Effects of virus infection and smoking on binding of bacteria to epithelial cells

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Declaration

I declare here that the work for this thesis was carried out by myself or under my direct supervision

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I dedicate this work to my father Ramadan, my mother Halima, my wife Fadwa and all my family for the inspiration they have provided and for all they have done. Also to my gorgeous daughters: Essra, Sara and Malak

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Abstract

Both smoking and virus infection are risk factors for bacterial infections of the lower respiratory tract. Serious secondary bacterial disease can occur following illness due to respiratory viruses, and viral infections have also been suggested to be predisposing factors for bacterial meningitis.

Previous studies found enhanced binding of pathogenic bacteria to cells infected with Respiratory Syncytial Virus (RSV) which is an important cause of infection in infants, young children and the elderly. While smoking is usually thought of as a risk factor for viral infection, this study was designed to examine separately the effects of these two factors on bacterial binding. The objectives of the study were: (1) to determine if there is a similar pattern of enhanced binding of *Neisseria meningitidis*, *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Bordetella pertussis* to RSV subgroup B infected cells as observed with RSV subgroup A infection; (2) to determine if there is increased binding of other species of bacteria associated with meningitis and those associated with secondary respiratory infections or exacerbation of chronic bronchitis to RSV infected cells; (3) to determine if there was a similar pattern of increased bacterial binding to influenza virus infected cells; (4) to determine if as with RSV infected cells there was an increase in expression of native cell surface antigens which can act as receptors for bacteria; (5) to determine if there is enhanced binding of bacteria associated with meningitis or respiratory disease to cells of smokers;

(6) to assess cells of smokers and non-smokers for differences in level of expression of molecules proposed to act as bacterial receptors.

With the exception of an antibiotic-sensitive strain of *Moraxella catarrhalis* (MC2) infection of an epithelial cell line (HEp-2) with RSV (subgroups A or B) enhanced binding of all bacterial strains tested. Compared with the antibiotic-resistant strain, MC2 and other antibiotic-sensitive isolates of *M. catarrhalis* were found to express differences in outer membrane proteins, sensitive to complement-mediated killing. With human monocytes or the THP-1 monocyte cell line, phagocytosis or intracellular survival studies showed no differences between the two isolates of *M. catarrhalis*.

Cells infected with human influenza A virus showed increased adherence of each of the species tested, including the antibiotic-sensitive isolates of *M. catarrhalis*. Compared with uninfected cells, influenza virus infected HEp-2 cells showed significantly increased binding of monoclonal antibodies for the cell surface antigens CD14 and CD18 that appeared to act as receptors for Gram-negative species tested. Pre-treatment of HEp-2 cells with neuraminidase showed increased bacterial binding compared with untreated HEp-2 cells, but the increase was less than that observed for influenza infected cells.

Buccal epithelial cells from smokers bound more of each of the bacterial species tested, but there were no differences between cells from smokers compared with non-smokers in expression of blood group antigens, fibrinogen or fibronectin. Treatment of cells

with an extract of cigarette smoke enhanced binding. Although the extract contained material cross reactive with the H type 2 blood group antigen which acts as a receptor for some strains, this could not completely explain the enhanced binding.

The results suggest that while smoking is a predisposing factor for viral infection, it can independently enhance bacterial binding of strains associated with meningitis or respiratory infection on its own.

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Chapter 1

General Introduction

1.1 Bacterial infection of the respiratory tract

Two environmental factors, viral infection and exposure to cigarette smoke, have been associated with susceptibility to bacterial infection of the respiratory tract. It has been reported that both virus infection and active smoking increase the levels of bacterial colonisation (Musher and Fainstein, 1981). The importance of colonisation of mucosal surfaces as the first step in development of bacterial infection has been recognised for many years. Most of the early work concentrated on the bacterial components involved in binding to the host cell, and it became clear that density of colonisation reflected in numbers of bacteria attached to epithelial cell surfaces was an important factor in pathogenesis (Table 1.1) (Beachey, 1981). More recently, host factors that could affect colonisation have been explored. Many of the clues to these factors have come from epidemiological studies in which genetic, developmental and environmental factors associated with susceptibility to bacterial diseases have been identified (Haneberg *et al.*, 1983; Pershagen, 1986; Stuart *et al.*, 1988; Blackwell, 1989; Blackwell *et al.*, 1995; Blackwell *et al.*, 1997).

Both active smoking and passive exposure to cigarette smoke are associated with an increased risk of viral respiratory infection in adults and children (Hall *et al.*, 1984). In

Table* 1.1 Relationship between adherence of bacteria to epithelial cells in *vitro* and bacterial infectivity in *vivo*

Bacteria	Bacterial variants	Relative adherence in vitro	Relative infectivity in vivo	References
Gonococci	T1 (fimbriated) T4 (nonfimbriated)	Good Poor	High Low	Swanson (1977); Buchanan and Pearce (1976); Watt and Ward (1980); Pearce and Buchanan (1980)
Escherichia coli (enterotoxigenic)	CF positive CF negative	Good Poor	High Low	Satterwhite, DuPont, Evans (1978)
Streptococci	Dextran positive Dextran negative	Good Poor	High Low	Scheld, Valone, Sande (1978); Ramirez-Ronda (1978)
Salmonella	Fimbriated Nonfimbriated	Good Poor	High Moderate	Duguid and Old (1980)
E. coli	K88 positive K88 negative	Good Poor	High Low	Jones and Rutter (1974)
Proteus mirabilis	Fimbriated Nonfimbriated	Good Poor	High Low	Silverblatt (1974); Silverblatt and Ofek (1978)
Bordetella pertussis	Fimbriated Nonfimbriated	Good Poor	High Low	Sato, Izumiya, Oda, Sato (1979)

* adapted from Beachey, 1981

this project, the separate effects of virus infection and exposure to cigarette smoke on bacterial adherence were investigated. Two models were used. The first was based on the well established associations between virus infection, exposure to cigarette smoke and secondary bacterial infection of the respiratory system, *e.g.*, pneumonia, exacerbations of chronic bronchitis and otitis media. The second model was based on more speculative associations between virus infection, exposure to cigarette smoke and increased susceptibility to bacterial meningitis.

The classic example of influenza virus infection as a predisposing factor for bacterial pneumonia has been studied in both epidemiological and experimental investigations (Nugent and Pesanti, 1983). Influenza has also been implicated in epidemiological studies of bacterial meningitis reflected in timing of outbreaks of meningococcal disease in relation to prevalence of influenza in the community and antibodies to influenza A in sera of patients with meningitis (Cartwright *et al.*, 1991). Because influenza is less common in infants and young children in which the major peak of meningitis occurs, respiratory syncytial virus (RSV) was also examined in the model system. It is implicated in susceptibility of children to several bacterial infections of the respiratory tract (Hall, 1980) and annually affects the age range in which the majority of bacterial meningitis cases occur.

1.2. Influenza viruses

Influenza viruses belong to the family *Orthomyxoviridae* and are separated into types A, B, and C. Type C might represent a separate genus, as it does not demonstrate the

genetic variability seen in types A and B and rarely causes clinical disease. These enveloped, negative stranded segmented RNA viruses are of similar size and morphology and contain two types of membrane glycoproteins which project from the virion surface. One glycoprotein, haemagglutinin, is responsible for attachment of infecting viruses to host cell membranes. The other, neuraminidase, is involved in release of virus from infected cells.

The most distinctive feature of the influenza viruses is the ability to change their antigenic characteristics at irregular intervals. The antigenic changes are accounted for by alterations in the amino acid sequence of haemagglutinin and / or neuraminidase. With small changes (antigenic drift), previously formed antibodies can offer partial protection; however, with major changes (antigenic shift), previously formed antibodies do not "recognise" the variant and hence do not provide protection against reinfection (Kilbourne, 1987 a; Glezen and Couch, 1989).

The influenza viruses are responsible for yearly outbreaks of infections that occur in the winter or early spring. These outbreaks are usually widespread but vary considerably in their extent and in the severity of clinical infections. Some epidemics are associated with sharp increases in mortality, especially in the elderly. These yearly outbreaks occur world-wide (Kilbourne, 1987 a; Glezen and Couch, 1989).

1.2.1 Disease caused by influenza virus

The influenza viruses are responsible for a wide spectrum of clinical disease. The characteristic "flu" symptoms (fever, headache, myalgia, malaise, anorexia, and photophobia) occur early in the course of infection (Kilbourne, 1987 a; Glezen and Couch, 1989). Cough becomes prominent thereafter. These are the classic findings in influenza infections that aid identification of affected individuals during epidemics. The influenza viruses may cause infection indistinguishable from those due to other viruses. Such infections range from undifferentiated upper respiratory tract infections to those that involve the lower respiratory tract in the form of croup, bronchiolitis, or pneumonia. In small children, the primary site of involvement in most influenza infections is the tracheobronchus. Clinical infections differ widely in severity and are accompanied by a variety of complications.

Secondary bacterial infection is the most common, life-threatening complication of influenza infection (Kilbourne, 1987 a; Glezen and Couch, 1989). The general clinical picture is one of gradual improvement during convalescence with a sudden relapse characterized by recurrence of fever, chest pain, and productive cough. At times this biphasic pattern does not appear, and the secondary infection blends into the initial viral infection. *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* are the most common pathogens isolated in influenza-associated bacterial pneumonia; *Klebsiella pneumoniae*, *Streptococcus pyogenes*, and *Neisseria meningitidis* superinfections have also been reported (Leigh *et al.*, 1991).

Experimental studies in which animals were used have shown that bacterial superinfection occurs after simultaneous inoculation of virus and bacteria or after primary influenza virus infection followed by inoculation of bacteria into the respiratory tract (Pio *et al.*, 1985). Influenza-associated bacterial infections is particularly prominent in developing countries where nasopharyngeal colonisation with *S. pneumoniae* and *H. influenzae* is higher than in developed countries (Pio *et al.*, 1985).

1.2.2 Factors that influence the course and outcome of influenza virus infections

1.2.2.1 Immunity

Innate and immune responses to influenza virus infection are essential for limiting the severity and duration of illness, as well as for protecting against subsequent infection by an antigenically similar strain. Individuals who are immunocompromised from congenital or acquired defects in the immune system or from immunosuppression by a variety of conditions are more likely to have severe and prolonged illnesses with influenza virus infection (Leigh *et al.*, 1991).

1.2.2.2 Age

The influenza viruses commonly infect patients of all ages, probably because of the changing antigenic structure of the viruses and the absence of protective immunity to the new strains. For reasons that are not completely understood, individuals at the extremes of age (infants and the elderly) are at increased risk for serious and even fatal influenza infections (Kilbourne, 1987a; 1987b). The increase in mortality is

particularly prominent in the elderly. Possible factors that could be important in this group are waning of immune responsiveness and underlying cardiopulmonary insufficiency. The clinical presentation of influenza infection in infants and young children differs from that in older children and adults and often resembles that of bacterial sepsis. Some studies have suggested that the course of illness and virus shedding are more prolonged in infants than in older age groups. Studies in ferrets with influenza infection also have demonstrated increased mortality among younger animals (Collie *et al.*, 1980). One of the proposed explanations for this high mortality is that congestion of the small nasal passages of infant ferrets leads to complete nasal obstruction and obstructive apnea (Sweet *et al.*, 1988).

1.2.2.3 Cardiovascular disease

Several studies have demonstrated that underlying cardiovascular disease is a major risk factor for severe influenza illness. In the 1957 epidemic, patients with heart disease, most notably those with rheumatic heart disease, experienced a high mortality rate (as high as 50%) and a high incidence of pneumonia (50%), both primary influenza pneumonia and secondary bacterial pneumonia (Kilbourne, 1987a). Little is known about the course of influenza in patients with congenital heart disease, but RSV infections are particularly severe in patients with congenital heart defects, especially those with pulmonary hypertension (MacDonald *et al.*, 1982).

1.2.2.4 Chronic lung disease

Patients with chronic lung diseases, specifically chronic bronchitis, bronchiectasis, asthma, emphysema, and bronchopulmonary neoplastic disease, are at increased risk for pneumonia and death from influenza infection (Kilbourne, 1987a). Several mechanisms might be playing a role in increasing the severity of disease. The underlying lung disease could limit respiratory reserve, thereby increasing the risk of respiratory failure during influenza infection. In addition, many of these patients have airway hyper-reactivity and impaired mucociliary clearance, which might increase the likelihood of their developing bronchospasm, mucus plugging, and secondary bacterial pneumonia in association with influenza virus infection (Leigh *et al.*, 1991).

1.2.2.5 Malnutrition

Malnutrition is a major risk factor for acute respiratory illnesses, leading to increased incidence of disease and mortality (Pio *et al.*, 1985; Stansfield, 1987). The effects of malnutrition on immune function have been reviewed extensively (Rochester, 1984; Martin, 1987). Protein-calorie malnutrition results in major impairment of cell-mediated immunity and virus infections that are controlled by cell-mediated responses are more prevalent and severe among the malnourished. Humoral immunity and phagocytic function of polymorphonuclear leukocytes are impaired in severely malnourished individuals but not to the extent that cell-mediated immunity is impaired.

Vitamin A deficiency has been identified as a nutritional determinant influencing incidence of disease and mortality from acute respiratory illnesses in developing

countries (West *et al.*, 1989). Vitamin A is important for maintenance of epithelial integrity; experimental vitamin A depletion results in squamous metaplasia of airway epithelium and loss of ciliated and goblet cells. In addition, vitamin A deficiency impairs humoral and cell-mediated immunity, phagocytosis, and delayed hypersensitivity. Other nutritional factors that appear to be important are prenatal nutrition and breast feeding. The risk of mortality from respiratory infections is higher in infants of low birth weight than in infants of higher birth weight (Pio *et al.*, 1985). Likewise, infants who are not breast-fed are more likely to die of a respiratory illness than are those who are breast-fed (Stansfield, 1987).

1.2.2.6 Infections due to other micro-organisms

Preceding or coexisting infections with other micro-organisms could influence the course of influenza infections. Colonisation or infection of the airways with bacteria during influenza infection increases the risk of a secondary bacterial pneumonia. Other infections could impair host defense mechanisms that are important in controlling influenza disease. Primary infections by viruses, most notably human immunodeficiency virus (HIV), can cause immunosuppression (Rouse and Horohov, 1986). HIV infection is prevalent world-wild and has been shown to influence immunity to influenza (Ragni *et al.*, 1987). Other viruses capable of inducing immunosuppression include measles, Epstein-Barr virus (EBV), cytomegalovirus (CMV), and herpes simplex virus (HSV), but their association with influenza virus has not been documented.

1.3 RSV

RSV belongs to the genus *Pneumovirus* in the family of *Paramyxoviridae*. It is a large enveloped, negative-stranded RNA virus. The virus particles are pleomorphic, approximately 80-350 nm in diameter or filaments of up to 10 um in length. The virions assemble at circumscribed regions on the plasma membrane of the infected cell and mature by budding during which intracellular nucleocapsid is packaged within an envelope that is derived from the host cell membrane (Bachi, 1988). The envelope contains spike-like projections spaced at 6-10 nm intervals composed of the major structural glycoproteins G and F which mediate viral attachment (G) (Levine *et al.*, 1987) and fusion and penetration (F) (Walsh and Hrusks, 1983). These glycoproteins are transported to the cell membrane and inserted in the lipid bilayer (Satake *et al.*, 1985). Two antigenically distinct types of RSV, designated A and B subgroups based on variation in G glycoprotein of RSV have been recognised (Anderson *et al.*, 1985; Johnson *et al.*, 1987).

1.3.1 Age

RSV is a major cause of respiratory infections in infants and young children (Avila *et al.*, 1989; Monto and Lim, 1986). In children with respiratory disease, RSV is the most frequently isolated virus (Huq and Rahman, 1990; Nicolai and Pohl, 1990). Over 90% of all these RSV infections are observed in children less than 2 years of age (Avila *et al.*, 1989; Huq and Rahman, 1990). Most children are infected with RSV by the age of 2 years, but the infections can occur throughout life and have been reported in adolescents, adults and elderly people (Finger *et al.*, 1987; Belshe *et al.*, 1978; Englund

et al., 1991). Taylor and colleagues (1989) studied over 500 infants from two epidemics and found that patients with subgroup A infections tended to be significantly younger (22 weeks) than those with subgroup B infections (28 weeks). Tsutsumi and colleagues (1988) studied nine epidemics of RSV in Japan and also found that children infected with virus of subgroup A tended to be younger. The study by Hall and colleagues (1990), a longitudinal study over 15 years on RSV isolates from 1209 hospitalised and ambulatory children, did not find a significant age difference between patients infected with subgroup A and those infected with subgroup B. All the studies used the same methods for detection of the virus (direct immunofluorescent antibody technique and ELISA).

RSV is also a significant cause of serious lower respiratory tract infection in immunocompromised adults and the elderly (Falsey *et al.*, 1995).

1.3.2 Geographical distribution and seasonality

RSV infections have been reported world wide (Finger *et al.*, 1987; Sung *et al.*, 1987; Hall *et al.*, 1990; Huq and Rahman, 1990; Nicolai and Pohl, 1990). There is a definite seasonal periodicity in RSV infections. In zones with a temperate climate, the peak incidences of RSV infections usually occur yearly during the autumn, winter and early spring (Anderson *et al.*, 1990). The epidemic lasts 5 to 6 months and peaks during the third or fourth month, the actual month of peak infection varies a little from year to year. Fewer studies have been reported from sub-tropical countries (Sung *et al.*, 1987; Nwanko *et al.*, 1988; Tantivanich *et al.*, 1984). In these countries, correlations of

incidence of infection with rainfall have been observed. It could be that factors such as cold weather in temperate zones or rain in sub-tropical areas lead to close contact indoors and enhanced transmission of the virus.

1.3.3 Incidence and prevalence of primary infection

Among children in hospital with pneumonia, RSV could be recovered in 9 to 39% of cases (Sung *et al.*, 1987; Nicolai and Pohl, 1990; De Silva and Hanlon, 1986; Kim *et al.*, 1973). Among those with bronchiolitis, RSV was recovered in 27-70% of the children. Most infants become infected with RSV during their first year of life (Kim *et al.*, 1973), and 25-50% have antibodies to RSV (Parrot *et al.*, 1966). Others studies reported that 95-98% of children have antibodies to RSV at the end of their third year of life (Brussow *et al.*, 1991; Fernald *et al.*, 1983). Differences in reliability and accuracy of tests may underlie these different observations, particularly results of earlier studies with less accurate means of diagnosis of infection.

1.3.4 Transmission

RSV infections are transmitted by large droplets through fomite contamination, or by direct contamination with infected secretions. Close contact appears to be necessary for infection to spread from one person to another. The most important route of transmission appears to be self-inoculation with fingers contaminated with infected secretions (Hall, 1990). The fingers transmit the virus to the nasal mucosa or conjunctivae from which the virus spreads to the upper respiratory tract.

The incubation period is usually 2-8 days (Hall, 1990). The infection spreads to the lower respiratory tract within a few days of the onset of symptoms. In a study by Waris and colleagues (1992), 40-60% of the patients stopped shedding RSV 8 to 10 days after the onset of illness. Some infants can shed RSV for up to 3 or 4 weeks or longer. Longer periods of virus shedding have been noted in immunosuppressed children, and shorter durations in older children and adults (Hall *et al.*, 1976).

1.3.5 Risk factors for RSV infection

Several risk factors for RSV lower respiratory tract infection have been identified. In the early months of life, the infection is more common in males (Ruuskanen and Ogra, 1993). RSV infection is more common in: children born during the summer months approximately 6 months before the outbreak; those sharing a bedroom with other children (especially when there are two or more sharing the room); day-care settings; and infants of mothers with lower educational levels (Holberg *et al.*, 1991; McConnochie and Roghmann, 1986; Anderson *et al.*, 1988). Importantly, breast-feeding for longer than 1 month has a protective role, especially for those infants whose mothers are of lower socio-economic status. Infants with a low titre of RSV antibody in cord serum and minimal breast-feeding are especially at risk for RSV infection of the lower respiratory tract (Holberg *et al.*, 1991).

1.4 Virus infection and susceptibility to bacterial disease

While influenza and RSV affect different age ranges, there are several bacteria that are commonly associated with diseases for which these viruses are predisposing factors. These can be found as part of the normal respiratory flora; therefore, it is important to examine ways in which virus infection could lead to disease, *e.g.*, enhanced density of colonisation.

RSV is a major cause of hospitalisation in infants during the first 6 months of life and secondary bacterial infections such as acute otitis media and pneumonia are common (Ruuskanen and Ogra, 1993). In a Finnish study, RSV infections were associated with significant rises of antibody titres to *H. influenzae*, *Moraxella catarrhalis* and pneumococcal antigens (Korppi *et al.*, 1989). In the United States, RSV infection was associated with isolation of *Bordetella pertussis* from infants during a winter outbreak (Nelson *et al.*, 1986), and recent studies indicate that RSV infection was significantly associated with invasive pneumococcal disease in both adults and children (Kim *et al.*, 1996).

Many of the host's defenses against bacterial infections of the respiratory tract can be altered by influenza virus infections (Couch, 1981). Influenza infection causes desquamation of ciliated cells and altered mucous secretion, compromising the efficiency of mucociliary clearance by the airways. Bacterial adherence to influenza-infected cells is enhanced by mechanisms that are not clear. The combination of impaired mucociliary clearance and enhanced binding of micro-organisms, inhaled or

aspirated coincident with an influenza infection, will be retained in the lung and cause pneumonia.

Influenza virus infection causes impairment in the function of alveolar macrophages (Jakab, 1982), reduction in chemotaxis of monocytes (Kleinerman *et al.*, 1975) and reduction in lymphocyte responsiveness and proliferation of T-suppressor cells (Gardner, 1981). It also interferes with the chemotactic and phagocytic function of polymorphonuclear leucocytes (PMN) (Larson *et al.*, 1980; Martin *et al.*, 1981; Leigh *et al.*, 1991). Similar effects on the function of PMN were found with RSV (Craft *et al.*, 1976).

1.5 Air pollutants and infectious diseases

Exposure to air pollutants, especially indoor air pollutants, has been identified as a risk factor for respiratory illnesses (Samet *et al.*, 1987; Samet *et al.*, 1988). This is particularly important in the developing world (Pio *et al.*, 1985; Stansfield, 1987). Products of combustion from fossil fuels, wood, and dung are major indoor air pollutants in developing countries where exposure levels to indoor air pollutants, especially for children, are much greater than in technologically advanced countries (Samet *et al.*, 1987). The incidence of both acute respiratory infections and chronic respiratory symptoms is increased in individuals exposed to certain agents. Many pollutants, such as ozone, cigarette smoke, wood smoke, and nitrogen dioxide, a product of burning fuels, increase airway reactivity, which in turn might increase the bronchospasm associated with respiratory infections. Chronic irritation of the airways

by pollutants results in chronic bronchitis and impaired mucociliary clearance, predisposing the airways to infection. A study by Goings and colleagues (1989) demonstrated that exposure to nitrogen dioxide increases susceptibility to influenza virus infection.

Environmental tobacco smoke, a complex mixture of exhaled mainstream smoke and non-inhaled side-stream smoke, also contributes to respiratory morbidity of children (Chen *et al.*, 1986). Tobacco combustion produces multiple toxic compounds (Guerin *et al.*, 1992). Although environmental tobacco smoke differs from mainstream smoke in several ways, it contains many of the same toxic substances. Infants and toddlers may be especially at risk when exposed to environmental tobacco smoke (US National Research Council, 1986). Exposure to toxic compounds in infancy is particularly problematic because early lung development appears to be a critical determinant of respiratory health (Martinez *et al.*, 1988).

1.5.1 Cigarette smoke and susceptibility to infection

1.5.1.1 Respiratory infection in adults

Active smoking is associated with an increased risk of respiratory tract infections in adults. Aronson and colleagues (1982) showed that young smokers had an approximately two fold greater risk of developing an infection compared to non-smokers. Smokers were also more likely to have a lower respiratory tract infection and they remained symptomatic for a 25% longer period. Kark and colleagues (1982) suggested a link between smoking and contracting H1 N1 influenza virus in a

susceptible population of young men. Within this population, the severity of disease was considerably greater among smokers. Smokers also have an increased risk of developing the common cold, smoking being related to both the probability of infection and of developing symptoms (Cohen *et al.*, 1993). Cigarette smoking has been shown to be the most common cause of chronic bronchitis, and heavy smokers are particularly susceptible to lower respiratory tract infections caused by *H. influenzae*, *S. pneumoniae*, *S. aureus* and *M. catarrhalis* (Cazzola *et al.*, 1990).

An association has also been demonstrated between susceptibility to several non-respiratory infections and smoking. Smoking has been shown to confer a two fold increase on a woman's risk of developing pelvic inflammatory disease, a severe upper genital tract infection caused mainly by *Streptococcus intermedius* or *Neisseria gonorrhoeae* (Scholes, 1992). It has also been shown that cigarette smoking will accelerate progression to AIDS in HIV positive individuals, and that heavy smokers are over three times more likely to develop *Pneumocystis carinii* pneumonia than light smokers (Nieman *et al.*, 1993).

1.5.1.2 Adults and Passive Smoking

There is very little information about the effects of passive smoking related to adult respiratory infections, but a strong association has been shown between passive smoking, especially in the home, and lung cancer (Spitzer *et al.*, 1990). Passive smoking has been related to a higher incidence of both colds and chronic obstructive pulmonary disease in adult non-smokers (Cazzola, 1990). Shepherd (1992)

hypothesised that a non-smoker who lives with a smoker will be more likely to develop a respiratory tract infection as the smoker himself has an increased risk of disease, thus increasing the exposure of the non-smoker to respiratory pathogens. Chronic exposure of a non-smoker to environmental tobacco smoke is thought to produce effects similar to those associated with active smoking, *e.g.*, chronic bronchitis (Robbins *et al.*, 1993).

1.5.1.3 Children and Passive Smoking

Substantially more information has been published concerning the association between passive smoking and children's respiratory health. The strongest associations appear to be found between maternal smoking and the respiratory health of children less than two years old (Pershagen, 1986); but even in later childhood (5-11 years old), the risk of respiratory tract infections due to passive smoking is still increased (Chen and Rona 1991). A child's exposure to cigarette smoke, especially in the home, might be for up to eighteen years and this early exposure to smoke is believed to be crucial in determining the long term health of the child (Whidden, 1993).

Respiratory infections are frequent in childhood, and about 30 % of all infants are treated for bronchiolitis, croup or pneumonia (Wright *et al.*, 1989). Bacterial and viral infections of the respiratory tract are more common in children exposed to cigarette smoke (Colley *et al.*, 1974, Ogston *et al.*, 1987), and they are four times more likely to be admitted to hospital than children whose parents do not smoke (Berg *et al.*, 1991). Ogston and colleagues (1987) found parental smoking was associated with higher respiratory and alimentary illnesses in the first year of life, and there was evidence of

lower incidences of infection among the breast fed compared with bottle fed infants. Alimentary illness was associated with mother's smoking habit, whereas respiratory illness was associated with smoking by either parent. Lower respiratory tract infections such as pneumonia and bronchitis are also more common in a child whose parents smoke (Chen *et al.*, 1986; Weiss *et al.*, 1983). Ear infections, especially otitis media caused by *H. influenzae* and *S. pneumoniae* have been demonstrated to be more common in children of smokers than those of non-smokers (Kraemer *et al.*, 1983).

Maternal smoking during pregnancy might predispose a child to respiratory infections in early life by causing congenital damage to the developing respiratory system, interfering with the immune system or causing a secondary immunodeficiency (Taylor and Wadsworth, 1987). Cigarette smoking during pregnancy had been associated with reduced birth weight, and low birth weight was associated with a wide range of defective immune functions that might predispose to respiratory infections (Taylor and Wadsworth, 1987). Infants exposed to maternal smoking had an increased incidence of lower respiratory tract infection (Fergusson *et al.*, 1981). This effect showed a dose-response relationship to maternal smoking and decreased after the first year of life. Infants with bronchiolitis before the age of 2 years were 2.4 times more likely to have been exposed to maternal smoking than infants who did not develop a lower respiratory tract infection (McConnochie *et al.*, 1986).

A study by Wright and colleagues (1991) found that infants whose mothers smoked at least one package of cigarettes per day had 2.8 times the risk of developing a lower

respiratory infection. Children hospitalized for acute lower respiratory illness before the age of 2 are 1.8 times as likely to live with smokers than control subjects hospitalised for non-respiratory illness (Hall *et al.*, 1984). Considering the substantial morbidity, and even mortality, of acute respiratory illness in childhood, a doubling in risk attributable to passive smoking clearly represents a serious paediatric health problem (US. National Research Council, 1986).

1.5.2 How does cigarette smoke affect the respiratory tract ?

1.5.2.1 Prenatal effect of smoking

The manner in which passive exposure to environmental tobacco smoke leads to increased lower respiratory infection risk is unknown. Prenatal effects of maternal smoking on the lung have been demonstrated by Hanrahan and colleagues (1992) who found that infants born to mothers who smoke have reduced forced expiratory flows (measured in litres / min with a Peak Flow Meter). The degree of reduction was correlated with increasing maternal urine cotinine/creatinine ratios during pregnancy. Subsequent lung dysfunction and respiratory illness could thus begin by *in utero* exposure to cigarette smoke, with alteration of the developing lung (Martinez *et al.*, 1988). Children whose mothers smoked only after pregnancy were still more likely to develop acute respiratory illness (Woodward *et al.*, 1990).

1.5.2.2 Bacterial adherence

Bacterial adhesion is an important factor favouring persistence and colonisation of the respiratory tract (Reynolds, 1987; Plotkowski *et al.*, 1993). Most studies have

investigated bacterial adhesion of *S. pneumoniae* and *H. influenzae* to oropharyngeal and buccal cells, either to exfoliated cells or to nasopharyngeal mucosal biopsies. Adhesion has also been demonstrated to exfoliated human bronchial cells for *B. pertussis* (Toumanen *et al.*, 1988).

Smokers have been shown to have an enhanced adherence of *S. pneumoniae* to buccal cells (Raman *et al.*, 1983; Mahajan and Panhotra, 1989), and smokers with chronic bronchitis appear to have increased adherence of *H. influenzae* to their pharyngeal cells (Fainstein and Musher, 1979).

1.6 Smoking and Meningococcal Disease

The risk factors for meningococcal disease include close contact with a case, overcrowded accommodation, poverty and lower social class, and passive exposure to cigarette smoke (Stuart *et al.*, 1988). In Britain, smoking is more prevalent among both men and women in partly skilled or unskilled occupations compared with those in professional classes (Wald *et al.*, 1988).

Although there is no direct association between meningitis and smoking, there is a connection between cigarette smoking and meningococcal colonisation of the nasopharynx which is not dependent on age, sex, or social class. The association between smoking and meningococcal colonisation is highly significant and appears to be greater among heavier smokers (Stuart *et al.*, 1989; Blackwell *et al.* , 1992).

While there is no association with active smoking and meningitis, children who live in households in which there are smokers are at increased risk of invasive meningococcal disease (Stuart *et al.*, 1988).

Cigarette smoking is suggested to reduce nasopharyngeal mucosal defences against meningococcal invasion (Stuart *et al.*, 1988; Haneberg *et al.*, 1983). Since cigarette smokers are more likely to be meningococcal carriers (Stuart *et al*, 1989; Blackwell *et al.*, 1990; Blackwell *et al.*, 1992), young children living in households with smokers would have more frequent contact with carriers, greater chances of acquiring pathogenic meningococci, and hence higher attack rates (Stanwell-Smith *et al.*, 1994). Studies among Greek school children found that in children in households where the mother or other "carer" smoked, the proportion of carriers was significantly higher compared with children from households where no one smoked (Kremastinou *et al.*, 1994).

1.7 Experimental models for examination of bacterial binding

Many experimental models have been used to investigate virus induced alterations in attachment and invasion by bacteria. Since human volunteers are rarely used as models for studies of bacterial pathogens, *in vitro* assays on human cells and cultures of cells and organs from human sources are employed, but there are some limitations.

1.7.1 Buccal and pharyngeal epithelial cells

Human buccal epithelial cells have been used in many studies of bacterial attachment (Craven *et al.*, 1980; Salit and Morton, 1981; Stephens, 1989). Easy availability of the cells is offset by the fact that they are not uniform in size, degree of maturity or viability. They might already have bacteria attached to their surface from oral flora which can affect the results of binding assays. The cells are not the normal site of attachment of many bacterial pathogens. Bacteria can attach to these cells, but unlike *in vivo* target cells, they do not penetrate the membrane of these cells. Different samples of buccal epithelial cells can vary in surface chemistry due to molecules from the food taken just before sampling or to inherited differences between individuals, *e.g.*, blood groups. Variations in binding of bacteria to cells from the same donor have been shown (Tramont and Wilson, 1977). Use of buccal epithelial cells to study the effect of virus infection on bacterial binding is further hindered by the inability of these cells to be infected with respiratory viruses.

Pharyngeal cells can also be obtained relatively easily. Their use for *in vitro* bacterial attachment assays is limited by the factors above. Pharyngeal cells, however, are relevant to the study of the viral-bacterial association since virus-infected cells can be obtained from virus-infected hosts. Fainstein and colleagues (1980) compared bacterial binding to pharyngeal cells obtained from 21 individuals who had nasopharyngeal symptoms of infection but were culture negative for influenza virus with cells from 10 healthy subjects infected with an experimental vaccine strain of influenza virus and cells from 21 age-matched, symptom-free controls to study the effect of viruses on bacterial

binding. There was increased binding of *S. aureus* to the cells of virus infected subjects compared with those from the uninfected group.

1.7.2 Tissue / organ cultures

Nasopharyngeal tissues from adenoids (Stephens, 1989; Stephens and Farley 1991) and nasal tissues from inferior turbinates resected from patients with non-allergic symptoms (Read *et al.*, 1991) were obtained for the study of pathogenesis of *N. meningitidis* and *H. influenzae* infections. This model might accurately reflect the events happening in natural infections. Its major disadvantages that the tissues are difficult to obtain. The features related to genetic make up of the donor and the disease for which the donor was treated are reflected in the resected tissue. Patients undergoing surgery are presumably free of viral infections. So far, no attempt has been made to infect these organ cultures with viruses *in vitro*.

Organs, however, can be used from animals naturally or experimentally infected with viruses. Tracheas from mice experimentally infected with influenza virus A and killed on 2^{nd}, 4^{th} or 6^{th} day post-infection were exposed to a suspension of radiolabelled *S. pneumoniae* for 90 minutes. In comparison with radioactivity detected on the tracheas from uninfected mice, that on the influenza infected mice was significantly increased. The bacteria were attached to flat-surfaced epithelial cells which replaced ciliated columnar epithelial cells in the mucosa of the infected mice and to basement membrane on the denuded areas of the tracheas, as shown by electron microscopy (Plotkowski *et al.*, 1986). Organ cultures of tracheas from chinchillas infected with influenza A virus

have been used to study the effect of virus infection on the tracheal histopathology and on binding of *H. influenzae*. Non-typable strains bound more effectively compared with type b *H. influenzae* (Hib). Increased numbers of Hib were recorded in the nasal washings from infant rats that had been inoculated with influenza A virus followed by bacteria 48 hours post-infection (Michaels and Myerowitz, 1983). Binding of non-typable *H. influenzae* to chinchilla tracheal tissue infected with influenza A virus was decreased at 72 hr post-infection (Bakaletz *et al.*, 1988).

Use of organs dissected from animals and animal models is, however, not relevant for assessment of the effect of viral infections on the bacteria causing meningitis since most common bacteria responsible for the disease (Hib, meningococci and pneumococci) are exclusively human pathogens. Animals, however, have been used to study isolated events in the pathogenesis of the disease.

1.7.3 Cell culture models

Cell culture models have been used for the study of cytotoxicity caused by bacteria, bacterial attachment, and the effect of viral infections on this attachment. Primary cells have been employed for these studies but are difficult to establish and maintain. No significant difference was observed in the binding of *S. aureus* and other bacteria causing bovine respiratory diseases to cells from bovine embryonic lung infected with bovine parainfluenza virus (Toth and Gates, 1983). A primary endothelial cell culture established from newborn umbilical cord was used to examine the binding of *S. aureus* to human endothelium in the pathogenesis of bacterial endocarditis (Ogawa *et al.*,

1985). Complications such as variations in the source of the tissues and, often, pathology of the organ from which the tissue in question is resected make the primary cell culture model less ideal for studies of virus-bacterium interaction.

Continuous cell lines of tumour origin are easy to handle and can be maintained aseptically in standard conditions. Stephens (1989) reviewed the use of different models including HeLa and the HEp-2 cells (human epithelial cell lines) in studies of the binding of *Neisseria* species. Continuous cell lines were found suitable for the studies. Chang epithelial cells (derived from human conjunctiva) were used to determine the role of bacterial pili in the attachment of *H. influenzae* (St. Geme and Falkow, 1990). HeLa and Detroit cells (human epithelial cell line) were used to study the effect of cellular infection with measles virus and adenovirus on binding of bacteria (Selinger *et al.*, 1981).

Sanford and colleagues (1978) used a continuous line of Madin-Darby canine kidney (MDCK) cells to examine the effect of influenza virus infection of the cells on the attachment of streptococci. The bacteria attached to the infected cells only. The attachment could be blocked by pre-treatment of the infected cells with an antibody to the viral glycoproteins expressed on the surface of the infected cells indicating that the viral glycoproteins might be the receptors for the bacteria. Staphylococci did not attach to MDCK cells in this study, but attachment was observed in a later study by the same group (Sanford *et al.*, 1986). The cells in this study were used to analyse the effect of influenza virus infection, the effect of enzymes and changes in other conditions in the

assay on the attachment of staphylococci. The reason for the discrepancy between the two findings for the attachment of staphylococci to MDCK cells was not discussed in their report.

Previous work in this department found that compared with uninfected HEp-2 cells, HEp-2 cells infected with RSV-A showed enhanced binding of *N. meningitidis* and *H. influenzae* type b (Raza *et al.*, 1993). In addition it was found that glycoprotein G of RSV subgroup A is an additional receptor for meningococci (Raza *et al.*, 1994). As the glycoprotein G of the two subgroups vary (Anderson *et al.*, 1985; Johnson *et al.*, 1987), examination of the effect of RSV-B on bacterial binding was needed.

1.7.4 Detection of bacterial binding

Methods commonly used for assessing bacterial binding to cell cultures include: light microscopy of stained monolayers of the cells (Selinger *et al.*, 1981; Musher and Fainstein, 1981); fluorescence microscopy (Sanford *et al.*, 1978); interference contrast microscopy (Andersson *et al.*, 1986); electron microscopy (Stephens *et al.*, 1983); radioassays (Ogawa *et al.*, 1985; Sanford *et al.*, 1986); and the pour plate method, counting colony forming units of bacteria in the supernatant after incubation with cell monolayers (Ogawa *et al.*, 1985).

Direct microscopy is simple, cheap and allows visualisation of the process being studied. It is, however, time consuming, not accurate for quantitation of large numbers of bacteria or for large experiments. Radioassays are also limited in value since the

manipulation of bacteria for the uptake of the label can affect bacterial binding. Leakage of radioactive material from the labelled bacteria and background disturbance in detection of radioactivity in these assays also make the assessment more complicated. The use of electron microscopy is mainly limited to the study of mechanisms of bacterial attachment to the cells and bacterial pathogenicity. It cannot be used to demonstrate adherence with confidence since the results cannot be generalised due to patchy distribution of bacteria bound to cells and the area of tissue visualised by the microscope is limited.

Flow cytometry has been applied to studies of binding of both Gram-negative and Gram-positive bacteria (Rahat, 1990; Raza *et al.*, 1993; Saadi *et al.*, 1993; Saadi *et al.*, 1996; Alkout *et al.*, 1997). The flow cytometer can detect fluorescein-labelled bacteria bound to cells. The proportion of cells with FITC-labelled bacteria attached and mean level of fluorescence can be obtained using this method. Although mean fluorescence recorded on cells is proportional to the numbers of bacteria attached, it cannot be expressed in terms of numbers of bacteria because the level of fluorescence obtained with one bacterium on a cell is difficult to determine accurately. The method is, however, rapid, objective, precise and analyses a large number of cells in each sample and large numbers of samples can be assessed in each experiment.

1.8 Aims of the study

Epidemiological studies on nasopharyngeal flora of infants found no association between frequency of isolation of a number of species associated with respiratory tract infection or *N. meningitidis* with symptoms of virus infection or exposure to cigarette smoke (MacKenzie *et al.*, 1994; MacKenzie *et al.*, 1996). The main aim of this study was to assess the effects of virus infection and smoking on density of bacterial binding as this has been demonstrated to be associated with disease (Beachey, 1981). Smoking and exposure to smoke are both predisposing factors for respiratory virus infection, but effects of virus infection or exposure to cigarette smoke on bacterial attachment to epithelial cells were assessed separately. Two groups of bacteria were examined:

1) respiratory bacteria for which there is epidemiological and experimental evidence that exposure to cigarette smoke and viral infection contribute to susceptibility to infection;

2) bacteria associated with meningitis for which epidemiological studies have provided evidence that these two factors enhance susceptibility to disease.

The objectives of the study were:

1-to determine if, as found with RSV subgroup A, infection of HEp-2 cells with RSV subgroup B enhances bacterial binding;

2-to determine if there is increased binding of Gram-positive species associated with meningitis and species associated with secondary respiratory infections or exacerbation of chronic bronchitis to RSV infected cells;

3-to determine if binding of bacteria to influenza virus infected cells was increased in the HEp-2 cells model used for RSV;

4-to assess changes in surface components of virus infected cells that could affect bacterial binding;

5-to determine if there is enhanced binding of bacteria associated with meningitis or respiratory diseases to cells of smokers;

6-to assess cells of smokers and non-smokers for levels of antigens proposed to act as bacterial receptors.

Chapter 2

General Materials and Methods

All chemicals were of Analytical Grade and were obtained from BDH Chemicals Ltd., UK unless otherwise indicated.

2.1 Phosphate-buffered saline (PBS) for washing bacteria

PBS contained 8 mM $NaHPO_4$, 1 mM KH_2PO4, 3 mM KCl and 0.15M NaCl. The pH was adjusted to 7.2

2.1.1 Dulbecco's PBSA for washing cell lines

Dulbecco's PBS prepared from concentrated PBS (10X) (Gibco) without calcium and magnesium was used for washing the cells.

2.2 Reagents used for determination of bacterial binding

2.2.1 Fluorescein isothiocyanate (FITC) buffer

The buffer contained sodium carbonate (0.05 M) and sodium chloride (0.1 M). The pH was adjusted to 9.2

2.2.2. FITC Labelling of bacteria

FITC (Sigma, Poole, Dorset, UK) was used for labelling the bacteria in all assays. The solution was prepared as described by Rahat (1990). FITC (0.4% w/v) in FITC buffer immediately before use.

2.2.3 Buffered paraformaldehyde

Buffered paraformaldehyde (1% w/v) was prepared by adding sodium cacodylate (1% w/v) to paraformaldehyde (1% w/v). The pH was adjusted to 7.2

2.3 Bacterial culture

2.3.1 Media

Modified New York City medium (MNYC) (Cherwell Laboratories Ltd., UK) was used to culture *Neisseria* species. *S. pneumoniae* was grown on Columbia blood agar, *H. influenzae* isolates on chocolate agar with horse blood, *B. pertussis* on charcoal agar and *S. aureus* on nutrient agar. The prepared media were obtained from Oxoid Unipath Ltd, UK.

2.3.2 Maintenance and storage of bacteria

Bacterial cultures were prepared by reconstituting lyophilized strains in distilled water for 20 min and plating them on appropriate media. For storage, colonies were emulsified in Microbank beads (Pro-Lab Diagnostic, Ontario, Canada) and kept at -20^{o}C. A fresh bead was used to inoculate plates for each set of experiments.

2.4 Tissue culture cell lines and growth media

2.4.1 HEp-2 cells

HEp-2 cell line (Flow Laboratories) was used in the studies. This continuous cell line consists of transformed epithelial cells originating from a human laryngeal carcinoma.

2.4.2 MDCK cells

The MDCK cell line (European Collection of Animal Cell Cultures, CAMR, Salisbury, UK) was used in the studies for the growth of influenza virus.

2.4.3 THP-1 cells

The THP-1 (human monocyte / macrophage) cell line used in some experiments was obtained from European Collection of Animal Cell Cultures, CAMR, Salisbury, UK.

2.4.4 THP-1 growth medium

RPMI 1640 medium (Gibco, Paisley, UK) supplemented with foetal calf serum (FCS) (Gibco) (10%), 100 U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin sulphate (Sigma, Poole, Dorset) and 1m M L-glutamine (Gibco) was used to grow the THP-1 cells.

2.4.5 HEp-2 cells growth medium (GM)

HEp-2 cells were grown in GM which consisted of Eagle's minimal essential medium (Gibco) supplemented with FCS (Gibco) (10%), $NaHCO_3$ (0.85gl^{-1}), L-Glutamine (2mM), streptomycin (200 μgl^{-1}) and penicillin (100 IU ml^{-1}). The pH of the final preparation was adjusted to 7.4 with 1 N NaOH.

2.4.6 Cell maintenance medium (MM)

MM consisted of the same constituents as GM except the quantity of FCS was reduced to 1%. In some assays involving live bacteria, MM without antibiotics was used.

2.4.7 Growth of HEp-2 cell line

Confluent monolayers of HEp-2 cells were obtained in tissue culture flasks by growth in GM. The monolayer was rinsed twice with PBSA. A solution (2 ml) of 0.05% trypsin (w/v) and 0.02% EDTA (v/v) (Gibco) was applied to the monolayer of cells in a 75 cm^3 flask for 4-6 min at 37°C to prepare a suspension for the next passage. The effect of trypsin / EDTA on the cells was terminated by suspending the cells in 5-10 ml of GM. Cells were counted by preparing a dilution (1/10) of the cell suspension in 0.5% trypan blue (w/v) in physiological saline (Northumbria Biological, UK) and viable cells counted by light microscopy using an improved Neubauer counting chamber. Cells from freshly confluent monolayers ($4x10^6$ ml^{-1}) were suspended in 1 ml of GM with 10% dimethyl sulphoxide (DMSO) (Sigma) and stored in liquid nitrogen following gradual cooling to -70°C. Frozen cells were resuscitated by rapid thawing at 37°C for further use.

2.5 Standardisation of respiratory syncytial virus (RSV)

2.5.1 Plaque assay

2.5.1.1 Solutions

Overlay medium (OLM) was prepared by adding methyl cellulose 3% w/v (Sigma) in Hank's buffered salt solution (Gibco) and $NaHCO_3$ (2 g l^{-1}) to maintenance medium in a proportion of 1 : 3

2.5.1.2 Method

HEp-2 cell monolayers were obtained by seeding 24-well tissue-culture plates (Costar) which were incubated in 5% CO_2 in air for 24 hr. Ten-fold dilutions of the virus suspension to be assayed were distributed to wells in quadruplicate (200 µl / well) and adsorbed to monolayers for 1 hr at 37°C. The supernatant was removed from the wells and 1 ml of overlay medium added to each well. The plates were incubated in 5% CO_2 at 37°C for 3-4 days until syncytia / plaques appeared in the monolayers. The monolayers were fixed with formol saline (10% v/v) for 10 min and staining solution was used to examine cell monolayers for syncytia and plaque formation. The staining solution contained crystal violet (0.13% w/v) and formalin (5% v/v) in normal saline. After 20 min the wells were washed with tap water. The monolayers were examined for plaques by inverted light microscopy.

2.6 Immunofluorescence reagents for detection of RSV or influenza infected cells

2.6.1 RSV

The RSV reagent (Imagen, Dako Diagnostics Ltd, UK) contains monoclonal antibodies conjugated to FITC. The conjugated antibodies bind specifically to viral antigens present in all human RSV. The reagent was used in a one-step direct immunofluorescence technique. Coverslips of fixed cell preparations (fixed in acetone for 10 minutes) were incubated with 10 µl of the reagent for 15 minutes at 37°C in a moist chamber. The excess reagent was removed by gently washing the slide in an

agitating bath containing PBS for 5 minutes. The coverslip was mounted and examined using fluorescence microscopy. Within the cells, RSV antigens showed bright green granular cytoplasmic fluorescence which contrasts with the red background staining of uninfected cells revealed by the Evans blue counterstain in the reagent. Positive control slides containing fixed HEp-2 cells infected with RSV provided with the reagent were used as control.

2.6.2 Influenza virus

The reagent for detection of influenza A (Imagen, Dako) contains monoclonal antibodies specific for the virus conjugated to FITC. The reagent was used in a one-step direct immunofluorescence assay. Coverslips of fixed cell preparations were incubated with 10 µl of the reagent for 15 minutes at 37°C in a moist chamber. The excess reagent was removed by gently washing the slide in an agitating bath containing PBS for 5 minutes. The stained area was mounted and examined using fluorescence microscopy. Within the cytoplasm and nucleus of the cells, the influenza A virus antigens were detected by characteristic bright green granular fluorescence which contrasts with the red background staining of uninfected cells. Positive control slides containing fixed monkey kidney cells infected with influenza A provided with the reagent were used as control.

2.7 Bradford reagent and standard for protein estimation

Bradford reagent was prepared from Commasie Blue G250 (0.01% w/v) (Sigma), ethanol (4.7% w/v) and phosphoric acid (8.5% w/v) in distilled water. Bovine serum albumin was used as the standard for each experiment.

2.8 Buffers and solutions used for enzyme linked immunosorbent assays (ELISA)

2.8.1 Coating buffer

Coating buffer consisted of sodium carbonate (15 mM), sodium bicarbonate (35 mM) and sodium azide (3 mM) (pH 9.6).

2.8.2 Washing buffer

Washing buffer was prepared by adding bovine serum albumin (BSA) (Sigma) 0.1 % (w/v) and Tween-20 (0.05 % v/v) to PBS (pH 7.2). The buffer was used for all washing procedures during the assay.

2.8.3 Blocking buffer

Blocking buffer contained 1 % (w/v) BSA in 0.01 M PBS (pH 7.2).

2.8.4 Phosphate citrate buffer

Phosphate citrate buffer contained sodium hydrogen phosphate (0.1M) and citric acid (0.1 M) (pH 5).

2.8.5 Substrate solution

The substrate solution used to detect horseradish peroxidase (HRP) labelled antibodies contained 40 mg O-phenylenediamine in 100 ml of 0.1 M phosphate citrate buffer (pH 5.0) activated immediately before use by adding 40 µl H_2O_2 (30 % v/v).

2.8.6 Stopping solution

The stopping solution contained 12.5 % (v/v) H_2SO_4.

2.9 Monoclonal and polyclonal antibodies

Monoclonal and polyclonal antibodies, the animal from which they were obtained, isotype and source are listed in Tables 2.1 and 2.2.

Table 2.1 Monoclonal antibodies used to detect host cell antigens

Monoclonal antibody	Host	Isotype	Source
Anti-Lewis^{a}	mouse	IgM	SAPU*
Anti-Lewis^{b}	mouse	IgM	SAPU
Anti-Lewis^{x} (Anti-CD15)	mouse	IgM	SAPU
Anti-CD14	mouse	IgG	SAPU
Anti-CD18	rat	IgG	Dako
Anti-H type 2	mouse	IgM	Serotec

* SAPU Scottish Antibody Production Unit

Table 2.2 Polyclonal antibodies used to detect monoclonal antibodies

Polyclonal antibody	Host	Isotype	Source
FITC anti-mouse	rabbit	IgM	Sigma
FITC anti-mouse	rabbit	IgG	Sigma
FITC anti-rat	rabbit	IgG	Serotec

Chapter 3

Effect of infection with subgroups A or B of RSV on binding of bacteria associated with meningitis to a human epithelial cell line (HEp-2)

3.1 Introduction

Three species account for the majority of cases of bacterial meningitis, *N. meningitidis*, *H. influenzae* and *S. pneumoniae* (Salih, 1990). For epidemiological studies, these bacteria have been classified based on their cell surface antigens, mainly their capsular polysaccharides, which must be considered in studies on host-parasite interactions, as particular antigenic types are associated with meningitis.

3.1.1 *N. meningitidis*

N. meningitidis is an encapsulated Gram-negative diplococcus with a flattened shape and an average size of 0.6 x 0.8 μm. Three different groups of surface structures of the meningococci have been used for strain differentiation : capsular polysaccharides (CPS); outer membrane proteins (OMP); lipooligosaccharide (LOS). An extensive phenotypic classification system has been developed on the basis of these surface components (Table 3.1). Serogroups are based on differences in the structure of the CPS. Serotypes and subtypes are based on differences in class 2/3 and class 1 OMP respectively. Immunotypes are based on differences in the oligosaccharide structure of

Table 3.1 Phenotypic classification system of *N. meningitidis*

category	antigens	no. of groups or types
Serogroups	CPS	12
Serotypes	Class 2/3 OMP	20
Subtypes	Class 1 OMP	10
Immunotypes	LOS	12

CPS = capsular polysaccharide
OMP = outer membrane protein
LOS = lipooligosaccharide

the meningococcal LOS (Frasch *et al.*, 1985; Griffiss *et al.*, 1988; Kim *et al.*, 1988; Peltola, 1983; Poolman *et al.*, 1982; Tsai *et al.*, 1987; Zollinger and Mandrell, 1980).

3.1.2 *H. influenzae*

H. influenzae produce a polysaccharide capsule which is used to classify this species into six distinct antigenic types, designated a-f. The most important of these is type b (Hib). In the UK prior to development of an effective vaccine for infants, more than 95% of all invasive diseases attributable to *H. influenzae*, including meningitis, septicaemia, pneumonia, epiglottis, cellulitis, arthritis, osteomyelitis, and pericarditis, were due to *H. influenzae* type b (Wilfert, 1990).

The majority of all systemic *H. influenzae* type b infections occurred in children younger than 18 months of age. The peak incidence of meningitis and other invasive Hib disease occurs in children 6 to 7 months of age. Children younger than 18 months of age are more likely to have meningitis than other forms of invasive disease. Epiglottitis, however, is more likely to occur in older children, with a peak occurrence among 3 year olds (Wilfert, 1990).

There is seasonal variation in the incidence of *H. influenzae* type b infections. Unlike the other major causes of bacterial meningitis (*S. pneumoniae* and *N. meningitidis*), the peak incidence of which regularly occurs between January and March, there was a bimodal pattern to the occurrence of *H. influenzae* type b meningitis with peaks in

October-November and March-May (Ward *et al.*, 1986; Ward and Cochi, 1988; Broome, 1987).

3.1.3 *S. pneumoniae*

Pneumococci are the second most frequent cause after *H. influenzae* type b of bacterial meningitis (Dagan *et al.*, 1994) and otitis media (Musher, 1992) in children. The incidence of pneumococcal disease is highest in infants under 2 years of age and in people over 60 years of age (Garcia-Leoni *et al.*, 1992; Gray *et al.*, 1979). Because of the introduction of conjugate vaccines for *H. influenzae* type b, pneumococcal meningitis is likely to become increasingly prominent. In adults, *S. pneumoniae* is the most important etiological agent of community-acquired pneumonia and is the second most common cause of bacterial meningitis after *N. meningitidis*. In spite of the availability of antibiotics, the mortality of pneumococcal disease remains high between 25 and 29% (Gillespie, 1989).

Pneumococci are classified on the basis of differences in capsular polysaccharide structure into 90 serotypes. The Danish nomenclature classifies serotypes according to structural and antigenic characteristics; *e.g.*, serotypes 6A and 6B differ only slightly from each other. The American nomenclature, assigns the numbers in sequence of first isolation, so types 6A and 6B are types 6 and 26, respectively (Alonso DeVelasco *et al.*, 1995).

The distribution of types isolated from adults differs substantially from that of types isolated from children. Serotypes 14, 6, 18, 19, 23, 1, 4, and 9 are associated with most

of the serious infections in children; these types accounted for about 85 % of all infections (Gray and Dillon, 1986; Jacobs *et al.*, 1979). In adults, however, serotypes 1, 3, 4, 7, 8, 9, 12, 14, and less often 6, 18, and 19 as a group have been associated with most serious bacteremic disease (Smart *et al.*, 1987; Gray and Dillon, 1986; Jacobs *et al.*, 1979

3.1.4 Host cell receptors involved in enhancement of bacterial binding by virus infection

Because the peak incidence of RSV infection coincides with the winter peak of bacterial meningitis and is common among children in the age range at greatest risk, this virus was used in a model system to assess the effect of the viral infection on binding bacterial species that cause meningitis. There are two antigenically distinct types of RSV, designated subgroups A and B based on variation in the attachment glycoprotein G (Akerlind *et al.*, 1988. Anderson *et al.*, 1991; Cane *et al.*, 1991). RSV-A infection of HEp-2 cells was shown to enhance significantly binding of *N. meningitidis* and Hib (Raza *et al.*, 1993). Other work by this group demonstrated that other respiratory pathogens followed a similar pattern in which binding to RSV infected cells was significantly enhanced, *S. aureus* (Saadi *et al.*, 1993) and *B. pertussis* (Saadi *et al.*, 1996).

There are two ways in which virus infection could enhance bacterial binding by altering the cell surface: 1) production of virus encoded antigens that act as new receptors for bacteria; 2) enhancement of native host cell receptors.

Monoclonal antibodies (Mabs) generated against RSV distinguish the two subgroups (A and B) according to epitope variations in the G proteins (Anderson *et al.*, 1985; Mufson *et al.*, 1985). Variations in the G protein appear to be the most common, whereas the other major surface protein (F) has relatively minor differences (Akerlind *et al.*, 1988; Anderson *et al.*, 1985; Cane *et al.*, 1991). The study by Raza and colleagues (1994) was carried out with RSV subgroup A, and it was demonstrated that the glycoprotein G, but not glycoprotein F, was an additional receptor for meningococci on RSV infected cells.

Initial studies on host cell antigens of RSV-A infected cells indicated that CD14, CD15 and CD18 were enhanced compared with uninfected cells. Pre-treatment with monoclonal antibodies to CD14 and CD18 significantly reduced binding of meningococci (Raza *et al.*, 1994); however the effects of virus infection on binding of Gram-positive bacteria were not examined.

3.1.5 Bacterial components involved in enhanced binding to RSV infected cells

In the initial studies by Raza *et al.* (1993), meningococcal strains of different serogroups, serotypes, and subtypes were examined. Differences in these antigenic characteristics did not have an effect on the enhanced binding to RSV infected cells. The effect of differences in LOS structures were not examined as immunotyping is not routinely carried out even by most reference laboratories.

Immunotypes L8, L9, L10, and L11 are found within group A meningococci; of these, L10 and L11 are prevalent and are uniquely associated with this serogroup (Kim *et al.*, 1989; Salih *et al.*, 1990; Zollinger and Mandrell, 1977). Immunotypes L1 through L9 are identified within serogroups B and C meningococci. Studies by Zollinger and Mandrell (1977) and Poolman and colleagues (1982) suggest that immunotype L3,7,9 is the most prevalent within these serogroups, followed by immunotypes L2 and L1,8. The distribution of LOS immunotypes among serogroup B meningococci might become of special interest, because the LOS is a potential vaccine candidate (Poolman, 1990).

Although pili are expected to be involved in binding to epithelial cells (Virji *et al.*, 1992), the procedures used in the preparation of bacteria for the binding assays remove these structures; therefore, they are not likely to be involved in the enhanced binding to virus infected cells observed in previous studies.

3.1.6 Aims of the study

The aims of this part of the study were:

1) to determine if there is a similar pattern of enhanced binding of meningococci and *H. influenzae* type b to RSV B infected cells as observed with RSV A;

2) to determine if there is increased binding of *S. pneumoniae* serotypes associated with meningitis to RSV infected cells;

3) to assess the role of meningococcal oligosaccharide structures (immunotypes) in binding to RSV infected cells;

4) to compare the expression of host cell antigens that can act as receptors for bacteria on uninfected and RSV-infected HEp-2 cells.

3.2 Materials and methods

3.2.1 Cells

HEp-2 cells were used between passages 151-184 for the binding assays. HEp-2 cells were grown for 24 hr in 75 cm^2 flasks in GM (2.4.1). Monolayers of uninfected and RSV-infected HEp-2 cells were maintained for an additional 24 hr in MM (2.4.2).

3.2.2 Viruses

RSV subgroup A (Edinburgh strain) (Ogilvie *et al.*, 1981) and subgroup B (strain 18573) were used at passages 10-12 and 6-8 respectively to infect the HEp-2 cells. Stock aliquots of supernatant fluid from infected cell lysates were stored at -70°C and contained approximately $1x10^6$ plaque forming units (p.f.u) ml^{-1} as described in 2.3.1.

3.2.3 Bacteria

Strains of *N. meningitidis* were obtained from several sources: C:2b:P1.2 was provided by Dr. R. J. Fallon the Meningococcal Reference Laboratory, Ruchill Hospital, Glasgow, Scotland; NG:2b:P1.10, and B:2b:P1.10 was obtained from Dr. G. Tzanakaki, National School of Public Health, Athens, Greece. Twelve meningococcal immunotype strains were provided by Dr. W. D. Zollinger, Walter Reed Army Medical Institute, Washington DC, USA. One strain of *Neisseria lactamica* (LO1) was obtained from Dr. H. Young of this department. Three strains of *S. pneumoniae* associated with meningitis (types 7F, 12F, and 18C) were kindly provided by Dr. L. E. Smart, The

Meningococcal and Pneumococcal Reference Laboratory, Stobhill Hospital, Glasgow, Scotland. There was one strain of *H. influenzae* type b isolated from a local patient. Bacterial cultures were prepared by reconstituting lyophilized strains in distilled water and plating on appropriate media. *N. meningitidis* and *N. lactamica* were grown on MNYC (Young, 1978). Pneumococci were grown on Columbia blood agar with horse blood (BA). *H. influenzae* were grown on boiled blood agar (BBA). Each of the strains were grown overnight at 37°C in a humidified atmosphere with 10% CO_2, harvested, washed three times in PBS by centrifugation at 2500 x *g* for 15 min and resuspended in MM without antibiotics by vigorous pipetting to disperse clumps.

The bacterial concentrations were determined by measuring optical density (OD) at 540 nm. For each strain the linear relationship between OD reading and total count assessed microscopically with a Thoma counting chamber was determined. A standard curve was prepared for each strain used.

3.2.4 Infection of HEp-2 cells with RSV-subgroups A or B

Overnight monolayer cultures of HEp-2 cells in culture flasks ($75cm^3$) were infected with RSV-subgroups A or B at a multiplicity of infection (MOI) of 1.0 p.f.u / cell. Virus (2.5ml in MM) was adsorbed for 1 hr, the fluid was replaced with 25 ml MM and incubated overnight at 37°C. Cultures were rinsed twice with sterile DPBS+A and harvested by adding 5ml of 0.05% ethylenediaminetetraacetic acid (EDTA) per flask at 37°C for 5-10 min. MM (5ml) was added to the cells to counteract EDTA activity.

The cells were centrifuged at 460 x *g* for 7 min and resuspended in MM without antibiotics, counted and adjusted to $1x10^6$ cells ml^{-1} (Raza *et al.*, 1993).

3.2.5 Bacterial binding to RSV-infected HEp-2 cells

The bacterial suspensions were labelled with FITC (Sigma) freshly prepared as a 0.4 % (w/v) solution in NaH_2CO_3 (0.05M) and NaCl (0.1M) (pH 9.2). The washed bacterial pellet obtained from two culture plates was suspended in 2 ml of the FITC solution by gentle shaking and incubated at 37°C for 30 min (Raza *et al.*, 1993). FITC-labelled bacteria were washed three times in PBS and resuspended in MM without antibiotics. The concentration of the suspension was determined by OD at 540 nm and adjusted to provide a range of ratios of bacteria per cell (Raza *et al.*, 1993).

The bacterial suspensions (200 µl) were incubated with equal volumes of uninfected HEp-2 cells, or HEp-2 cells infected with RSV-A or RSV-B at 37°C in an orbital incubator (Gallenkamp) for 30 min at 60 rpm. The cells were washed three times at 460 x *g* with PBS, resuspended in 200 µl of PBS and fixed with 100 µl of 1% buffered paraformaldehyde and stored in the dark at 4°C until analysed by flow cytometry within 48 hours of preparation.

3.2.6 Detection of RSV infected cells

3.2.6.1 Fluorescence microscopy

Coverslips with monolayers of HEp-2 cells were prepared and infected with RSV subgroups A or B at a MOI of 1.0 following incubation for 16-24 hr, the cells were

fixed. The coverslips were incubated with 10 µl of the direct immunofluorescence reagent as described in (2.6.1). For each coverslip, 100 cells were counted.

3.2.7 Flow cytometry

Flow cytometry is capable of analysing and sorting single cells with great speed and efficiency in their natural form. The major components of the EPICS-XL (Coulter Electronics, Luton, UK) include: (1) flow cell into which a stream of labelled cells was injected; (2) a laser light source (15 mW argon ion laser at 488 nm) to excite fluorescence of the cells in the stream formed by the flow system; (3) photomultiplier tube system to measure the emitted fluorescence as well as to determine the degree of laser light scattered by each cell; (4) a mechanism for placing a given cell into a droplet which can be separated from other droplets on the basis of the fluorescence, size, and viability characteristics of the cell; (5) a computer system for the analysis and storage of data (Figure 3.1).

3.2.7.1 The principles of flow cytometry

The flow cytometry measures the light scatter of cells as they passed one by one through a laser bean. Cells scatter the laser light at low-angle in the forward direction according to their size; small cells scatter small amounts of light and large cells scatter greater amounts of light. Cellular granularity is directly proportional to the amount of laser light scattered at a 90^{o} angle (also referred to a side scatter). Data are displayed graphically by plotting forward versus side scatter to produce a two parameter histogram or scattergram (Fig 3.2a). The instrument electronically selects the desired cell

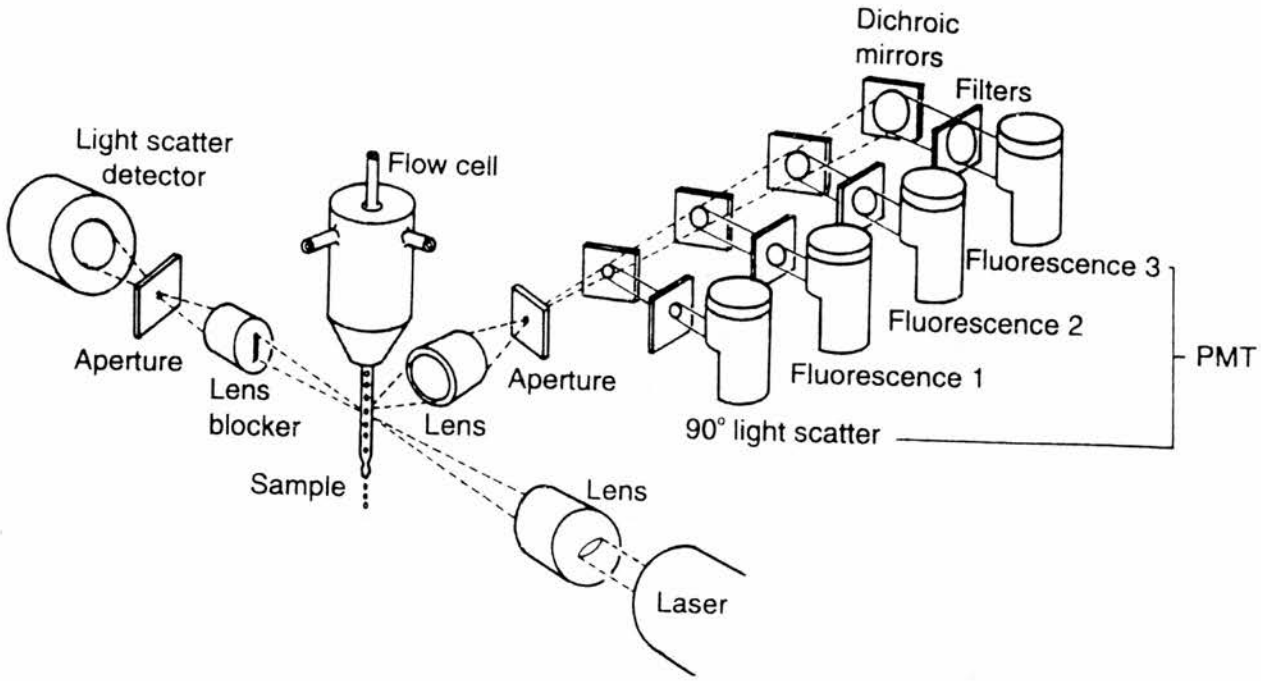

Figure 3.1 The major components of the flow cytometry.

(adapted from Clinical Immunology, Catherine Sheehan, 1997)

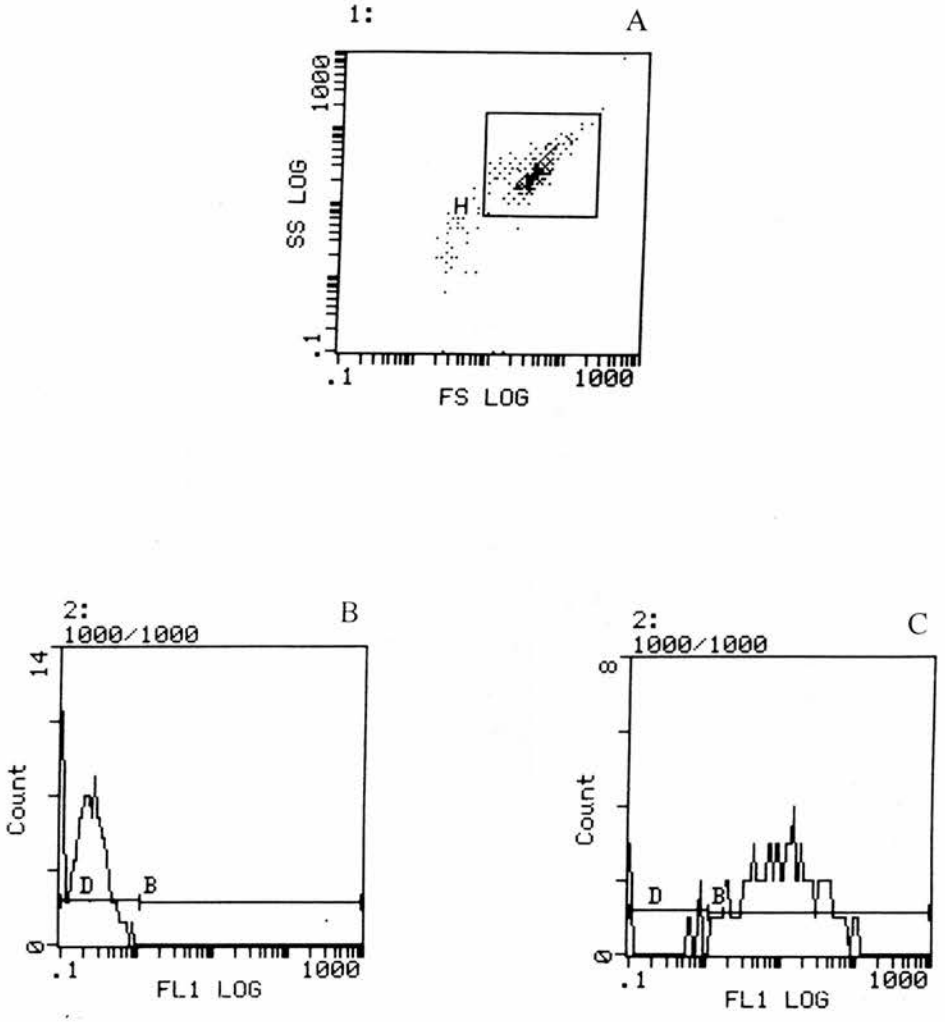

Figure 3.2 Flow cytometric analysis showed population of interest (the bitmap) (A), control cells with no bacteria (B), and FITC-conjugated bacteria attached (C).

population for analysis by drawing a gate or window around the population of interest (the bitmap) (Fig 3.2a).

In addition to detectors for forward angle scatter and side scatter, there are detectors to measure the fluorescence emitted from labelled bacteria attached to cells. These data are displayed as a single parameter histogram that plots the number of cells versus fluorescence (Fig 3.2c). Cells with no bacteria or cells with FITC-conjugated secondary antibody were analysed first to establish background fluorescence. Figures 3.2b and 3.2c are examples for the control and test samples respectively from a single experiment.

For each sample analysed, two parameters were obtained: 1) the percentage of cells in the population that showed fluorescence levels higher than the background (cells incubated without FITC-labelled bacteria); 2) the mean levels of fluorescence in the population of cells with bound bacteria reflecting the mean number of bacteria bound per cell on a logarithmic scale. The mean fluorescence channel values for positive cells were obtained from a conversion table of log fluorescence supplied by the manufacturer (Coulter). Some individuals cells showed bound FITC-conjugated secondary antibody (control). A computer programme (Immunoanalysis, Coulter) was applied to recalculate the percentage and the mean of the test by assessing overlap of the two histograms (control and test) (Figure 3.3).

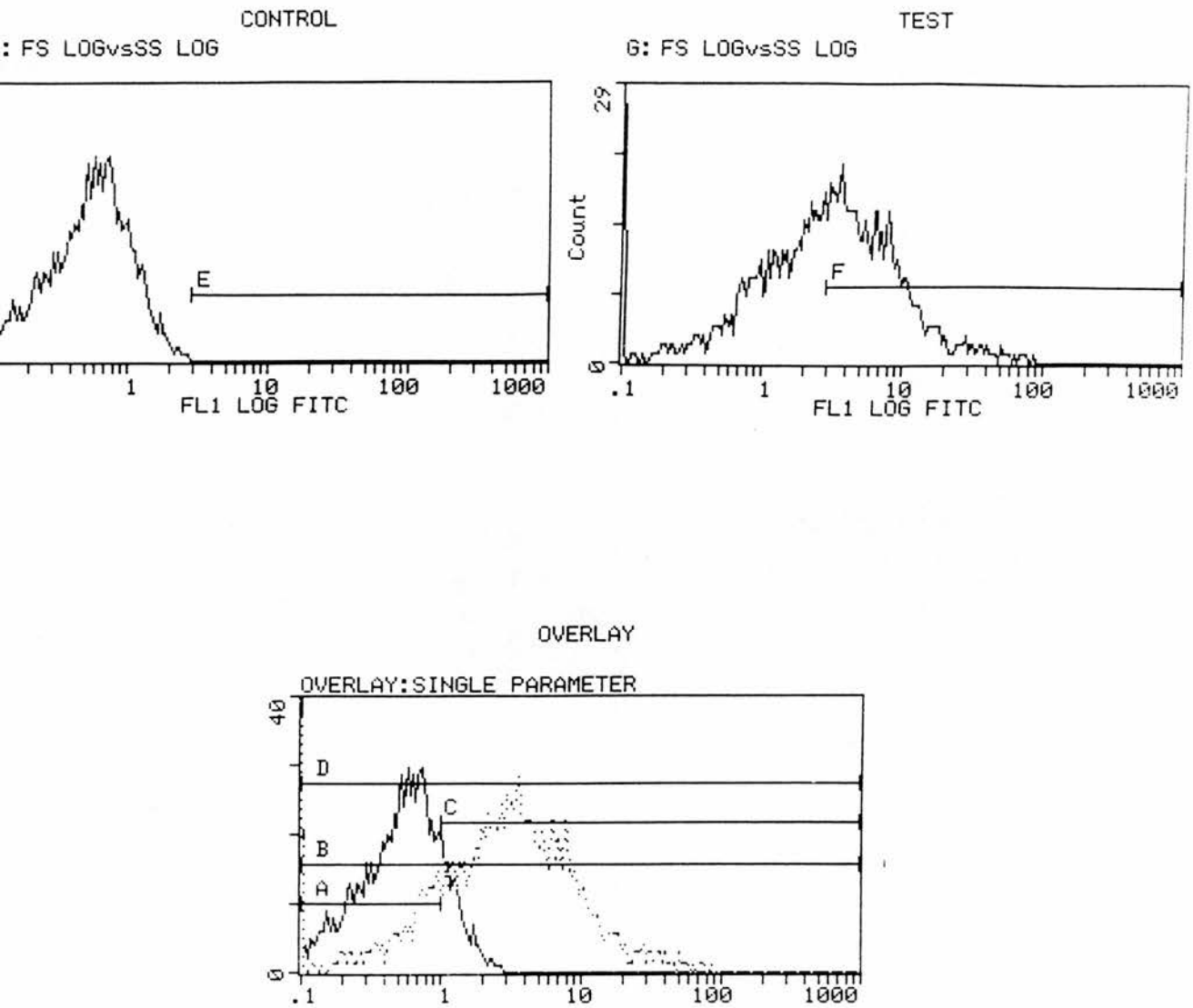

Figure 3.3 Examples of immunoanalysis of the control, test, and overlap of the two histograms.

3.2.7.2 Calculation of binding index (BI)

A binding index for each sample in the study was calculated by multiplying the percentage of positive cells and the mean fluorescence of positive cells. It has been suggested that mean numbers of bacteria attached to whole population of cells provide a move accurate estimate of adhesiveness. In this study the data were presented as binding indices, since the virus infection might affect the percentage of cells binding bacteria and the mean numbers of bacteria bound.

3.2.8 The effect of FITC on bacterial binding

HEp-2 cells ($1x10^5$ ml^{-1}) in GM were seeded on to glass coverslips (1ml / well) in 24 well culture plates (Costar) and incubated in 5% CO_2 in air overnight at 37°C. The GM was removed and 200 µl of FITC-labelled or unlabelled bacteria (200 bacteria : cell) were added to the coverslips for 30 min at 37°C in an orbital incubator. The coverslips were washed three times with PBS, fixed in absolute ethyl alcohol for five minutes at room temperature, stained by Gram's stain and examined by light microscopy with an oil immersion lens. The number of cells counted, number of cells binding bacteria and number of bacteria per positive cell were recorded. For each coverslip, 100 cells were counted. BI for each coverslip was obtained by multiplying the proportion of cells which bound bacteria by the mean number of bacteria per positive cell.

3.2.9 Detection of host cell surface antigens on HEp-2 cells infected with RSV-subgroups A or B

HEp-2 cells and HEp-2 cells infected with RSV-A or RSV-B (200 µl, $1x10^6$ ml-1) were incubated for 60 min at 37°C with the following antibodies to the host cell surface antigens listed in tables 2.1 and 2.2: CD14 (1/2); CD18 (1/20) and CD15 (1/10). The cells were washed twice with PBS and incubated for 30 min in an orbital shaker at 37°C with FITC-labelled secondary antibodies used to detect binding of their respective primary antibody: rabbit anti-mouse IgM (Sigma) diluted 1 in 100 in PBS; rabbit anti-mouse IgG (Sigma) diluted 1 in 200 in PBS; or rabbit anti-rat IgG (Serotec) diluted 1 in 100 in PBS. The FITC-labelled second antibodies were incubated with the cells which were not exposed to the primary antibody to control for non-specific binding.

Fluorescence was analysed with an EPICS 'XL' flow cytometer (Coulter, UK), and the BI was calculated from the percentage of the cells with fluorescence greater than the background (cells treated only with the FITC-labelled second antibody) multiplied by the mean fluorescence for the positive population.

3.2.10 Inhibition of bacterial binding by antibodies to host cell antigens.

Sources of monoclonal antibodies binding to cell surface antigens are listed in Table 2.1. RSV-A infected HEp-2 cells (200 µl) were incubated with PBS, mouse monoclonal antibody to CD14 diluted 1 in 2; rat monoclonal antibody to CD18 diluted 1

in 20; Lewis ^{x} (CD15) diluted 1 in 10, RSV monoclonal anti-glycoprotein G diluted 1 in 100 (Prof. P. J. Watt, Southampton University) at 37^{o}C for 60 min. After washing twice by centrifugation at 300 x *g* for 10 min, the attachment assay with FITC-labelled bacteria were performed and analysed as described in (3.2.7).

3.2.11 Statistical methods.

The data were assessed by estimate of relative binding of the bacteria to RSV-infected HEp-2 cells compared with uninfected cells based on paired *t*-tests applied to logarithms of the binding indices.

3.3 Results

3.3.1 Determination of total bacterial count in relation to OD

For each strain, the bacterial concentration was determined by OD at 540 nm and in relation to total count obtained by light microscopy with a Thoma counting chamber. Graphs of the range of readings of OD values having a linear relationship with the total counts were prepared for each of the isolates: *N. meningitidis* (Figure 3.4); *H. influenzae* type b (Figure 3.5); *N. lactamica* (Figure 3.6); *S. pneumoniae* (Figure 3.7) and *N. meningitidis* LOS immunotype strains (Figure 3.8).

3.3.2 The effect of FITC on bacterial binding:

To examine the effect of FITC on binding of the bacteria to HEp-2 cells, binding of all the strains, FITC-labelled and unlabelled, were examined by light microscopy. Binding of each strain was tested twice for binding to monolayers of HEp-2 cells and the averages of the two experiments are summarised in (Tables 3.2 and 3.3). At a ratio of 200 bacteria : cell, there were no significant differences in BI of FITC-labelled bacteria compared with unlabelled bacteria.

3.3.3 Determination of binding to HEp-2 cells

Dose response experiments were carried out with HEp-2 cells for each strain: *N. meningitidis* and *N. lactamica* (Figure 3.9); *S. pneumoniae* isolates associated with meningitis (Figure 3.10); *N. meningitidis* LOS immunotype strains (1-4) (Figure 3.11);

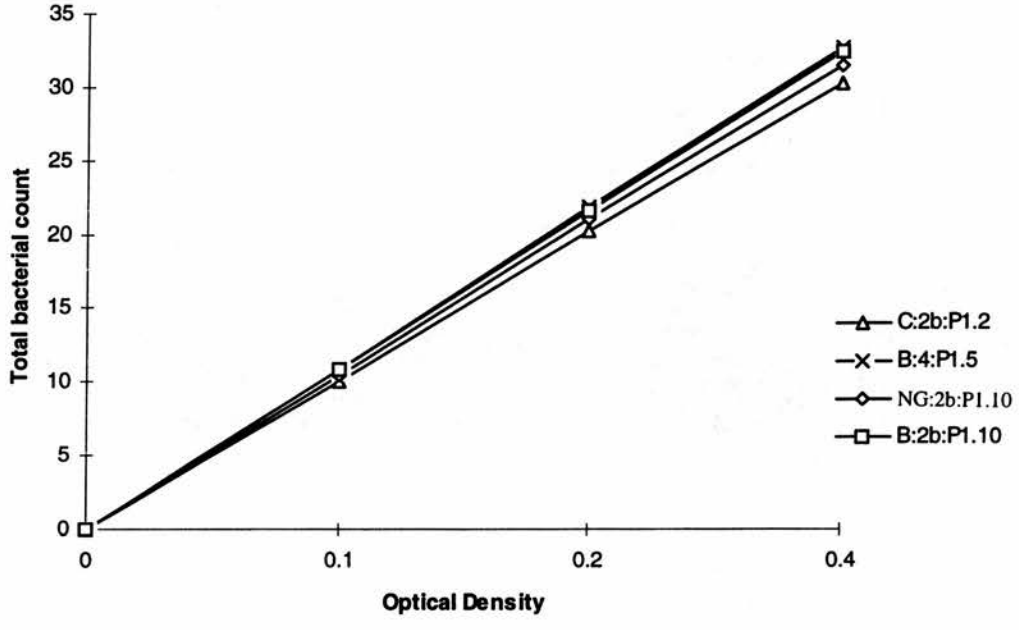

Figure 3.4 Total bacterial count (x 10^7) determined by microscopy versus optical density of *N. meningitidis* isolates.

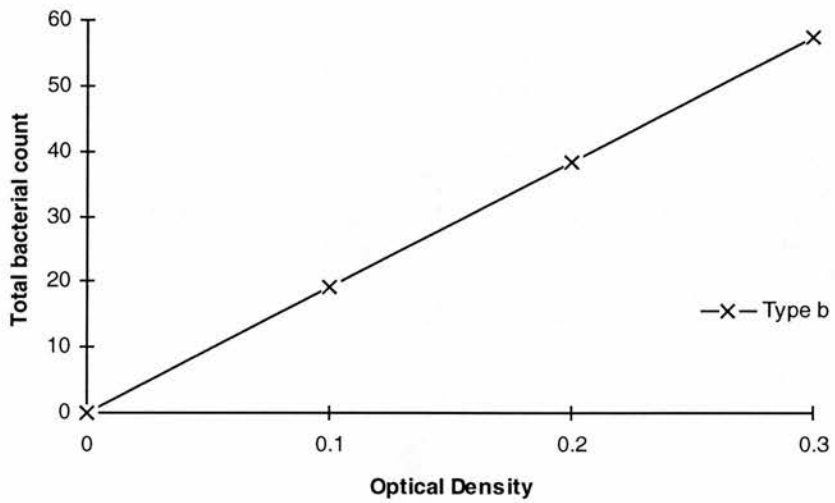

Figure 3.5 Total bacterial count (x 10^7) determined by microscopy versus OD of *H. influenzae* serotype b.

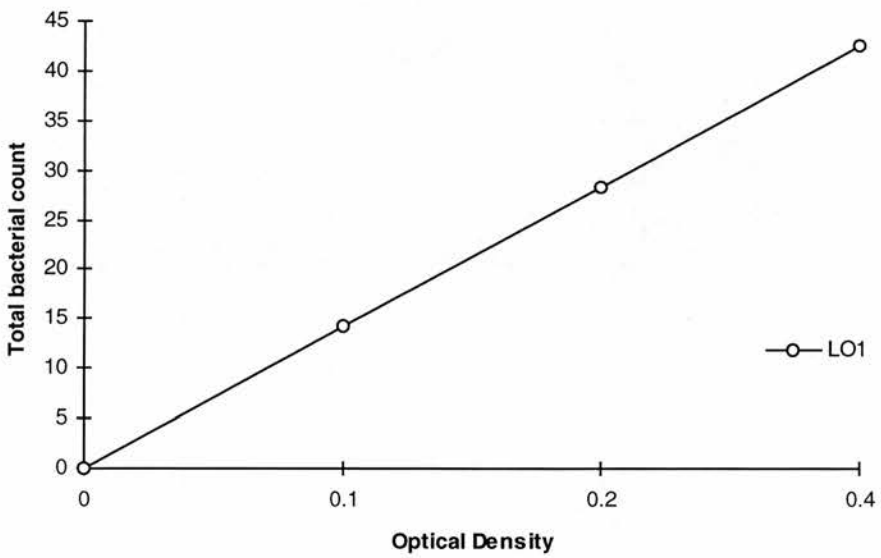

Figure 3.6 Total bacterial count (x 10^7) determined by microscopy versus OD of *N. lactamica*.

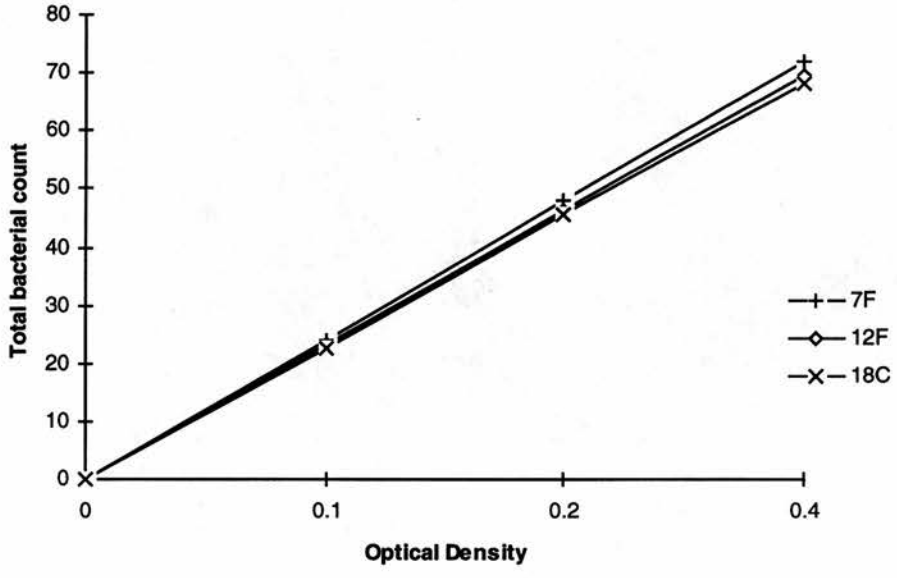

Figure 3.7 Total bacterial count (x 10^7) determined by microscopy versus OD of *S. pneumoniae* serotypes associated with meningitis.

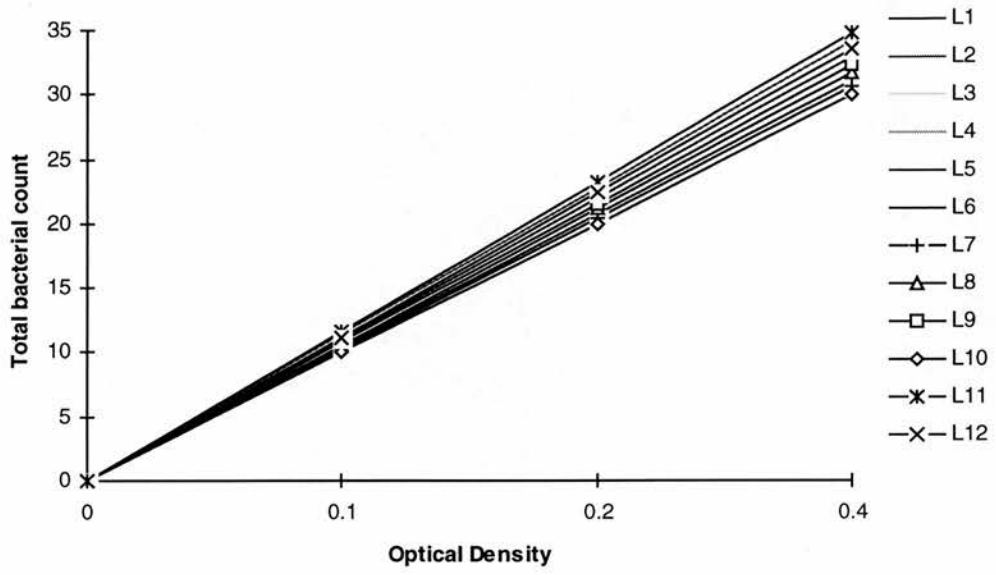

Figure 3.8 Total bacterial count (x 10^7) determined by microscopy versus OD of *N. meningitidis* LOS immunotype strains.

Table 3.2 Comparison of binding indices of FITC-labelled and unlabelled bacteria to HEp-2 cells assessed by light microscopy (200 bacteria : cell).

Species	Strain	non-labelled	FITC-labelled
N. meningitidis	C:2b:P1.2	421	378
	NG:2b:P1.10	501	460
	B:2b:P1.10	485	429
H. influenzae	type b	582	515
N. lactamica	LO1	475	424
*S. pneumoniae**	7F	494	481
	12F	450	432
	18C	448	387

(*) Strains associated with meningitis

Table 3.3 Comparison of binding indices of labelled and unlabelled *N. meningitidis* LOS immunotype strains to HEp-2 cells assessed by light microscopy (200 bacteria : cell).

Species	Strain	non-labelled	FITC-labelled
N. meningitidis LOS immunotype	L1	601	560
	L2	580	534
	L3	594	519
	L4	489	410
	L5	542	490
	L6	610	564
	L7	579	503
	L8	623	571
	L9	650	604
	L10	468	418
	L11	589	512
	L12	501	478

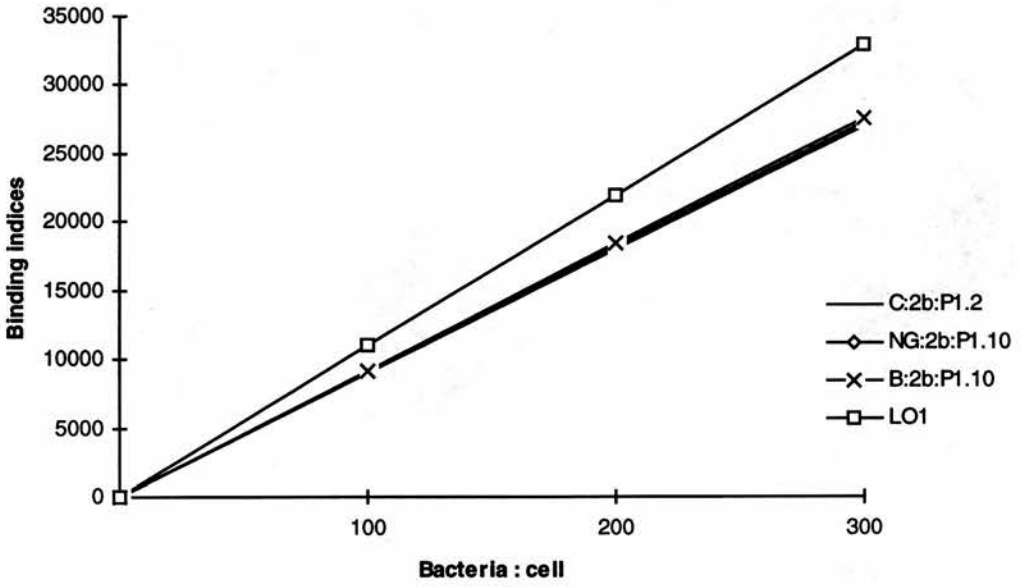

Figure 3.9 Representative dose response curves obtained with 3 *N. meningitidis* isolates and an isolate of *N. lactamica* (LO1).

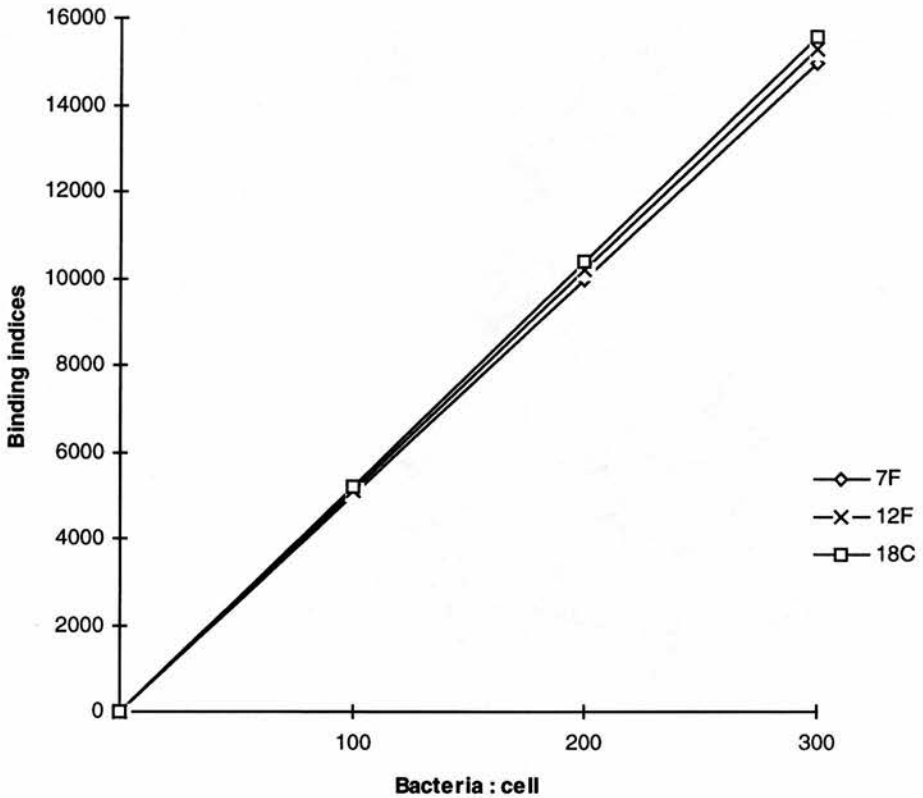

Figure 3.10 Representative dose response curves obtained with 3 *S. pneumoniae* serotypes associated with meningitis.

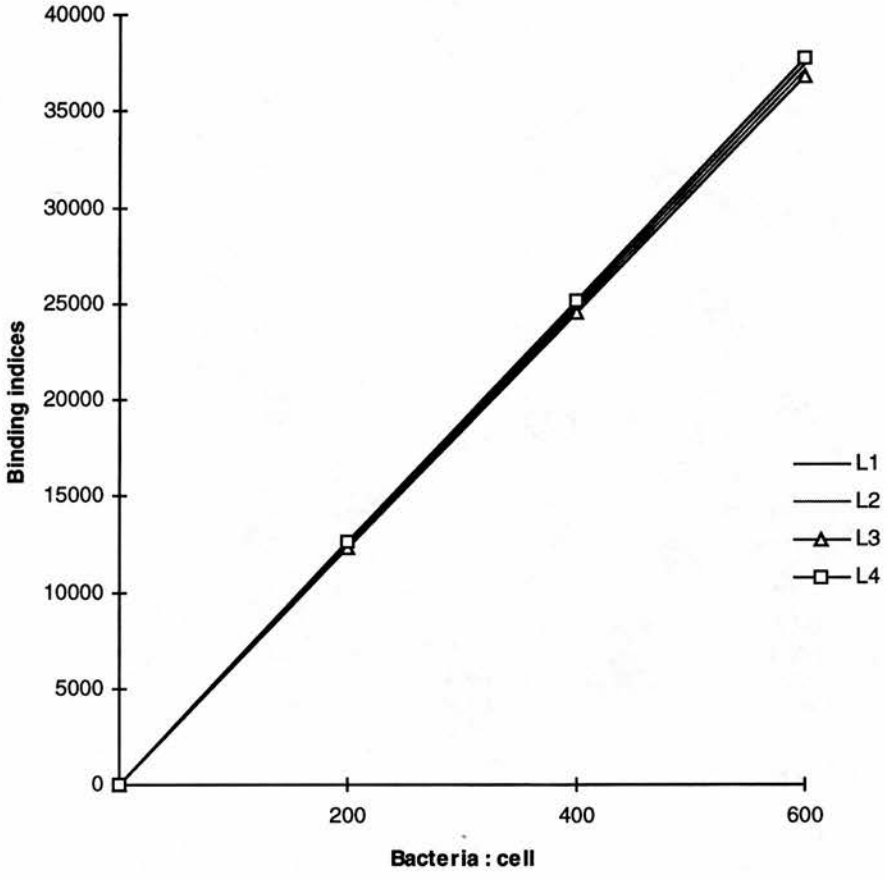

Figure 3.11 Representative dose response curves obtained with 4 *N. meningitidis* LOS immunotype strains.

and *H. influenzae* type b (Figure 3.12). The results for the isolates of each species were similar. The binding of the non-capsulate *N. lactamica* was higher than that observed for the capsulate meningococci (Figure 3.9). For experiments in which binding of bacteria to RSV-infected and uninfected HEp-2 cells was assessed, a ratio of bacteria : cell at the midpoint of the dose response curve was used.

3.3.4 Attachment of Gram-negative species to RSV infected cells:

Table 3.4 summarises the results of 6 experiments in which a ratio of 200 bacteria : cell was used to assess the effect of infection with RSV-A or RSV-B on binding to HEp-2 cells by *N. meningitidis*, *H. influenzae*, and *N. lactamica*. All the bacterial species showed enhanced binding to cells infected with RSV-A or RSV-B compared to uninfected cells. For each strain binding was greater to cells infected with RSV-A; however, except for the *N. lactamica* isolate ($P < 0.01$), the experiments showed no significant differences between RSV-A and RSV-B in binding to *N. meningitidis*, or *H. influenzae* (Table 3.5).

3.3.5 Attachment of *S. pneumoniae* strains to RSV-infected HEp-2 cells.

At a ratio of 200 bacteria : cell, the three serotypes of *S. pneumoniae* associated with meningitis had significantly greater binding indices to HEp-2 cells infected with either RSV-A or RSV-B compared with their binding indices for uninfected HEp-2 cells. The results for six assays with *S. pneumoniae* were combined for analysis (Table 3.6).

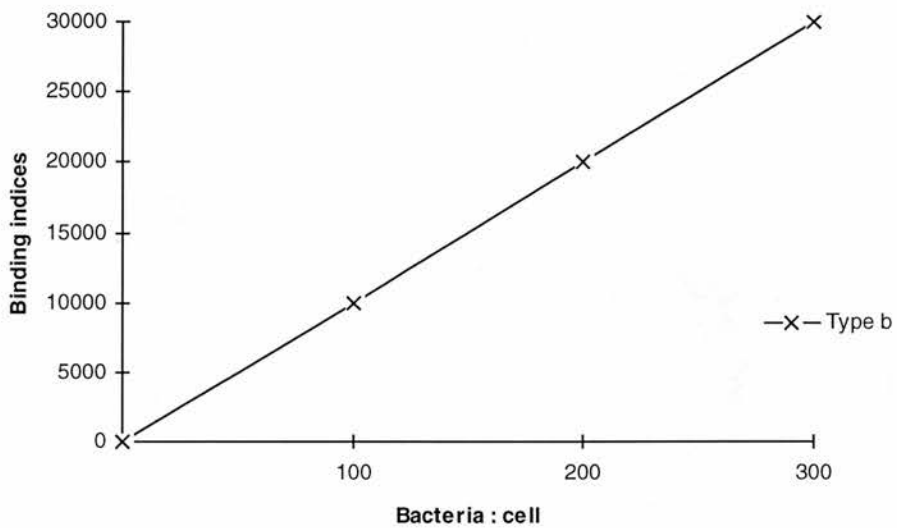

Figure 3.12 Representative dose response curves obtained with *H. influenzae* type b.

Table 3.4 Results of 6 experiments on binding of Gram-negative species to HEp-2 cells and HEp-2 cells infected with RSV-A or RSV-B (200 bacteria : cell).

Species	Strain	mean BI of uninfected cells	mean BI of RSV-A infected cells	RSV-A as % of uninfected cells (95% CI)	P	mean BI of RSV-B infected cells	RSV-B as % of uninfected cells (95% CI)	P
N. meningitidis	C:2b:P1.2	15971	54260	335 (280-400)	0.001	45660	286 (263-310)	0.001
	NG:2b:P1.10	16769	53452	320 (255-400)	0.001	52245	301 (277-327)	0.001
	B:2b:P1.10	13604	49028	352 (225-548)	0.001	38765	292 (225-379)	0.001
H. influenzae	type b	20670	56171	267 (199-359)	0.001	40035	197 (157-246)	0.001
N. lactamica	LO1	23675	64410	271 (250-295)	0.001	40686	167 (139-201)	0.001

Table 3.5 The differences in binding of RSV-A or RSV-B infected HEp-2 cells with Gram-negative species

Species	Strain	95% CI for RSV-A as % of RSV-B	P
N. meningitidis	C:2b:P1.2	83-166	NS
	NG:2b:P1.10	81-139	NS
	B:2b:P1.10	75-195	NS
H. influenzae	type b	93-199	NS
N. lactamica	LO1	129-203	0.01

Table 3.6 Results of 6 experiments on binding of *S. pneumoniae* serotypes associated with meningitis (200 bacteria : cell) to HEp-2 cells and HEp-2 cells infected with RSV-A or RSV-B.

Species	Strain	mean BI of uninfected cells	mean BI of RSV-A infected cells	RSV-A as % of uninfected cells (95% CI)	P	mean BI of RSV-B infected cells	RSV-B as % of uninfected cells (95% CI)	P
S. pneumoniae	7F	12586	20406	164 (128-210)	0.01	19054	153 (122-193)	0.01
	12F	15374	24417	154 (124-192)	0.01	18848	152 (131-177)	0.001
	18C	15330	25523	163 (127-207)	0.01	25388	163 (133-200)	0.01

Although the binding indices were greater for RSV-A infected cells, there was no significant difference between cells infected with RSV-A or RSV-B in binding of *S. pneumoniae* (Table 3.7).

3.3.6 Attachment of *N. meningitidis* LOS immunotype strains to RSV-infected HEp-2 cells.

Table 3.8 summarises the results of 6 experiments in which a ratio of 400 bacteria : cell was used to assess the binding of strains with different LOS immunotype antigens to virus infected cells. Each of the 12 immunotype strains exhibited enhanced binding to HEp-2 cells infected with RSV-A or RSV-B compared to uninfected HEp-2 cells. Table 3.9 summarises the differences between RSV-A or RSV-B infected cells in binding of the 12 immunotype strains tested; 8 bound in significantly greater numbers to RSV-B infected cells.

3.3.7 Detection of host cell surface antigens on HEp-2 cells infected with RSV-subgroups A or B

Table 3.10 summarises the results of 4 experiments in which binding indices for monoclonal antibodies directed toward cell surface antigens indicate there was no significant difference in binding of anti-CD15 to HEp-2 cells or HEp-2 cells infected with RSV-subgroups A or B. There were significant increases in binding of anti-CD14 ($P < 0.001$) and anti-CD18 ($P < 0.001$) to HEp-2 cells infected with RSV-A, the same

Table 3.7 The differences in binding of RSV-A or RSV-B infected HEp-2 cells with *S. pneumoniae* associated with meningitis

Species	strain	95% CI for RSV-A as % of RSV-B	P
S. pneumoniae	7F	97-118	NS
	12F	81-106	NS
	18C	89-111	NS

Table 3.8 Results of 6 experiments on binding of *N. meningitidis* LOS immunotype strains to HEp-2 cells and HEp-2 cells infected with RSV-A or RSV-B (400 bacteria : cell).

strain	phenotype		mean BI of uninfected cells	mean BI of RSV-A infected cells	RSV-A as % of uninfected cells (95% CI)	P	mean BI of RSV-B infected cells	RSV-B as % of uninfected cells (95% CI)	P
L1	C : NT : P1.2	L1,8	28352	40828	145 (133-156)	0.001	46390	164 (153-176)	0.001
L2	C : 2C : P1.1	L2	25978	36750	143 (125-164)	0.01	41500	161 (139-187)	0.001
L3	B : 2a : P1.5,2	L (3,7),7	26842	38300	143 (125-164)	0.001	40846	152 (141-164)	0.001
L4	C : 11 : P1.16	L4	25295	37393	149 (114-164)	0.001	40803	162 (140-187)	0.001
L5	B : 4 : NT	L5	23745	35620	151 (136-167)	0.001	38971	165 (139-195)	0.001
L6	B : 5 : P1.7,1	L6,7	28226	38778	137 (127-149)	0.001	42006	149 (138-161)	0.001
L7	B : 9 : P1.7,1	L(3,7),7	28347	39365	140 (123-159)	0.01	43302	153 (132-178)	0.001
L8	B : 8,19 : P1.7,1	L(3,7),7,8	31566	47839	153 (132-177)	0.001	50528	162 (139-187)	0.001
L9	A : 21 : P1.10	L (3,7),7	36272	49686	137 (119-159)	0.01	54269	150 (142-158)	0.001
L10	A:	L10	29483	38042	129 (123-136)	0.001	42530	144 (133-156)	0.001
L11	A : 21 : P1.10	L11	29580	47626	163 (138-192)	0.001	52485	179 (156-207)	0.001
L12	A : 21 : NT	L12	28062	37087	134 (117-152)	0.01	43199	155 (137-175)	0.001

Table 3.9 The differences in binding of HEp-2 cells infected with RSV-A or RSV-B with *N. meningitidis* LOS immunotype strains

strain	phenotype		RSV-A as % of RSV-B (95% CI)	P
L1	C : NT : P1.2	L1,8	88 (81-96)	0.05
L2	C : 2C : P1.1	L2	89 (82-95)	0.01
L3	B : 2a : P1.5,2	L (3,7),7	94 (87-101)	NS
L4	C : 11 : P1.16	L4	92 (85-99)	0.05
L5	B : 4 : NT	L5	92 (82-102)	NS
L6	B : 5 : P1.7,1	L6,7	92 (86-99)	0.05
L7	B : 9 : P1.7,1	L(3,7),7	91 (84-98)	0.05
L8	B : 8,19 : P1.7,1	L(3,7),7,8	94 (89-100)	NS
L9	A : 21 : P1.10	L (3,7),7	92 (82-102)	NS
L10	A:	L10	90 (86-93)	0.05
L11	A : 21 : P1.10	L11	91 (84-99)	0.05
L12	A : 21 : NT	L12	86 (79-94)	0.01

Table 3.10 Binding indices for monoclonal antibodies to host cell surface antigens on HEp-2 cells and RSV-subgroups A or B infected HEp-2 cells

Antibodies	mean BI of uninfected HEp-2 cells	mean BI of RSV-A infected cells	mean BI of RSV-B infected cells	P
CD14	1549	6885	7008	0.001
CD18	3629	8640	8594	0.001
CD15	1587	1565	1517	NS

pattern was observed with the RSV-B infected HEp-2 cells anti-CD14 ($P < 0.001$) and Anti-CD18 ($P < 0.001$) compared with uninfected HEp-2 cells.

3.3.8 Inhibition of binding of *S. pneumoniae* by treatment of HEp-2 cells with monoclonal antibodies

3.3.8.1 Host cell antigens

Previous work in this laboratory found that binding of meningococci to HEp-2 cells and HEp-2 cells infected with RSV-A could be inhibited by pre-treatment of the cells with monoclonal antibody to CD14 or CD18 (Raza *et al.*, 1994). These studies were repeated with Gram positive strains.

In four experiments, pre-treatment of RSV-A infected HEp-2 cells with monoclonal antibody to CD14, CD18 or CD15 did not significantly reduce binding of pneumococcal strain 12F: anti-CD14 (95% CI 96-112); anti-CD18 (95% CI 101-106); and anti-CD15 (95% CI 99-102) (Figure 3.13). The same pattern were observed with the strain 18C: anti-CD14 (95% CI 98-101); anti-CD18 (95% CI 96-105); and anti-CD15 (95% CI 95-106) (Figure 3.14).

3.3.8.2 Glycoprotein G of RSV

Data from 4 experiments indicated that monoclonal anti-glycoprotein G bound to the viral glycoprotein G on the surface of infected cells (Figure 3.15), but there was no evidence the glycoprotein G of RSV is an additional receptor for *S. pneumoniae*.

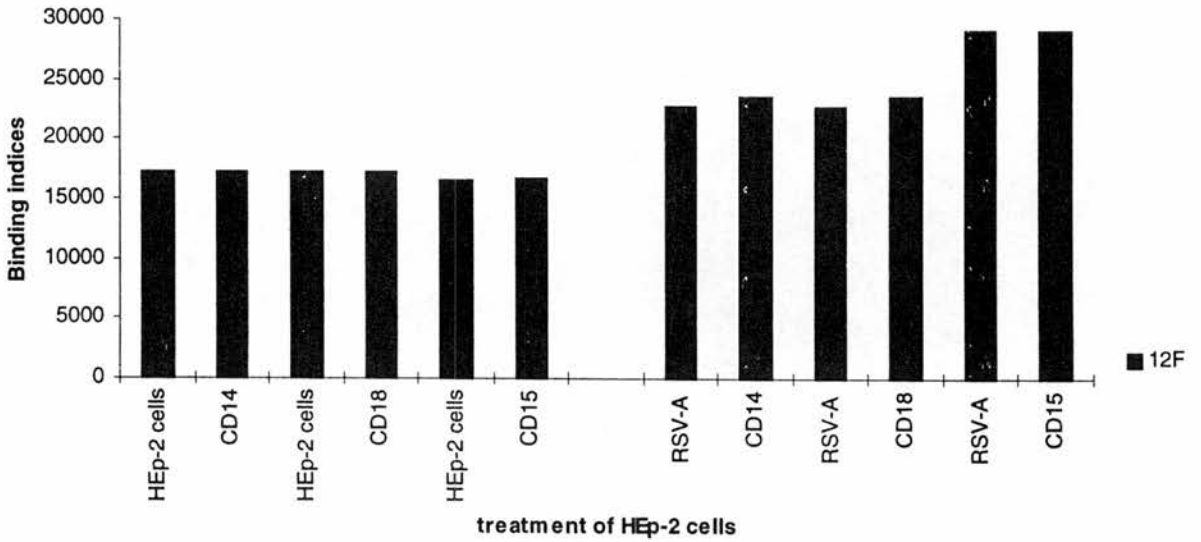

Figure 3.13 The effect of pre-treatment of HEp-2 cells and RSV-A infected HEp-2 cells with monoclonal antibody to CD14, CD18 and CD15 on binding of *S. pneumoniae* type 12F

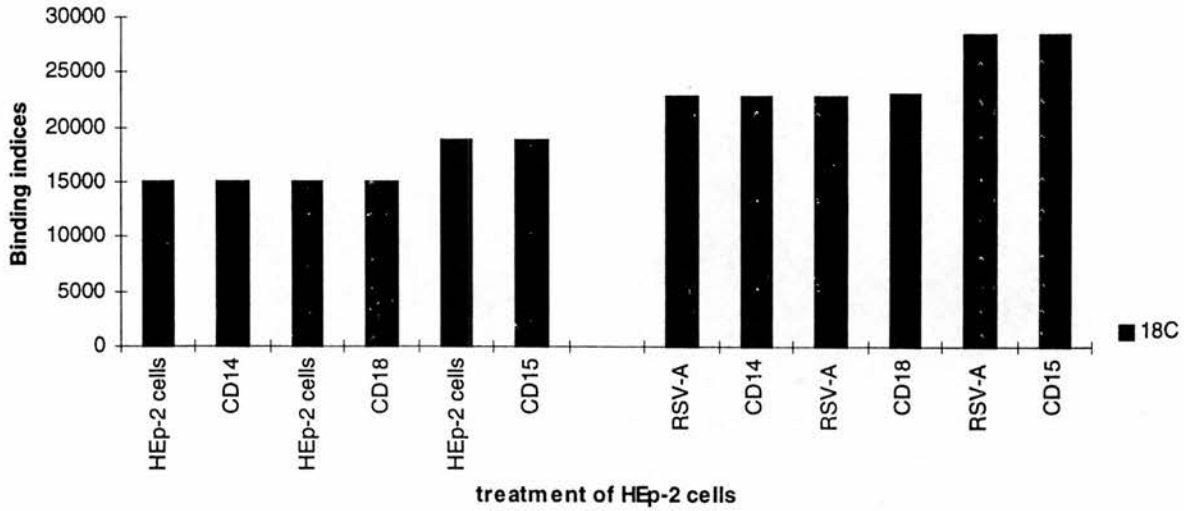

Figure 3.14 The effect of pre-treatment of HEp-2 cells and RSV-A infected HEp-2 cells with monoclonal antibody to CD14, CD18 and CD15 on binding of *S. pneumoniae* type 18C

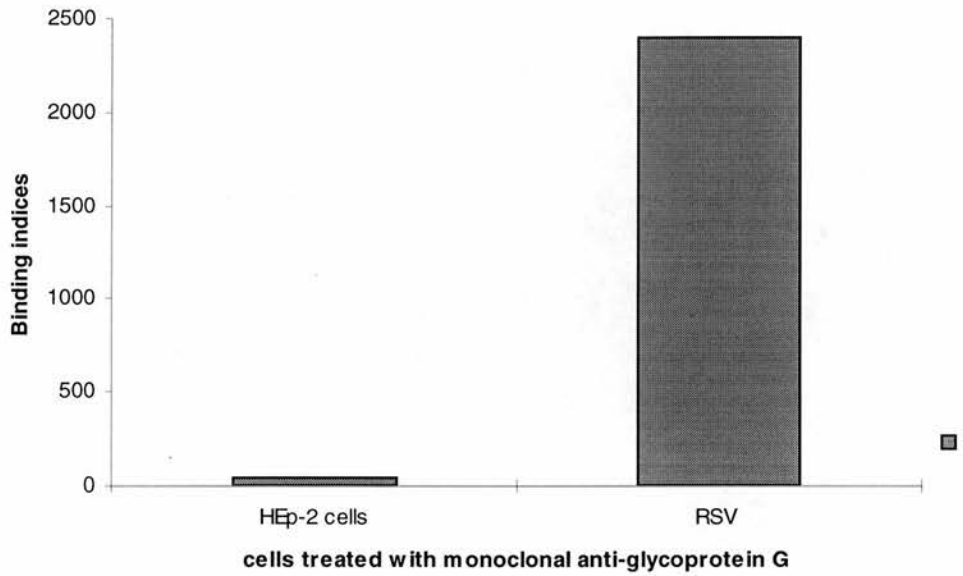

Figure 3.15 Binding of monoclonal antibody to RSV glycoprotein G with HEp-2 cells or RSV infected HEp-2 cells.

Pre-treatment of HEp-2 cells or RSV-A infected HEp-2 cells with the monoclonal antibody had no effect on binding of either pneumococcal isolate tested (Table 3.11).

Table 3.11 The effect of pre-treatment of HEp-2 cells and RSV-infected HEp-2 cells with monoclonal antibody to glycoprotein G of RSV on binding of *S. pneumoniae* species.

Antibody	Strain	mean BI of uninfected cells	mean BI of uninfected cells with anti-G	mean BI of RSV-infected cells	mean BI of RSV-infected cells with anti-G	95% CI for treated uninfected cells with anti-G as % of uninfected cells	95% CI for treated RSV-infected cells with anti-G as % of RSV-infected cells	P
monoclonal Anti-G	12F	16550	16770	29075	29124	99-103	99-101	NS
	18C	18862	18824	28515	28554	98-102	99-101	NS

3.4 Discussion

The results provided evidence to answer the questions posed in the introduction. Variations in glycoprotein (G) between RSV subgroups A and B had been reported (Akerlind *et al.*, 1988; Anderson *et al.*, 1991; Cane *et al.*, 1991), and previous work by our group demonstrated that glycoprotein G of RSV-A was an additional receptor for meningococci (Raza *et al.*, 1994). This study demonstrated that infection of HEp-2 cells with RSV-B also enhanced bacterial binding. Initial studies showed there were higher levels of binding observed on cells infected with subgroup A compared with subgroup B (Table 3.4).

There was significantly enhanced binding of meningococci, Hib, *N. lactamica*, and *S. pneumoniae* strains associated with meningitis to cells infected with either RSV-A or RSV-B in experiments in which both subgroups of the virus were tested at the same time. The *N. lactamica* isolate bound more to RSV-A infected cells (Tables 3.5; and 3.7) and some meningococcal immunotype strains bound more to RSV-B infected cells (Table 3.9); however, for most strains, statistical analysis showed no significant of differences in binding to cells infected with RSV-A or RSV-B.

Previous studies indicated that enhanced binding of *N. meningitidis* strains was not associated with particular serogroup (capsular), serotype (outer membrane protein 2/3) or subtypes (outer membrane protein 1) antigens on the bacteria (Raza *et al.*, 1993). Strains with different LOS immunotype antigens were assessed for binding to determine if the carbohydrate composition of the endotoxin affected binding to virus infected cells.

Each of 12 immunotype strains exhibited enhanced binding to HEp-2 cells infected with RSV-A or RSV-B compared to uninfected HEp-2 cells. With the exception of strains L4, L5, L8 and L9, the results showed that cells infected with the RSV subgroup B bound significantly more bacteria than those observed with RSV-A infected cells.

Previous work in this laboratory found RSV infected cells show increased binding of monoclonal antibodies to CD14, CD15 and CD18 (Raza *et al.*, 1994b). Pre-treatment of uninfected or RSV infected HEp-2 cells with monoclonal antibodies to CD14 and CD18 significantly reduced binding of meningococci (Raza *et al.*, 1994). This was not observed with the Gram-positive pneumococcal strains tested. This indicates that the enhanced binding of Gram-negative bacteria to RSV infected cells is partly due to enhanced expression of host cell components that bind endotoxin (Wright and Jong, 1986). Glycoprotein G of subgroup A has been shown to be an additional receptor for meningococci (Raza *et al.*, 1994a), but pre-treatment of HEp-2 cells or RSV-A infected HEp-2 cells with the monoclonal to glycoprotein G did not affect binding of the 2 pneumococcal strains. These results indicate that the changes in host cell antigens that enhanced binding of meningococci are not the same as those that enhance binding of pneumococci.

Colonisation of the upper respiratory tract with *H. influenzae*, *S. pneumoniae*, or *N. meningitidis* is thought to be the initial event in the pathogenesis of bacterial meningitis. The majority of children with bacterial meningitis had signs and symptoms of upper respiratory infection, suggesting that viral infection promotes bacterial spread and

invasion (Krasinski *et al.*, 1987). This study showed enhanced binding of species that cause meningitis to RSV A or RSV B infected cells might contribute to density of colonisation which is associated with development of disease (Beachey, 1981).

An epidemiological study published after this work began found no strong correlation between outbreaks of meningococcal disease and RSV infection (Stuart *et al.*, 1996). Screening of patients with meningococcal infection for evidence of RSV infection, either identification of the virus or serological response, is needed to determine if infection by these viruses plays a significant role in susceptibility to meningitis. There is, however, good evidence that RSV predisposes children to respiratory infection; therefore, the methods developed in this chapter were used to assess binding of bacterial respiratory pathogens.

Chapter 4

The effect of infection with RSV A or RSV B on binding of bacteria associated with respiratory tract infection to HEp-2 cells

4.1 Introduction

S. pneumoniae, non-typable *H. influenzae* and *M. catarrhalis* are the three species most commonly associated with bacterial respiratory tract infection in both children and adults. These species are also isolated from children with acute otitis media (AOM) and adults with acute exacerbation of chronic bronchitis and chronic obstructive pulmonary disease (COPD).

4.1.1 Infection in children

The infection of the respiratory tract by viruses and subsequent bacterial colonisation and infection, for instance bacterial pneumonia associated with measles or influenza, are potentially fatal problems. RSV was chosen for these studies because it is recognised as an important viral respiratory pathogen in infants and in young children causing bronchiolitis and pneumonia. It is a major cause of hospitalisation in infants less than six months of age and secondary bacterial infections such as acute otitis media and pneumonia are common (Ruuskanen and Ogra, 1993). In a Finnish study, RSV infections were associated with a significant rise of antibodies to *H. influenzae*, *M. catarrhalis* and pneumococcal antigens (Korppi *et al.*, 1989). A recent study of

invasive pneumococcal disease was associated with RSV infection in both adults and children (Kim *et al.*, 1996).

Several studies have indicated a crucial role of respiratory viruses in the pathogenesis of AOM which is the most common respiratory tract infection of infancy and early childhood (Klein, 1994). Increased rates of AOM have been noted especially during epidemics of RSV, influenza A virus and rhinovirus (Ruuskanen *et al.*, 1991). Bacterial pathogens isolated from approximately two thirds of children with AOM are *S. pneumoniae* followed by non-typable strains of *H. influenzae* and *M. catarrhalis*.

A high frequency of mixed viral and bacterial infections has been documented in developing countries. In Papua New Guinea two-thirds of documented viral infections occurred concurrently with a bacterial infection (Shann *et al.*, 1984). In Pakistan, 26% of children infected with RSV also had bacteraemic infection with *Haemophilus* species or *S. pneumoniae* (Ghafoor *et al.*, 1990). Fifty-four percent of the cases of bactereamic *Haemophilus* infection and 47% of bacteraemic infection with *S. pneumoniae* were associated with viral infection (Berman, 1991).

In the United States simultaneous infections with RSV and *B. pertussis* have been demonstrated. In one study of 29 children with pertussis, 14 also had RSV infection (Nelson *et al.*, 1986). The temporal sequence of these two infections could not be determined. The symptoms and signs of RSV infection or pertussis alone were similar.

4.1.2 Infection in adults

RSV is a significant cause of serious lower respiratory tract infection in immunocompromised adults and the elderly (Falsey *et al.*, 1995). In a family study, 17% of the adults living with infected children also became infected (Hall *et al.*, 1976). In adults, RSV infection can be asymptomatic or can induce mild to moderate upper respiratory tract symptoms. In healthy adults, the infection is rarely severe or fatal (Aylward and Burdge, 1991). The average duration of virus shedding is 5 days (Hall *et al.*, 1991). Based on clinical features, RSV infection cannot be differentiated from the common cold induced by other etiologic agents.

4.1.2.1 Pneumonia

Pneumonia due to either bacterial or non-bacterial causes is characterised by prolonged respiratory symptoms and persistently abnormal chest X ray (Kirtland and Winterbauer, 1991). Prior infection by respiratory viruses has long been regarded as one factor that predisposes to pneumococcal pneumonia. Studies have shown the rate of nasopharyngeal colonisation by *S. pneumoniae* to be greatest in autumn, winter, and early spring among families, adults in a closed environment (such as military recruits), and in the population at large (Gray *et al.*, 1982). The observed association between season and pneumococcal carriage or pneumococcal infection might be mediated through the seasonal incidence of viral illness.

The study by Kim and colleagues (1996) using the novel approach of a community-based viral surveillance system found a distinct temporal association between invasive

pneumococcal disease in adults and viral-type respiratory illnesses of all kinds. They also found that pneumococcal disease was associated with isolation of RSV.

4.1.2.2 Acute exacerbation of chronic bronchitis (AECB)

By definition, chronic bronchitis is the production of sputum on most days of 3 consecutive months in at least 2 consecutive years (Wilson, 1995). Manifestations of acute bacterial exacerbations are predominantly bronchopulmonary and include greater frequency and severity of cough, increased sputum production, purulent sputum, haemoptysis, chest congestion and discomfort, and increased dyspnea and wheezing.

Many micro-organisms have been associated with exacerbations of chronic bronchitis, however, the predominant pathogen is non-typable *H. influenzae* which has been recognised to cause more than 50 % of all bacteriologically defined exacerbations (Ball, 1995). The proportions of major bacterial respiratory pathogens isolated from patients in clinical trials indicated that *M. catarrhalis* and *S. pneumoniae* account for approximately a further third of isolates from AECB (Chodosh, 1992). These bacteria are persistently present in respiratory secretions of established chronic bronchitic patients. If, as suggested by Beachey (1981), density of colonisation is a major determinant for disease, factors that enhance the bacterial load might contributes to exacerbation of symptoms among these patients.

4.1.2.3 Chronic obstructive pulmonary disease (COPD)

COPD is characterised by periodic exacerbation in which the patient experiences worsening of symptoms, including increased cough and sputum production, increased sputum purulence and shortness of breath. Infection was the most common observable cause of death in a prospective study of patients with COPD (Burrows and Earle, 1969). The role of bacterial infection in COPD has been the subject of intense investigation over the past 40 years (Murphy and Sethi, 1992).

Bacteria may be involved in the pathogenesis of exacerbations in two ways. They could be the primary cause of the exacerbation; alternatively, they could act as secondary invaders after acute viral or mycoplasmal infection. Studies using viral cultures and serologic assays have established that viral and to a lesser extent, mycoplasmal infections cause approximately one third of these exacerbations (Buscho *et al.*, 1978; Tager and Speizer, 1975). Many studies have identified *S. pneumoniae* and nontypable *H. influenzae* with purulent sputum and exacerbations of COPD (Tager and Speizer, 1975; Murphy and Apicella, 1987). In addition, *M. catarrhalis* has emerged as a pathogen in this setting (Doern,1986; Verghese *et al.*, 1990).

4.1.3 Aim of the study

In the present study, the hypothesis tested was that HEp-2 cells infected with RSV subgroups A or B might exhibit enhanced binding of the bacteria associated with respiratory infections or exacerbation of chronic bronchitis.

4.2 Materials and methods

4.2.1 Cells (3.2.1)

4.2.2 Growth of RSV viruses (3.2.2)

4.2.3 Bacteria

Five isolates of *S. pneumoniae* (types 42, 23, 10, 6, and 33) were obtained from respiratory specimens by the Clinical Bacteriology Diagnostic laboratory, Department of Medical Microbiology. Two isolates of *M. catarrhalis* (MC1 and MC2) were obtained from Dr. H. Young, Department of Medical Microbiology, Edinburgh University; MC1 grew on Modified New York City Medium (MNYC) and MC2 did not grow on this medium which contained selective antibiotics. Two *B. pertussis* strains, 8002 (fimbriate, type 1,2) and 250815 (non-fimbriate, type 1,3), were supplied by Dr. N. W. Preston, Department of Microbiology, University of Manchester.

S. aureus strain NCTC 10655 was obtained from The National Type Culture Collection. Two non-typable *H. influenzae* isolates were obtained from throat cultures of infants (Infection and Immunity Laboratory).

Pneumococci were grown on Columbia blood agar with horse blood (BA). The MC1 isolate was grown on MNYC (Young, 1978), the antibiotic-sensitive isolate (MC2) and non-typable *H. influenzae* were grown on boiled blood agar (BBA). *B. pertussis* was grown on charcoal agar (Difco, UK). *S. aureus* was cultured on nutrient agar. Except for *B. pertussis* which required 3-5 days incubation, each strain was grown overnight at 37°C in a humidified atmosphere with 10% CO_2. The bacteria were harvested, washed

three times in phosphate buffered saline (PBS) by centrifugation at 2500 x *g* for 15 min and resuspended in MM without antibiotics by vigorous pipetting to disperse clumps.

The bacterial concentrations were determined by measuring OD at 540 nm. For each strain the linear relationship between OD reading and total count assessed microscopically with a Thoma counting chamber was determined, and a standard curve was prepared for each strain used.

4.2.4 Infection of HEp-2 cells with RSV-subgroups A or B (3.2.4)

4.2.5 Bacterial Binding to RSV-infected HEp-2 cells (3.2.5)

4.2.6 Detection of RSV-infected cells (3.2.6)

4.2.6.1 Fluorescence microscopy (3.2.6.1)

4.2.7 Flow cytometry (3.2.7)

4.2.8 The effect of FITC on bacterial binding (3.2.8)

4.2.9 Statistical methods.(3.2.11)

4.3 Results

4.3.1 Determination of total count in relation to OD

For each isolate, the bacterial concentration was determined by OD at 540 nm in relation to the total count obtained by light microscopy with a Thoma counting chamber. The range of OD readings providing a linear relationship with the total counts were used as standard curves: *S. pneumoniae*, respiratory isolates (Figure 4.1); *H. influenzae* (non-typable) isolates (Figure 4.2); *M. catarrhalis* (Figure 4.3); *S. aureus* (Figure 4.4); and *B. pertussis* (Figure 4.5). The bacterial suspensions were adjusted to provide a range of ratios of bacteria per cell for use in the assays.

4.3.2 The effect of FITC on bacterial binding

The effect of FITC on bacterial binding was examined by light microscopy with each of the FITC-labelled and unlabelled bacterial species used in this study. The results in Table 4.1 in which a ratio of 200 bacteria : cell (except for *M. catarrhalis* 400 bacteria : cell and *B. pertussis* 500 bacteria : cell) was tested represent the mean of 2 experiment. There were no differences observed in binding indices of FITC-labelled bacteria compared with unlabelled bacteria.

4.3.3 Determination of bacterial binding to uninfected cells

Dose response experiments were carried out with HEp-2 cells for each strain: *S. pneumoniae* respiratory isolates (Figure 4.6); *H. influenzae* non-typable isolates (Figure 4.7); *M. catarrhalis* (Figure 4.8); *S. aureus* (Figure 4.9); and *B. pertussis*

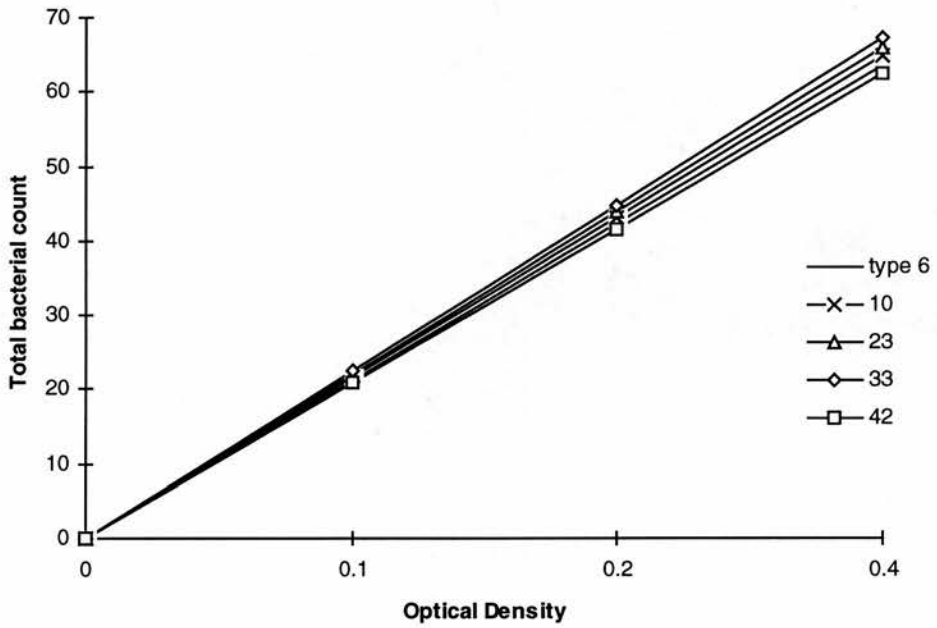

Figure 4.1 Total bacterial count (x 10^7) determined by microscopy versus OD of *S. pneumoniae* respiratory isolates.

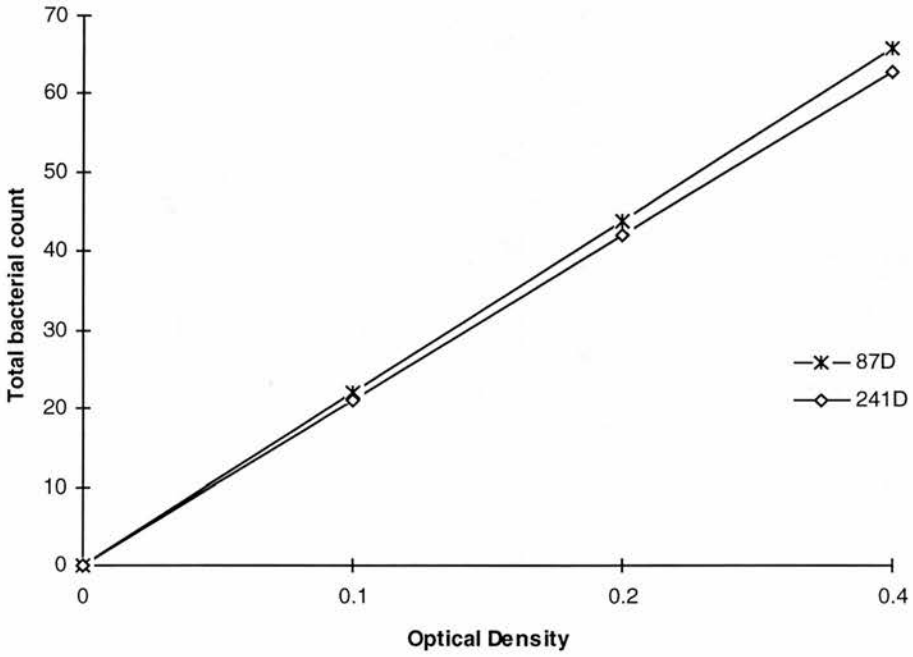

Figure 4.2 Total bacterial count (x 10^7) determined by microscopy versus OD of *H. influenzae* (non-typable) isolates.

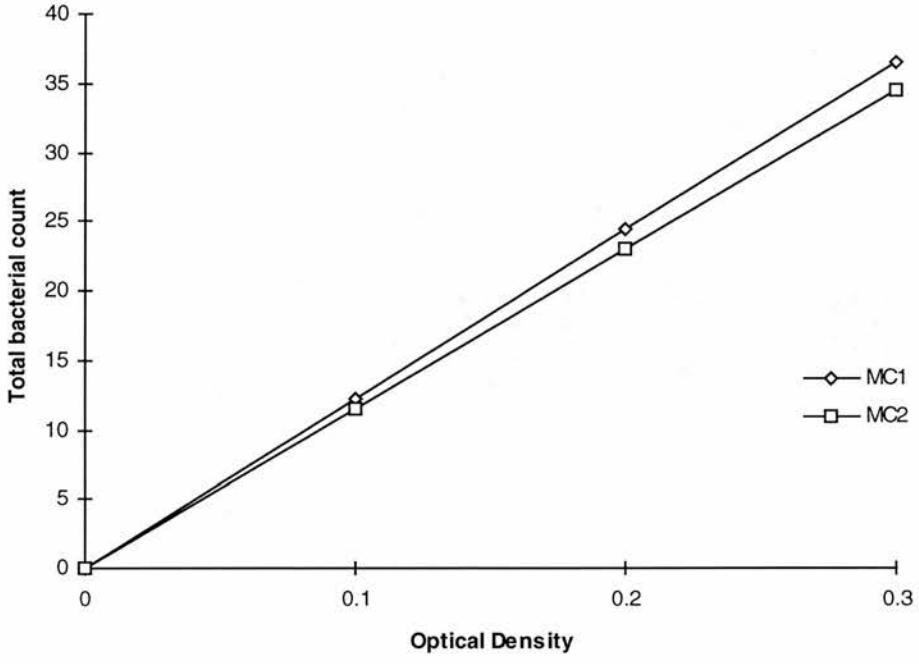

Figure 4.3 Total bacterial count (x 10^7) determined by microscopy versus OD of *M. catarrhalis* isolates.

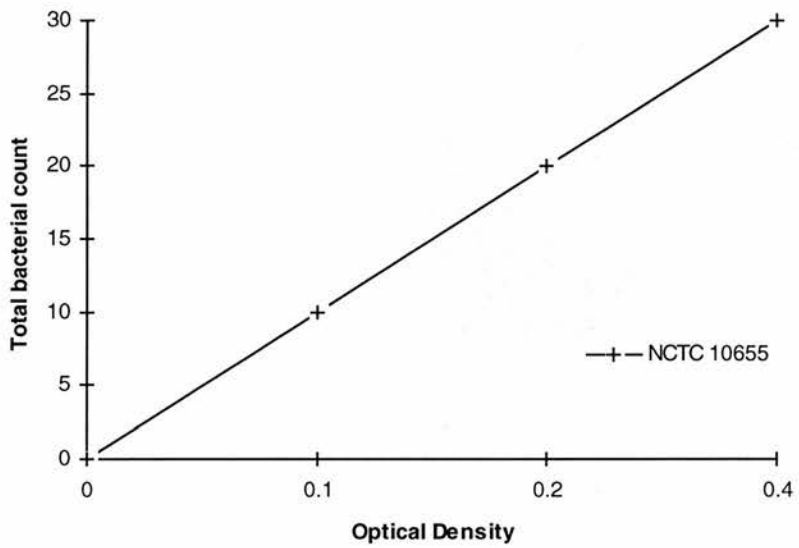

Figure 4.4 Total bacterial count (x 10^7) determined by microscopy versus OD of *S. aureus* strain NCTC 10655.

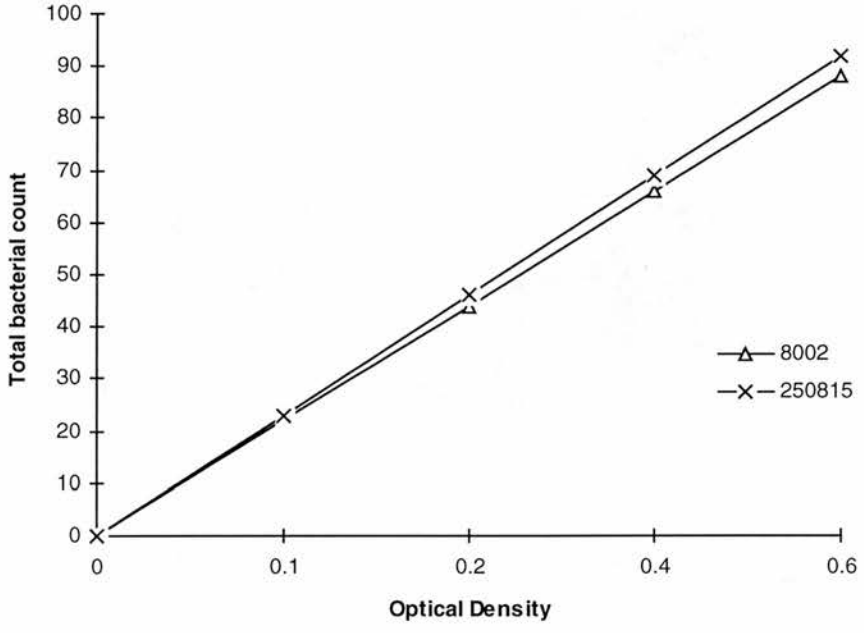

Figure 4.5 Total bacterial count (x 10^7) determined by microscopy versus OD of *B. pertussis* strains.

Table 4.1 Comparison of binding indices of FITC-labelled and unlabelled bacteria to HEp-2 cells assessed by light microscopy.

Species	Strain	non-labelled	FITC-labelled
M. catarrhalis	MC1	546	507
	MC2	539	499
H. influenzae (nontypable)	87D	409	396
	241D	431	401
B. pertussis	type 1,2	397	387
	type 1,3	412	369
S. pneumoniae *	type 6	489	472
	type 10	501	469
	type 23	531	502
	type 33	490	476
	type 42	526	494
S. aureus	10655	563	549

(*) Respiratory isolates

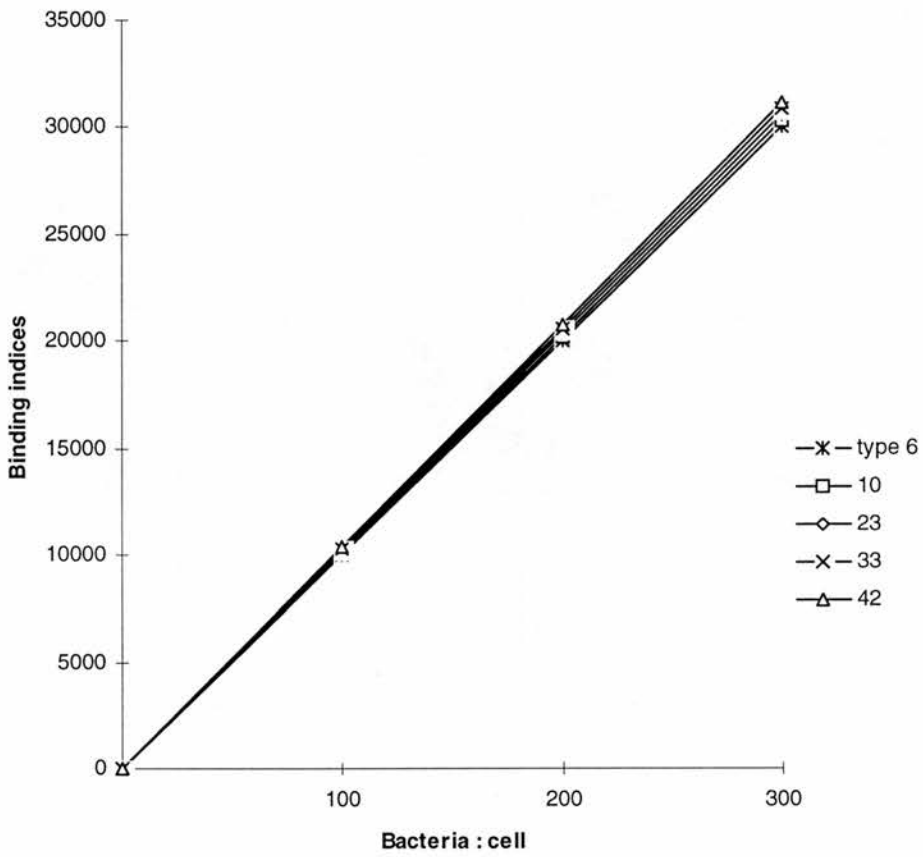

Figure 4.6 Representative dose response curves obtained with 5 *S. pneumoniae* isolates.

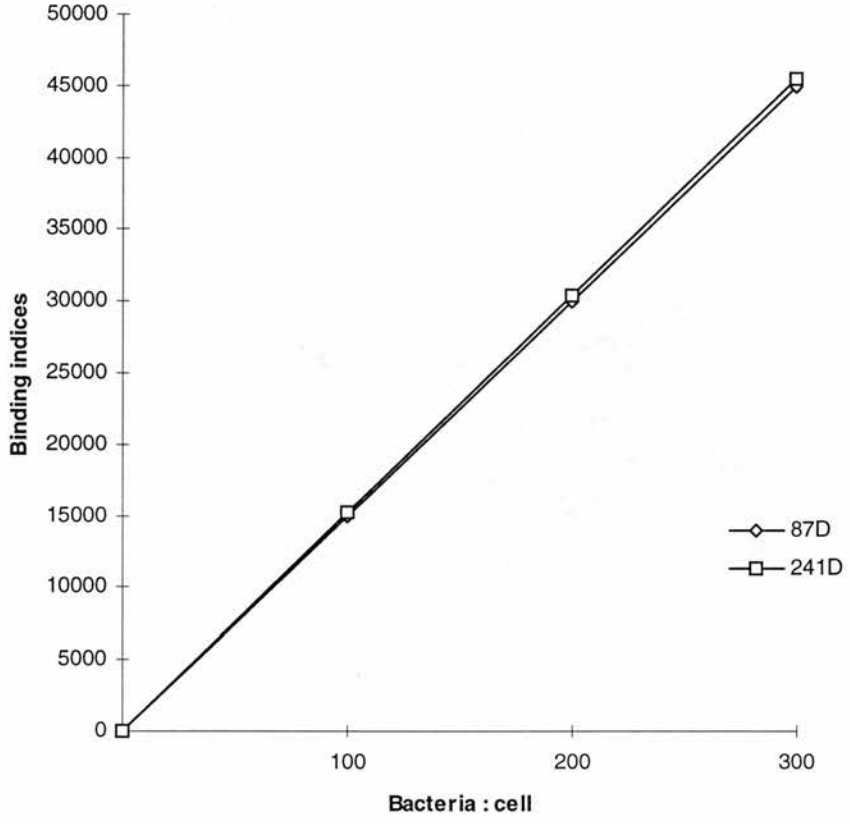

Figure 4.7 Representative dose response curves obtained with 2 non-typable *H. influenzae* isolates.

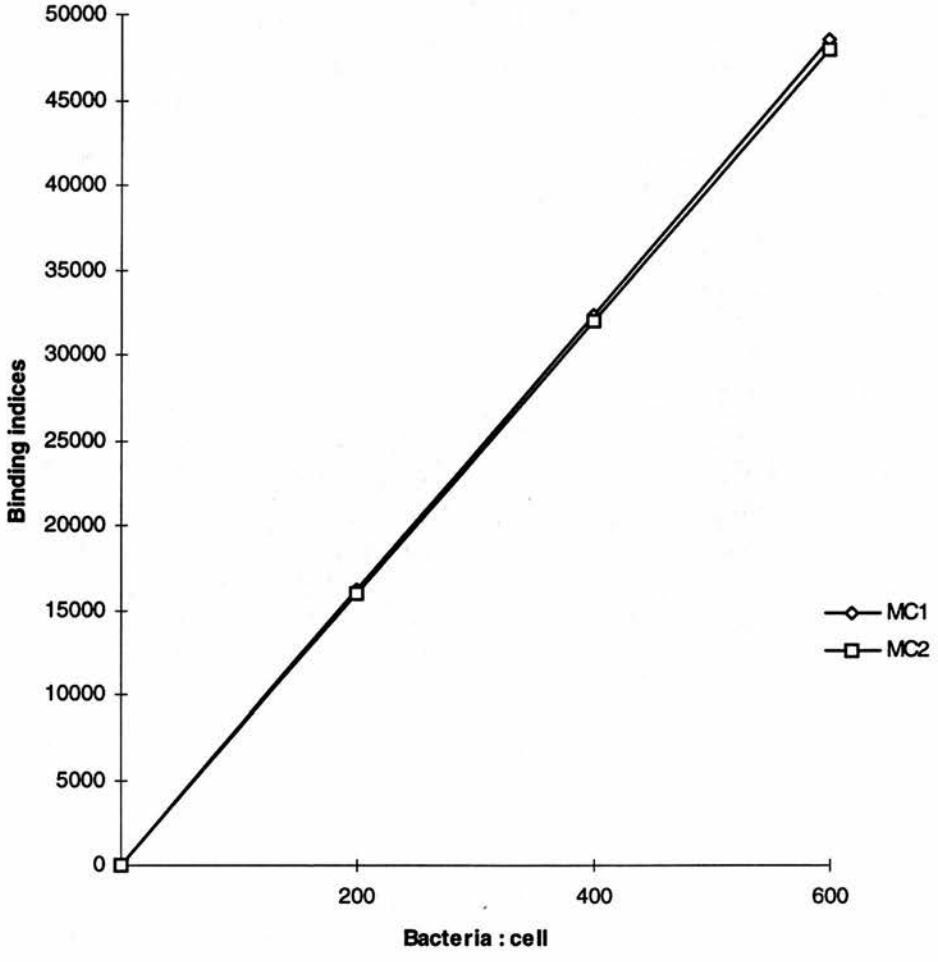

Figure 4.8 Representative dose response curves obtained with 2 *M. catarrhalis* isolates.

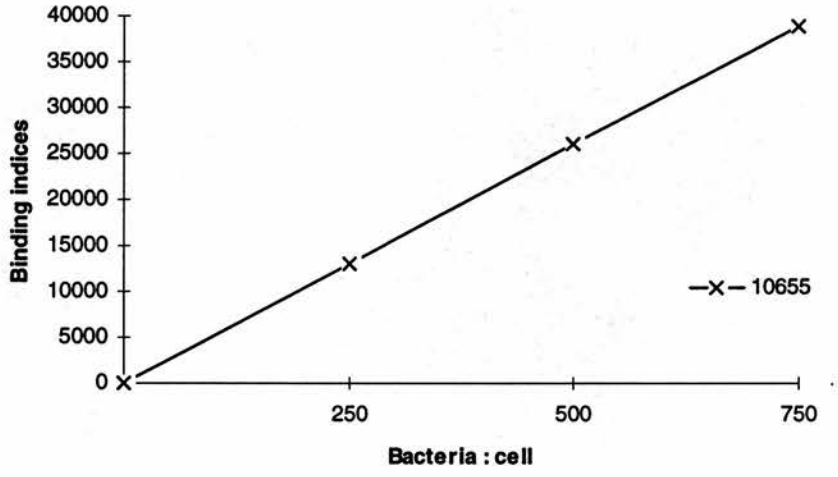

Figure 4.9 Representative dose response curves obtained with *S. aureus* strain NCTC 10655.

(Figure 4.10). For experiments in which binding of bacteria to RSV infected and uninfected HEp-2 cells was assessed, a ratio of bacteria : cell at the mid point of the dose response curve of the test strain was used.

4.3.4 Binding of Gram-negative bacteria to RSV-infected HEp-2 cells

Table 4.2 summarises the results of 6 experiments in which the effect of infection with RSV subgroups A or B on binding of *M. catarrhalis* (400 bacteria : cell), non-typable *H. influenzae* (200 bacteria : cell), and *B. pertussis* (500 bacteria : cell) to HEp-2 cells was assessed. Except for *M. catarrhalis* (MC2) which bound in significantly lower levels to HEp-2 cells infected with either RSV-A or RSV-B, all the bacterial species showed significantly enhanced binding to cells infected with RSV subgroups A or B. For non-typable *H. influenzae* and *B. pertussis*, the binding to RSV-A infected cells was significantly greater than to RSV-B infected cells (Table 4.3).

4.3.5 Binding of Gram-positive species to RSV infected HEp-2 cells

In 6 experiments, all the serotypes of *S. pneumoniae* isolated from patients with respiratory infections and the *S. aureus* isolate demonstrated significantly greater binding indices to HEp-2 cells infected with either RSV subgroups A or B compared with their binding indices for uninfected HEp-2 cells (Table 4.4). For *S. pneumoniae* strains there was no significant difference in binding to the RSV A-infected

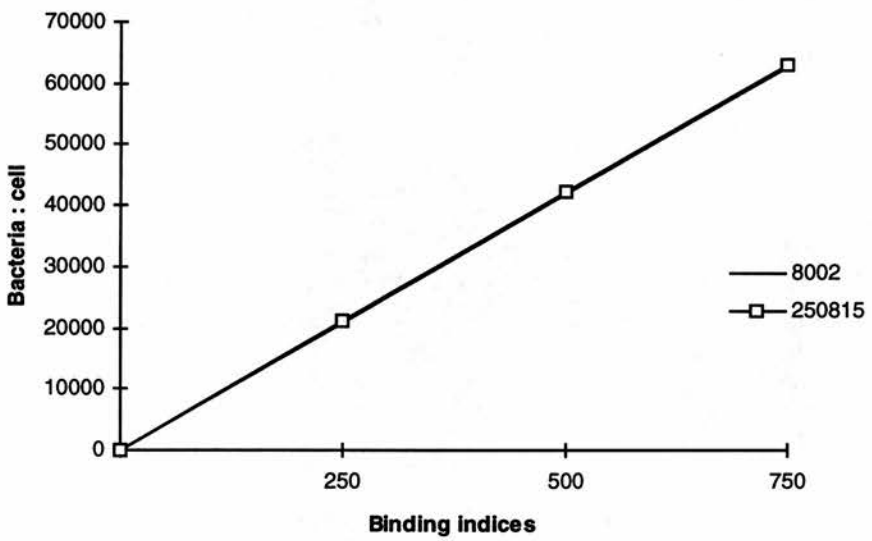

Figure 4.10 Representative dose response curves obtained with 2 *B. pertussis* strains.

Table 4.2 Results of 6 experiments on binding of Gram-negative species to HEp-2 cells and HEp-2 cells infected with RSV-A or RSV-B (200 bacteria : cell, except for *M. catarrhalis* 400 bacteria : cell and *B. pertussis*, 500 : cell).

Species	isolate / Strain	mean BI of uninfected cells	mean BI of RSV-A infected cells	RSV-A as % of uninfected cells (95% CI)	P	mean BI of RSV-B infected cells	RSV-B as % of uninfected cells (95% CI)	P
M. catarrhalis	MC1	21726	42886	209 (126-349)	0.05	33736	163 (101-267)	0.05
	MC2	**37758**	**31144**	**80 (64-99)**	**0.05**	**23559**	**55 (31-95)**	**0.05**
H. influenzae (NT)	87D	31810	50358	157 (154-162)	0.001	45030	142 (136-148)	0.001
	241D	30570	49099	161 (151-171)	0.001	42228	138 (127-151)	0.001
B. pertussis	type 1,2	46554	55825	120 (112-127)	0.001	50580	109 (105-112)	0.001
	type 1,3	49580	57614	115 (106-125)	0.01	53675	108 (105-111)	0.001

Table 4.3 The differences between RSV-A and RSV-B on binding with Gram-negative species

species	isolate / strain	RSV-A as % of RSV-B (95% CI)	P
M. catarrhalis	MC1	130 (97-174)	NS
	MC2	186 (86-390)	NS
H. influenzae (NT)	87D	112 (110-114)	0.001
	241D	116 (113-121)	0.001
B. pertussis	type 1,2	110 (105-115)	0.01
	type 1,3	107 (100-114)	NS

Table 4.4 Results of 6 experiments on binding of Gram-positive species (200 bacteria : cell) to HEp-2 cells and HEp-2 cells infected with RSV-A or RSV-B.

Species	strain	mean BI of uninfected cells	mean BI of RSV-A infected cells	RSV-A as % of uninfected cells (95% CI)	P	mean BI of RSV-B infected cells	RSV-B as % of uninfected cells (95% CI)	P
S. pneumoniae	type 6	21987	30349	138 (129-147)	0.001	31478	143 (131-157)	0.001
	type 10	25851	33407	130 (117-145)	0.01	33434	131 (115-149)	0.01
	type 23	19340	28088	146 (127-167)	0.001	27081	141 (122-161)	0.01
	type 33	20014	28735	145 (119-176)	0.01	28024	141 (125-160)	0.001
	type 42	22376	31161	140 (123-159)	0.01	30549	137 (122-153)	0.001
S. aureus	NCTC 10655	27634	53637	194 (177-213)	0.001	48079	174 (160-189)	0.001

cells compared with RSV-B infected cells; however, *S. aureus* bound in significantly greater numbers to the RSV-A infected cells (Table 4.5).

Table 4.5 The differences between RSV-A and RSV-B on binding with Gram-positive species

species	strain	RSV-A as % of RSV-B (95% CI)	P
S. pneumoniae	type 6	96 (88-105)	NS
	type 10	100 (88-105)	NS
	type 23	104 (101-106)	0.01
	type 33	102 (89-117)	NS
	type 42	102 (96-108)	NS
S. aureus	NCTC 10655	112 (102-122)	0.05

4.4 Discussion

There is extensive evidence that virus infections are predisposing factors for bacterial disease or carriage of respiratory pathogens. Most information has come from studies of influenza virus and bacteria causing pneumonia such as pneumococci and staphylococci. While RSV is primarily a cause of infection in children, recent studies indicate it is an important predisposing factor for bacterial respiratory disease in older age groups (Kim *et al.*, 1996). The objective of this part of the study was to test the hypothesis that HEp-2 cells infected with RSV-A or RSV-B would bind greater numbers of bacteria associated with secondary respiratory infection or exacerbation of chronic respiratory conditions. With the exception of the isolate MC2 of *M. catarrhalis*, the results showed enhanced binding of each of the bacterial species tested. As with pneumococcal serotypes associated with meningitis, differences in capsular antigens of strains associated with respiratory infections did not affect the enhanced binding to RSV infected cells. If there were significant differences in binding of these bacteria to cells infected with the two subgroups, RSV-A infected cells were observed to have higher binding indices (Tables 4.3 and 4.5).

There are many epidemiological studies of subgroups A and B RSV infections in the United States and other countries. Monto and Ohmit (1990) showed that the two RSV subgroups existed in one community since at least 1965. In almost all epidemics, subgroups A and B have been found. The occurrence of outbreaks with predominantly subgroup A or predominantly subgroup B varied according to the year and country studied (McIntosh *et al.*, 1993). Many studies showed that different groups can

predominate in different geographic locations during the same year. A study from 14 laboratories in United States and Canada found 63% of 483 RSV isolates to be within subgroup A and 24% within subgroup B (Anderson *et al.*, 1991).

The identification of the two groups has led to the speculation that there might be a relationship between severity of infection and RSV subgroup. McConnochie and colleagues (1990) reported that subgroup A infections were more severe. Hall and colleagues (1990) found in a study on 1,209 hospitalized children those with subgroup A RSV infections required intensive care more often. These observations are not in agreement with those that found no differences in the severity of disease relative to subgroup A or B RSV infections (Mufson *et al.*, 1991; Taylor *et al.*, 1989; Salomon *et al.*, 1991; Russi *et al.*, 1989; Monto and Ohmit, 1990; Hendry *et al.*, 1989; Stark *et al.*, 1991; Tsutsumi *et al.*, 1991; Kneyber *et al.*, 1996). The present study found increased bacterial binding to cells infected with either RSV subgroups, indicating that there is probably no association with secondary bacterial infection and subgroups of the virus.

With the two isolates of *M. catarrhalis* tested in the assays, only the isolate able to grow in the presence of selective antibiotics in modified New York City Medium (MC1) showed enhanced binding to RSV-infected cells. The other isolate (MC2) was sensitive to the antibiotics and bound in significantly lower levels to the viruses infected cells. This was the first exception to the general pattern observed for bacterial binding to RSV infected cells. The characteristics of the two isolates and additional isolates of *M. catarrhalis* were examined further in the next chapter.

Chapter 5

Characterisation of antibiotic-resistant and antibiotic-sensitive strains of *M. catarrhalis*

5.1 Introduction

M. catarrhalis is a normal inhabitant of the upper respiratory tract that is increasingly recognized as a pathogen in certain clinical conditions (Berk, 1990; Catlin, 1990). *M. catarrhalis* is the third most common cause of otitis media in children after *S. pneumoniae* and non-typable *H. influenzae* (Van Hare *et al.*,1987). It also commonly causes sinusitis and conjunctivitis in otherwise healthy children and elderly people (Catlin, 1990). In addition to *S. pneumoniae* and *H. influenzae*, it is associated with acute exacerbation of chronic bronchitis. In immunocompromised hosts, *M. catarrhalis* can cause a variety of severe infections, including pneumonia, endocarditis, septicaemia, and meningitis (Boyle *et al.*, 1991; Catlin, 1990; Hager *et al.*, 1987).

Prevalence studies suggest that as many as 50% of children are colonised with *M. catarrhalis* (Ingvarsson *et al.*, 1982; van Hare *et al.*, 1987). The rate of colonisation is highest in the early years and then steadily declines to < 5% in adulthood (Aniansson *et al.*, 1992; Ejlertsen, 1991). Increased colonisation with *M. catarrhalis* often occurs during acute respiratory illness (Brodsky *et al.*, 1991; Van Hare *et al.*, 1987).

With the recognition of *M. catarrhalis* as a human pathogen, studies of its surface antigens are important to identify virulence factors and to understand pathogenesis (Murphy, 1990). The presence of endotoxin in the *M. catarrhalis* outer membrane undoubtedly contributes to its pathogenic potential, especially in situations in which inflammation plays a major role (DeMaria, 1988).

It has been shown that complement is important in host defenses against infection with *Neisseriae* (Densen, 1989); individuals with inherited complement deficiencies, particularly those with a late complement component deficiency, have a markedly increased risk (about 8,000-fold) of acquiring neisserial infections (Densen, 1989; Densen, 1991). The role of complement in anti-neisserial defenses is emphasized by the recognition of complement resistance among strains involved in systemic infections (Densen, 1989). Studies on the resistance of *M. catarrhalis* strains to complement-mediated killing found a wide variation in proportions of serum-resistant strains ranging from 0 to 95% (Brorson *et al.*, 1976; Chapman *et al.*, 1985; Jordan *et al.*, 1990; Soto-Hernandez *et al.*, 1989). A study by Verduin and colleagues (1994) found only 10% of clinical isolates obtained from adults were complement sensitive. In contrast, most isolates (58%) from healthy schoolchildren were sensitive to complement-mediated killing. Some of the variation in the proportion of complement resistant isolates could be due to differences in methods, source of complement, or definition of "resistance" and "sensitive" by individual investigators (Hol *et al.*, 1993).

The decreased binding of the antibiotic-sensitive strain of *M. catarrhalis* MC2 (Chapter 4) was the only exception to the pattern of enhanced binding to RSV-infected cells observed for all the species tested; therefore, differences in other characteristics associated with the two strains were examined.

5.1.1 Aims of the study

The objectives of this part of the project were:

1) to test a larger number of antibiotic-sensitive strains for their binding to RSV infected cells to determine if this was a general pattern or an exceptional strain;

2) to determine if the differences in binding to RSV-infected cells were due to differences in outer membrane proteins of the bacteria;

3) to compare the complement-mediated killing of the antibiotic-sensitive isolates of *M. catarrhalis* with that resistant to antibiotics;

4) to assess the phagocytosis and intracellular survival of antibiotic resistant and sensitive isolates.

5.2 Materials and methods

5.2.1 Binding to RSV infected cells

The bacterial binding assays to HEp-2 cells and HEp-2 cells infected with RSV-A or RSV-B were carried out as described in chapters 3 and 4.

5.2.2 Isolates and culture

In addition to the two isolates in chapter 4, 6 isolates of *M. catarrhalis* (MC3-MC8) were obtained from the Clinical Bacteriology Laboratory, Department of Medical Microbiology; each failed to grow on MNYC containing antibiotics. MC1 was grown on MNYC and antibiotic-sensitive isolates (MC2-MC8) were grown on boiled blood agar (BBA). Isolates were grown overnight at 37°C in a humidified atmosphere with 10% CO_2, harvested, washed three times in PBS by centrifugation at 2500 x *g* for 15 min and resuspended in MM without antibiotics by vigorous pipetting to disperse clumps.

The bacterial concentrations were determined by measuring OD at 540 nm. For each isolate the linear relationship between OD reading and total count assessed microscopically with a Thoma counting chamber was determined, and a standard curve was prepared for each isolate used.

5.2.3 Isolation of *M. catarrhalis* outer membrane proteins (OMP)

The outer membrane proteins of each of the isolates was obtained from bacteria grown on Columbia agar with horse blood grown overnight at 37°C in a humidified atmosphere

with 10% CO_2. The bacteria were harvested, washed 3 times in Tris buffer 0.01M (pH 7.4) and resuspended in 50 ml Tris buffer 0.01M. Bacterial cells were disrupted by sonication (Probe-sonicator, MSE, Fisher scientific) for 15 min at 4000 microns. Unbroken cells were removed by centrifugation twice at 3000 x *g* for 20 min. The supernatant was then centrifuged at 100,000 x *g* (Ultra centrifuge, Beckman, USA) for 60 min at 4°C. The pellet was mixed with 20 ml of 1% (v/v) sodium N-lauryl sarcosinate (Sarkosyl, Sigma) for 60 min at 37°C with shaking. This detergent selectively solubilizes the inner membrane from the crude envelope preparations and provides a method for obtaining the outer membrane (Hancock and Poxton, 1988). The remaining outer membrane-peptidoglycan complex was sedimented by centrifugation at 60,000 *x g* for 60 min at 4°C, reconstituted in pyrogen free water and stored at -20°C. The protein concentration was estimated by the method described by Bradford (1976).

5.2.4 Determination of OMP concentration

The outer membrane-peptidoglycan complex (0.1 ml) or bovine serum albumin as standard were added to 5 ml of Bradford reagent as described in (2.7). The tubes were mixed by inversion, and after 10 min the OD at 595 nm against reagent blank (reagent only) were recorded. The OD of outer membrane sample was plotted with the standard curve to calculate the protein concentration which was adjusted to 8 µg protein before separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

5.2.5 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of M. catarrhalis OMP

Outer membrane proteins were separated by SDS-PAGE using the SDS-discontinous system of Laemmli (1970) on a mini-protein II cell (Bio-Rad Laboratories Ltd., Watford, Hertfordshire, UK). Equal volumes of protein sample and sample buffer [pH 6.8 (double-strength) containing Tris 0.125M, SDS 4% (w/v), 2-mercaptoethanol 2% (v/v), glycerol 20% (v/v), and bromophenol blue 0.002% (w/v)] were mixed and heated to 100°C for 5 min. Sample (20 µl) was applied to each lane and electrophoresis carried out at a constant voltage of 100 V through the stacking gel (4% acrylamide) and a constant voltage of 60 V through the separating gel (10% acrylamide). Proteins were visualised by staining overnight with Coomassie blue (0.5% w/v) Coomassie brilliant blue in (25% v/v) propan-2-ol + glacial acetic acid (10% v/v) followed by destaining with 10% (v/v) glacial acetic acid 4 times for 1 hr intervals. Molecular weight markers (Sigma) in the range of 29 to 205 kDa were run in parallel. The *M. catarrhalis* outer membrane proteins were identified by their molecular weight previously reported by Murphy (1990).

5.2.6 Complement-mediated killing assays

5.2.6.1 Sera

Sera from 14 post-mortem autopsies with no history of hospital admission before death were obtained from the Forensic Medicine Unit, Edinburgh University. There were 10

sera from individuals 60 years of age or over (5 male and 5 female), and 4 sera from individuals under 20 years of age (2 male and 2 female).

5.2.6.2 Complement

Serum from a donor of blood group AB was absorbed twice over a period of 24 h at 4°C with a suspension of the 8 test isolates to remove any specific antibodies. The serum was centrifuged (1500 x *g*) at 4°C and the remaining bacteria removed by filtration, first with a filter with a pore diameter of 5 µm (Nucleopore Corporation, Pleasanton, USA). The absorbed serum was then passed through a sterilising filter with pore diameter of 0.22 µm (Nuflow cellulose acetate membrane, Oxoid). The minimum haemolytic titre of the serum with sensitized sheep red blood cells (5.2.6.3) was determined by the method described by Zorgani *et al.* (1996).

5.2.6.3 Preparation of sensitized sheep red blood cells

Whole blood taken aseptically from healthy sheep was supplied as a 50% (v/v) mixture in Alsever's solution (SAPU). Fresh sheep red blood cells were washed twice in saline by centrifugation at 450 x *g* for 5 min and the supernatant was removed. The red blood cells were resuspended in saline (4%); this was checked by placing some of the suspension in a haematocrit tube and centrifugation at 450 x *g* for 5 min. The donkey anti-sheep antiserum (SAPU) was diluted 1 in 100 with saline and equal volumes of donkey antiserum and sheep red blood cells were mixed and incubated at 37°C for 30 min. The sensitized sheep red blood cells were stored at 4°C until used.

5.2.6.4 Bactericidal activity assay

Serum samples were heated at 56°C for 30 min to inactivate endogenous complement. Each of the 96 wells of an 8 x 12 "U" well microtitre plate (Sterilin) was filled with 40 µl of Dulbecco's complete phosphate buffered saline containing supplement B (DPBS+B) (pH 7.1). Each 100 ml DPBS was supplemented with 0.5 ml Dulbecco's B containing calcium chloride (20 g l^{-1}) and magnesium chloride (20 g l^{-1}) (DPBS+B). Heated inactivated neat serum (40 µl) was added to the first well in each column. A series of seven two-fold dilutions was then made; 8 sera were tested per plate.

Overnight cultures of the two *M. catarrhalis* isolates (MC1 and MC2) were harvested from the medium and suspended in DPBS+B, and the number of bacteria estimated by OD of the suspension at 540 nm (Zorgani *et al.*, 1996). The bacteria were diluted to provide a concentration of approximately $1x10^4$ colony forming units (c f u ml^{-1}). To each well in a plate was added 40 µl of one of the 2 bacterial suspensions. A 1/8 dilution of the absorbed complement source (20 µl) was added to all the test wells.

For each isolate and each serum specimen (dilution 1 in 5), there were 3 controls: one in which the complement was replaced by PBS; one in which the serum was replaced by PBS; one in which both serum and complement were replaced by PBS. The plates were covered and incubated at 37°C for 30 min. Three 20 µl drops from each well were then plated onto a BBA plate and incubated at 37°C overnight. The reciprocal of the highest serum dilution at which there was at least an 80% decrease in viable count compared

with the controls was recorded as the bactericidal titre. The bactericidal titres for MC1 and MC2 were determined under the same conditions at the same time.

5.2.7 Phagocytosis assay

5.2.7.1 Preparation of monocytes

Buffy coats from 7 donors were obtained from Scottish National Blood Transfusion Service, Edinburgh. The samples were inverted to ensure thorough mixing, diluted 1 in 2 with 0.15 M saline and a 10 ml aliquot layered onto 3 ml cushions of Histopaque (Sigma) in a 50 ml conical centrifuge tube. The cells were then centrifuged at 200 x *g* for 25 min. Leukocytes were collected at the plasma / Histopaque interface by suction through a Pasteur pipette and washed once at 100 x *g* for 10 min in PBS. The cells (50 µl) were diluted in white cell diluting fluid (450 µl) containing gentian violet 1% (w/v) in glacial acetic acid 1% (v/v). The cells were counted with a haemocytometer and adjusted to give a concentration of $1x10^6$ ml^{-1} for the assay.

5.2.7.2 THP-1 cells

THP-1 cells (2.4.3) were grown routinely in RPMI 1640 with 10% FCS at 37°C in a humidified 5% CO_2 incubator in tissue culture flasks. THP-1 cells were harvested by centrifugation at 300 x *g* for 10 min and resuspended in PBS. The cell count was adjusted to give a concentration of $1x10^6$ ml^{-1} for the assay.

5.2.7.3 Binding assay

Two sets of tubes were prepared for each donor sample, the assay was carried out at two different temperatures: 4°C (to examine binding) and 37°C (to examine binding and ingestion). RSV infection of the cells (monocytes or THP-1) was assessed following infection of the cells overnight at 37°C. Uninfected and RSV-A infected monocytes or THP-1 cell suspensions (200 µl) and FITC conjugated bacteria (200 µl) (400 bacteria : cell) were mixed and incubated for 30 min at each temperature. After incubation, the unbound bacteria were removed and washed twice by centrifugation at 4°C with chilled PBS (450 x *g*) and the cells fixed with buffered paraformaldehyde. The tubes were kept in the dark at 4°C until analysis by flow cytometry.

5.2.7.4 Intracellular survival assays

Monocyte suspensions from 4 donors (200 µl, $1x10^6$ cells ml^{-1}) and 200 µl of the unlabelled *M. catarrhalis* isolates (MC1 and MC2) at a ratio of 200 bacteria : cell were mixed together in a 5 ml tube. The tubes were incubated for 30 min at 37°C in an orbital incubator and washed three times at 300 x *g* for 10 min to remove non-bound bacteria. Gentamicin (500 µl, 10mg ml^{-1}) was added to the cells and incubated for 60 min at 37°C to kill bacteria attached to the cell surface. The cells were washed twice with PBS, and 500 µl SDS (0.05% w/v) was added to lyse the cells and release the intracellular bacteria. This preparation was further diluted 1/10 and 1/100 with PBS and 3 (20 µl) drops of each dilution was plated onto BBA plates and incubated at 37°C

for 48 hours. A colony count was then performed. The average of the 3 drops determined and actual numbers of bacteria calculated by multiplying by 50 then by the appropriate dilution factor.

5.2.8 Statistical methods (3.2.11)

5.3 Results

5.3.1 Determination of infection of monocytes and THP-1 cells by RSV

Infection of monocytes or THP-1 by RSV was assessed by direct immunofluorescence assay as described in 3.2.6.1. The percentage of the positive cells detected per 100 cells counted was 40-50 %.

5.3.2 Attachment of *M. catarrhalis* isolates to RSV infected cells

Table 5.1 summarises the results of 6 experiments in which a ratio of 400 bacteria per cell was used to assess the effect of infection with RSV-A or RSV-B on binding of *Moraxella* isolates to HEp-2 cells. As the results observed with MC2 indicated, decreased binding to RSV-infected cells was observed for the other 6 antibiotic-sensitive isolates. The antibiotic-resistant *M. catarrhalis* isolate that grew on MNYC showed significantly higher binding indices with RSV-A or RSV-B infected cells. The binding indices of each of the antibiotic-sensitive isolates were significantly decreased for the RSV-infected cells.

5.3.3 Comparison of OMPs among *M. catarrhalis* isolates.

The OMP of *M. catarrhalis* contains eight major proteins, the molecular weights of these proteins range from 21 to 98 kDa, a typical OMP pattern for Gram-negative bacteria. The OMP profiles of the antibiotic-resistant and the seven antibiotic-sensitive isolates of *M. catarrhalis* are shown in (Figure 5.1).

Table 5.1 Results of 6 experiments on binding of *M. catarrhalis* isolates to HEp-2 cells and HEp-2 cells infected with RSV-A or RSV-B (400 bacteria : cell).

Bacteria	isolates	mean BI of uninfected cells	mean BI of RSV-A infected cells	RSV-A as % uninfected cells (95% CI)	P	mean BI of RSV-B infected cells	RSV-B as % uninfected cells (95% CI)	P
M. catarrhalis	**MC1**	**21726**	**42886**	**209 (126-349)**	**0.05**	**33736**	**163 (101-267)**	**0.05**
	MC2	37758	31144	80 (64-99)	0.05	23559	55 (31-95)	0.05
	MC3	43192	37137	85 (80-92)	0.01	30516	69 (53-90)	0.05
	MC4	48462	40446	83 (76-91)	0.01	30853	57 (36-91)	0.05
	MC5	56003	51476	91 (89-94)	0.001	47061	81 (70-94)	0.05
	MC6	38587	31005	81 (74-90)	0.01	22937	50 (29-86)	0.05
	MC7	51789	48236	93 (89-97)	0.01	44735	83 (71-97)	0.05
	MC8	41376	30125	74 (62-88)	0.01	23630	51 (28-92)	0.05

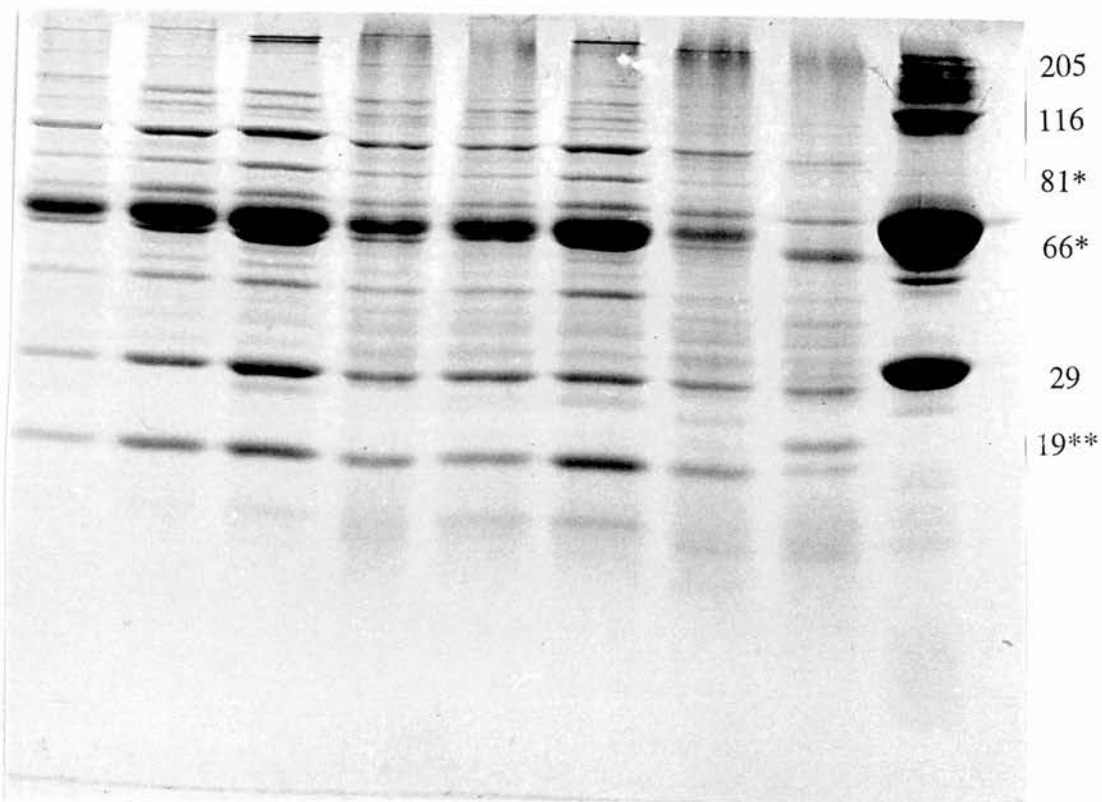

Fig 5.1 Coomassie blue-stained 10% SDS-polyacrylamide gel containing OMP preparations of 8 different strains of *M. catarrhalis* with low molecular-weight standard on the right.

* 81 & 66 kDa proteins present in antibiotic-sensitive strains MC2-MC8

** 19 kDa protein present in antibiotic-resistant strain MC1

Despite the diversity of the isolates, the results showed a high degree of similarity in OMP patterns; however, the antibiotic-resistant isolate of *M. catarrhalis* lacked bands at 81 kDa and 66 kDa which were present in the seven isolates which were antibiotic-sensitive. The antibiotic-resistant isolate of *M. catarrhalis* had a distinct band at 19 kDa which was not present in the antibiotic-sensitive isolates.

5.3.4 Assessment of complement-mediated killing assay

The effects of complement killing of MC1 and MC2 were assessed with serum samples from 14 individuals. The antibiotic-sensitive isolate (MC2) was killed by dilutions of serum ranging from 1/4-1/32. The antibiotic-resistant (MC1) was resistant at each of the dilutions tested for the 14 sera (Table 5.2).

5.3.5 Assessment of phagocytosis and intracellular survival of antibiotic-resistant and sensitive isolates

Assays were carried out to assess the differences in binding of the two isolates of *M. catarrhalis* by uninfected or RSV-A infected monocytes or the THP-1 cell line. Experiments were carried out at 4°C to assess binding separately from binding and phagocytosis at 37°C. At 4°C there was a significant increase in binding of both MC1 and MC2 to monocytes from buffy coats or THP-1 cells infected with RSV-A. A similar pattern was observed at 37°C (Tables 5.3 and 5.4).

Table 5.2 Distribution of bactericidal titres against the two *M. catarrhalis* isolates in the population tested

bactericidal titre	serum killing ≥ 80% of control			
	MC1		MC2	
	no	%	no	%
0	14	(100)		0
2		0		0
4		0	3	(21)
8		0	5	(36)
16		0	5	(36)
32		0	1	(7)
64		0		0
128		0		0

Table 5.3 Mean binding of MC1 and MC2 to uninfected and RSV-infected monocytes or THP-1 cells at 4°C (3 experiments)

Cells	isolate	mean BI of uninfected cells 4°C	mean BI of infected cells 4°C	RSV infected as % uninfected (95% CI)	P
Monocytes	MC1	817	1357	163 (103-258)	0.05
	MC2	1233	1809	146 (116-185)	0.05
THP-1	MC1	1388	3134	226 (195-261)	0.01
	MC2	1209	2139	266 (197-361)	0.01

Table 5.4 Mean binding of MC1 and MC2 to uninfected and RSV-infected monocytes or THP-1 cells at 37°C (3 experiments)

Cells	isolate	mean BI of uninfected cells 37°C	mean BI of infected cells 37°C	RSV-infected as % of uninfected	P
Monocytes	MC1	583	1006	168 (117-243)	0.05
	MC2	877	1158	134 (116-155)	0.01
THP-1	MC1	641	1712	177 (122-257)	0.05
	MC2	719	1120	156 (137-179)	0.01

There were no differences found between the two isolates of *M. catarrhalis* on intracellular survival within uninfected and RSV-A infected monocytes (Tables 5.5 a and b).

Tables 5.5 Comparison of intracellular survival of *M. catarrhalis* MC1 and MC2 phagocyted by uninfected and RSV-infected monocytes from 4 donors

A) MC1 isolate

donors	mean survival uninfected monocytes	mean survival RSV-infected monocytes	% infected / uninfected
1	2.1×10^4	1.6×10^4	76
2	1.3×10^4	2.2×10^4	169
3	1.8×10^4	1.9×10^4	105
4	2.5×10^4	2.5×10^4	100

initial inoculm = 4×10^7

B) MC2 isolate

donors	mean survival uninfected monocytes	mean survival RSV-infected monocytes	% infected / uninfected
1	2.0×10^4	1.6×10^4	80
2	1.2×10^4	2.0×10^4	166
3	1.6×10^4	2.1×10^4	131
4	2.4×10^4	2.5×10^4	104

initial inoculm = 4×10^7

5.4 Discussion

5.4.1 Results in relation to objectives of the chapter

The results obtained provided data to answer the questions posed in the introduction. Since the decreased binding of the antibiotic-sensitive isolate of *M. catarrhalis* was the only exception to the pattern of enhanced binding to RSV-infected cells observed with all other species tested, 6 additional antibiotic-sensitive isolates were tested for their binding to RSV-infected HEp-2 cells. The same pattern of significantly decreased binding to RSV-infected cells compared to uninfected cells was observed.

OMP profiles of the antibiotic-resistant and the antibiotic-sensitive isolates of *M. catarrhalis* showed a high degree of similarity in OMP patterns but there were differences between the two groups. The antibiotic-resistant isolate lacked bands at 81 kDa and 66 kDa which were present in the seven antibiotic-sensitive isolates (MC2-MC8). The antibiotic-resistant isolate (MC1) had a distinct band at 19 kDa which was not present in the antibiotic-sensitive isolates (MC2-MC8).

All of the sera tested had bactericidal activity against the antibiotic-sensitive isolate (MC2) with >80% reduction in viable bacteria compared with the control. None of the 14 sera showed complement mediated killing of the antibiotic-resistant isolate.

In contrast to results with the HEp-2 epithelial cell line, binding of both MC1 and MC2 was significantly enhanced for RSV-infected monocytes or THP-1 cells. The antibiotic-resistant isolate and antibiotic-sensitive isolate showed no differences in

binding or phagocytosis compared to uninfected cells with either monocytes or the THP-1 cell line. Although a high ratio of bacteria per cell was used in these experiments, there was no differences observed between the two isolates of *M. catarrhalis* on intracellular survival within uninfected and RSV-infected monocyte cells obtained from buffy coats.

5.4.2 Results in relation to epidemiological and clinical reports

There is epidemiological evidence to suggest viral infection enhances colonisation by *M. catarrhalis*. In a study in Denmark, significantly more children in the 1-48 month age range with upper or lower respiratory tract infections were colonised with *M. catarrhalis* (68%) compared with children without such infections (36%, $P< 0.001$). After recovery, the isolation rate in the infected group fell to that of the uninfected group (Ejlertsen *et al.*, 1994). *M. catarrhalis* has been associated with various severe infections in infants with chronic lung disease or in older compromised children (Cook *et al.*, 1989 and Korppi *et al.*, 1990). Serological evidence suggests that *M. catarrhalis* might be a potential bronchopulmonary pathogen even in children with no underlying disease. Patients with serological evidence of *M. catarrhalis* infection often had a concomitant viral infection caused by RSV (Korppi *et al.*, 1992). In another study, colonisation by *M. catarrhalis* was associated with other viral infections, mainly parainfluenza infections (Nohynek *et al.*, 1991).

5.4.3 OMP profile and serum sensitivity

The 81 kDa protein (*Cop*B) is associated with serum sensitivity and clearance of this organism from the lungs of mice. A mutant strain that did not express this protein expressed wild type levels of serum resistance and the ability to resist pulmonary clearance *in vivo* (Helminen *et al.*, 1993a). A monoclonal antibody to the protein bound to the majority (70%) of 23 *M. catarrhalis* strains which were obtained from individuals at five different research centres across the United States (Helminen *et al.*, 1993b).

Helminen and colleagues (1993a) studied the effect of the *Cop*B mutation on serum resistance of *M. catarrhalis* to determine whether this mutation might also affect the ability to survive *in vivo* in the lung of mice. The availability of this model facilitated the *in vivo* evaluation of the isogenic *Cop*B mutant, which was found to be much less able to survive in the lungs of mice than was the wild-type parent strains. This diminution of survival ability *in vivo* is likely directly related to the serum-sensitive phenotype of the *Cop*B mutant. The study did not assess the phagocytosis differences between the serum resistance strain and its mutant. The results in this chapter showed no differences in phagocytosis and killing between antibiotic-resistance and sensitive strains by human monocytes.

Resistance to complement is an important virulence factor in certain Gram-negative infections (Densen *et al.*, 1987). The study by Smith and colleagues (1992) indicated that resistance of gonococci to complement mediated killing by human serum was due

to sialylation of their lipopolysaccharide which prevents bactericidal antibody from reacting with target sites. Regardless of the role other components of the *M. catarrhalis* outer membrane might play in serum resistance, the absence of *Cop*B in the outer membrane of the *Cop*B mutant had a significant effect on serum resistance of *M. catarrhalis*. Reintroduction of *Cop*B into this mutant abolished the serum resistant phenotype, confirming that *Cop*B expression play a direct or indirect role in serum resistance (Helminen *et al.*, 1993a).

Published reports concerning the resistance of *M. catarrhalis* to killing by normal human serum indicate that many strains of this pathogen are resistant to complement-mediated lysis (Jordan *et al.*, 1990; Soto-Hernandez *et al.*, 1989; Chapman *et al.*, 1985). The study by Jordan and colleagues (1990) attempted to correlate serum resistance and disease production by *M. catarrhalis* strains. It suggested that strains isolated from infected sites are more likely to be serum-resistant than are strains isolated from the sputum of healthy persons.

5.4.4 Future studies

The antibiotic sensitivity, adherence characteristics and complement resistance properties of the *M. catarrhalis* antibiotic-resistant isolate (MC1) might contribute to its pathogenicity in the respiratory tract. In the context of other studies, the present finding suggests that the 81 kDa protein is associated with serum sensitivity and possibly antibiotic-sensitive and reduced binding to RSV-infected epithelial cells. The antibiotic-sensitive strains with these characteristics might be less likely to contribute to

serious disease following RSV infection. The effect of prolonged antibiotic treatment needed for patients with chronic lung disease on selection of resistant organisms needs to be examined in complementary clinical and laboratory studies.

Chapter 6

Binding of bacteria to HEp-2 cells infected with influenza A virus

6.1 Introduction

Secondary bacterial infection is the most common, life threatening complication of influenza infection (Kilbourne, 1987c). Influenza A virus infections in children are often complicated by otitis media (Henderson *et al.*, 1982; Wright *et al.*, 1977; Wright *et al.*, 1980). It has been suggested that viral respiratory tract infections might predispose to meningococcal disease (Moore *et al.*, 1990). Epidemiological studies suggest that influenza virus infection increases the risk of subsequent meningococcal infection (Cartwright *et al.*, 1991; Harrison *et al.*, 1991).

While many studies have been carried out on binding of bacteria in animal models (Plotkowski *et al.*, 1986), there were no reports on the effect of influenza virus infection on binding of bacteria to human respiratory epithelial cell lines. These studies were initiated to determine if, like RSV, infection of epithelial cells with influenza A might enhanced bacterial binding.

6.1.1 Aims of the study

The objectives of this part of the study were:

1) to test the hypothesis that HEp-2 cells infected with human influenza A virus might bind bacteria associated with meningitis or species associated with pneumonia, otitis media and exacerbation of chronic bronchitis;

2) to determine if as reported for RSV-infected cells (Raza *et al.*, 1994), there was an increased expression of native cell surface antigens which can act as receptors for bacteria;

3) to determine if neuraminidase affects bacterial binding to HEp-2 cells.

6.2 Materials and methods

6.2.1 HEp-2 Cells (3.2.1)

6.2.2 Growth of influenza virus

6.2.2.1 MDCK cells (2.4.2)

6.2.2.2 Method

Confluent monolayers of MDCK cells were obtained in tissue culture flasks by incubation in GM with 10% FCS. The monolayer was rinsed twice with DPBS+A to remove the serum which contained substances that reduce virus infectivity. Influenza virus type A, a recent isolate obtained from the Clinical Virology Laboratory, Edinburgh University, was adsorbed to cells for 60 min at 37°C. The inoculum was removed and replaced with serum free MM supplemented with 2 μg ml^{-1} trypsin (crystalline, Sigma) which is required for virus replication in this cell line. The cells were incubated at 37°C for 16-24 hr, the maximum time for the virus release. About two thirds of the medium in the flask was discarded and the flask was frozen at -70°C and thawed to lyse the cells. The suspension was centrifuged at 800 x *g* for 10 min. Aliquots of the supernatant were stored at -70°C.

6.2.3 Titration of influenza A virus by haemagglutination assay

The samples of supernatant fluid were tested for presence of influenza virus by the haemagglutination technique. Guinea pig erythrocytes obtained from the Transgenic Unit, Department of Medical Microbiology, Edinburgh University were washed and suspended (0.5% v/v) in Complement Fixation Test (CFT) buffer, prepared from tablets

(Oxoid) (pH 7.2). Diluted guinea pig erythrocytes (25 µl) were added to 25 µl of two fold serial dilutions of influenza supernatant fluids in a microtitre plate containing 96 "V" shaped wells with the same buffer and incubated for 1 h at room temperature. The virus preparation with the highest haemagglutination titer was frozen in 2 ml aliquots at -70°C until used.

6.2.4 Detection of influenza infected cells

6.2.4.1 Direct immunofluorescence

Coverslips with confluent monolayers of HEp-2 cells in a 24-well plate were prepared and infected with influenza virus aliquots (200 µl) at different dilutions (1/2, 1/5 and 1/10) for 1 hr at 37°C, and the inoculum was replaced with 1 ml serum free MM. The cells were incubated at 37°C for 16-24 hr.

Monolayers were fixed with acetone (BDH) for 5 min. The percentage of infected cells was determined for each dilution by incubating the coverslips with 25 µl of the FITC-labelled reagent for detection of influenza A virus (2.6.2). The slides were dried and mounted with 50% glycerol in PBS. They were examined with an ultraviolet microscope (Microstar IV, Reichert, Buffalo, New York, USA) for the proportion of fluorescent cells present. The percentage of positive cells per 100 cells counted was recorded.

6.2.5 Bacteria

Sources of the following strains of *N. meningitidis*, *S. pneumoniae*, *H. influenzae*, *B. pertussis*, *N. lactamica*, *M. catarrhalis* and *S. aureus*, their culture conditions and enumeration were described in (3.2.3, 4.2.3 and 5.2.2).

6.2.6 Infection of HEp-2 cells with influenza A virus

Overnight monolayer cultures of HEp-2 cells in culture flasks (75 cm^3) were infected with influenza A, 5 ml of a 1 : 5 dilution of the virus suspension in serum free MM was adsorbed for 1 hr. The fluid was replaced with 25 ml serum free MM and incubated overnight at 37°C. The monolayers were rinsed twice with sterile DPBS+A and harvested by adding 5 ml of EDTA (0.05% v/v) per flask at 37°C for 5-10 min. MM (5ml) was added to the cells to counteract EDTA activity. The cells were centrifuged at 460 x *g* for 7 min and resuspended in MM without antibiotics, counted and adjusted to $1x10^6$ cells ml^{-1}.

6.2.7 Bacterial Binding to influenza A-infected HEp-2 cells

Bacterial binding studies were carried out and analysed by flow cytometry as described previously.

6.2.8 Detection of host cell surface antigens on HEp-2 cells infected with influenza virus

Binding of monoclonal antibodies to host cell surface antigens to HEp-2 cells and HEp-2 cells infected with influenza virus (200 µl, $1x10^6$ ml^{-1}) was assessed by the method described in 3.2.9.

6.2.9 Inhibition of bacterial binding by pre-treatment of Flu-A infected HEp-2 cells with antibodies to host cell antigens

Influenza A-infected HEp-2 cells were treated with monoclonal antibody to CD14 or CD18 as described in 3.2.10. The attachment assays with FITC-labelled Gram-negative species (*N. meningitidis*, *M. catarrhalis*, *H. influenzae* type b) or Gram-positive species (*S. pneumoniae* and *S. aureus*), were performed and analysed as described in 3.2.7.

6.2.10 The effect of pre-treatment of HEp-2 cells with neuraminidase on bacterial binding

HEp-2 cell (200 µl, 1x 10^6 cells ml^{-1}) were incubated with neuraminidase type III from *Vibrio cholerae* (Sigma) (30 µl, 1 unit ml^{-1}) for 60 min at 37°C. The cells were washed twice with PBS. The FITC-labelled *N. meningitidis* strain (C: 2b: P1.2) (200 bacteria : cell) or *S. aureus* strain (NCTC 10655) (200 bacteria : cell) were incubated with equal volumes of HEp-2 cells and HEp-2 cells pre-treated with neuraminidase at 37°C for 30 min. The cells were washed three times at 460 x *g* with PBS and analysed by flow cytometry (3.2.7).

6.2.11 The effect of pre-treatment of HEp-2 cell with neuraminidase on detection of host cell surface antigens

HEp-2 cell (200 µl, 1x 10^6 cells ml^{-1}) were incubated with neuraminidase as described in 6.2.10 for 60 min at 37°C. The cells were washed twice with PBS and incubated for 60 min at 37°C with the same dilutions of antibodies as in 3.2.9 and antibody to H type 2 (1/10). The cells were washed twice with PBS and incubated for 30 min in an orbital shaker at 37°C with FITC-labelled secondary antibodies used to detect binding of their respective primary antibody. FITC labelled secondary antibodies were incubated with HEp-2 cells or neuraminidase treated HEp-2 cells incubated with PBS but no primary antibody as a control for non-specific binding. The cells were washed three times at 460 x *g* with PBS and analysed by flow cytometry (3.2.7).

6.2.12 Statistical methods.

The data were assessed by estimate of relative binding of the bacteria to influenza A-infected HEp-2 cells compared with uninfected cells based on paired *t*-tests applied to logarithms of the binding indices.

6.3 Results

6.3.1 Determination of influenza A-infected HEp-2 cells

The proportion of influenza A-infected HEp-2 cells at 24 h post inoculation assessed by direct immunofluorescence at the time of the attachment assay was >75 %.

6.3.2 Attachment of Gram-negative species to influenza A-infected HEp-2 cells

The ratios of bacteria per cell used in these studies were the same as those used in experiments with RSV-infected cells in chapters 3 and 4 as the uninfected HEp-2 cells were the standard for comparison with virus infected cells. Tables 6.1 and 6.2 summarise the results of 6 experiments to assess the effect of infection with influenza A virus on binding to HEp-2 cells by *Neisseria*, *Haemophilus*, *Moraxella*, and *Bordetella* species. Each isolate was tested at a ratio of 200 bacteria per cell except for *M. catarrhalis* 400 bacteria : cell and *B. pertussis* which was used at 500 bacteria per cell. All the bacterial species showed enhanced binding to cells infected with influenza A compared with uninfected HEp-2 cells, including the antibiotic-sensitive isolate of *M. catarrhalis*.

6.3.3 Attachment of Gram-positive species to uninfected and influenza A-infected HEp-2 cells

Binding of *S. aureus*, *S. pneumoniae* strains associated with meningitis and pneumococcal strains associated with respiratory infections to uninfected and influenza-infected cells was assessed in 6 experiments in which ratios of 200 bacteria per cell

Table 6.1 Results of 6 experiments on binding of Gram-negative species to uninfected HEp-2 cells and HEp-2 cells infected with influenza A virus (200 bacteria : cell, except for *B. pertussis*, 500 : cell).

species	type	mean BI of uninfected cells	mean BI of Flu-A infected cells	Flu-A as % uninfected cells (95% CI)	P
N. meningitidis	C:2b:P1.2	27832	47300	552 (402-759)	0.001
	NG:2b:P1.10	31756	47472	490 (361-665)	0.001
	B:2b:P1.10	28906	50794	656 (493-873)	0.001
H. influenzae	type b	37635	54588	540 (463-630)	0.001
	87D (NT)	31810	52785	166 (158-175)	0.001
	241D (NT)	30570	47900	157 (145-169)	0.001
N. lactamica	LO1	38972	53087	396 (336-467)	0.001
B. pertussis	type 1,2	39742	56948	387 (286-524)	0.001
	type 1,3	42937	59330	367 (270-500)	0.001

Table 6.2 Results of 6 experiments on binding of *M. catarrhalis* isolates to uninfected HEp-2 cells and HEp-2 cells infected with influenza A virus (400 bacteria : cell).

isolates	mean BI of uninfected cells	mean BI of Flu-A infected cells	Flu-A as % uninfected cells (95% CI)	P
MC1	37419	52442	288 (261- 318)	0.001
MC2	39743	60986	421 (354- 500)	0.001
MC3	47743	56143	197 (165- 234)	0.001
MC4	49491	68211	321 (258- 399)	0.001
MC5	54145	66768	335 (299- 375)	0.001
MC6	41145	62384	323 (260- 402)	0.001
MC7	54416	67595	314 (252- 392)	0.001
MC8	38276	63355	442 (256- 764)	0.001

were used. Influenza virus infected HEp-2 cells had significantly higher binding indices compared with binding indices for uninfected cells (Table 6.3).

6.3.4 Binding of *N. meningitidis* LOS immunotype strains to influenza A-infected HEp-2 cells

Strains with different LOS immunotype antigens, were assessed for binding to determine if the carbohydrate composition of the endotoxin affected binding to virus infected cells. In 5 experiments each of 12 immunotype strains exhibited significantly enhanced binding to cells infected with influenza A virus compared to uninfected cells (Table 6.4).

6.3.5 Expression of cell surface antigens on HEp-2 cells infected with influenza virus

In 3 experiments the binding indices for monoclonal antibodies directed towards cell surface antigens indicate there was no significant difference in binding of monoclonal antibodies to Le^b, Le^x or H type 2 to HEp-2 cells or HEp-2 cells infected with influenza virus. There were significant increases in binding of anti-CD14 ($P< 0.05$, 95% CI 182-2188) and anti-CD18 ($P< 0.001$, 95% CI 514-684) monoclonal antibodies to HEp-2 cells infected with influenza virus compared to uninfected HEp-2 cells (Table 6.5).

Table 6.3 Results of 6 experiments on binding of *S. pneumoniae* to uninfected HEp-2 cells and HEp-2 cells infected with influenza A virus (200 bacteria : cell).

species	type/strain	mean BI of uninfected cells	mean BI of Flu-A infected cells	Flu-A as % uninfected cells (95% CI)	P
*S. pneumoniae**	7F	25249	32099	233 (162-335)	0.01
	12F	23415	33237	254 (171-379)	0.01
	18C	15526	30267	309 (254-378)	0.001
*S. pneumoniae***	6	12675	24442	275 (228-333)	0.001
	10	14224	31165	289 (254-329)	0.001
	23	13206	28681	263 (238-290)	0.001
	33	29415	44974	213 (200-227)	0.001
	42	12078	28551	262 (236-290)	0.001
S. aureus	NCTC 10655	32756	45330	362 (292-450)	0.001

(*) Meningitis strains
(**) Respiratory isolates

Table 6.4 Results of 5 experiments on binding of *N. meningitidis* LOS immunotype strains to uninfected HEp-2 cells and HEp-2 cells infected with influenza A virus (400 bacteria : cell).

strains	phenotype		mean BI of uninfected cells	mean BI of Flu-A infected cells	Flu-A as % uninfected cells (95% CI)	P
L1	C : NT : P 1.2	L1,8	20071	40747	360 (298-436)	0.001
L2	C : 2C : P 1.1	L2	15232	38222	471 (328-531)	0.001
L3	B : 2a : P 1.5,2	L (3,7),7	19477	49190	716 (493-1070)	0.001
L4	C : 11 : P 1.16	L4	17592	37622	318 (163-384)	0.001
L5	B : 4 : NT	L5	16825	38237	412 (277-615)	0.001
L6	B : 5 : P 1.7,1	L6,7	19326	39217	407 (282-587)	0.001
L7	B : 9 : P 1.7,1	L(3,7),7	21243	41701	414 (303-564)	0.001
L8	B : 8,19 : P 1.7,1	L(3,7),7,8	26127	45959	372 (295-471)	0.01
L9	A : 21 : P 1.10	L (3,7),7	20828	43547	691 (482-991)	0.01
L10	A:	L10	21095	37424	322 (224-462)	0.001
L11	A : 21 : P 1.10	L11	19610	36935	376 (231-612)	0.01
L12	A : 21 : NT	L12	20195	35556	327 (220-485)	0.01

Table 6.5 Binding indices for monoclonal antibodies to host cell surface antigens on HEp-2 cells and influenza-A infected HEp-2 cells

Antibodies	mean BI of uninfected cells	mean BI of flu-A infected cells	95% CI	P
$Lewis^{b}$	1782	1496	64-111	NS
$Lewis^{x}$ (CD15)	1241	1143	69-123	NS
H type 2	790	680	67-126	NS
CD 14	1933	10984	182-2188	0.05
CD 18	2173	12901	514-684	0.001

6.3.6 Inhibition of bacterial binding

In 4 experiments binding of *N. meningitidis* (C: 2b: P1.2), *M. catarrhalis* strains MC1 and MC2 and the type b *H. influenzae* isolate to influenza A-infected HEp-2 cells was inhibited by anti-CD14 or anti-CD18. The results showed significant reduction in binding by anti-CD14 for each species tested: *N. meningitidis* ($P< 0.001$, 95% CI 55-62); *M. catarrhalis* (MC1) ($P< 0.001$, 95% CI 47-61); MC2 ($P< 0.01$, 95% CI 52-69); and *H. influenzae* type b ($P< 0.001$, 95% CI 43-52). A similar pattern was observed for anti-CD18: *N. meningitidis* ($P< 0.001$, 95% CI 45-53); *M. catarrhalis* (MC1) ($P< 0.01$, 95% CI 37-62); MC2 ($P< 0.001$, 95% CI 50-64); and *H. influenzae* type b ($P< 0.001$, 95% CI 39-45) (Fig 6.1).

With Gram-positive species, no significant reduction in binding was observed. Binding indices for *S. aureus* to influenza A infected cells treated with anti-CD14 (95% CI 87-106) or anti-CD18 (95% 90-106) showed no differences compared with untreated cells. A similar pattern was observed with *S. pneumoniae* (12F) with anti-CD14 (95% CI 95-107) or anti-CD18 (95% CI 96-108) (Fig 6.2).

6.3.7 The effects of neuraminidase treatment on bacterial binding

In 3 experiments the binding indices of *S. aureus* strain (NCTC 10655) and *N. meningitidis* strain (C:2b:P1.2) to HEp-2 cells pre-treated with neuraminidase were compared with their binding indices to untreated HEp-2 cells (Fig 6.3 and 6.4). The statistical analysis showed the increases observed were not significant for *N. meningitidis* strain

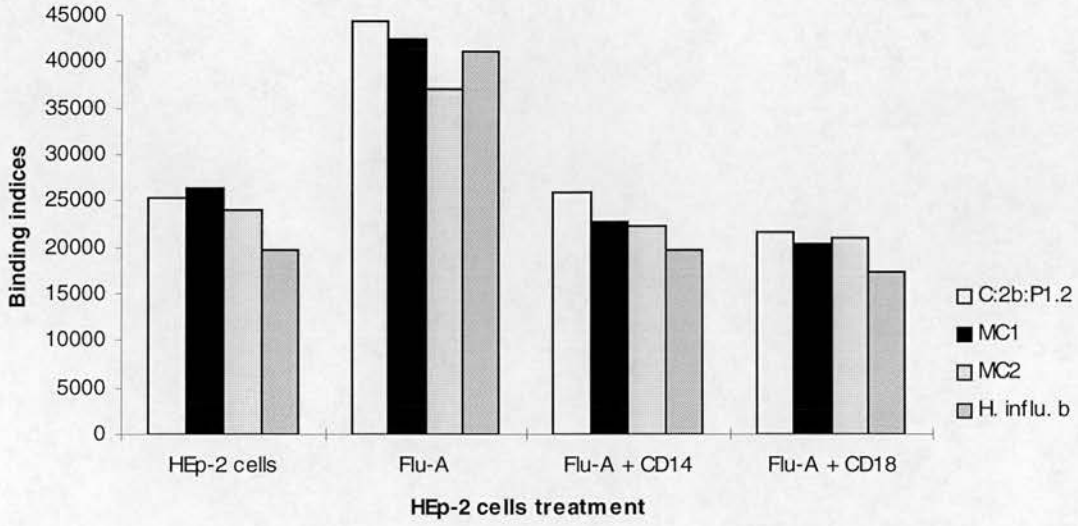

Figure 6.1 The effect of pre-treatment of influenza A infected HEp-2 cells with monoclonal antibody to CD14 or CD18 on binding of Gram-negative species.

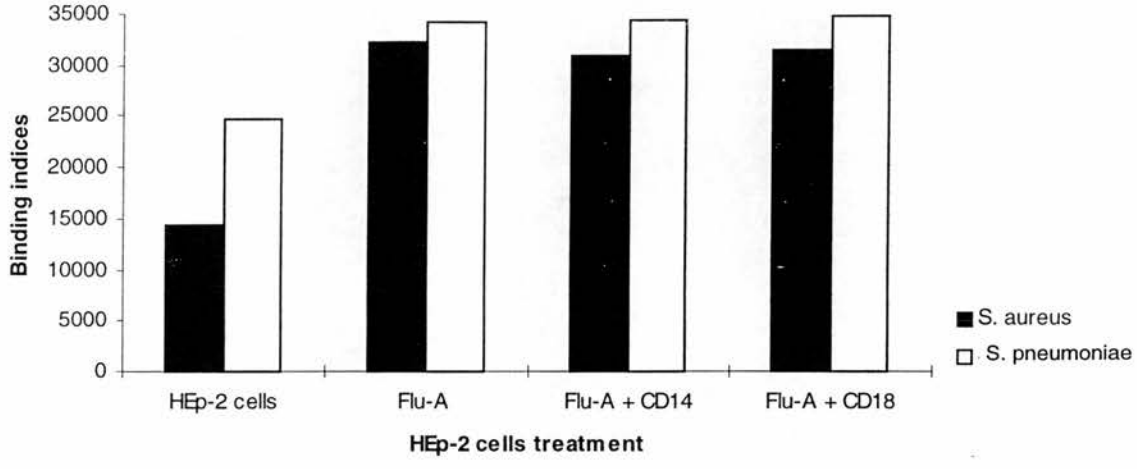

Figure 6.2 The effect of pre-treatment of influenza A infected HEp-2 cells with monoclonal antibody to CD14 or CD18 on binding of Gram-positive species.

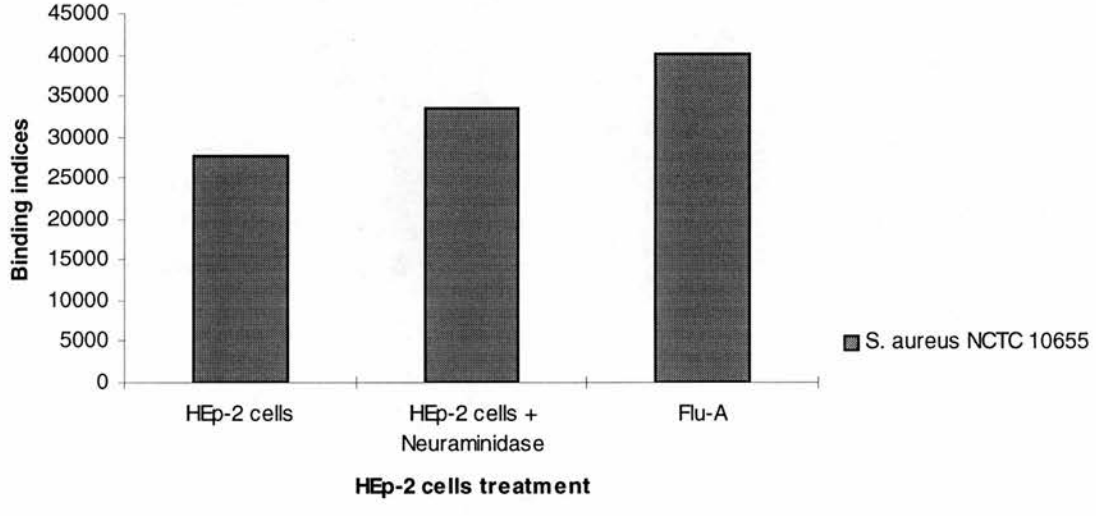

Figure 6.3 The effect of pre-treatment of HEp-2 cell with neuraminidase on binding of *S. aureus* strain (NCTC 10655) compared with their binding to uninfected HEp-2 cells and influenza A-infected cells.

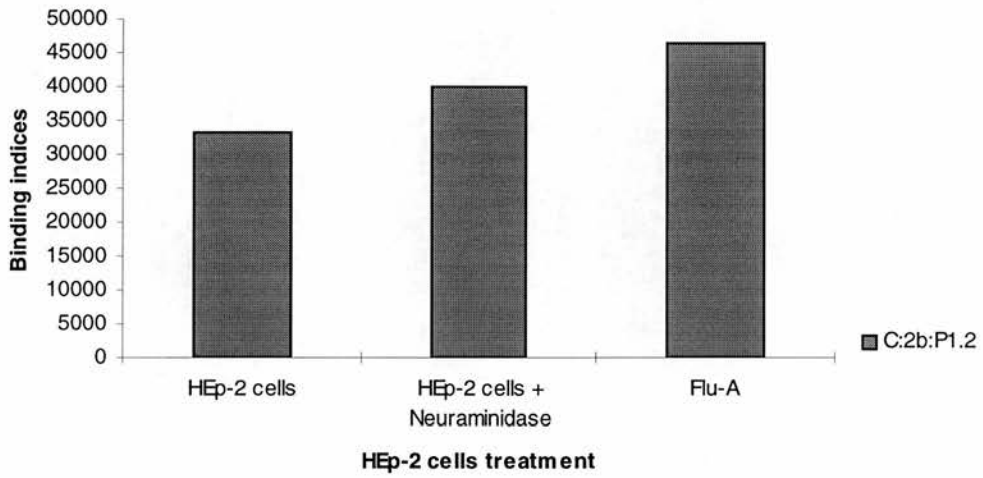

Figure 6.4 The effect of pre-treatment of HEp-2 cell with neuraminidase on binding of *N. meningitidis* (C:2b:P1.2) compared with binding to uninfected HEp-2 cells and influenza A-infected cells.

(NS, 95 % CI 99-125), but marginally significant (P< 0.05, 95 % CI 105-139) for *S. aureus*.

6.3.8 The effects of neuraminidase treatment on detection of host cell surface antigens

In 3 experiments the binding indices for monoclonal antibodies directed to cell surface antigens indicate there was no significant difference in binding of monoclonal antibodies to Le^b, Le^x or H type 2 to HEp-2 cells or HEp-2 cells treated with neuraminidase. There were significant increases in binding of anti-CD14 (P< 0.01, 95% CI 220-350) and anti-CD18 (P< 0.01, 95% CI 256-359) monoclonal antibodies to HEp-2 cells treated with neuraminidase compared to untreated HEp-2 cells (Figure 6.5).

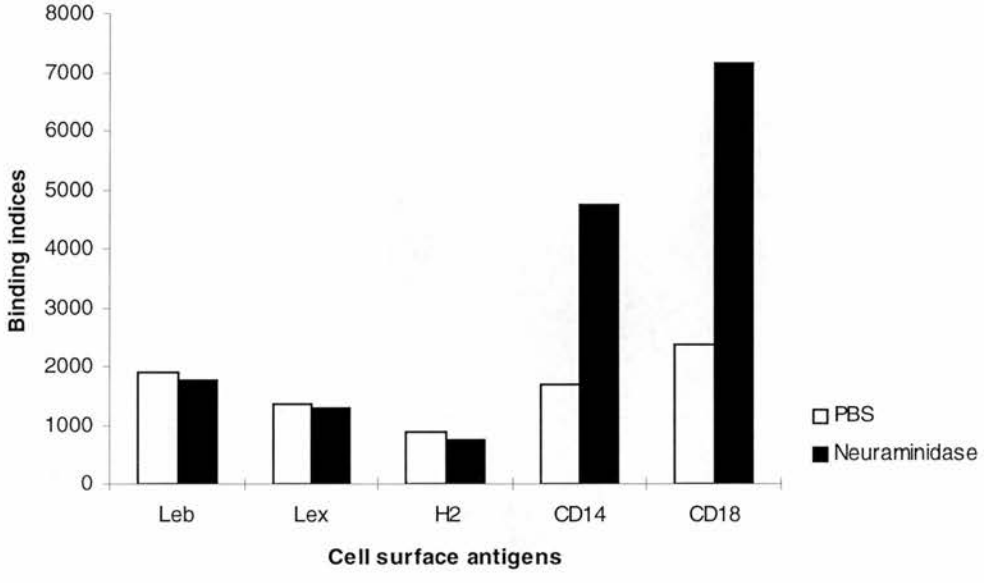

Figure 6.5 Binding of monoclonal antibodies to host cell surface antigens on HEp-2 cells treated with PBS or neuraminidase.

6.4 Discussion

6.4.1 Results in relation to objectives of the chapter

The results of this study answered the questions posed in the introduction. HEp-2 cells infected with human influenza A virus showed increased adherence of respiratory tract bacteria associated with meningitis, pneumonia and exacerbation of chronic bronchitis. In contrast to studies with RSV, the antibiotic-sensitive isolates (MC2-MC8) of *M. catarrhalis* also showed significantly increased binding to cells infected with influenza virus. Influenza virus infected HEp-2 cells showed no changes in expression of Lewis antigens or H type 2, but significantly increased binding of monoclonal antibodies for the cell surface antigens CD14 and CD18 compared to uninfected HEp-2 cells. In contrast, RSV infection enhanced binding of Lewis^{x} as well as to CD14 and CD18 (Raza *et al.*, 1994).

Pre-treatment of the virus infected cells with anti-CD14 or anti-CD18 significantly reduced binding of Gram-negative species tested but not the Gram-positive species. Treatment of uninfected HEp-2 cells with neuraminidase enhanced bacterial binding which might be an additional mechanism by which influenza infection increases the accessibility of host cell antigens to which bacteria bind. Neuraminidase treatment significantly enhanced binding of *S. aureus*. This was suggested to be due to removal of sialic acid from sialyl Lewis^{x} as Lewis^{x} has been shown to be a receptor for *S. aureus* (Saadi *et al.*, 1996); however there was no changes in binding of anti-Lewis^{x} to neuraminidase treated cells. The enhanced binding observed with *N. meningitidis* and

neuraminidase treated cells was not significant. This could be due to small number of experiments. Binding of anti-CD14 and anti-CD18 to the neuraminidase treated cells was significantly enhanced.

Monoclonal antibodies to influenza antigens such as the haemagglutinin were not available, so these were not tested.

6.4.2 Secondary respiratory infection and influenza

Several possible mechanisms for promoting bacterial superinfection by influenza have been proposed. These include: decreased tracheal clearance function (Sweet and Smith, 1980; Nugent and Pesanti, 1983); impairment in pulmonary and systemic bacterial clearance mechanisms (Warshauer *et al.*, 1977). Studies by Sanford and colleagues (1978; 1979), and Davison and Sanford (1981) suggest the possibility that influenza infection predisposes the host to bacterial colonisation, and subsequent disease by enhancing the adherence of some bacteria to virus-infected respiratory tract cells. This hypothesis is, in fact, consistent with clinical, epidemiological, and experimental evidence which indicates that influenza A infection predisposes the lungs to secondary pneumonia due to *S. aureus*, *S. pneumoniae*, and to a lesser extent, *H. influenzae* (Nugent and Pesanti, 1983). Vaccination against influenza virus type A before an influenza A epidemic has been shown to be effective in preventing the development of AOM in children aged between 1-3 years (Heikkinen *et al.*, 1991). In the present study each of the bacterial strains associated with AOM or pneumonia showed enhanced binding to influenza infected cells.

6.4.3 Bacterial meningitis and influenza

Many children with bacterial meningitis had signs and symptoms of upper respiratory infection, suggesting that viral infection could lead to secondary bacterial invasion (Fulginiti and Sieber, 1975). While recent epidemiological studies found little evidence to suggest RSV is associated with outbreaks of meningococcal infection (Stuart *et al.*, 1996), there is evidence that influenza virus infection might increase the risk of subsequent meningococcal infection (Cartwright *et al.*, 1991; Harrison *et al.*, 1991). In the experiments carried out in this project, both viruses enhanced binding of all meningococcal isolates tested, regardless of serogroup, serotype, subtype or immunotype.

Influenza virus is also known to affect cellular immunity adversely (Notkins *et al.*, 1970; Kantzler *et al.*, 1974; Jakab, 1982). It has been suggested that influenza-induced coughing causes aerosolization and, therefore, increased transmission of *N. meningitidis*. All of these findings could, alone or in combination, account for the association between these two infections.

6.4.4 Host cell receptors for bacteria and influenza infection

6.4.4.1 CD14 and CD18

CD14 and CD18 are receptors for Gram-negative bacteria such as *Escherichia coli* (Wright and Jong, 1986; Wright *et al.*, 1989). Influenza virus infected HEp-2 cells showed significantly increased binding of monoclonal antibodies for the cell surface

antigens CD14 and CD18 compared to uninfected HEp-2 cells. This might partly explain how virus infection contributes to enhanced binding by upregulation of expression of host cell surface antigens that can act as receptors for the Gram-negative bacteria.

Pre-treatment of influenza A virus infected HEp-2 cells with monoclonal antibodies to CD14 or CD18 significantly inhibited binding of Gram-negative species tested (*N. meningitidis*, *M. catarrhalis*, and *H. influenzae* type b). While it could be argued that this effect was due to stearic hinderance, there was no effect on inhibition of binding of Gram-positive species tested. This indicates that the endotoxin of Gram-negative bacteria are involved in enhanced binding of Gram-negative species observed for virus infected cells.

6.4.4.2 Effect of neuraminidase

HEp-2 cells pre-treated with neuraminidase showed significantly increased binding of *S. aureus* compared to untreated HEp-2 cells while the binding of meningococci was increased, the results were not significant. Differences in binding to enzyme treated cells and influenza infected cells indicates neuraminidase contributes to but is not the only effect on cells that could enhance binding.

Sialic acid is present on the structure of both CD14 molecules (Stelter *et al.*, 1996) and CD18 molecules (Kerr and Stocks, 1992) and neuraminidase treatment appears to make the epitope to which monoclonal reagents bind more accessible. Studies by Spooncer

and colleagues (1984) and Fukuda and colleagues (1984) had shown by carbohydrate analysis that two of the major carbohydrate structures carried by neutrophil glucoproteins are large highly branched N-linked structures: a polyfucosylated lactosaminoglycan and a sialylated fucosyl-lactosaminoglycan; both of these carry the CD15 determinant. The sialylated derivative sLe^x is not recognized by the CD15 antibodies unless the sialic acid is remove. Removing of sialic acid from the host cell antigens molecules by neuraminidase treatment might expose epitopes masked by sialic acid residues; however, enhanced binding of monoclonal anti CD15 was not observed.

An addition to the various mechanisms proposed to explain why influenza A is a predisposing factor for bacterial disease of the respiratory tract and meningitis, these studies indicate that virus infected epithelial cells bind more bacteria. If a similar phenomenon occurs in *vivo*, this could contribute to density of colonisation which is an important factor in development of bacterial disease.

Chapter 7

The effect of smoking on adherence of respiratory pathogens to epithelial cells

7.1 Introduction

Smoking is associated with an increased risk of respiratory tract infection in adults (Aronson *et al.*, 1982) and also with carriage of some potentially pathogenic species of bacteria (Musher and Fainstein, 1981; Blackwell *et al.*, 1990; Blackwell *et al.*, 1992). Young smokers had an approximately two fold greater risk of developing infection compared to non-smokers. Smokers were more likely to develop a lower respiratory tract infection, and they were also symptomatic for a 25% longer period (Aronson *et al.*, 1982).

Although there is no association between meningococcal disease and active smoking, isolation of *N. meningitidis* from the nasopharynx is significantly greater among smokers, and heavier smokers are more likely to be carriers of meningococci (Stuart *et al.*, 1989; Blackwell *et al.*, 1990; Blackwell *et al.*, 1992). Passive exposure to cigarette smoke is a risk factor for meningococcal infection in children (Stuart *et al.*, 1988; Haneberg *et al.*, 1983). Passive exposure to cigarette smoke is associated with respiratory tract infections in children, and in many studies the strongest association was

with maternal smoking (Pershagen, 1986). Greek schoolchildren from households in which the mother or other members of the family traditionally involved in child care smoked were more likely to be carriers of meningococci than children from households in which no one smoked (Kremastinou *et al.*, 1994).

Cigarette smoking remains the most common cause of chronic bronchitis, although recurrent childhood respiratory infections and air pollution play important roles in the pathogenesis of this disease (Cazzola, 1990). Bacteria commonly isolated from the sputum of patients with chronic bronchitis include non-typable *H. influenzae*, *S. pneumoniae*, *S. aureus* and *M. catarrhalis*. These bacteria are found at both exacerbation and times of remission (Lampe *et al.*, 1982).

Several studies showed that smokers have increased bacterial adherence to pharyngeal and epithelial cells (Musher and Fainstein, 1981; Mahajan and Panhotra, 1989). *H. influenzae* (CCUG21594) and *S. pneumoniae* (CCUG10175) have been shown by interference contrast microscopy to attach more readily to pharyngeal cells from smokers with chronic bronchitis (Riise *et al.*, 1994). While a number of host cell antigens have been identified to act as receptors for bacteria (Table 7.1), the effect of smoking on expression of these antigens has not been assessed.

Table 7.1 Bacterial species and host cell antigens which act as receptors

Species	Antigen	References
B. pertussis	Lewis^{x}, Lewis^{a}	van t'Wout *et al*, 1992 Saadi *et al*, 1996
S. aureus	Lewis^{x}, Lewis^{a} Fibronectin	Saadi *et al*, 1993 Ljungh and Wadstrom 1996
H. pylori	Lewis^{b} H type 2, Lewis^{a}	Boren *et al*, 1993 Alkout *et al*, 1997
E. coli	CD14, CD18	Wright and Jong, 1986

7.1.1 Aims of the study

This study used the flow cytometry method to determine:

1) if there was enhanced binding to cells of smokers by bacteria in which the epidemiological studies found disease or carriage of the bacteria was associated with smoking or passive exposure to cigarette smoke;

2) if cells of smokers expressed higher levels of antigens proposed to act as bacterial receptors;

3) if cells of non-smokers treated with a water soluble extract of cigarette smoke could enhance bacterial binding.

7.2 Materials and Methods

7.2.1 Collection of epithelial cells

Buccal epithelial cells (BEC) were obtained from donors in the Department of Medical Microbiology by rubbing the inside of the oral cavity with cotton swabs. To remove the cells, the swabs were agitated in 10 ml PBS. They were washed twice in PBS in a Sorvall RT 6000 centrifuge at 300 x *g* for 10 min and the concentration adjusted to 2.5 x 10^5 ml^{-1} after determination of the number of cells microscopically in an improved Neubauer haemocytometer (Weber Scientific International Ltd, UK).

7.2.2 Bacteria

Sources of strains of *N. meningitidis*, *S. pneumoniae*, *H. influenzae*, *B. pertussis*, *N. lactamica*, *M. catarrhalis* and *S. aureus* and their culture conditions were described in chapters 3, 4 and 5. The bacteria were harvested, washed three times in PBS by centrifugation at 2500 x *g* for 15 min and resuspended in MM without antibiotics by vigorous pipetting to disperse clumps. The bacterial concentrations were determined by measuring OD at 540 nm (3.3.1 and 4.3.1).

7.2.3 Bacterial binding to buccal epithelial cells of smokers & non-smokers

Bacteria were labelled with FITC as described in chapter 3 and the binding studies with cells from smokers and non-smokers carried out as described for HEp-2 cells. Dose response experiments were carried out with cells of non-smokers to determine the optimal ratios of each bacterial strain.

7.2.4 Preparation of water soluble components of cigarette smoke

A hand operated vacuum pump was used to draw cigarette smoke through sterile PBS. The apparatus consisted of a 250 ml Duran bottle with two holes drilled in the cap. Through one of these holes was placed a length of sterilised tubing with a connection to a piece of larger bore tubing which held the cigarette. Another length of tubing was run from the other hole to the vacuum pump. The cigarette smoke was drawn through the medium by the change in pressure caused by extracting the air from the upper part of the bottle (Figure 7.1). The smoke from 10 cigarettes (Regal King Size) was infused into 100 ml of the PBS to provide a stock solution of 0.1 cigarettes / ml. The medium was filter sterilised using a 0.2 μm Millipore syringe filter and frozen in aliquots at -20°C prior to use.

7.2.5 Treatment of BEC from non-smokers with water soluble components of cigarette smoke

BEC from non-smokers (200 μl, 2.5 x 10^5 ml^{-1}) were incubated with various dilutions the cigarette smoke extract (CSE) (500 μl) for 30 min at 37°C. The cells were washed twice with PBS by centrifugation at 300 x *g* for 10 min, then the cells were used for bacterial binding as described in chapter 3.

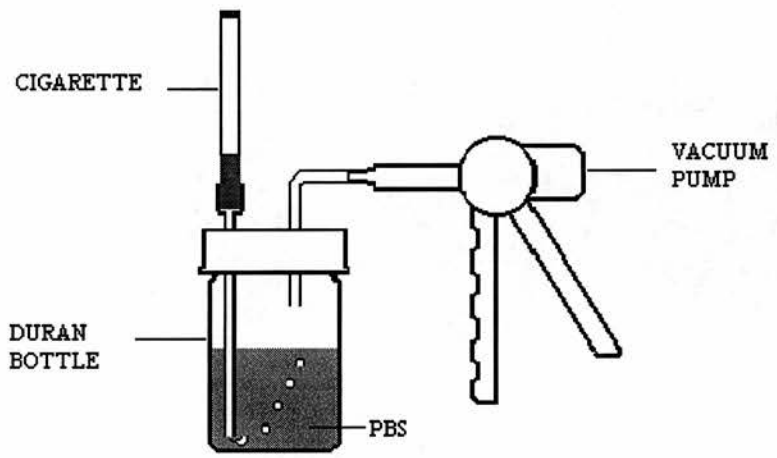

Figure 7.1 Apparatus used in the production of the water soluble components of cigarette smoke.

7.2.6 Detection of host cell surface antigens on BEC of smokers and non-smokers

BEC from smokers and non-smokers were assessed for expression of CD14, CD18, H type 2, and Lewis antigens as described in 3.2.9 and 6.2.11. For detection of fibrinogen and fibronectin, BEC from smokers and non-smokers were incubated with sheep polyclonal anti-human fibrinogen (Sigma) diluted 1 in 10 or sheep polyclonal anti-fibronectin (Sigma) diluted 1 in 10 for 60 min at 37°C. The cells were washed twice with PBS and incubated with donkey anti-sheep / goat IgG conjugated with FITC diluted 1 in 100 for 30 min in an orbital shaker at 37°C. The cells were washed twice with PBS by centrifugation, resuspended in 1% buffered paraformaldehyde and analysed with an EPICS-XL flow cytometer. Controls included cells incubated without either primary or secondary antibodies and cells incubated only with the secondary antibody.

7.2.7 Detection of host cell antigens on BEC of non-smokers treated with CSE

BEC (200 µl, 2.5 x 10^5 ml^{-1}) from non-smokers treated with PBS or CSE (7.2.5) were added to Falcon tubes containing 200 µl of monoclonal antibodies to Le^a (1/5), Le^x (1/10), Le^b (1/10), or H type 2 (1/10). The tubes were incubated at 37°C for 60 min, washed twice with PBS by centrifugation at 300 x *g* for 10 min and incubated with 200 µl rabbit anti-mouse IgM conjugated with FITC diluted 1 in 200. The FITC-labelled antibody was also added to 200 µl of cells which had not been treated with the first

antibody as a control. The tubes were incubated at 37°C for 60 min with continuous shaking. The cells were washed twice with PBS and fixed with 200 µl of 1% buffered paraformaldehyde and stored in the dark at 4°C until analysed. The cells were analysed with an EPICS-XL flow cytometer (3.2.7).

7.2.8 Enzyme linked immunosorbent assay (ELISA) to detect H antigen

Microtitre plates (96 wells) were coated with 100 µl of CSE (undiluted, 1/10, 1/20, 1/40 and 1/80) diluted in coating buffer and incubated overnight at 4°C. The plates were washed with washing buffer (2.8.2), blocked with blocking buffer (2.8.3) and incubated at room temperature for 20 min. The blocking buffer was removed and the plates washed with washing buffer.

Biotinylated *Ulex europaeus* lectin (which detects H antigen) was diluted to 5 µg ml^{-1} in blocking buffer (2.8.3) and 100 µl was added to the wells and incubated at 37°C for 30 min. The plates were then washed 6 times with washing buffer (2.8.2). Streptavidin-HRP (Sigma) (100 µl) diluted 1 in 100 in blocking buffer was added to the plates and incubated for 60 min at room temperature then washed 6 times in washing buffer. The substrate (2.8.5) (100 µl) activated by 40 ml H_2O_2 (30 % v/v) immediately before use was added. The colour change was stopped after 10 min by adding 100 µl of H_2SO_4.(12.5%). The optical density at 490 nm was determined by an ELISA reader

(Dynatech) and corrected by subtracting the OD of the corresponding blank well containing smoke extract, streptavidin-HRP and substrate. Saliva from an individual of blood group O who secreted H type 1 and Lewis blood group antigens was used as positive control.

7.2.9 Statistical analysis

The assessment of the data on bacterial binding to cells of smokers and non-smokers was based on paired *t*-test applied to logarithms of the binding indices.

7.3 Results

7.3.1 Determination of the ratio of bacteria per cell

Dose response experiments were carried out with buccal epithelial cells for each strain: *N. meningitidis* (Figure 7.2); *S. pneumoniae* (Figure 7.3); *H. influenzae* (Figure 7.4); *M. catarrhalis* (Figure 7.5); *N. lactamica* (Figure 7.6). For experiments in which binding of bacteria to cells from smokers and non-smokers was assessed, a ratio of bacteria : cell at the midpoint of the dose response curve for the non-smoker was used. Data from previous studies on bacterial binding to BEC of smokers and non-smokers in the laboratory indicated optimal levels of *S. aureus* and *B. pertussis* were 500 : cell and 640 : cell respectively (Saadi *et al.*, 1994; Saadi *et al.*, 1996).

7.3.2 Binding of Gram-negative species to buccal epithelial cells of smokers and non-smokers

Compared with binding indices for cells from non-smokers, those obtained for *N. meningitidis* strains with epithelial cells from smokers were significantly increased. A similar pattern was found with the two non-typable isolates of *H. influenzae* (87D), (241D) and the serotype b isolate. For the two *Moraxella* isolates (MC1) and (MC2), binding to cells of smokers was significantly increased compared to binding to cells of non-smokers. Both *N. lactamica* and *B. pertussis* showed significantly higher binding indices with cells from smokers (Table 7.2).

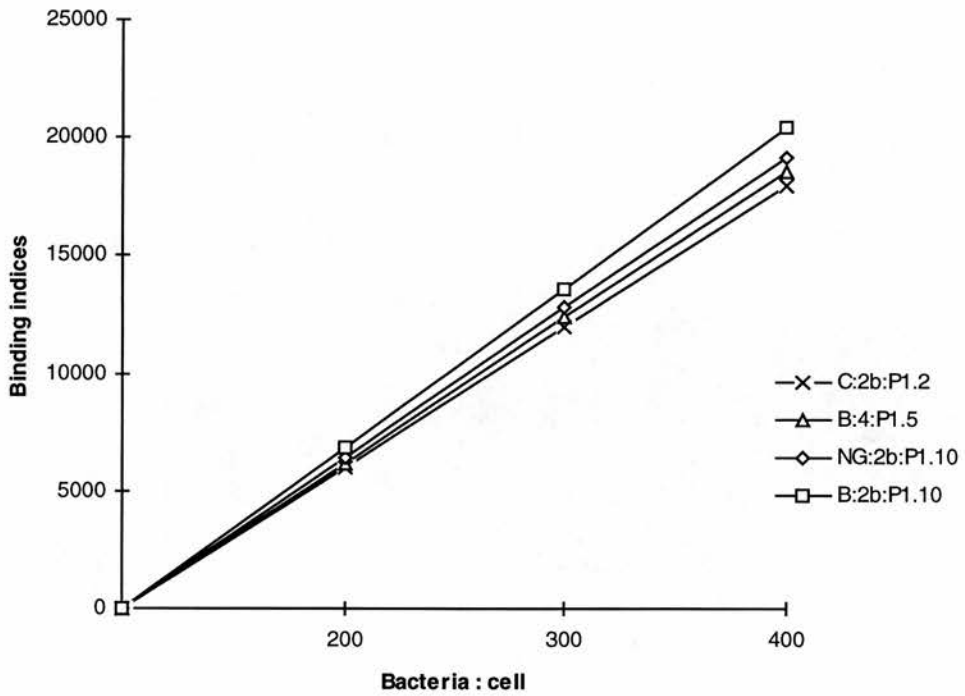

Figure 7.2 Representative dose response curves obtained with 4 *N. meningitidis* strains for binding to BEC of non-smokers.

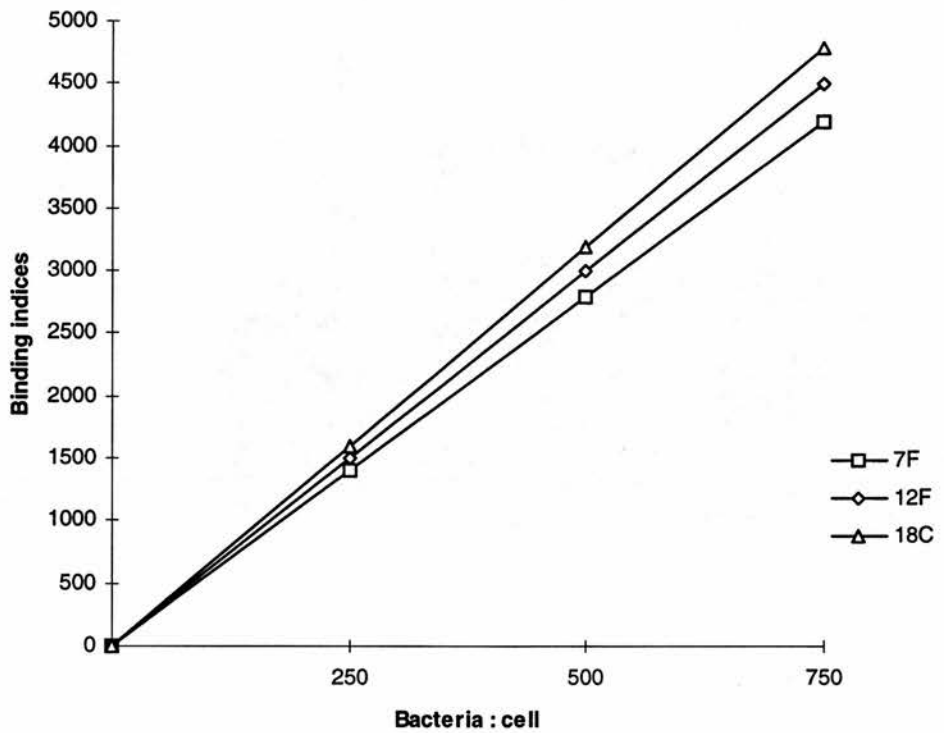

Figure 7.3 Representative dose response curves obtained with 3 *S. pneumoniae* strains for binding to BEC of non-smokers.

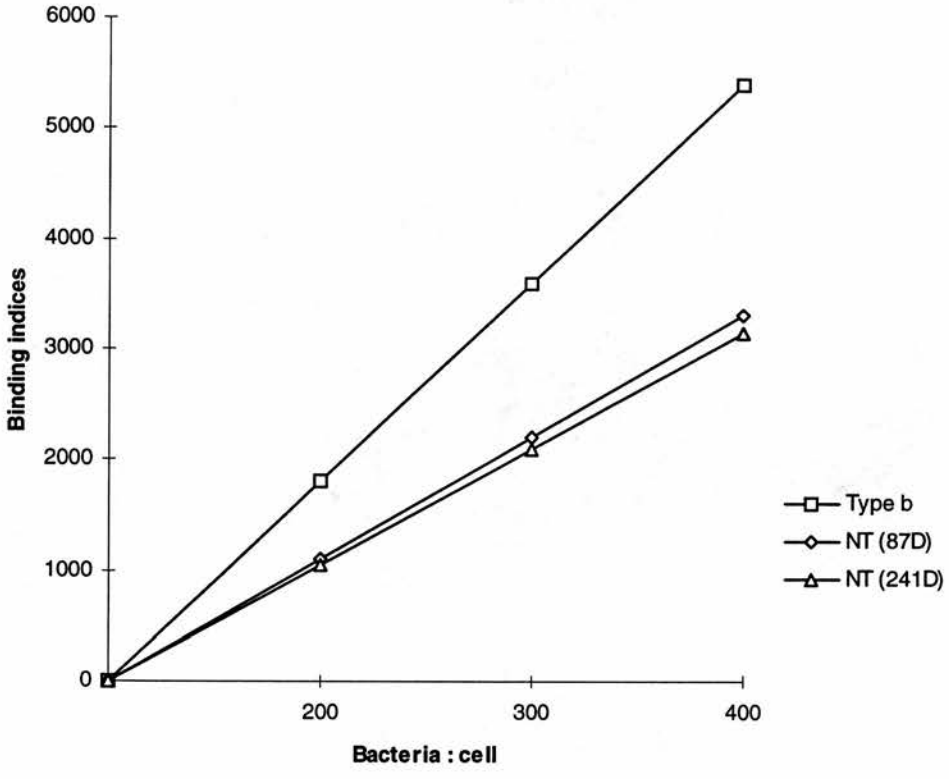

Figure 7.4 Representative dose response curves obtained with 3 *H. influenzae* (type b and non-typable) isolates for binding to BEC of non-smokers.

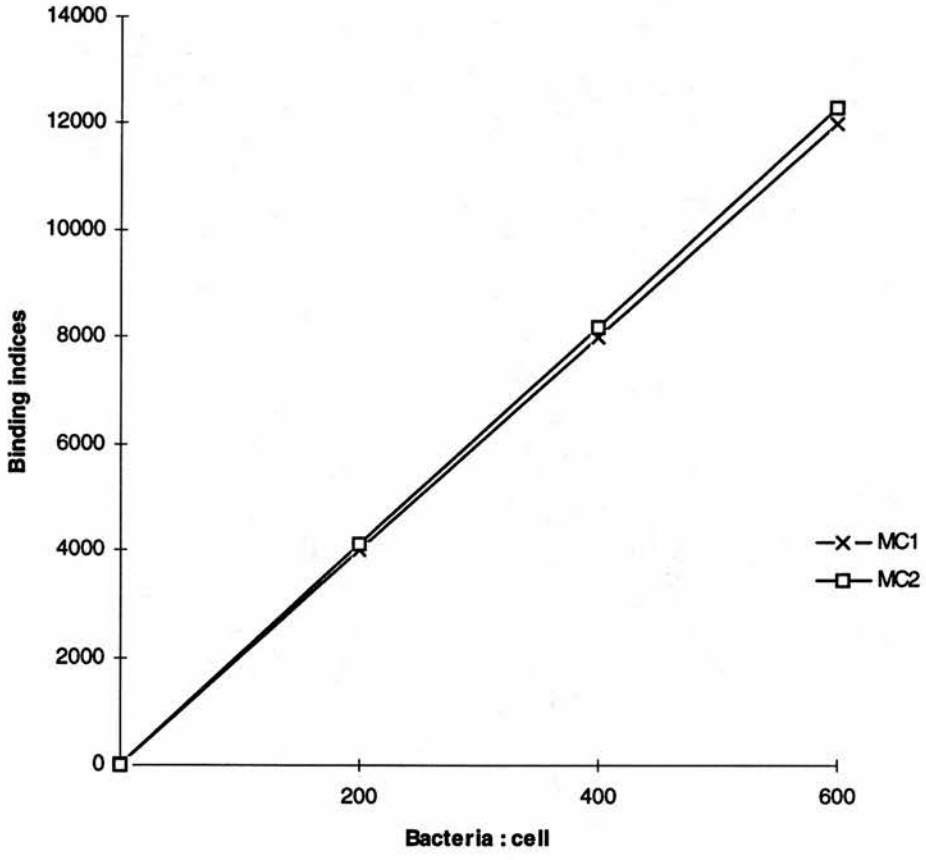

Figure 7.5 Representative dose response curves obtained with 2 *M. catarrhalis* isolates for binding to BEC of non-smokers.

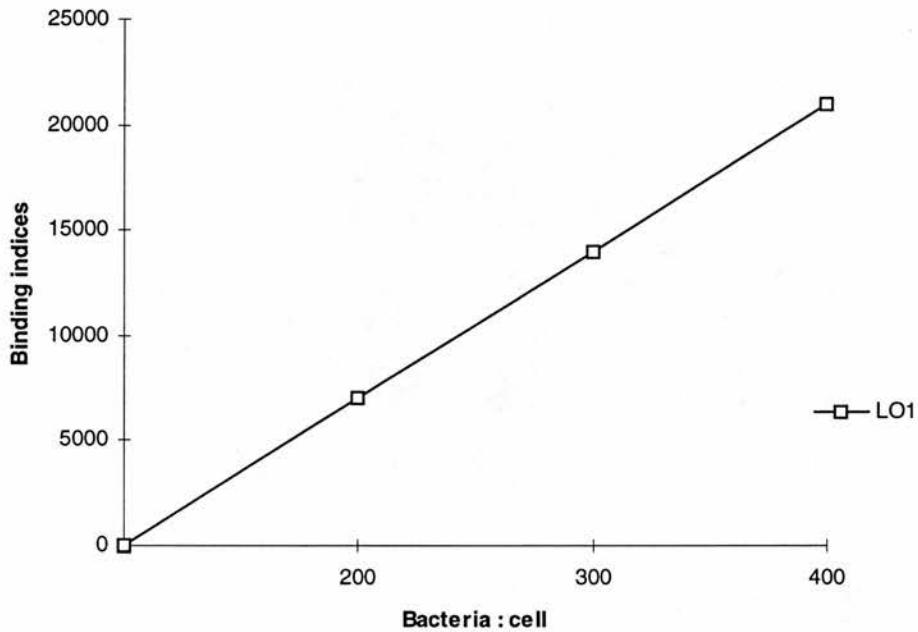

Figure 7.6 Representative dose response curve obtained with *N. lactamica isolate* (LO1) for binding to BEC of non-smokers.

Table 7.2 Binding of Gram-negative species to buccal epithelial cells from smokers & non-smokers

Species	strain	pairs	ratio[a]	mean BI of non-smokers BEC	mean BI of smokers BEC	smokers as % of non-smokers BEC (95% CI)	P
N. meningitidis	C:2b:P1.2	8	300	11105	14718	132 (115-151)	0.01
	B:4:P1.15	8	300	7172	15707	219 (183-262)	0.001
	NG:2b:P1.10	8	300	14899	21711	145 (122-173)	0.001
	B:2b:P1.10	8	300	13016	17751	136 (115-160)	0.01
H. influenzae (b)	type b	5	400	5229	9139	169 (127-223)	0.01
H. influenzae (NT)	87D	6	400	1701	2204	130 (121-139)	0.001
	241D	6	400	1338	1976	160 (138-185)	0.001
N. lactamica	LO1	10	300	12226	28780	235 (214-257)	0.001
M. catarrhalis	MC1	6	400	3492	4348	129 (109-152)	0.01
	MC2	6	400	6182	7714	124 (107-145)	0.01
B. pertussis	8002	15	640	1982	2846	140 (108-183)	0.05

(a) ratio of bacteria: cell

7.3.3 Binding of Gram-positive species to buccal epithelial cells of smokers and non-smokers

S. aureus, S. pneumoniae serotypes associated with meningitis and those associated respiratory infections were examined for binding to cells of smokers and non-smokers. The binding indices of the each isolate was significantly greater for cells from smokers compared with non-smokers (Table 7.3).

7.3.4 Effects of undiluted water soluble components of cigarette smoke on bacterial attachment

BEC from non-smokers were treated with undiluted water soluble components of cigarette smoke as described in (7.2.5). The bacterial species tested in these assays were three strains of *N. meningitidis* (C: 2b: P1.2, NG: 2b: P1.10 and B: 2b: P1.10), one strain of *N. lactamica* (LO1), one strain of *H. influenzae* (type b) and the two isolates of *M. catarrhalis* (MC1 and MC2). There was significantly decreased binding of each isolate to BEC treated with undiluted extract of smoke components compared to untreated BEC of the same non-smokers (Table 7.4).

7.3.5 Effects of dilution of water soluble components of cigarette smoke on bacterial binding

To determine the effect of concentration of the cigarette smoke extract on bacterial binding, in three experiments BEC from a non-smoker were treated with different dilutions of the water soluble components of cigarette smoke (1/10 to 1/1280). From the 1/10 dilution to the 1/320 dilution, there was enhanced binding of *S. pneumoniae*

Table 7.3 Binding of Gram-positive species to buccal epithelial cells from smokers and non-smokers.

Species	strain	pairs	ratio [a]	mean BI of non-smokers BEC	mean BI of smokers BEC	smokers as % non-smokers BEC (95% CI)	P
S. pneumoniae [b]	7F	8	500	2794	6705	238 (178-317)	0.001
	12F	8	500	2846	6765	257 (178-372)	0.001
	18C	8	500	2771	6810	262 (168-411)	0.001
S. pneumoniae [c]	6	7	500	562	1620	124 (103-149)	0.01
	10	7	500	523	1906	158 (112-223)	0.01
	23	7	500	893	1324	150 (116-174)	0.01
	42	7	500	2650	4436	159 (115-243)	0.01
S. aureus	NCTC 10655	8	500	579	835	156 (105-223)	0.05

(a) ratio of bacteria: cell
(b) Meningitis strains
(c) Respiratory isolates

Table 7.4 Binding of bacterial species to untreated BEC of non-smokers and the cells treated with undiluted water soluble components of cigarette smoke

Species	strain	Donors	ratio[a]	mean BI of untreated BEC	mean BI of treated BEC	treated BEC as % untreated BEC (95% CI)	P
N. meningitidis	C:2b:P1.2	3	300	38049	19434	61 (37-70)	0.05
	NG:2b:P1.10	3	300	43555	26148	60 (48-76)	0.05
	B:2b:P1.10	3	300	38753	22456	58 (43-78)	0.05
N. lactamica	LO1	3	300	49024	33027	67 (53-85)	0.05
H. influenzae (b)	type b	3	400	61264	43525	71 (61-82)	0.05
M. catarrhalis	MC1	3	400	56056	41051	73 (62-86)	0.05
	MC2	3	400	52279	37573	72 58-89)	0.05

(a) ratio of bacteria : cell

(12F) (Figure 7.7). At the 1 in 640 dilution the effect of the extract was no longer observed.

7.3.6 Effects of diluted water soluble components of cigarette smoke on bacterial binding to BEC from additional non-smokers

To determine if the effect of the cigarette smoke extract was limited only to the cells of the non-smokers in 7.3.5, BEC from 7 other non-smokers were treated with the cigarette smoke extract diluted 1 in 10 (7.2.5). Binding of different bacterial species including *N. meningitidis*, *M. catarrhalis*, *H. influenzae* type b, *N. lactamica*, *S. pneumoniae* and *B. pertussis* to cells treated with the extract diluted 1 in 10 was compared with that for untreated cells (7.2.5). The results indicate there was significantly increased bacterial attachment to BEC treated with the diluted extract (Table 7.5).

7.3.7 Detection of surface antigens on BEC of smokers and non-smokers

In experiments with 8 pairs of smoker and non-smoker donors, there were no significant differences in binding of monoclonal or polyclonal antibodies to Le^a, Le^b, Le^x, H type 2, fibrinogen or fibronectin to BEC from smokers and non-smokers. The amount of CD14 or CD18 detected was too small to analyse (Table 7.6).

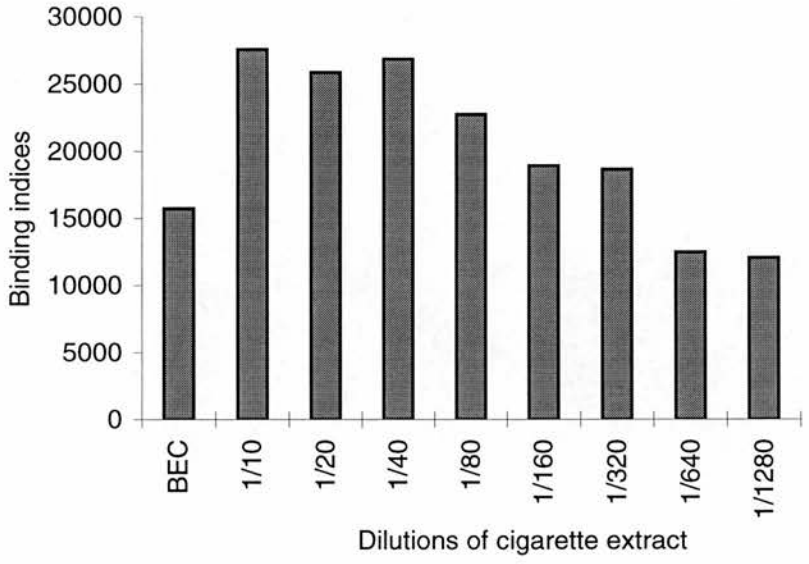

Figure 7.7 The effect of different dilutions of cigarette extract on binding of *S. pneumoniae* type 12F compared with untreated BEC.

Table 7.5 Binding of bacterial species to BEC of non-smokers or treated with diluted (1/10) water soluble components of cigarette smoke

Species	strain	Donors	ratio[a]	mean BI of untreated BEC	mean BI of treated BEC	treated BEC as % untreated BEC (95% CI)	P
N. meningitidis	C:2b:P1.2	7	300	19403	34247	175 (137-224)	0.01
	NG:2b:P1.10	7	300	21935	37418	173 (131-228)	0.01
	B:2b:P1.10	7	300	18667	31109	171 (136-215)	0.01
N. lactamica	LO1	7	300	13289	30695	234 (166-330)	0.001
H. influenzae (b)	type b	7	400	24900	55016	224 (194-260)	0.001
S. Pneumoniae	12F	7	500	15092	28380	183 (147-228)	0.001
	18C	7	500	26582	50025	188 (179-198)	0.001
M. catarrhalis	MC1	7	400	32313	52300	161 (133-194)	0.001
	MC2	7	400	36746	59190	161 (134-193)	0.001
B. pertussis	8002	7	640	28091	59259	208 (175-248)	0.001
	250815	7	640	29099	56347	194 (188-200)	0.001

(a) ratio of bacteria : cell

Table 7.6 Expression of surface antigens on buccal epithelial cells of smokers and non smokers

Antibodies	No of pairs	mean BI of non-smokers	mean BI of smokers	P
Lewis^a	8	761	840	NS
Lewis^b	8	1314	1118	NS
Lewis^x	8	625	458	NS
H type 2	8	2263	2089	NS
Fibronectin	8	1249	1105	NS
Fibrinogen	8	1265	1655	NS

Binding of monoclonal antibodies for CD14 and CD18 was too low to analyse

7.3.8 Effects of water soluble components of cigarette smoke on detection of cell surface antigens

Cells of 7 non-smoker donors were treated with PBS or CSE diluted 1 in 10. The binding indices of monoclonal antibodies to the following antigens were significantly decreased for the cells treated with CSE compared with untreated BEC of the same donors, Le^a (63%, P< 0.01, 95% CI 49-82); Le^b (80%, P< 0.05, 95% CI 66-97); Le^x (47%, P< 0.01, 95% CI 34-67). The only exception was the binding index of the monoclonal antibody to H type 2; this was significantly increased (120%, P < 0.01, 95% CI 133-180) for the CSE treated cells compared with untreated BEC of the same donors (Fig 7.8).

7.3.9 ELISA to detect H type 2 in CSE

The result of the spectrophotometer assays showed that a fucose containing substance which bound the biotinylated *Ulex europaeus* lectin was present in the CSE. The OD values decreased with dilution of the CSE : non-diluted CSE (0.165, 0.166) 1/10 (0.084, 0.086), 1/20 (0.035, 0.038). At the 1/40 and 1/80 dilutions the OD values were not above those of the controls (Fig 7.9).

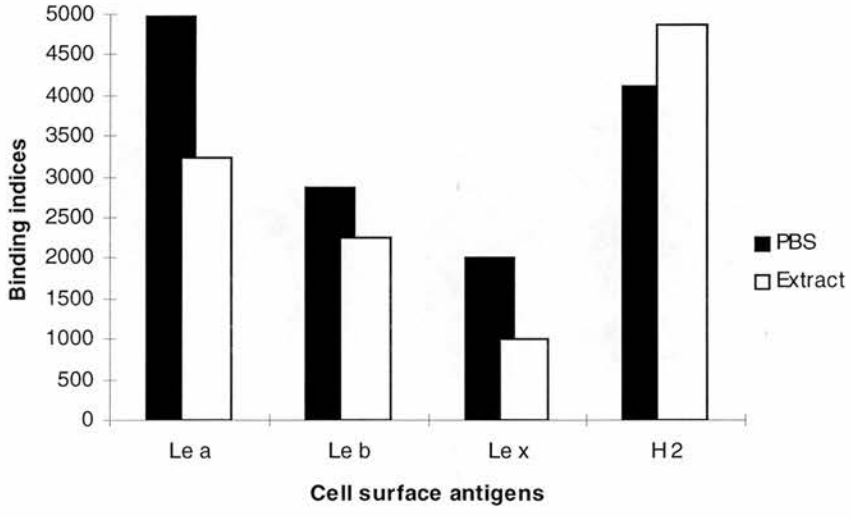

Figure 7.8 The binding of monoclonal antibodies to host cell surface antigens on BEC treated with PBS or water soluble components of cigarette smoke.

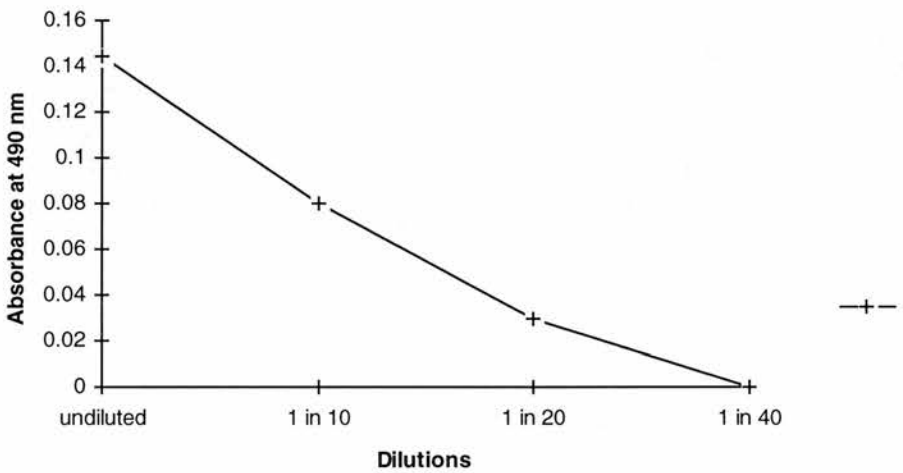

Figure 7.9 OD values for detection of fucose containing components in smoke extract.

7.4 Discussion

The results of the experiments provided data to answer the question posed in the introduction. Epidemiological observations found disease or carriage of several bacterial species was associated with smoking or passive exposure to cigarette smoke. Cells from smokers bound significantly more of each of the species tested, *N. meningitidis*, *H. influenzae*, *M. catarrhalis*, *N. lactamica*, *S. pneumoniae*, *S. aureus*, and *B. pertussis*. Buccal epithelial cells of non-smokers treated with undiluted CSE exhibited decreased binding compared to untreated BEC but at dilutions of 1/10 - 1/320 there was increased bacterial binding.

One hypothesis to explain the enhanced bacterial binding to cells of smokers was that smoking increased expression of some of the host cells antigens used as receptors for some bacteria. There were no significant differences in binding of monoclonal or polyclonal antibodies to Lewis antigens, H type 2, fibrinogen or fibronectin to cells of 8 smokers compared with 8 non-smokers. The amount of CD14 and CD18 identified as receptors on HEp-2 cells was too small to analyse on BEC. BEC of non-smokers treated with CSE at a dilution 1 in 10 exhibited decreased binding of monoclonal antibodies to Le^a, Le^b and Le^x compared to untreated BEC. Decreased binding could be explained if some components of cigarette smoke masked the cell surface antigens which have been shown to bind some of the bacteria tested *e.g*, Le^a and Le^x for *S. aureus* and *B. pertussis* (Saadi *et al.*, 1993; Saadi *et al.*, 1996) The only exception was increased binding of the monoclonal antibody to H type 2 antigen on the CSE treated

cells. This might be due to the presence of substances in CSE containing fucose epitopes that cross-react with H antigen. Binding of the biotinylated *Ulex europaeus* lectin to the extract was demonstrated in the spectrophotometric assay, but the material was not detected at dilutions ≥ 1/40; therefore, other components in the CSE must contribute to the enhanced bacterial binding.

Exposure to cigarette smoke could contribute to frequency or density of colonisation. This study indicates there might be increased density of colonisation by several bacterial species among smokers. In a longitudinal study of bacterial flora of infants, *N. lactamica* was isolated more frequently from infants whose mothers smoked (Mackenzie *et al.*,1996), and in this study, these bacteria bound in greater numbers to cells of smokers. Mothers smoking was also associated with isolation of meningococci, but the numbers of isolates were too small for analysis. No associations between frequency of isolation and exposure to cigarette smoke were noted for *S. aureus*, *H. influenzae* or *S. pneumoniae* (Mackenzie *et al.*,1996). There was, however, a significant correlation between isolation of each species examined from both mother and baby.

Shepherd (1992) suggested that a non-smoker who lives with a smoker will be more likely to develop a respiratory tract infection. As the smoker himself has an increased risk of disease, this will tend to increase the exposure of the non-smoker to respiratory pathogens. Chronic exposure of a non-smoker to environmental tobacco smoke is thought to produce effects similar to those associated with active smoking, for example

chronic bronchitis (Robbins *et al.*, 1993). In the survey of infants, there was a highly significant correlation between cotinine levels in respiratory secretions and the number of cigarettes smoked in the household each day (Mackenzie *et al.*,1996). If passive coating of epithelial cells by components of cigarette smoke occurs *in vivo* as in the experiments with CSE, it is possible that passive exposure to cigarette smoke could enhance density of colonisation.

Specific adherence of micro-organisms to epithelial cells is an important step in the colonisation process. Although non-specific factors such as surface charge, pH, ionic bridging and hydrophobic interactions might be important (Watt and Ward, 1980), it has been argued that the attachment of bacteria to host cells is facilitated by specific interactions. Specific interaction might be necessary since the surface of both host and bacteria have a net negative charge. This mutual repulsion could be overcome by interaction of surface molecules which act as ligands (Ofek and Beachey, 1980). The current study found no difference in expression of specific ligands known to act as receptors for several bacterial species on cells of smokers and non-smokers: blood group antigens; fibrinogen; or fibronectin. Enhanced binding noted with dilutions of the CSE might reflect changes in charge or other non-specific effects on cells.

Although exposure to cigarette smoke enhances susceptibility to respiratory viral infection, this study indicates that smoking or passive exposure to cigarette smoke alone can enhance bacterial binding to epithelial cells. Virus infection upregulated expression of some host cell antigens involved in bacterial binding, but smoking did not. While

the components in the water soluble extract of cigarette smoke that enhanced bacterial binding have not been identified, the material cross-reactive with the H antigen might be one factor. Blood group antigens with an immunodominant fucose have been demonstrated to be a receptor for at least one bacterial species *Helicobacter pylori* (Alkout *et al.*, 1997) and for some strains of *Candida albicans* (Cameron and Douglas, 1996).

Chapter 8

General discussion

This chapter summarises the findings and conclusions with reference to the original objectives set out in the general introduction, explores the limitations of the experimental methods of analysis used throughout this study, and makes suggestions for future work.

8.1 Objectives of the project

Active smoking, passive exposure to cigarette smoke and viral infection are risk factors for respiratory infection. The general aim of the project was to assess virus infection and exposure to cigarette smoke for their individual effects on bacterial attachment.

The results of this study provided answers for the questions set at the start of the investigation. Because the glycoprotein G expressed on RSV-A infected cells was found to be an additional receptor for meningococci and variations in glycoprotein G between RSV subgroups A and B had been reported (Akerlind *et al.*, 1988; Anderson *et al.*, 1991; Cane *et al.*, 1991), the first objective of the study was to determine if there is a similar pattern of enhanced binding of *N. meningitidis*, *H. influenzae* type b, *S. aureus*, and *B. pertussis* to cells infected with RSV subgroup B as observed with RSV subgroup A. This study showed that HEp-2 cells infected with RSV subgroup B also enhanced bacterial binding.

The second aim of the study was to determine if there is increased binding of other species of bacteria associated with meningitis and those associated with secondary respiratory infections or exacerbation of chronic bronchitis to RSV-infected cells. For both RSV A and RSV B infected cells, there was significantly enhanced binding of the majority of species and strains tested. The only exception was the antibiotic-sensitive isolates of *M. catarrhalis* which bound in significantly lower levels to RSV infected cells. This unusual finding was further explored and significant differences in the OMP profile and complement sensitivity of antibiotic-sensitive and resistant strains were found.

The third objective was to test the hypothesis that influenza A virus infection of HEp-2 cells might enhance binding of bacteria associated with meningitis or bacterial species associated with pneumonia, otitis media and exacerbation of chronic bronchitis. The results in this study showed that HEp-2 cells infected with human influenza A virus significantly increased adherence of respiratory tract bacteria tested in the assays. In contrast to the finding with RSV, the antibiotic-sensitive isolates (MC2-MC8) of *M. catarrhalis* also showed significantly increased binding to cells infected with influenza virus.

The fourth objective was to examine changes in host cell components resulting from viral infection. As with RSV infection, there was increased binding of monoclonal antibodies to CD14 and CD18 to HEp-2 cells infected with influenza A; but, in contrast

with RSV infected cells binding of monoclonal antibody to CD15 was not enhanced. Neuraminidase treatment also enhanced bacterial binding but not to same extent as influenza infection, indicating other changes were associated with enhanced binding. Factors associated with enhanced binding of Gram-negative bacteria (enhanced CD14 and CD18) differed from those associated with Gram-positive bacteria (neuraminidase activity).

The fifth and sixth objectives were to examine the effects of smoking on bacterial binding, expression of host cell antigens that act as receptors for some species. Cells of smokers bound more bacteria associated with meningitis or respiratory disease compared with cells of non-smokers. While cells from smokers bound significantly greater numbers of all the species tested, there was no difference in level of host cell antigens which act as receptors for some bacteria on cells of smokers compared with cells of non-smokers. There appear to be substances in cigarette smoke that enhance binding of bacteria to epithelial cells (substances similar to H antigen) or perhaps alter the charges on the cells.

8.2 Applications and limitation of methods and results

8.2.1 Assessment of bacterial binding by flow cytometry

The majority of studies on attachment of bacteria to epithelial cells *in vitro* have been carried out by light microscopy (Tramont and Wilson, 1977; Taylor *et al.*, 1990; Riise *et al.*, 1994). The basic method consists of mixing epithelial cells with bacteria and incubating the mixture for an appropriate period. Excess bacteria are removed, the cells

are resuspended, a drop of suspension dried on a glass slide and examined under the light microscope.

There are several problems associated with the light microscopy method for assessment of bacterial binding. Bacteria were not evenly distributed over the BEC. There were background commensal organisms which affected the counting of bound bacteria and the number of bacteria observed varied greatly (Andersson *et al*, 1981). It is a very laborious method, prone to subjective errors in counting and samples few epithelial cells. These limitations have been overcome in this study by the use of fluorescein-labelled bacteria and analysis of the adherence by flow cytometry.

The advantages of flow cytometry are considerable and it provides a powerful tool for analysing bacterium-cell interactions. It is a reliable and practical method. Large numbers of cells were analysed in a short period of time. Over a thousand BEC can be analysed within a few minutes. It is less prone to subjective errors, and, unattached bacteria are not analysed by the instrument as they are represented in another area of the bitmap.

8.2.2 The effect of FITC-labelled bacteria on attachment assays

Direct labelling of the bacterial species tested in the study with FITC was the method chosen for these studies. The FITC does not alter binding to epithelial cells. The results in the preliminary studies by light microscopy showed there were no differences between the number of labelled and unlabelled bacteria bound to the cells.

8.2.3 Detection of host cell surface antigens by flow cytometry

Flow cytometry was used to determine if infection by influenza virus might increase the expression of host cell surface antigens which can act as receptors for bacteria as reported for RSV-infected cells (Raza *et al.*, 1994). Influenza virus infected HEp-2 cells showed no changes in expression of Lewis antigens but significantly increased binding of monoclonal antibodies for the cell surface antigens CD14 and CD18 compared to uninfected HEp-2 cells. This might explain how virus infection contributes to enhanced binding by upregulation of expression of host cell surface antigens that can act as receptors for Gram-negative bacteria. These results need to be interpreted with caution. HEp-2 cells are a transformed cell line and could be expressing more CD14 and CD18 than epithelial cells *in vivo* as evidenced by the low levels of anti-CD14 and anti-CD18 bound to BEC. While the neuraminidase of influenza virus might make some epitopes more accessible, it is not the only factor that contributes to enhanced binding of the bacterial species examined.

8.3 Characteristics of antibiotic-sensitive and antibiotic-resistant *M. catarrhalis* isolates

M. catarrhalis is increasingly recognised as an important pathogen in the lower respiratory tract, producing exacerbation of chronic bronchitis and pneumonia in adults, especially in patients with compromised respiratory function (Malkamaki *et al.*, 1983). This study showed that binding of *M. catarrhalis* to RSV-infected cells was enhanced for the isolate (MC1) which grew in the presence of the selective antibiotics in NYC medium. Isolate MC2 and 6 additional antibiotic-sensitive isolates did not grow on the

selective medium. These were the only exceptions to the pattern of increased binding observed with RSV-infected HEp-2 cells among all the species tested. The outer membrane protein profiles of the antibiotic-resistant isolate and the antibiotic-sensitive isolates differed at three bands. The antibiotic-resistant isolate MC1 lacked bands at 81 kDa and 66 kDa which were present in the seven antibiotic-sensitive isolates, while the antibiotic-resistant isolate had a distinct band at 19 kDa which was not present in the antibiotic-sensitive isolates.

In the assays for complement-mediated killing, all sera tested had bactericidal activity against the MC2 isolate but no killing was observed for MC1. While in animal models the strain expressing the 81 KDa protein was more rapidly cleared from the lungs (Helminen *et al.*, 1993a), with human monocytes or the THP-1 monocyte cell line phagocytosis or intracellular survival studies showed no differences between the two isolates of *M. catarrhalis*.

If the 81 kDa protein is associated with both sensitivity to complement killing and reduced binding to RSV-infected cells, these isolates might be less likely to cause serious disease following RSV infection. The effects of prolonged use of antibiotics or selection of strains similar to MC1 with characteristics that could contribute to virulence need to be examined. This might be of particular relevance to children treated for recurrent otitis media or patients with chronic lung diseases.

8.4 Smoking and bacterial binding

Cigarette smoking increases the incidence and severity of respiratory infections among patients with chronic bronchitis and obstructive airways disease. In addition, some evidence suggests that it directly affects the incidence and severity of respiratory infections in both healthy smokers and children exposed passively to cigarette smoke.

In addition to physical and physiological damage to mucosa of the respiratory tract, the results obtained in this study indicate it is an independent factor contributing to density of colonisation. BEC from smokers attached significantly more of each of the bacteria species tested. The underlying interactions are not yet clarified, but analysis of expression of host cell antigens that act as receptors for some species did not differ for smokers compared with non-smokers.

8.5 Conclusion

Two environmental factors that affect the respiratory tract, viral infection and cigarette smoke have been associated with susceptibility to invasive bacterial disease. While both active smoking and passive exposure to cigarette smoke are associated with increased risk of respiratory virus infection, this study concludes that smoking and viral infection are two separate risk factors for enhancement of bacterial binding.

8.6 Further studies

1- Additional studies are needed to determine the receptors involved in enhanced binding of Gram-positive species to RSV or influenza infected HEp-2 cells.

2- The components of cigarette smoke which enhanced binding of the bacterial species to epithelial cells from smokers or BEC treated with water soluble components of cigarette smoke need to be identified. For example, CSE from cigarettes with different levels of tar might be examined in the model system to assess the effect of these components on bacterial binding.

3- The results obtained with the two isolates of *M. catarrhalis* in chapter 5 indicate that antibiotic usage might contribute to selection of more virulent strains. This hypothesis could be tested by longitudinal studies of *M. catarrhalis* from patients with chronic pulmonary disease on long term antibiotic treatment and studies on mutants of *M. catarrhalis* derived from selection by antibiotics commonly used in treatment of respiratory tract infections. It has been observed that over prescribing of antibiotics such as erythromycin is more prevalent in area in which there are higher levels of meningococcal disease. Similar studies on selection and characterisation of erythromycin resistant isolates of meningococci might provide evidence to explain the epidemiological observations.

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