximal inflammation, as defined by pro-inflammatory cytokine and chemokine production, in the SAS during bacterial meningitis. Therefore, it may be appropriate in future experiments to extend this model of inflammation by investigating the inflammatory response elicited by the various meningeal pathogens from meningioma cells co-cultured with tissue macrophages.

5.3.3 The stimulation of inflammatory mediators from Chang epithelial cells

The mucosal epithelium plays a very important role early in the pathogenesis of bacterial pathogens, providing a mechanical barrier to initial microbial penetration. In addition, epithelial cells also act as environmental sensors with the ability to modulate the host immune response through the secretion of cytokines and chemokines, expression of adhesion molecules and activation of NO (Khair *et al.*, 1996; Kagnoff & Eckmann, 1997). As with meningioma cells, in the current study the secretion of a specific subset of pro-inflammatory cytokines and chemokine proteins secreted by Chang cells was detected in response to bacterial challenge.

The challenge of Chang epithelial cells with *N.meningitidis* and *H.influenzae* resulted in the secretion of IL-6, IL-8, and to a lesser extent GM-CSF. These data are consistent with previous studies in which respiratory epithelial cell lines have been shown to actively participate in the inflammatory response following bacterial challenge (McCormick *et al.*, 1998; Frick *et al.*, 2000; Clemans *et al.*, 2000). By contrast, the challenge of Chang cell monolayers with both *E.coli* K1 strains did not stimulate the secretion of either IL-6 or IL-8 protein, although very low levels of GM-CSF were detected later on. These data are consistent with several other studies, which have demonstrated that the challenge of airway epithelial and tubuloepithelial cell lines with *E.coli* was insufficient to stimulate the production of inflammatory mediators (Koyama *et al.*, 2000; Leeker *et*

al., 2001). However, intestinal and bladder epithelial cell lines have been shown to produce significant levels of IL-6 and IL-8 in response to *E.coli* infection (Hosoi *et al.*, 2003; Schilling *et al.*, 2003). Low levels of TNFα and GM-CSF secretion have been also been observed in urinary tract and renal cortical epithelial cell lines (Brauner *et al.*, 2001; Funfstuck *et al.*, 2001). These contradictory data suggest that the production of pro-inflammatory cytokines and chemokines is not only dependent on the nature of the bacterial infection, but is also specific to each individual epithelial cell line.

In contrast to the leptomeninges, mucosal surfaces are constantly exposed to large amounts of LPS, predominantly released from gram-negative commensal flora. Although these epithelial cell lines express TLR4, they are commonly CD14 negative ensuring they are not continuously activated by free LPS (Hedlund *et al.*, 1999; Cario *et al.*, 2000). Significantly, CD14 was expressed on intestinal and bladder epithelial cells, suggesting that the secretion of inflammatory mediators from these cell lines was probably in response to stimulation with *E.coli* LPS involving the co-receptor TLR-4. These data suggest that the lack of pro-inflammatory cytokine and chemokine secretion from Chang cells challenged with *E.coli* K1 may be due to the cell line being CD14 negative. However, this is unlikely since the challenge of Chang epithelial cells with meningococci, *H.influenzae*, *S.pneumoniae* and *N.lactamica* resulted in the secretion of significant levels of IL-6 and IL-8. By contrast, the induction of IL-6 and IL-8 secretion from epithelial cell lines challenged with uropathogenic *E.coli* has been demonstrated to be dependent on the expression of P-fimbriae and not LPS (Hedlund *et al.*, 1999). However, the absence of IL-6 and IL-8 secretion from Chang epithelial cells in the current study may be explained by the absence of P-fimbriae on the *E.coli* K1 strains IH3080 and DSM.

In the current study, the challenge of Chang cells with *S.pneumoniae* stimulated the secretion of low levels of IL-6 and IL-8. By contrast, several other studies in NCI respiratory tract (Hakansson *et al.*, 1996) and pulmonary alveolar epithelial cell lines (Madsen *et al.*, 2000) did not observe IL-6 and IL-8 secretion following challenge with pneumococci. However, the stimulation of IL-8 from pulmonary alveolar epithelial cells by the pneumococcal surface protein CbpA and constituents of the pneumococcal cell wall (Bruyn *et al.*, 1992; Murdoch *et al.*, 2002) suggest that immune activation can occur following the autolysis of *S.pneumoniae*.

The commensal organism *N.lactamica*, which induced low levels of inflammatory mediators from meningioma cell lines, stimulated the secretion of large amounts of IL-6 and IL-8, and lower levels of RANTES and GM-CSF from Chang epithelial cells. These data are unexpected since the mucosal epithelium should remain immunologically silent in response to commensal flora to protect against continuous chronic inflammation. Indeed, urinary tract, intestinal, and renal cortical epithelial cell lines have been shown to be unresponsive to their commensal bacteria and LPS (Hedlund *et al.*, 1999; Cario *et al.*, 2000; Backhed *et al.*, 2002). However, while intestinal epithelial cells are unresponsive to commensal bacteria of the gut, these

gut commensals induce a potent inflammatory response in urogenital and pulmonary epithelial cell lines (Michel *et al.*, 1997; Rackley *et al.*, 1999). Indeed, *N.lactamica* is a commensal of the upper respiratory tract and Chang cells derive from the conjunctiva, an observation that may explain the induction of pro-inflammatory cytokines and chemokines from Chang cells following challenge with *N.lactamica.*

The relatively low immune activation of Chang cells by meningeal pathogens compared to *N.lactamica* demonstrates a possible suppression of the inflammatory response by pathogenic bacteria. This suggests pathogenic bacteria utilize a strategy of suppressing the host defence mechanisms in order to successfully colonise the mucosal surfaces, which is a prerequisite for invasive disease. By contrast, once the bacteria have gained entry into the SAS, the absence of effective humoral or cellular host defences in the SAS (Simberkoff *et al.*, 1980; Quagliarello & Scheld, 1992; Brandtzaeg *et al.*, 1992) possibly negates the need for this immune suppression. This may in part explain the significantly increased secretion of inflammatory mediators from meningioma cell lines compared to Chang epithelial cells. However, it is also possible that the meningioma cell lines are more sensitive to bacterial stimuli than Chang epithelial cells. The molecular mechanisms for the possible immune suppression by meningeal pathogens are unclear, but studies with other pathogens may provide us with a better understanding of this modulation of the inflammatory response. A recent study by Hauf and Chakraborty demonstrated the suppression of bacteria-induced cytokine expression in epithelial cells by shiga toxin-producing *E.coli*, through the interference of NF-κβ binding to target gene DNA (Hauf & Chakraborty, 2003). Indeed, this mechanism is very similar to that seen with *Yersinia pseudotuberculosis* and *Y.enterocolitica*, in which the activation of NF-κβ is inhibited by the presence of secreted prokaryotic effector proteins, YopJ or YopP, in the target cell cytoplasm (Ruckdeschel *et al.*, 1998; Schesser *et al.*, 1998).

5.3.4 Biological effects of pro-inflammatory cytokines and chemokines

The challenge of both leptomeningeal and epithelial cell lines with pathogenic bacteria results in the induction of a host inflammatory response, mediated by pro-inflammatory cytokine and chemokine molecules. IL-6 is a pleiotrophic cytokine that is capable of inducing leukocytosis and activating the complement and clotting cascades (Hirano *et al.*, 1990). In addition, there is a strong correlation between IL-6 levels and fever (Rothwell, 1994). In the CSF, IL-6 participates in the induction of the characteristic influx of PMNLs into the SAS and is responsible for a number of crucial functions in the CNS, which include the coordination of neuroimmune functions and the protection of neurons from damage (Gruol & Nelson, 1997). The chemokines IL-8 and RANTES have been reported to up-regulate adhesion molecules on the vascular endothelium (Inaba *et al.*, 1997; Adams & Lloyd, 1997), and thus may promote the recruitment of PMNL into the CSF or to the mucosal epithelium. In addition, MCP-1 is a chemoattractant for monocytes, which induces

their migration from the bloodstream resulting in the gradual change of infiltrating inflammatory cells in the SAS from PMNL to monocytes (Lahrtz *et al.*, 1998). The late production of GM-CSF from leptomeningeal and epithelial cell lines may be responsible for the subsequent maturation of infiltrated monocytes into tissue macrophages (Geissler *et al.*, 1989).

In summary, meningioma cell lines challenged with meningococci produced large amounts of the pro-inflammatory cytokine IL-6, the chemokines IL-8, MCP-1 and RANTES, and the cytokine growth factor GM-CSF. Although the challenge of meningioma cells with other meningeal pathogens also elicited the secretion of these five inflammatory mediators, the type and concentration of these inflammatory mediators was influenced by the different bacteria. In addition to differences between the individual pathogens in the levels of pro-inflammatory cytokine and chemokine produced from Chang cells, the overall levels of protein secretion from Chang cells were greatly reduced when compared to the corresponding meningioma values. These differences may suggest a modulation of the host innate immune response by meningeal pathogens, which is specific to each human cell line. Indeed, future studies could investigate the possible role of NF-κβ and other cell signalling pathways in the innate immune modulation identified in this thesis. In the following chapter, the expression and regulation of an additional innate immune effector molecule, human β-defensins, from cell lines challenged with meningeal pathogens will be investigated.

CHAPTER 6 THE PRODUCTION OF β-DEFENSINS BY HUMAN CELLS IN RESPONSE TO CHALLENGE WITH MENINGOCOCCI

In this study, cells of leptomeningeal origin have been shown to secrete a distinct group of pro-inflammatory, growth-factor related and chemoattractant cytokines in response to challenge with various bacterial pathogens (Chapter 4). However, several data suggest that the interactions of bacteria with these cells may modulate the innate inflammatory response. Recently another component of the innate immune response, the human β-defensins, have been reported to play a protective role at mucosal surfaces during bacterial infection (Goldman *et al.*, 1997; McCray & Bentley, 1997). In the current study, the potential modulation of these human β-defensins by meningococci was investigated in both Chang epithelial and meningioma cell lines.

6.1 β-defensin mRNA expression from human cells challenged with meningococci

Previous studies have identified the presence of β-defensin mRNA in a range of epithelia including the female reproductive tract (Zhao *et al.*, 1996), the gastrointestinal tract (Stolzenberg *et al.*, 1997; O'Neil *et al.*, 1999), the skin (Harder *et al.*, 1997; Liu *et al.*, 1998), the upper respiratory tract (McCray & Bentley, 1997) and the ocular surface (Haynes *et al.*, 1999). In the present study, the effect of challenge with meningococci on the expression of hBD-1 and hBD-2 mRNA transcripts was initially investigated in monolayers of Chang conjunctival epithelial cells.

6.1.1 Semiquantitative TaqMan® analysis for hBD mRNA expression

The relative quantification of β-defensin and ribosomal 18S gene expression was investigated by real time TaqMan®, using a standard curve method (Anonymous, 1997). Standard curves for hBD-1, hBD-2 and 18S were created by plotting the concentration of β-defensin plasmid or cDNA stock against the corresponding cycle threshold (C_T) value, which represented the onset of the exponential phase during the PCR reactions. The high correlation coefficients of these standard curves (≥0.99), from which the C_T values for experimental unknowns were extrapolated, demonstrated the large dynamic range and accuracy of real-time semiquantitative PCR. All experimental samples were subsequently expressed as a ratio of either hBD-1 or hBD-2 copy number over the relative ribosomal 18S concentration. The relative expression of the 18S gene could therefore be used to control for variations in the efficiency of each reverse-transcriptase reaction, where low 18S values corresponded to low RT efficiency.

6.1.2 Expression of β-defensin mRNA from Chang cells challenged with meningococci

The expression of hBD-1 and hBD-2 mRNA transcripts were initially investigated in control, uninfected Chang cells and from cells challenged with piliated and non-piliated meningococci (~$1x10^6$ cfu) over a time course of 1,3,6,9 and 24 hours. Although, hBD-1 mRNA was identified in both stimulated and non-stimulated Chang cells, suggesting constitutive expression, no discernible pattern could be seen over the experimental time course (data not shown). In contrast, neither constitutive nor bacterial-induced hBD-2 mRNA expression could be detected from Chang cell monolayers. In addition, the incubation of Chang cell monolayers with TNFα, a known stimulator of hBD-2 from epithelial cell lines (Harder *et al.*, 1997; Krisanaprakornkit *et al.*, 2000), did not induce the expression of hBD-2 mRNA.

The expression of hBD-1 mRNA by Chang cells was then investigated in more detail over a shorter time course with samples taken every hour. Expression of hBD-1 mRNA transcripts in the form of β-defensin / 18S ratios is shown in Figure 6.1. The constitutive expression of hBD-1 from control, unchallenged Chang cells formed a transverse wave pattern, the cycles of which were followed for twelve hours (data not shown). The challenge of Chang cell monolayers with piliated meningococci resulted in a two-fold down-regulation of hBD-1 mRNA at one ($P=0.01$) and two ($P=0.003$) hour post-challenge. By three hours, there was no significant difference between the levels of hBD-1 mRNA expression from Chang cells challenged with piliated meningococci and controls ($P\geq0.7$). However, preliminary data from 6-11 hours post-challenge suggests the continued down-regulation of hBD-1 mRNA expression from Chang cells challenged with piliated meningococci compared to the control. In contrast, the challenge of Chang cells with non-piliated meningococci resulted in a two-fold up-regulation of hBD-1 mRNA expression at two hours compared to that of control cells ($P=0.004$), but these differences were no longer significant by four hours post-challenge ($P=0.6$).

To investigate the effect of concentration of bacterial inocula on the modulation of hBD-1 mRNA expression, Chang cells were challenged with various concentrations of piliated meningococci from $1x10^2$ to $1x10^8$ cfu per monolayer (Figure 6.2). The challenge of Chang cell monolayers with between $1x10^4$ and $1x10^8$ cfu of piliated meningococci resulted in approximately a two-fold down-regulation of hBD-1 mRNA expression compared to control levels at one and two hours ($P\leq0.05$). In addition, there were no significant differences ($P\geq0.05$) in the down-regulation induced by these concentrations of bacteria (Figure 6.2A-C). By contrast, the levels of hBD-1 mRNA expression from Chang cell monolayers challenged with approximately $1x10^2$ cfu of piliated meningococci were not significantly different to those of unchallenged cells over the first two hours ($P\geq0.1$). However, by three hours, the characteristic two-fold down-regulation of hBD-1 mRNA expression was observed ($P=0.004$) (Figure 6.2D). Significantly, the accompanying bacterial association data identified three hours as the time point at which the lowest concentration of piliated meningococci began to associate with Chang cell monolayers, as shown in Figure 4.36A, Section 4.3.1.1.

Figure 6.1: Effect of challenge with piliated and non-piliated meningococci on the constitutive expression of hBD-1 mRNA from monolayers of Chang epithelial cells. Monolayers were infected with approximately $1x10^6$ cfu per monolayer of bacteria. The data points represent the mean hBD-1 / 18S ratio and the standard deviation of triplicate samples.

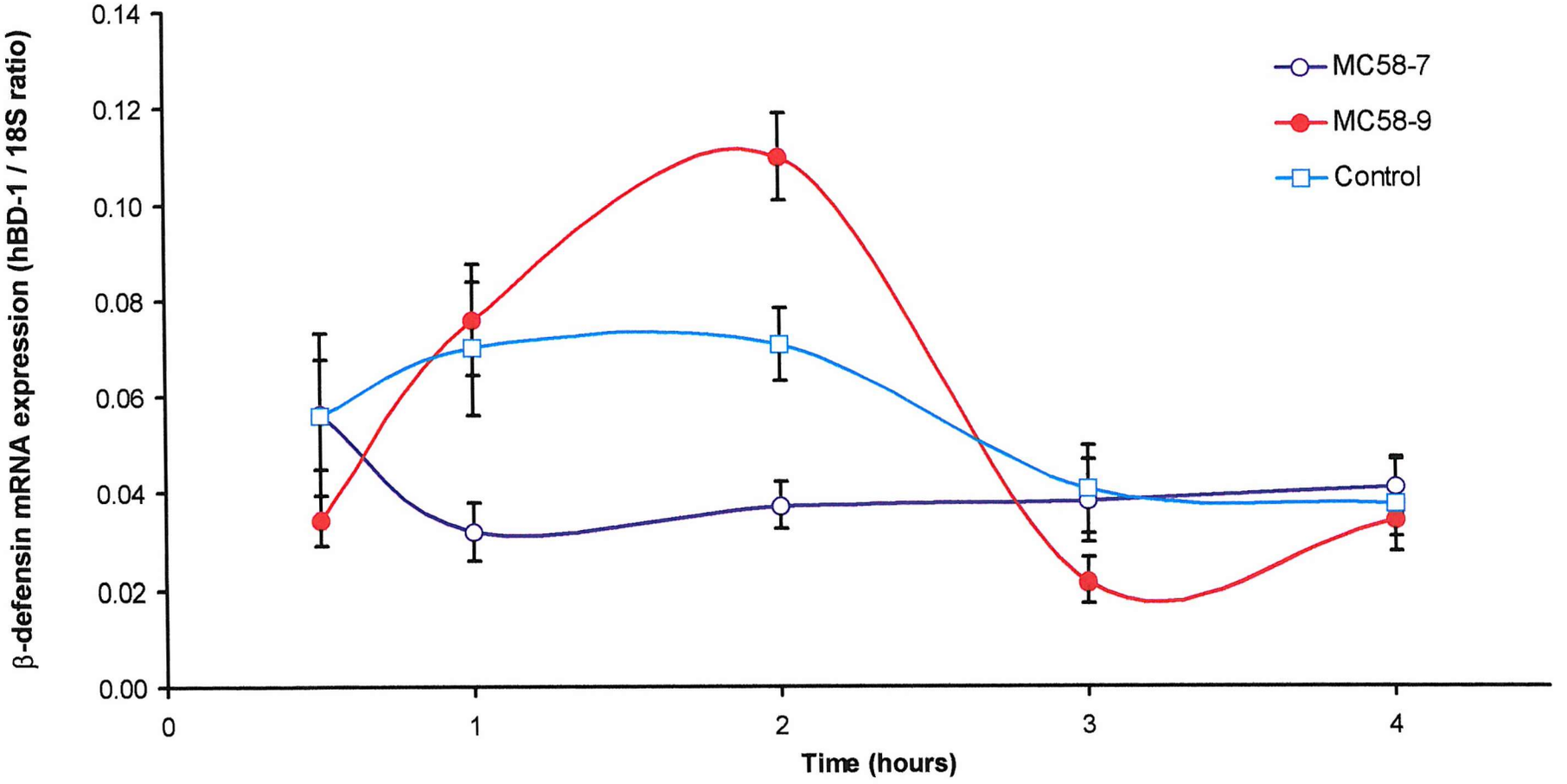
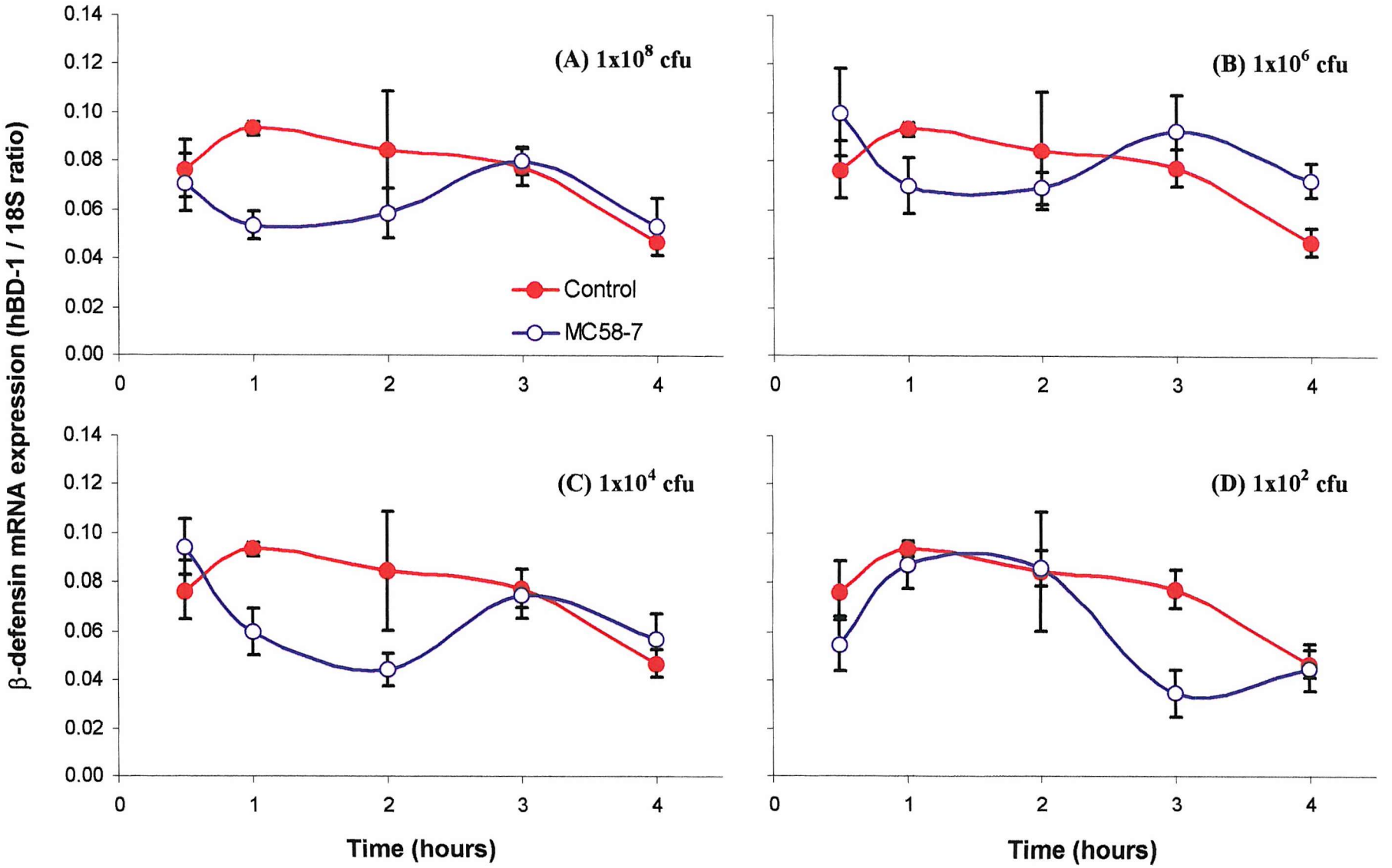


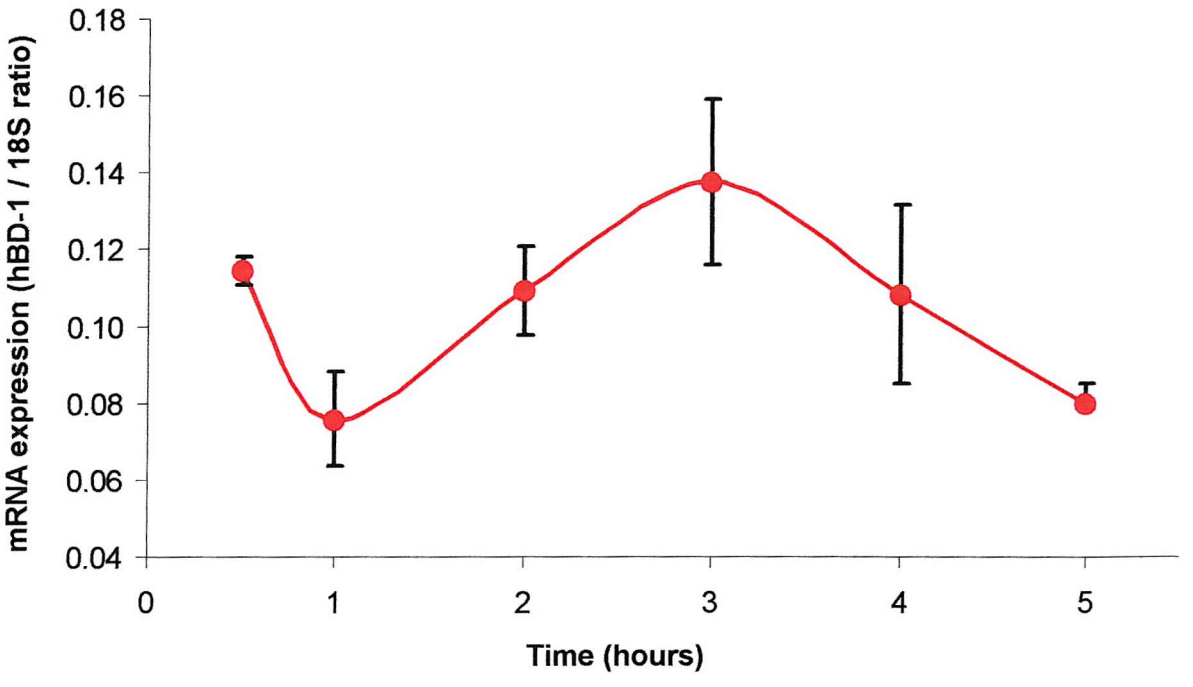
Figure 6.2: The effect of bacterial concentration on the expression of hBD-1 mRNA from monolayers of Chang epithelial cells. Monolayers were challenged with between $1x10^2$ and $1x10^8$ cfu of piliated meningococci. The data points represent the mean hBD-1 / 18S ratio and standard deviation of triplicate samples.



6.1.3 Expression of β-defensin mRNA from meningioma cell lines

Preliminary experiments demonstrated that a control, unchallenged meningioma cell line (M27) constitutively expressed hBD-1 (Figure 6.3), but not hBD-2. Furthermore, both the levels and the transverse wave pattern of hBD-1 mRNA expression from meningioma cells was similar to those observed with unchallenged Chang cells (Figure 6.1).

Figure 6.3: The constitutive expression of hBD-1 mRNA from naïve meningioma cells. The data points represent the mean hBD-1 / 18S ratio and standard deviation of triplicate samples.



6.2 Chapter discussion

6.2.1 Summary of results

The constitutive expression of hBD-1 mRNA, but not hBD-2 mRNA, was identified in meningioma and Chang epithelial cell lines. The levels of hBD-1 mRNA expression were apparently down regulated in Chang cells following challenge with piliated, but not non-piliated meningococci.

6.2.2 Expression of β-defensin mRNA from human cells

Human β-defensins are cationic, cysteine-rich antimicrobial peptides, predominantly expressed at various epithelial sites, where their protective role against microbial infection has been well-established (Goldman *et al.*, 1997; McCray & Bentley, 1997). Although the production of β-defensins in response to meningococcal challenge has not been previously studied, their expression could potentially play a critical role in meningococcal pathogenesis, especially during the interaction and traversal of the mucosal epithelium of the nasopharynx. However, the identification of β-defensin mRNA up-regulation in the brains of diseased cattle (Stolzenberg *et al.*, 1997)) and also their presence in the human brain, particularly the choroid plexus (Bals *et al.*, 1998; Nakayama *et al.*, 1999; Hao *et al.*, 2001), has suggested a potentially wider role for these antimicrobial peptides in innate immunity in the CNS.

In the current study, constitutive expression of hBD-1 mRNA was identified in Chang epithelial cells. This finding is consistent with other reports in the literature identifying hBD-1 mRNA expression in gastrointestinal (Bals *et al.,* 1998; O'Neil *et al.*, 1999), respiratory (Goldman *et al.,* 1997; McCray & Bentley, 1997) and genitourinary (Bals *et al.,* 1998; Valore *et al.*, 1998) epithelial cell lines. These data suggest that hBD-1 may be an integral component of innate host defences at exposed epithelia. Significantly, the current data also demonstrate that the constitutive expression of hBD-1 mRNA is not at a constant level, but is cyclic and follows a transverse wave pattern. However, in contrast to the majority of other studies that have demonstrated regulation of only hBD-2, and not hBD-1 mRNA expression upon bacterial challenge (Harder *et al.*, 1997; O'Neil *et al.,* 1999), the current study demonstrates that piliated meningococci can modulate the constitutive expression of hBD-1. Indeed, the challenge of Chang cells with piliated meningococci resulted in the rapid down-regulation of hBD-1 mRNA expression compared to the corresponding non-infected controls. These data were subsequently confirmed in experiments that varied the concentration of bacteria used to challenge the cell monolayers. However, at very low concentrations of piliated meningococci (~100 cfu / monolayer) the down-regulation of hBD-1 mRNA expression in Chang epithelial cells did not occur within the first 30 minutes, as observed with the intermediate and high doses of bacteria. Significantly, the characteristic down-regulation of hBD-1 mRNA expression was not observed until higher numbers of meningococci were detected in association with the cell monolayers, approximately three hours after challenge. However, challenge of Chang cells with non-piliated meningococci did not result in the suppression of hBD-1 mRNA expression. These data suggest that down-regulation of hBD-1 mRNA expression by meningococci is dependent on the expression of pili and positively correlated with bacterial adherence.

Only two other studies have demonstrated the down regulation of hBD-1 expression. A recent study has demonstrated the down regulation of hBD-1 expression in the gut biopsies of patients with Shigella infections. Plasmid DNA is thought to interfere with signal pathways regulating β-defensin expression, although this is unlikely in meningococci since they contain no plasmids (Islam *et al.*, 2001). The down regulation of hBD-1 mRNA expression has also been reported in

human synovial membranes during pyogenic joint inflammation (Paulsen *et al.*, 2002). Conversely, the up-regulation of hBD-1 gene expression has been demonstrated in gastric epithelial cell lines during infection with *Helicobacter pylori* and in periodontal tissue during inflammation (Dale *et al.*, 2001; Bajaj-Elliott *et al.*, 2002). The molecular mechanisms induced by bacterial infections that modulate the expression of hBD-1 mRNA remain unclear. It is unlikely that the NF-κβ signalling pathway is involved, since the hBD-1 gene lacks the NF-κβ motif which is present in the inducible hBD-2 (Harder *et al.*, 2001).

Previous reports have demonstrated the induction of hBD-2 mRNA from gastric epithelial cells challenged with *Helicobacter pylori* and *Salmonella typhimurium* (Wada *et al.*, 1999), and intestinal epithelium cells challenged with *E.coli* and *Salmonella dublin* (O'Neil *et al.*, 1999). In addition, TNF-α and IL-1α stimulated the up-regulation of hBD-2 mRNA expression in human gingival and intestinal epithelial cell lines respectively (O'Neil *et al.*, 1999; Krisanaprakornkit *et al.*, 2000). However, in the current study, the up-regulation of hBD-2 mRNA from Chang cells by challenge with meningococci or TNFα, a known stimulator of hBD-2 production (Harder *et al.*, 1997; O'Neil *et al.*, 1999), could not be demonstrated. This lack of hBD-2 mRNA expression may be due to the differences between Chang cells and other epithelial cell lines and epithelia *in vivo,* with respect to the expression of surface receptors and subsequent activation of intracellular signalling pathways.

To our knowledge, the data from the current study are the first to demonstrate the constitutive expression of hBD-1 mRNA from human meningeal cell lines. Although the expression of hBD-1 mRNA has been detected previously in meningeal fibroblasts (Hao *et al.*, 2001), these cells do not exhibit the major characteristics of leptomeningeal cells, with respect to cytological structure and expression of specific cellular markers (Feurer & Weller, 1991; Hardy *et al.*, 2000). Preliminary experiments have demonstrated that this constitutive expression of hBD-1 mRNA expression follows a cyclic transverse wave pattern, which was also observed with Chang epithelial cells. The identification of this cyclic pattern of hBD-1 mRNA can be attributed to the high sensitivity of the real-time TaqMan® PCR method used in the current study, compared with the traditional PCR utilised in other studies (Zhao *et al.*, 1996; Harder *et al.*, 1997; O'Neil *et al.*, 1999; Haynes *et al.*, 1999). Our data suggest that β-defensins, previously only found at sites of constant microbial challenge, may also be expressed in the meninges, which is normally an immunologically privileged site sheltered from the circulating effector cells and proteins of the immune system. The lack of functional opsonisation and bactericidal activity in the CSF in conjunction with the low numbers of invading bacteria required to cause meningitis (Brown *et al.*, 1981) may explain why meningeal cells constitutively express hBD-1 and not the inducible hBD-2. Therefore, β-defensin expression may function as a primary defence against invading pathogens, before the induction of pro-inflammatory cytokine and chemokine secretion results in the influx of PMNL into the CSF.

The expression of β-defensins in the meninges may potentially have a wider role in meningococcal pathogenesis than through its action as an antimicrobial compound. Recent studies have shown that cationic defensins are capable of binding to and neutralising LPS, preventing the stimulation of pro-inflammatory cytokines, including TNFα, IL-1β and IL-6 (Sawa *et al.*, 1998; Yang *et al.*, 1999). This ability to neutralise LPS could play a major role in meningococcal meningitis since it has been suggested that the release of LPS and not meningococcal adhesion to the meninges may be responsible for the stimulation of some cytokines in the SAS (Christodoulides *et al.*, 2002).

CHAPTER 7 CONCLUSIONS

Despite the introduction of effective bactericidal antibiotics and supportive care over the last fifty years, pyogenic bacterial meningitis still remains a substantial cause of morbidity and mortality worldwide (Schuchat *et al.*, 1997; Anonymous, 2000). At present and exclusive of epidemics, over one million cases of bacterial meningitis are estimated to occur annually worldwide resulting in 135,000 deaths (Anonymous, 1998), with up to 50% of survivors presenting with serious psycho-neurological sequelae (Unhanand *et al.*, 1993; Naess *et al.*, 1994; Daoud *et al.*, 1995; van de Beek *et al.*, 2002). A greater understanding of the mechanisms that occur during the interaction of meningeal pathogens with leptomeningeal cells may potentially highlight therapeutic strategies to alleviate the adverse outcome associated with bacterial meningitis.

The current study has investigated and compared the interactions of four major meningeal pathogens, namely *Neisseria meningitidis*, *Haemophilus influenzae*, *Escherichia coli* and *Streptococcus pneumoniae*, with human leptomeningeal and representative epithelial cell lines. The panel of meningeal pathogens were selected primarily on their phenotypic resemblance to bacteria commonly recovered from the CSF of individuals presenting with meningitis, while a non-pathogenic commensal bacterium *N.lactamica* was included for comparison.

7.1 What is the identity and expression of surface receptors on human cells that interact with meningeal pathogens?

Despite their differing type and expression of surface adhesins, the entire panel of meningeal pathogens adhered at high levels to a representative epithelial cell line, an ability consistent with the primary colonisation of the nasopharynx or gastrointestinal tract. In addition, the non-pathogenic bacterium *N.lactamica* exhibited equally high levels of adherence to Chang cells, which is to be expected for a commensal of the human nasopharynx. Although all of the pathogenic bacteria exhibited a tropism for the human leptomeninges, there were significant variations between them in the levels of adherence to meningioma cell lines. These differences may possibly be due to variations in the efficiency of the interactions between the individual surface adhesins and their corresponding receptors on leptomeningeal cells. Alternatively, these differences in adherence may be the result of varying levels of expression of bacterial adhesin receptors on the surface of leptomeningeal cells. It would therefore be of great relevance to investigate the surface expression of the receptors CD46, CD66, heparan sulfate proteoglycans, platelet-activating factor, α-D-mannosides, sialic acid containing glycoconjugates, and polymeric immunoglobulin receptors (pIgR) on the meningioma cell lines used in this study. By contrast, the similarity between the levels of association of the meningeal pathogens with Chang cells suggest the possible involvement of different bacterial adhesins than those implicated in interactions with meningioma cell lines. Indeed, the current study, which has investigated the roles of meningococcal pilus and *H.influenzae* capsule expression in the association with human cells,

could be extended to investigate the importance of other bacterial adhesins in adherence at the mucosa and meninges. For example, leptomeningeal and epithelial cell lines could be treated with neuraminidase, which cleaves sialic acid groups from glycoconjugates, to investigate the effect on subsequent S-fimbriae-mediated association.

7.2 How do invasive bacteria overcome the epithelial and leptomeningeal barriers?

The invasion of Chang epithelial cells by *E.coli* and *S.pneumoniae* in the current study was consistent with previous reports in the literature associating these bacteria with a transcellular mechanism of traversing the mucosal barriers (Geelen *et al.,* 1993; Cundell *et al.,* 1995a; Smith, 2003). Conversely, the absence of Chang cell invasion by *H.influenzae* is consistent with literature demonstrating that the traversal of the nasopharyngeal mucosa by *H.influenzae* is via the paracellular pathway (Stephens & Farley, 1991; Quagliarello & Scheld, 1992). The lack of invasion of meningioma cells observed with meningococci, *H.influenzae* and *S.pneumoniae* demonstrated that the leptomeninges is likely to provide a barrier *in vivo* to these pathogens. This is consistent with the presence of bacteria within the SAS with little or no spread to the underlying cerebral tissue, which is characteristic of bacterial meningitis (Brandtzaeg, 1995; Gray & Nordmann, 1997).

By contrast, *E.coli* IH3080 was internalised by meningioma cell lines, suggesting that traversal of the leptomeningeal barrier may occur *in vivo*. However, the lack of invasion by the faecal isolate *E.coli* DSM suggests the absence of an essential invasin in this strain, such as ibeA, which has a low prevalence in such isolates (Huang *et al.*, 1995; Bingen *et al.*, 1997). Although the host receptor for ibeA has been demonstrated to be specific to the surface of BMEC (Prasadarao *et al.,* 1999b), further investigation into its expression on meningioma cell lines would clarify its role, if any, in the internalisation of *E.coli* K1. Indeed, a future hypothesis to test would be what role does the ability to invade leptomeningeal cells contributes to the severity of neonatal meningitis associated with *E.coli* K1, when compared to other meningeal pathogens? In addition, further investigation into the mechanism of the rapid cytotoxicity of meningioma and Chang cell lines following challenge with *E.coli* K1 may identify other factors that contribute to the severity of this disease.

Although meningioma cells challenged with *E.coli* K1 and high doses of pneumococci exhibited the morphological characteristics of apoptosis as opposed to necrosis, further investigation is required to confirm this method of cell death. The demonstration of additional characteristics of apoptosis, such as the fragmentation of chromosomal DNA into small pieces (~50-300kbp), the reorganisation of cytokeratin intermediate filaments into granular structures, and caspase activity would confirm the occurrence of apoptosis in these cells (Studzinski, 1999). Alternatively, the presence of programmed cell death could be investigated further at a genetic level, through research into the modulation of apoptosis-related genes. Indeed, a recent study by

Wells and colleagues has demonstrated the ability of meningococci to induce rapid changes in the gene expression of meningioma cells that would, collectively, inhibit apoptosis (Wells *et al.*, 2001). This is consistent with the current study, in which no loss of viability of meningioma cell lines was observed during challenge with meningococci. These data suggest the direct interaction of meningococci with components of the apoptotic pathways, blocking apoptosis and subsequently maintaining the integrity of the leptomeningeal barrier. Conversely, *E.coli* and pneumococci may actively induce apoptosis in leptomeningeal cells through the activation of caspases and other proteases responsible for the proteolysis of numerous nuclear and cytoplasmic proteins (Studzinski, 1999). The consequent breakdown of the leptomeningeal barrier may allow the spread of these bacteria into the underlying brain contributing to the high mortality and morbidity associated with *E.coli* and *S.pneumoniae* meningitis. Indeed, the induction of apoptosis in host cells is an established virulence mechanism of intracellular pathogens, such as species of *Salmonella*, *Shigella*, *Listeria* and *Yersinia*, promoting their spread deeper into tissues (Gao & Kwaik, 2000).

7.3 What roles do the mucosal epithelia and leptomeninges play in the inflammatory response to bacterial pathogens?

Bacterial meningitis is characterised by an acute compartmentalised inflammatory response, and elevated levels of the pro-inflammatory cytokines TNFα, IL-1β, IL-6 and the chemokine IL-8 have been detected early in the CSF of individuals with bacterial meningitis (Leist *et al.*, 1988; Waage *et al.*, 1989; Brandtzaeg *et al.*, 1992; Matsuzono *et al.*, 1995; van Furth *et al.*, 1996). However, it is unclear precisely which cell types within the CNS are responsible for the production of these inflammatory mediators during bacterial meningitis. The current study has demonstrated that the leptomeninges plays an active and pivotal role in the propagation of this acute inflammatory response, through the secretion of the pro-inflammatory cytokine IL-6, the chemokines IL-8, MCP-1 and RANTES, and the cytokine growth factor GM-CSF, following challenge with meningeal pathogens. Indeed, the secretion of the pro-inflammatory cytokine IL-6 from leptomeningeal cell lines is associated with fever and leukocytosis. In addition, the induction of the chemokines IL-8, MCP-1 and RANTES aid the influx of PMNL in to the CSF, through the increased expression of adhesion molecules on the vascular endothelium, while GM-CSF aids in the maturation of resident and infiltrating macrophages present in the SAS. However, differences were observed in the type and levels of pro-inflammatory cytokines and chemokines induced by the various meningeal pathogens. This could be a direct consequence of the variations observed between the meningeal pathogens in their corresponding levels of adhesion to meningioma cells. However, this is unlikely since there were no significant differences between the meningeal pathogens in the levels of association with Chang epithelial cells, but variations in the type and levels of inflammatory mediators were still evident.

The possible mechanisms of this apparent modulation of the host immune response, which is specific to each human cell line, could be further investigated. Indeed, the identification of the cell signalling events involved during bacteria-host cell interactions, such as NF-kB and other transcription factors, may identify possible mechanisms of pro-inflammatory cytokine and chemokine regulation. Furthermore, the disruption of the interactions between meningeal pathogens and leptomeningeal cells, and hence the subsequent intracellular signally events, may offer potential targets for therapeutic intervention.

7.4 Do bacterial pathogens modulate β-defensins, a component of the innate immune response?

The current study has identified the constitutive expression of hBD-1 mRNA from both leptomeningeal and epithelial cell lines, which suggests a potential role for β-defensins as an initial host defence against pathogenic bacteria. Indeed, the ability of meningococci to down-regulate the expression of these antimicrobial molecules may be an important parameter of meningococcal virulence by establishing a temporary niche for bacterial survival at the mucosal surface, which might be necessary for subsequent interactions and invasion. However, the current study could be extended in order to establish whether the observed variations between the meningeal pathogens in the induction of pro-inflammatory cytokine and chemokines are mirrored in the modulation of hBD-1 mRNA expression from Chang cells. The identification of β-defensin expression by leptomeningeal cell lines in this study has a potentially important biological significance since the dominant host defence mechanisms, namely PMNL, immunoglobulins and complement components are virtually absent in the CSF. Again the current study could be extended to investigate whether the expression of hBD-1 mRNA from meningioma cell lines is capable of being modulated by meningococci, *H.influenzae*, *E.coli* or *S.pneumoniae,* and if this modulation is specific to leptomeningeal cells or similar to that observed in Chang epithelial cells. Indeed, it is possible that this bacterial modulation results in the antimicrobial barrier being overwhelmed both at the nasopharynx and in the meninges, although the *in vivo* levels of β-defensins would need to be identified to further investigate this hypothesis.

The modulation of β-defensin expression by bacterial pathogens could potentially have wider implications than just overwhelming antimicrobial barriers with the recent association of cationic defensins with the neutralisation of bacterial LPS (Sawa *et al.*, 1998; Yang *et al.*, 1999). Indeed, the down-regulation of β-defensins, and hence reduction of LPS neutralising molecules, at the meninges may indirectly contribute to the intense inflammatory response, and ultimately clinical severity and neurological outcome, associated with high levels of LPS in the CSF of patients with bacterial meningitis (Mertsola *et al.*, 1991; Brandtzaeg *et al.,* 1992; Brandtzaeg *et al.*, 2001).

CHAPTER 8 APPENDIX

Proteose peptone (PP) agar

Both meningococcal strains and *N.lactamica* were grown on PP agar, prepared according to the method of Zak and colleagues (Zak *et al.*, 1984).

Materials:	**Amount per litre (dH_2O)**
Proteose peptone (BD Biosciences)	10g
Bacto-agar (N°.1, Oxoid)	10g
Starch	1g
$K_2HPO_4.3H_2O$ (Fisher)	5.24g
$K_2H_2PO_4$	1g
Sodium Chloride (Fisher)	5g
Supplement A	8ml
Supplement B	2ml

All components except supplements A and B were sterilised by autoclaving at 1.05kg cm^{-2} for 15min. Supplements A and B were added to the autoclaved solution after cooling to 50°C.

Supplement A:	**Amount per 800ml (dH_2O)**
Glucose	100g
L-glutamine	10g
Para-amino-benzoic acid (Sigma)	13mg
β-nicotinamide adenine dinucleotide (Sigma)	250mg
Thiamine hydrochloride (Sigma)	3 mg
Co-carboxylase (Sigma)	100mg
Cyanocobalamin (Sigma)	10mg
Ferric nitrate	20mg

The components of supplement A were dissolved in dH_20, 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 components, except cysteine, were dissolved in 100ml of boiling 0.1M hydrochloric acid. The cysteine was added after cooling to 25°C and the final volume made up to 200ml with dH_2O. The solution was filter sterilised and stored at –20°C.

Luria-Bertani (LB) agar

Both *E.coli* strains were grown on LB agar.

Materials:	**Amount per litre (dH_2O)**
Tyrptone	10g
Yeast extract (Fisher)	5g
Sodium chloride (Sigma)	5g
Bacto-agar (N°.1, Oxoid)	10g

All components were sterilised by autoclaving at 15lb in^{-2} for 15min.

Haemophilus Test Medium (HTM) agar

Both *H.influenzae* strains were grown on HTM base medium (Oxoid) supplemented with 15µg ml^{-1} of both β-NAD and Hematin.

Materials:	**Amount per litre (dH_2O)**
HTM base (Oxoid)	43g
β-NAD supplement	2ml
Hematin supplement	2ml

The HTM base medium was dissolved in dH_2O and sterilised by autoclaving at 1.05kg cm^{-2} for 15min. β-NAD and Hematin supplements were added to the autoclaved solution after cooling to 50°C.

β-NAD supplement:	**Amount per 10ml (dH_2O)**
β-NAD (Sigma)	75mg

Hematin supplement:	**Amount per 10ml (dH_2O)**
Hematin (porcine) (Sigma)	75mg
Tri-ethanolamine (Sigma)	400µl

All components were dissolved in dH_2O by heating to 70°C for 20mins. The solutions were filter sterilised and stored at –20°C.

Todd Hewitt (TH) agar

S.pneumoniae was grown on TH agar, prepared according to the manufacturers' instructions.

Materials:	**Amount per litre (dH_2O)**
Todd Hewitt broth (Oxoid)	36.4g
Bacto-agar (N°.1, Oxoid)	10g

All components were sterilised by autoclaving at 1.05kg cm^{-2} for 15min.

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