Role of infections, cigarette smoke and cytokines in the pathogenesis of chronic obstructive pulmonary disease

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This work is dedicated to

Azra, my wife,

and my children, Hasan, Ali and Zeinab

Declaration

It is to be declared that the thesis presented here has been composed by me, the work therein has been conducted by me or under my direct supervision, and a substantial proportion of the work contributing to the thesis was conducted during my posting in the University of Edinburgh.

Dr. Muhammad Waqar Raza

Acknowledgements

I am certain that I could not have completed this work without inspiration from my wife Azra. Her intriguingly varying colours from showing encouragement to pressing coercion provided me patience and a driving force to complete this work. It was also my attempt towards setting targets for my children difficult to match. Hasan, my elder son, now reading Mathematics in Imperial College, is approaching me swiftly; I am also expecting fierce competition from the other two, Ali and Zeinab, both in AS level showing a promising performance. I am indebted to them for their humorous contributions to the family atmosphere and less demanding mind-set during the periods of intense work.

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As a Muslim I believe that only Allah grants the ability, capability and opportunity to make a difference and that compassionate people around are manifestations of His blessings. With a very humble background and coming from thousands of miles away, I was not expecting to find Kate, Sebastian or Caroline there to help me without Allah's mercy.

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Abstract

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide. The precise sequence of events in COPD is not completely understood. Inflammation in the airways has been unanimously seen by researchers as a pivotal factor, and cigarette smoking without doubt is the main cause. Large proportions of heavy smokers, however, do not suffer with COPD, suggesting a role of additional risk factors, *e.g.*, respiratory tract infections, in pathogenesis. The inflammatory response to cigarette smoke and infectious agents is determined by the host's genetic make up. Cigarette smoking, by altering the surface milieu of respiratory mucosa and by causing immunosuppression increases the susceptibility of individuals to infection with respiratory viral and bacterial pathogens. Virus infection has also been recognized as a susceptibility factor for secondary bacterial infection.

An investigation into the role of individual genetic variations in inflammatory cell and cytokine production and non-host factors involved in COPD is the basis for development of more effective strategies to intervene in pathogenesis, progression and exacerbation of COPD. The aims of this work was to review the evidence for predisposing factors for COPD, with a particular emphasis on respiratory tract infections, and to examine those findings in relation to individual genetic variations and their interactions for induction of pro-inflammatory cytokine production in the respiratory tract.

In vitro models were developed to measure cytokine responses to various agents implicated in COPD. These examined the interaction, antagonistic, indifferent, additive or synergistic, between cigarette smoke and infectious agents or their products on cytokine production. *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis* and respiratory syncytial virus are common bacterial and viral pathogens isolated from this group of patients. A human monocyte cell line in a model provided a consistent means to examine these interactions, and human peripheral blood monocytes from blood donors were used to study the individual variations in the responses. Effects of virus infection on bactericidal activity of human monocytes common bacterial respiratory pathogens were also examined. An epithelial cell line and monocytes were investigated for the effects due to virus infection on expression of some of the surface antigens relevant to bacterial binding and immune response.

The agents used in the study elicited inflammatory responses that could contribute to damage to the respiratory tract and these individual factors could be more harmful in combination. Monocytes from only a proportion of individuals exhibited extreme responses to these agents signifying the role of individual genetic make up in inflammatory processes. Virus-infected monocytes significantly decreased their ability to bind and kill bacteria. Compared with uninfected cells, fewer bacteria bound to virus-infected cells and intracellular bactericidal activity was also decreased. Virus-infected epithelial cells expressed more surface antigens that have been reported to

bind respiratory tract bacterial pathogens, while virus-infected monocytes expressed these antigens at lower levels, which offered an explanation to their decreased bacterial binding and bactericidal activity.

These experimental findings, taken together with the review of the literature presented in this work, suggested that exposure to a number of harmful factors for longer periods in individuals with certain genetic profiles for inflammation may cause significant damage to the respiratory tract resulting in COPD, and exacerbation in its course. Further work to examine the individual genetic make up and inflammatory cytokines in a population of patients with COPD compared with non-COPD smokers would be required to substantiate this hypothesis before investigating the possibility of therapeutic interventions to restrain or modify inflammatory process in COPD.

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Chapter 1

Introduction

1. 1 Chronic obstructive pulmonary disease (COPD): the magnitude of problem

Chronic obstructive pulmonary disease (COPD) is a destructive process in the lungs characterised by features of chronic bronchitis affecting the airways and emphysema affecting lung parenchyma. It is a major cause of morbidity and mortality world-wide. It affects 16 million people in the USA alone [Report by Centers for Disease Control and Prevention, 1993; Borson, *et al.,* 1998; Fiel 1996] and is the fourth leading cause of death [Wise, 1997]. In relation to other major causes, the death rates due to COPD and lung cancer have not declined over the past decades and not are expected to decline [Thom, 1989; Skrepnek & Skrepnek, 2004; Wouters, 2004].

Among the risk factors, cigarette smoking has been the primary determinant of pathogenesis and mortality due to COPD [Kuller, *et al.,* 1989; Bohadana *et al.*, 2004; Marlow & Staller, 2004]. The prevalence of COPD is increasing in countries and communities in which tobacco smoking is

aggressively promoted and marketed. Cessation of smoking does not result in complete reversal of pathological changes caused by chronic smoking. Deaths due to COPD resulted in a loss of 501,290 years of life per year before the expected average age in the USA, and it is feared that due to residual affects of smoking on health this burden would increase despite falling trends in smoking habits [Davis & Novotny, 1989; Wise, 1997; Skrepnek & Skrepnek, 2004; Wouters, 2004].

1. 2 Preview and definitions

1. 2. 1 Architecture of the lungs

Airways represent a serial branching system with which the parenchyma comprising gas-exchange units, called acini, in the lungs connect with the external air and gives rigidity and resilience to lungs. Airways are classified on the basis of calibre into large airways that include trachea and bronchi (about 5 generations of major branches), supported by the cartilage rings and small airways, called bronchioles, consisting of about 15-20 generations of minor branches, tapering into the terminal bronchioles. An acinus consists of a minute air sac, an alveolar duct and an apical bottleneck like structure, the respiratory bronchiole, with which it connects with the terminal bronchiole. With this anatomical arrangement the area of gas exchange for the given volume of the lungs is enormously increased.

Serous and mucous cells line the secretory glands that are present in the large airways. Basal cells, goblet (secretory) cells and ciliated cells are the main types present in the proximal parts of the lower airways while the distal part are lined with Clara cells (cells involved in detoxification) and ciliated cells [Bals, 1997]. The 'ciliary escalator' starts at the most distal parts of the tree and is responsible for bringing up the particulate deposits in the tract. Mechanical, non-specific mucosal and immune defence systems against particulate, chemical and microbial noxious agents that the lungs are continuously exposed to are located in the airways.

1. 2. 2 Emphysema

Emphysema is defined as an irreversible distension of acini distal to the terminal bronchioles, with destruction of alveolar septa without fibrosis [Thurlbeck & Muller, 1994]. Classification of emphysema is based on the pattern of involvement of acini. Centriacinar emphysema (CAE) involves the destruction of the respiratory bronchioles, while both the respiratory bronchioles and the peripheral parts of the acini are destroyed in panacinar emphysema (PAE). The two patterns may coexist in various proportions in the same patient, and result in imbalance of perfusion and ventilation in the lungs and in abnormalities in the lung compliance [Saetta *et al.,* 1994 a & b].

1. 2. 3 Chronic bronchitis

Chronic bronchitis is characterised by the presence of chronic productive cough for more than 3 months for at least two consecutive years in patients in whom other causes of chronic cough have been excluded [Martinez, 1998; Wilson *et al.*, 1996]. These symptoms are due to inflammation of the large airways. Similar changes of the varying severity in the peripheral airways, determine the degree of airflow obstruction, which often accompanies chronic bronchitis [Niewoehner, 1988]. Chronic bronchitis, however, can occur without obstruction although they often coexist.

1. 2. 4 Chronic obstructive pulmonary disease (COPD)

The clinical presentation of COPD is heterogeneous, composed of various combinations of features of emphysema and chronic bronchitis. COPD is characterised by increased airway resistance, hyperinflation and abnormal tests of expiratory flow that do not improve markedly over several months of observation, are worsened by episodes of infections and are only partially reversed by bronchodilator drugs [Senior & Anthonisen, 1998].

The most easily measured indices of obstruction are taken from volume-time plots of forced expiratory manoeuvres. The forced expiratory volume at 1 second (FEV1), after a deep inhalation, is reduced compared with

predicted value for a given sex, age and height. The forced vital capacity (FVC) is also reduced but to lesser degree than FEV1, hence the airway obstruction is characterised by reduced FEV1/FVC percentage. The forced expiratory time (the time taken to exhale fully after a full inspiration) is also invariably prolonged (Figure 1.1).

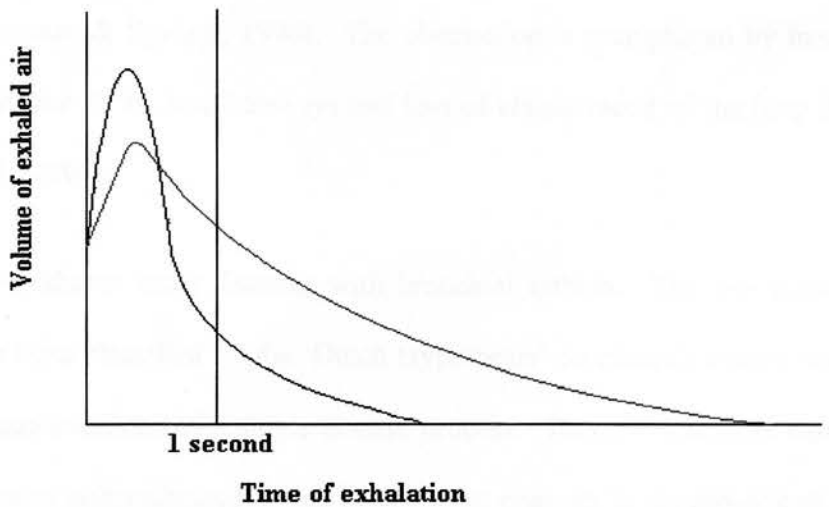


Figure 1.1. Forced exhalation is delayed (blue line) and the peak flow rate is lower in patients with COPD compared with normal individuals.

The mean flow rate over the middle of the vital capacity is frequently more severely diminished and is a more sensitive marker of the disease severity than FEV1. FEV1 is partially effort-dependent while the determinants of

maximal flow rates represent a complex and dynamic interplay among intrinsic airway calibre, elastic recoil properties of the lungs and expiratory collapse of intraparenchymal airways during forced exhalation and thus make the middle flow rate a more sensitive indicator of disease.

The airway obstruction is progressive, and may be accompanied by airway hyperirritability [Postma & Kerstjens, 1998]. The airway hyper-irritability, however, has been shown not to cause COPD [van Schayck *et al.,* 1994; Silverman & Speizer, 1996]. The obstruction is precipitated by increased resistance in the small airways and loss of elastic recoil of the lung [Saetta *et al.,* 1994].

COPD shares many features with bronchial asthma. The two conditions have been classified by the 'Dutch Hypothesis' on chronic airway diseases as manifestations of a single disease process. This hypothesis is based on common histopathological and biochemical changes in the airways of some asthmatic and some COPD patients [van Schayck *et al.,* 1994]. Further support for this hypothesis is sought from bronchial hyperirritability characteristic of bronchial asthma strongly associated with COPD [van Schayck *et al.,* 1994]. Airway inflammation is a hallmark of both conditions, although in asthma this is mainly due to eosinophils and mast cells, and in COPD due to neutrophils and macrophages [Sun *et al.,* 1998]. The differences in the infiltrating inflammatory cells and cytokines (see below) and the degree of hyper-irritability and reversibility of obstruction of

airways between the two conditions form the basis for the 'UK definitions' which classify COPD separately from asthma [van Schayck, *et al.,* 1994; Postma & Kerstjens, 1998; Magnussen *et al.,* 1998; Subramanian & Guntupalli, 1994; Vermeire & Pride, 1991].

The relationship between emphysema, chronic bronchitis and bronchial asthma is illustrated in Figure 1.2.

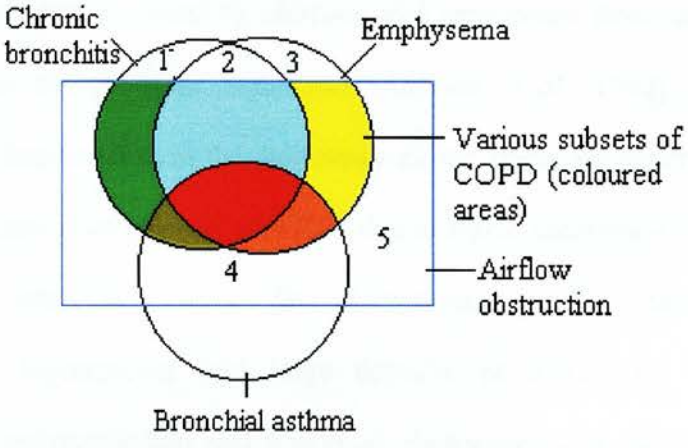


Figure 1.2. Schematic non-proportional representation showing subsets of COPD patients with emphysema, chronic bronchitis and/or bronchial asthma with features of airflow obstruction (coloured areas in the box). The patients without airflow obstruction are not classified as COPD (Areas 1, 2 and 3). Some bronchial asthmatic patients have reversible airflow obstruction and are not classified as COPD (Area 4). Airflow obstruction can be due to causes other than COPD, *e.g* ., cystic fibrosis (represented by the area in the box outside the circles). (Adapted from: No authors listed. Definitions, epidemiology, pathophysiology, diagnosis and staging of COPD. Am J Resp Crit Care Med 1995, 152: S78-S83, with permission of the American Thoracic Society, USA).

1. 3 Pathology

1. 3. 1 Emphysema

CAE, due to its effects on the respiratory bronchioles, causes more airflow limitation than PAE, where recoil abnormalities in the lungs predominate [Saetta *et al.,* 1994]. Damage and degradation of elastic fibres in the alveolar septa are mediated by elastases and proteinases from neutrophils [Hiemstra *et al.,* 1998] or monocytes [Abboud *et al.,* 1998]. Various patterns of degeneration in the pulmonary elastic fibres are observed from tissues obtained from patients with PAE due to $alpha_1$ antitrypsin deficiency or patients with CAE due to fine disruptions, vacuolar changes, fine amorphous degeneration, and large deposits of amorphous material. Incomplete polymerisation and abnormal elastogenesis of the fibres are presumed to be due to abnormal elastinolytic activity in the lungs [Fukuda *et al.,* 1989; Tomashefski *et al.,* 2004] which has been attributed to the presence of excessive numbers of neutrophils and monocytes in the tissue [Ofulue *et al.,* 1998].

1. 3. 2 Chronic bronchitis

The pathological changes in chronic bronchitis consist of abnormalities in the large and small airways and hypersecretion of mucous glands, The submucosa of the large airways is thickened due to hypertrophy of mucus glands [Tamaoki, 1998; Maestrelli *et al.,* 2001; Jeffery 2001].

Compared with normal large airways, the ratio of thickness of submucosa to that of bronchial wall is increased in chronic bronchitis. The small airways are the major site of obstruction are affected by Goblet cell hyperplasia, mucosal and submucosal inflammatory cell infiltration, loss of cilia, oedema, peribronchial fibrosis, smooth muscle hyperplasia and intraluminal plugs. Mucosal abnormalities, inflammatory cellular infiltrate, increased muscle and fibrosis in the bronchi are the hallmarks of chronic bronchitis in smokers [Saetta *et al.,* 1994; Shelhamer *et al.,* 1995; Camner *et al.,* 1973; McDonald 2001].

Growth of myocytes in inflammatory conditions in bronchi might be initiated due to their contact with inflammatory cells and their products [Panettieri, 1998]. Neutrophil defensins and serine proteinases cause injury to the pulmonary epithelium, decrease the frequency of ciliary beat and increase mucus production [Hiemstra *et al.*, 1998]. Neutrophil defensins exhibited chemotactic activity for human blood monocytes in a dose-dependent manner [Tkachenko *et al.,* 1993; Territo *et al.,* 1989] and caused release of tumour necrosis factor-α (TNF- α) from monocytes [Misuno *et al.,* 1992; Chaly *et al.,* 2000].

1. 4 Inflammation in COPD

Chronic inflammation has been widely considered as the underlying process causing damage to the airways and symptoms in patients with COPD.

Understanding the mechanisms and causative agents leading to inflammation in the airways is prerequisite to development of intervention strategies and control of exacerbation in the course of illness. Inflammation is initiated in bronchoalveolar epithelium, which also becomes its first main target [Takizawa, 1998]. The cytokines and chemokines secreted by the inflammatory cells in response to toxic and infectious stimuli are responsible for more inflammatory cells trafficking to the site of inflammation, tissue damage associated with fibrosis and hyperplasia, increased production of sputum and some of the systemic symptoms associated with COPD. A systemic review found higher levels of pro-inflammatory cytokines, C-reactive protein and leucocytosis in patients with COPD compared with healthy people [Gan *et al.,* 2004].

1. 4. 1 Inflammatory cells infiltrating the lungs

Bronchoalveolar lavage (BAL) and collagenase-dissociated lung tissue from rats exposed for one months to cigarette smoke showed infiltration by neutrophils. After one month of smoking, neutrophils were replaced by macrophages that stayed until the end of observation period of 6 months. The elastinolytic activity was demonstrated in samples with macrophages only [Ofulue *et al.,* 1998]. Lung biopsies obtained from non-smokers and smokers during surgery were evaluated for amounts of lung parenchyma per volume and the numbers of neutrophils, macrophages and T cells. Compared with non-smokers, smokers had less parenchyma and this was

negatively correlated with the numbers of macrophages and T cells but not with neutrophils [Finkelstein *et al.,* 1995]. BALs from non-smokers and smokers with or without chronic bronchitis were examined for inflammatory cells. Compared with healthy smokers and non-smokers, smokers with chronic bronchitis had increased macrophages, neutrophils and T cells, while non-smokers with asthmatic bronchitis showed more eosinophils and mast cells [Sun *et al.,* 1998]. Neutrophils are recruited in inflamed lung parenchyma and release of elastase by necrotic neutrophils has been shown responsible for the effect [Kim & Nadel, 2004].

1. 4. 2 Cytokines in the lungs

Cytokines are an intricate network of soluble signalling substances that dictate and control inflammation, immune responses, cellular growth and fibrosis. Cytokines in the lungs can be divided in to three categories, pro-inflammatory, anti-inflammatory and growth-stimulatory. Pro-inflammatory cytokines, *e.g.*, tumour necrosis factor α (TNF-α), interleukin-1 (IL-1) interferons (IFNs) and IL-6, are essential to combat infecting and invading agents [Ferrante, 1989; Nacy *et al.,* 1991; Kawakami *et al.,* 1999; van Schaik *et al.,* 1999; Neuzil *et al.,* 1996; Standiford 1997; Ehlers 2003]; however, their secretion in excessive quantities for prolonged periods can be detrimental to the tissue [Dinarello 2003; Beading & Slifka 2004]. While the primary role of anti-inflammatory cytokines, *e.g* ., IL-4 and IL-10, is to control the expression and effects of pro-inflammatory

cytokines, overproduction of these cytokines might hamper the protective role played by pro-inflammatory cytokines against noxious agents [Oswald *et al.,* 1992; Picard & Casanova 2004; Bastos *et al.,* 2004; Aleman *et al.,* 2000]. Anti-inflammatory effects of IL-4 are well recognised; however, pre-treatment, but not simultaneous treatment, with IL-4 potentiated production of TNF-α from mouse macrophages stimulated with LPS [Major *et al.,* 2002]. Chronic inflammation and fibrosis are often associated with over-production of tissue growth factors [Kim & Nadel, 2004]. TNF-α in most of the studies reported has been depicted as a central and main pro-inflammatory cytokine.

Compared with controls, patients with COPD had higher percentages of IFN-γ-producing CD4+ lymphocytes in their blood, characteristic of TH1 immune response, and lower percentages of IL-4-producing CD4+ cells, characteristic of TH2 immune response [Majori *et al.,* 1999]. TNF- α, IL-6 and IL-8 are involved in inflammation in COPD [Keatings *et al.,* 1996; Barnes, 2004]. Damaged proteins formed in lungs during the process might induce further secretion of pro-inflammatory cytokines [Koj *et al.,* 1994; Kim & Nadel 2004]. BAL from non-smokers with asthmatic bronchitis showed IL-5 and granulocyte-macrophage colony stimulating factor genes and proteins while samples from smokers with chronic bronchitis showed IL-2, TNF-α and IL-8 [Sun *et al.,* 1998]. Production of NO in patients with COPD is increased [Kanazawa *et al.,* 1998; Boulares *et al.,* 2003].

Smoking in young healthy individuals decreases IL-6 response [McCrea *et al.,* 1994; Soliman, 1992] while it increased IL-6 production [Carpegnano *et al.,* 2003; Bucchioni *et al.,* 2003] and IL-1 [Brown *et al.,* 1989; Yamaguchi *et al.,* 1989] in COPD patients. Production of IL-8 from neutrophils and eosinophils is enhanced in COPD in many studies [McCrea *et al.,* 1994; Pesci *et al.,* 1998; Yamamoto *et al.,* 1997; Perng *et al.,* 2004]. Some studies, however, have reported lower levels of IL-8 in COPD [Ohta *et al.,* 1998].

TNF- α is a pro-inflammatory and immune modulating mediator that plays a central role in the pathophysiology of inflammation due to infection, toxic or chemical injury to the tissues. Production of NO is increased in patients with COPD [Kanazawa *et al.,* 1998]. While NO produced at normal concentrations is protective to the airways, in higher concentrations it causes tissue injury and increases inflammation [Kienast *et al.,* 1996; Drumm *et al.,* 1999; Asano *et al.,* 1994]. TNF- α and NO have complex mutual interactions and their production is differentially altered in response to various conditions

1. 4. 2. 1 TNF-α

The suggestion that a substance with anti-tumour activity might exist was first made in the light of spontaneous regression of tumour in some patients following bacterial infections [Beutler, 1989]. This factor, designated as

TNF, was later found as a prototype of a family of molecules involved in regulation of inflammation and immunity [Gruss & Dower, 1995]. The inflammatory cells also exhibit receptors for the TNF superfamily comprising ten receptor proteins. TNF is secreted by many cell types including macrophages, CD4+ and CD8+ T cells [Ware *et al.,* 1992], adipocytes [Kern *et al.,* 1995], osteoblasts [Modrowski *et al.,* 1995], keratinocytes [Lisby *et al.,* 1995], colon epithelium [Jung *et al.,* 1995], mast cells [Bissonnette *et al.,* 1995], dendritic cells [Zhou & Tedder, 1995], pancreatic β cells [Yamada *et al.,* 1993], astrocytes [Lee *et al.,* 1993] and monocytes [Frankenberger *et al.,* 1996].

Human TNF-α is synthesised as a 233 amino acid transmembrane (extracellular C-terminus) or a soluble residue [Gruss & Dower 1995; Pennica *et al.,* 1984; Shirai *et al.,* 1985]. Expressed as membrane protein, TNF-α has an extracellular domain of 176 amino acids, a transmembrane region with 28 amino acids and an intracytoplasmic region of 29 amino acid [Yamaguchi, *et al.,* 1985]. The more potent soluble form [Decoster *et al.,* 1995] consisting of 157 amino acids is created by proteolytic conversion of the transmembrane form by the TNF converting enzyme [Black *et al.,* 1997; Moss *et al.,* 1997]. The soluble form is circulated in the blood as a homotrimer [Smith & Baglioni, 1987] in the range of 10-80 pg ml^{-1} [Spengler *et al.,* 1996].

TNF-α is a potent inducer of endothelial cells for production of intercellular adhesion molecule1 (ICAM-1), which is critical for recruitment of phagocytes [Kyan-Aung *et al.,* 1991]. TNF-α activates macrophages and neutrophils, increases cytotoxicity, releases oxygen and nitrogen radicals [Ferrante, 1989] and other cytokines, IL-6 and IL-8 [Kasahara *et al.,* 1991; Zoja *et al.,* 1991; Kim & Nadel 2004; Paulnock & Coller 2001].

1. 4. 2. 2 Nitric oxide

It has been suggested that NO plays a role in mediating pulmonary injury, and bronchial hyper-reactivity [Henriksen *et al.,* 1999; De Boer *et al.,* 1998]. In the cells, NO is produced by nitric oxide synthetase (NOS). NO in human airway tissue is localized to the airway epithelium, sensory nerves, endothelium, vascular and airway smooth muscles and inflammatory cells [Watkins *et al.,* 1997; Nijkamp & Folkerts, 1995]. It is generally agreed that at lower concentrations NO is protective and regulatory in function; at higher concentrations it acts as a toxic factor. The beneficial pulmonary vasodilatory, bronchodilatory, and bactericidal effects of NO in patients with COPD might be offset by exudate formation, DNA-toxicity and cytotoxicity which NO might cause in unbalanced state [Barnes & Belvisi, 1993; Nussler & Billiar, 1993].

Two distinct types of mRNA encoding for NOS have been described: constitutive, type I; and inducible, type II [Moncada & Higgs, 1993]. The

two types coexist in human alveolar and bronchial epithelial cells and co-ordinate in a complex manner in the epithelial cells to protect the host from microbial assault at the air/surface interface while shielding the host from the induction of airway hyper-reactivity [Asano *et al.,* 1994]. Constitutive NO is responsible for bronchodilation and is protective to the airways [Nijkamp & Folkerts, 1995]. Lack of constitutive NO production may be associated with bronchial hyper-reactivity observed in virus infection [Folkerts & Nijkamp, 1995]. The production of NO is much enhanced during inflammation [Nijkamp & Folkerts, 1995].

Nitric oxide production by a human alveolar type II epithelium-like cell line (A549) and a transformed human bronchial epithelial cell line (BEAS 2B) was enhanced by culture in the presence of interferon gamma, interleukin 1-β, TNF-α and lipopolysaccharide [Asano *et al.,* 1994].

Proinflammatory cytokines released in response to noxious agents modulate vascular contractility, primarily through regulation of inducible nitric oxide (NO), a potent vasodilatory factor [Geng & Hansson, 1992]. Vascular endothelial NO production is constitutively controlled and modulated by bradykinin, acetylecholine and epinephrine. Baseline tone is maintained in partial relaxation due to NO [Moncada & Higgs, 1993]. NO production from alveolar macrophages is enhanced in the presence of virus infections [Panuska *et al.,* 1995, Kharitonov *et al.,* 1995]. NO production is reduced

by episodic or habitual smoking [Kharitonov *et al.,* 1995], which returns to normal on cessation of smoking [Robbins *et al.,* 1997].

1. 5 Aetiology and risk factors

1. 5. 1 Cigarette smoking

Cigarette smoking is without doubt the main cause of COPD. Both cross sectional and longitudinal studies have shown that cigarette smoking causes decline in FEV1, in a dose-dependent response [Narayan *et al.,* 1996; Kuschner *et al.,* 1996]. COPD is associated with the total numbers of cigarettes smoked per year, current smoking status, smoking at an early age and duration of smoking [Sherrill *et al.,* 1990; Davis & Novotny, 1989; Sherrill *et al.,* 1994]. A large proportion of heavy smokers (80-90%) do not show significant decline in FEV1 and do not suffer with COPD [Sherman, 1991]. There are some clinical markers shown to be significantly associated with the risk of development of COPD, *e.g* ., a decreased FEV1/vital capacity with a high nitrogen slope of the alveolar plateau in smokers in their 50s [Stanescu *et al.,* 1998]. The factors that determine whether the disease is mild and short-lived or severe and chronic are, however, not clear. In some individuals, smoking causes well described histopathological [Adesina *et al.,* 1991; Ollerenshaw & Woolcock 1992], cellular [Schaberg *et al.,* 1992; Brown *et al.,* 1989] and biochemical [Soliman & Twigg, 1992; Nagai *et al.,* 1988; Rose *et al.,* 1992]

abnormalities in the airways without causing COPD. These observations indicate the existence of host and environmental factors that might determine the outcome of the injury caused by smoking.

1. 5. 1. 1 Effects of cigarette smoking on the respiratory system

Smoking causes three overlapping patterns of changes in the lungs: in the proximal airways, mucus glands hypertrophy with mucus hypersecretion; in the bronchioles, mucous hyperplasia and metaplasia and smooth muscle hypertrophy and fibrosis; and damage to respiratory bronchiolitis resulting in emphysema [Jeffery, 1991; Littman *et al.*, 2004].

Inhaling habits, smoking style, presence and type of filter and the kind of tobacco smoked might have effects on the pathology in the lungs in response to smoking [Wald *et al.,* 1980; Lange *et al.,* 1990; Wald & Watt, 1997]. The degree of smoking and depth of inhalation of smoke result in either high nicotine uptake and airway smoke particle deposition associated with chronic bronchitis or high alveolar smoke exposure with high CO absorption associated with emphysema [Clark *et al.,* 1998].

The alveolar epithelium is covered with an aqueous liquid, called surfactant, which reduces the surface tension of the alveoli [Higenbottam, 1989]. Surfactant proteins are collagen-like glycoprotiens synthesised by distal pulmonary epithelium. The surfactant also plays a protective role against noxious substances and microorganisms. It facilitates phagocytosis of

microorganism by macrophages and stores opsonins, *e.g.*, immunoglobulins and complement factors [Bisetti, 1989]. Thinning of the surfactant layer caused by smoking [Finley & Ladman, 1972; Betsuyaki *et al.*, 2004] impairs its protective role against toxic injury and microorganisms. The other important mucosal protective mechanism, ciliary movements, is slowed down by small quantities of whole smoke or its aqueous extract [Stanley *et al.*, 1986].

Cigarette smoking is associated with a decrease in natural killer (NK) cell [Nair *et al.*, 1990; Phillips *et al.*, 1985], and neutrophil functions [Venge *et al.*, 1991; Moszczynski *et al.*, 2001]. Alveolar macrophage functions in smokers are also impaired: Fc receptor affinity [Cosio *et al.*, 1982] and C3 receptor expression [Gomez *et al.*, 1982; Moszczynski *et al.*, 2001] are decreased resulting in altered bactericidal functions and defective expression of MHC class II molecules [Lensmar *et al.*, 1998; Mancini *et al.*, 1993]. This affects antigen presenting activity and eventually T-cell mediated responses. Smoking also reduces surface expression of various molecules, *e.g.*, lymphocyte function associated molecules, important in optimum immune and inflammatory response against microbial pathogens [Mancini *et al.*, 1993]. Cigarette smoke may contribute to persistent bacterial colonisation of the airways by immunosuppression and decreased production of cytokines with antibacterial activity, thus increasing likelihood of more frequent episodes of infection [Lean *et al.*, 2004].

1. 5. 2 Respiratory infections

Respiratory infection has been postulated as having a role in the pathogenesis and progression of COPD [Tager & Speizer, 1975; Murphy & Sethi 1992; Fagon & Chastre, 1996; Marin *et al.,* 1989; Verghese & Berk, 1991; Drannik *et al.,* 2004]. Treating exacerbations with antibiotics when bacteria are isolated is a uniform practice. There are, however, two difficulties. 1) While symptoms such as productive cough, purulent sputum and dyspnoea could be due to infection, a non-infectious increase in the underlying inflammatory process could cause similar symptoms. 2) The incidence of microbial isolates from the respiratory tract during exacerbation is not different from that during remission.

Whilst the airways are constantly bombarded with microbial agents, there are efficient microbicidal mechanisms keeping the lungs relatively sterile. Studies comparing bacterial isolates from patients with COPD and healthy subjects have not been reported, but it is likely that more bacteria colonise the lower airways and cause acute-on-chronic disease in COPD patients due to structural derangement and possible interference with the local bactericidal mechanisms. A proposed course of COPD highlighting the role of respiratory tract infections is illustrated in Figure 1.3. Alternatively, virulence of individual strains within a species might be responsible for disease in some patients compared with colonisation with less virulent strains in patients with stable COPD.

1. 5. 2. 1 Microorganisms associated with COPD

Non-typeable *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* are common bacterial species isolated from patients during episodes of exacerbation in the course of COPD and asthma [Calder & Schonell, 1971; Smith *et al.,* 1976; Nicotra *et al.,* 1986; Seddon *et al.,* 1992; Sethi *et al.,* 2000]. Infectious aetiologies in severely ill hospitalised patients with COPD, however, were found to be mostly viral and atypical bacteria; the above bacterial pathogens were responsible for minority of case [Lieberman *et al.*, 2001].

Two reviews [Fagon & Chastre, 1996; Zalacain *et al.,* 1997] did not find differences in isolation of bacterial flora between patients with exacerbation or with stable COPD. Other studies where samples were taken from the lower respiratory tract, however, demonstrated differences [Monso *et al.,* 1995; Pela *et al.,* 1998; Riise *et al.,* 1994; Cabello *et al.,* 1997]. Many of these studies did not measure the density of colonisation by bacterial flora [Beachey, 1981] of the respiratory tract during exacerbation and stable periods in a given patient populations, nor did they consider the effect of viral infections on the bacterial load or on the inflammatory response to resident flora. The presence of bacteria in the normally sterile lower respiratory tract, whether during stable COPD or acute exacerbations, warrants investigation into their role in inflammation. Possible changes in the growth patterns of bacteria might be due to the appearance of as yet

unrecognised host factor(s) in the respiratory tract, changes in the bacterial phenotype [van Alphen *et al.,* 1995] or virus infections. These changes in bacterial colonisation of the lungs during exacerbations are expected to disturb the fine-tuning of the cytokine production contributing to chronic inflammation.

Studies comparing the tracheobronchial microflora during acute exacerbation and during stable periods in the course of COPD showed significant differences in isolation rates of influenza virus, respiratory syncytial virus (RSV) [Mikhalchenkova *et al.,* 1987; Iakovleva *et al.,* 1987] and rhinovirus [Monto & Bryan, 1978]. Evidence of persistent infection with RSV [Krivitskaia & Iakovleva, 1992; Krivitskaia *et al.,* 1996; Iakovleva *et al.,* 1987] and adenovirus [Hogg, 2001] was found in the respiratory tract in patients with COPD.

1. 5. 2. 1. 1 *H. influenzae*

H. influenzae is a small (0.1 x 0.3 μm), non-spore-forming, Gram-negative, pleomorphic coccobacillus (Figure 1.3). Some strains produce a polysaccharide capsule, which determines the type a-f, type b being responsible for most of invasive diseases due to this bacterium. The other strains are non-typeable and non-invasive and cause disease only in subjects with underlying physiological, immunological or anatomical abnormalities. *H. influenzae* is an exclusively human pathogen, which resides principally in the upper respiratory tract of 25-80% of population [Faden *et al.,* 1996]. Non-typeable *H. influenzae* are implicated in infections affecting the course of COPD [Calder & Schonell, 1971].

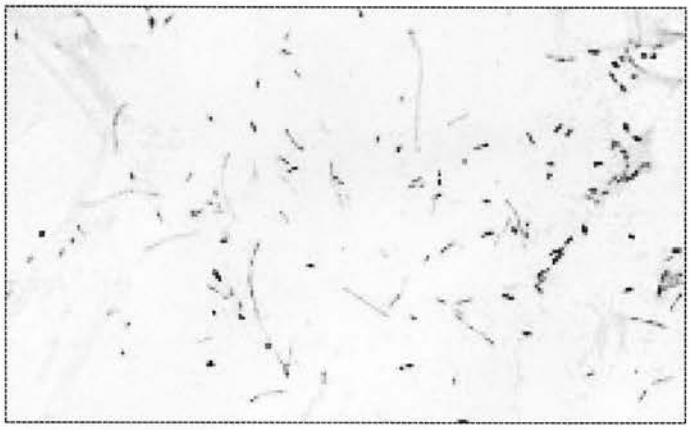


Figure 1.3. Gram negative coccobacilli of *H. influenzae* (x 1000).

1. 5. 2. 1. 2 *S. pneumoniae*

S. pneumoniae, commonly called the pneumococcus, is a member of normal pharyngeal flora of about 24% of the population [Sener *et al.*, 1998], and it is an important human pathogen causing ear, upper and lower respiratory tract infections and meningitis. It is a Gram-positive, non-motile, capsulate diplococcus (Figure 1.4). Virulence has been related to presence of capsule (Figure 1.5) and capsular serotypes have different propensities for types of tissues and different age group affected [Bedos *et al.*, 1999]. Of the 84 recognised capsular serotypes, 6, 19 and 15 were most common in children with chronic inflammatory disease of the respiratory tract [Katosova *et al.*, 1990]. Studies reporting most common serotypes in COPD have not been reported.

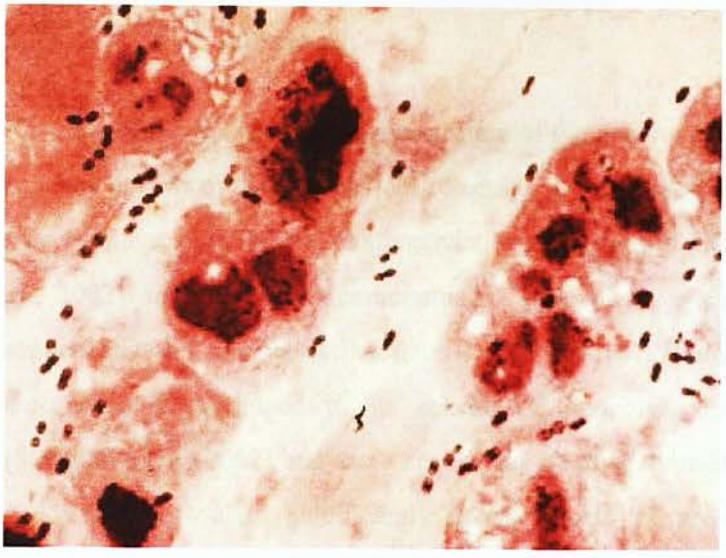


Figure1.4. *S. pneumoniae* in pairs from a sputum specimen (Gram stain, x1000)

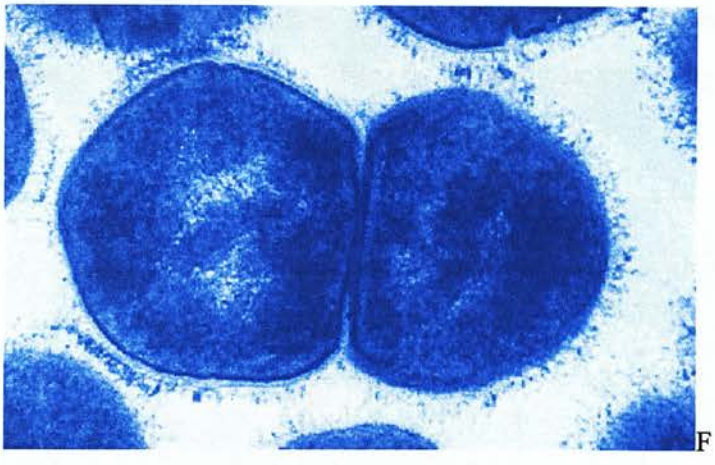


Figure 1. 5. *S. pneumoniae* in pair showing capsule (electron micrograph)

1. 5. 2. 1. 3 *M. catarrhalis*

M. catarrhalis is a part of the normal flora of the upper respiratory tract in as many as 50% of children and 5% of adults. It is responsible for a variety of conditions, otitis media, conjunctivits, exacerbation of COPD [Bakri *et al.*, 2002] and, in immunocompromised hosts, invasive diseases, *e.g* ., septicaemia and meningitis [Verduim *et al.,* 2002]. Pathogenicity of *M. catarrhalis* is attributed to its release of lipopolysaccharides (LPS) [Verduim *et al.,* 2002] and there are indications that some strains express endotoxins that elicit significantly greater levels of pro-inflammatory cytokines [Braun et al., 2002]. The bacteria are non-capsulate, non-motile short rods, and appear as Gram-negative diplococci with long axes parallel (Figure 1.6). Pathogenic strains grow on selective solid media containing antibiotics but most non-pathogenic strains do not. Differences in other attributes, *e.g.*, complement sensitivity and adherence characteristic, have been associated with the differences in antibiotic sensitivity [El Ahmer *et al*, 1996, 1997].

Figure 1.6. Gram negative coccibacilli of *M. catarrahlis* (x 1000)

1. 5. 2. 1. 4 Respiratory syncytial virus (RSV)

RSV is a ubiquitous, important viral respiratory tract pathogen. It causes disease in children [Falsey, 1998; Han *et al.*, 1999]. Half of children are infected in their first year of life and virtually all by the second year [Hall, 1980]. RSV is also an important pathogen in older age groups because complete immunity does not follow RSV disease and reinfections are common [Mlinaric-Galinovic *et al.*, 1996; Sullender *et al.*, 1998]. RSV belongs to the genus *Pneumovirus* in the family of *Paramyxoviridae*. It is an enveloped, large, negative-stranded single-stranded RNA virus, heterogenous in shape and size (Figure 1.7).

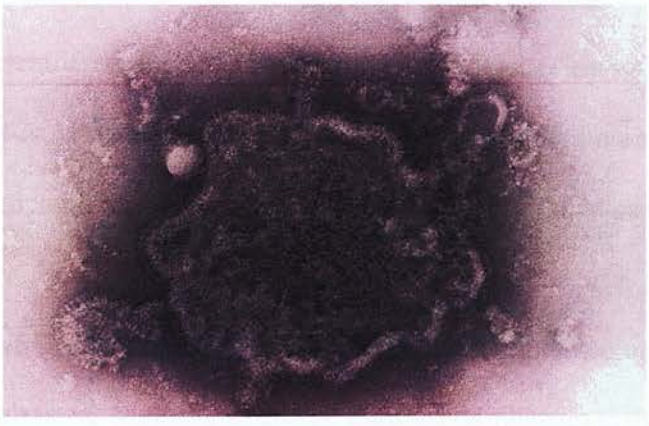


Figure 1.7. Respiratory syncytial virus (electron micrograph).

The virus assembles at circumscribed regions on the plasma membrane of the infected cells and matures by budding during which intracellular nucleocapsid is packaged within an envelope derived from the host cell

membrane [Bachi, 1988]. Two antigenically distinct types of RSV, designated A and B subgroups based on variation of the glycoprotein G, the attachment protein, have been described [Coggins *et al.,* 1998] and associated with severity of disease [Hornsleth *et al.,* 1998].

1. 5. 3 Virus infections predispose to bacterial disease

Clinical, epidemiological and experimental evidence indicates that virus infections can be predisposing factors for bacterial disease [Ramirez-Ronda *et al.,* 1981; Gwaltney *et al.,* 1975; Hament *et al.*, 1999; Levine *et al.*, 2004]. Prior virus infections can predispose patients to secondary bacterial diseases by a variety of mechanisms [Babiuk *et al.,* 1988]. Factors considered to contribute to this effect include: immune suppression [Babiuk *et al.,* 1988; Degre 1986]; local oedema formation and tissue injury; loss of mucocilliary function and decreased bacterial clearance [Camner *et al.,* 1973]; formation of exudate that enhances bacterial growth [Babiuk *et al.,* 1988]; increased bacterial binding to virus infected cells [Raza *et al.,* 1993; Peltola & McCullers, 2004]; diminished phagocytosis [Solano *et al.,* 1998; Franke-Ullmann *et al.,* 1995; Stockl *et al.*, 1999]; and increased production of inflammatory cytokines leading to immunopathology and tissue injury [Beadling & Slifka, 2004].

Infection of epithelial cells with RSV increase binding of respiratory bacterial pathogens to epithelial cells or respiratory mucosa [Raza *et al.,*

1993; Patel *et al.,* 1992; Jiang *et al.,* 1999; Ogra, 2004]. Patients with RSV infections showed a significant rise in antibodies to *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* [Korppi *et al.,* 1989]. Invasive disease due to pneumococci followed infection with RSV [Kim et al., 1996]. Invasive infections due to pneumococci or *H. influenzae* were identified in 26% children suffering with RSV. Similarly, 54% cases of invasive infection with *H. influenzae* and 47% with pneumococci also had evidence of viral infections [Takala *et al.,* 1993].

1. 5. 4 Smoking and viral infections

Smoking affects the respiratory tract by many ways. Smoking enhances the susceptibility of individuals to increased bacterial colonisation. Compared with non-smokers, buccal epithelial cells of smokers bound in higher numbers of *S. pneumoniae* [Mahajan & Panhotra, 1989; El Ahmer *et al.,* 1999; Raman *et al.,* 1983; Cazzola *et al.,* 1990], non-typeable *H. influenzae* [El Ahmer *et al.,* 1999; Piatti *et al.,* 1997; Cazzola *et al.,* 1990] and *M. catarrhalis* [El Ahmer *et al.,* 1999]. Smoking also increases susceptibility to viral and bacterial infection [Marcy & Merrill, 1987].

1. 5. 5 Genetic factors in pathogenesis of COPD

Susceptibility to and/or progressions of some inflammatory conditions have been associated with cytokine gene or major histocompatibility antigen polymorphisms. In a study of a group of patients with systemic lupus

erythematosus in South Africa, a TNF-2 variant at locus TNF-238 was shown to be significantly associated with white subjects and with HLA-DR3, which also showed strong independent association with white ethnicity compared with black [Rudwaleit *et al.,* 1996]. Ethnic differences were found in genotypes of TNF and there linking to MHC alleles [Gallagher *et al.,* 1997]. Associations have been found between certain TNF gene polymorphisms and rheumatoid disease [Mu *et al.,* 1999], myasthenia gravis [Skeie *et al.,* 1999], cystic fibrosis [Hull & Thomson, 1998], primary biliary cirrhosis [Jones *et al.,* 1999], and ulcerative colitis [Bouma *et al.,* 1999]. Similarly, the finding that many cigarette smokers are spared from progressing into COPD is likely to be explained by genetic polymorphisms. TNF2 (G to A replacement at –308 position) polymorphism was over-represented in patients with chronic bronchitis compared with school children and age and sex-matched control group [Huang *et al.,* 1997]. This finding was not, however, confirmed by Ferrarotti *et al.* (2003) in an epidemiological study. Kucukaycan *et al.* (2002) found +489G/A phenotype among several examined to be associated with COPD. In mice, cigarette smoke-related lung injury was not observed in animals with knocked-out TNF-α receptors [Churg *et al.*, 2002]. TNF1 homozygosity, however, was more associated with childhood asthma patients [Albuquerque *et al.,* 1998].

1. 5. 5. 1 Gene polymorphism in cytokine production and susceptibility to infections

Variations in individual susceptibility to infection have aroused interest in the investigation of polymorphism of relevant genes. Immune responses and inflammatory cytokines have been reported to differ in several ethnic groups. Several single nucleotide substitution polymorphisms in the TNF gene have been described: the more regularly reported –238 and –308 and newly identified –574, -856 and –862 [Uglialoro *et al.,* 1998]. A G to A exchange at position -308 of TNF promoter/enhancer region has been described and associated with alteration of TNF expression *in vitro* in some cell lines transfected by the altered gene [Kroeger *et al.,* 1997], but not in some other cell lines [Brinkman *et al.,* 1995] and in patients with sepsis compared with control [Stuber *et al.,* 1995]. It is proposed that single nucleotide mutation in the promoter region of TNF affects the binding of nuclear factors, *e.g.*, kappa B [Christman *et al.,* 1998].

Samples from patient with severe sepsis were genotyped as either homozygous for TNFB1 or TNFB2 or heterozygous, based on the polymorphic site of the restriction enzyme NcoI within the TNF locus. Compared with uneventful sepsis cases, increased TNF secretion and higher prevalence of NcoI-digestion+ alleles TNFB2 were found in patients with sepsis with multiple organ failure and higher death rates. The finding was

more significant for homozygous patients for TNFB2 compared with heterozygous patients [Stuber *et al.*, 1996]. The –308A TNF gene polymorphism has been implicated in a higher susceptibility to cerebral malaria [Wilson *et al.*, 1997] and in sepsis [Hedberg *et al.*, 2004]. Significant associations of the TNF gene promoter polymorphism at the –238 position with chronicity of hepatitis B infection and at the –238 and –308 positions with chronicity of hepatitis C infection [Lu *et al.*, 2004; Hohler *et al.*, 1998; Thio *et al.*, 2004] have been reported. TNF polymorphism at position –308 has also been associated with the severity and outcome of meningococcal disease [Nadel *et al.*, 1996] and with resistance to human cytomegalovirus infection [Hurme & Helminen, 1998]. Significant genetic components associated with both pro-and anti-inflammatory cytokine productions have been reported [Westendorp *et al.*, 1997].

1. 5. 5. 2 Host genetic factors for vulnerability to cigarette smoking and COPD

The finding that many cigarette smokers do not progress into COPD might indicate genetic polymorphism. $Alpha_1$-antitrypsin is an acute phase protein with anti-proteases properties. It is produced in many inflammatory conditions [Nelson *et al.*, 1998]. Deficiency of $alpha_1$-antitrypsin is genetically determined and has been associated with early onset panacinar emphysema [Sandford *et al.*, 1999]. There is some evidence of an

association between the heterozygous state that affects 5-14% of the population and lung function abnormalities [Sandford *et al.,* 1999; Silverman *et al.,* 1998]. It has been postulated that alpha$_1$-antitrypsin protects the lungs by degrading the proteolytic enzymes secreted by inflammatory cells [Prescott *et al.,* 1997]. Cigarette smoke is particularly deleterious to persons homozygous (and possibly heterozygous) for alpha$_1$-antitrypsin deficiency [Silverman *et al.,* 1998].

Analysis of incidence of COPD in affected families indicates the presence of other yet unknown genetic factors [Redline *et al.,* 1989; Rybicki *et al.,* 1990]. Similar conclusions have been drawn from studies of twins and first-degree relatives of patients of COPD [Silverman *et al.,* 1998; Tager *et al.,* 1976 234; Redline *et al.,* 1987]. Decreased prevalence of disease was noticed with increasing genetic distance from the patients with COPD [Fernandez *et al.,* 1994]. Genetic rather than environmental factors correlated with lung function in families [Kauffmann *et al.,* 1989]. Relatives of the cases of COPD showed an increased incidence of COPD, which was significantly associated with blood group A, but not with ABH secretor status [Khoury *et al.,* 1986].

1. 5. 6 Air pollution and occupation

The incidence and mortality rates of COPD are higher in heavily industrialised urban areas [Dutau & Charpin, 1998; Voisin, 1997; Bernstein

et al., 2004]. Associations have been found between the amount of sulphur dioxide and particulate matter in the air and exacerbation of bronchitis [Leuenberger 1995; Simpson *et al.,* 1998; Kappos *et al.*, 2004]. Coal dust exposure has been generally accepted as a causal factor in chronic bronchitis and COPD.

1. 5. 7 Sex, race, and socio-economic status

Men suffer more than women do with respiratory symptoms when the effects are measured independent of smoking [Sherrill *et al.,* 1990; Pride & Soriano, 2002]. Female smokers, however, suffer higher degree of morbidity compared with male smokers [Prescott *et al.,* 1997; Varkey, 2004]. Differences in mortality rates for COPD have been reported for different ethnic groups in Europe [Horne *et al.,* 1989] and in the USA [Hnizdo *et al.*, 2004]. Lower socio-economic status is associated with higher morbidity and mortality due to COPD [Sherrill *et al.,* 1990; Hnizdo *et al.*, 2004].

1. 5. 8 Childhood respiratory virus infections

Significant association between childhood respiratory infections and the later development of chronic bronchitis had been demonstrated [Ding, 1992]. Repeated childhood respiratory infections were shown to have a greater influence than cigarette smoking on the subsequent development of COPD in later life [Barker & Osmond, 1986]. Passive exposure to cigarette

smoke during childhood predisposes to recurrent respiratory infections [Omenaass et al., 1995; Alder *et al.*, 2001; Peat *et al.*, 2001; Sockrider, 2004].

1. 6 Hypotheses

The hypotheses tested in these studies were focused on assessment of the interactions of some of the factors perceived as responsible for contributing to COPD or to exacerbation in its course. Following hypotheses were tested:

- RSV and water soluble cigarette smoke extracts produce additive or synergistic effects on monocytes of an increase in production of TNF-α and/or a decrease in production of NO;
- RSV infection enhances expression of potential bacterial receptors on epithelial cells;
- RVS infection of monocytes decreases their capacity to bind and phagocytose bacterial pathogens of the respiratory tract. It also affects their ability to produce cytokine that are associated with bacterial killing.

1. 7 Objectives

The objectives of the study were

1) To develop an *in vitro* method for measuring cytokine response to water soluble components of cigarette smoke and infectious agents implicated in COPD;

2) To assess inflammatory responses from monocytes from healthy individuals to components of cigarette smoke extract and/or RSV;

3) To develop cell culture models to evaluate the effect of virus infection on cell surface expression of antigens and binding of bacteria;

4) To develop methods to assess the effect of virus infection on phagocytosis and killing of bacteria implicated in COPD.

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 Washing buffers

2. 1. 1 Phosphate-buffered saline (PBS) for washing bacteria

PBS contained 8 mM Na_2HPO_4, 1 mM KH_2PO4, 3 mM KCl and 0.15M NaCl. This composition yielded a pH 7.2±0.1.

2. 1. 2 Dulbecco's PBS for washing cell lines

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

2. 2 Bacterial pathogens

2. 2. 1 Bacterial isolates

There were two isolates each of non-typeable *Haemophilus influenzae* (HI1 and HI2), *Moraxella catarrhalis* (MC1 and MC2) and *Streptococcus pneumoniae* (serotype 3 and 6) from patients with exacerbation of COPD

obtained from the Division of Medical Microbiology, University of Edinburgh. *H. influenzae* and pneumococcal isolates were sensitive to ampicillin. *M. catarrhalis* strain MC1 was able to grow on New York City medium with antibiotics selective for the pathogenic *Neisseria* species (see under). *M. catarrhalis* MC2 did not grow on this medium.

2. 2. 2 Media

Modified New York City medium (MNYC) (Cherwell Laboratories Ltd., UK) (GC medium base (Difco, UK) was supplemented with 10% (v/v) horse blood lysed by saponin (0.5% v/v) yeast dialysate (2.5% v/v), glucose (0.1% w/v), lincomycin (1 μg/ml), colistin (6 μg/ml), amphotericin B (1 μg/ml) and trimethoprim lactate (6.5 μg/ml)) was used to culture *Neisseria meningitidis*. *S. pneumoniae* was grown on Columbia blood agar, and *M. catarrhalis and H. influenzae* isolates on chocolate agar with horse blood. The prepared media were obtained from Oxoid Unipath Ltd, UK.

2. 2. 3 Maintenance, storage and preparation of bacteria

Aliquots of heavy bacterial suspensions in maintenance medium (MM) (2.4.1.2) without antibiotics were stored at -20°C for up to 3 months for use in monocytes (THP-1) bactericidal assays. Concentrations of live bacteria in the frozen samples were determined by plating triplicate samples (5 μl) of appropriate dilutions in PBS on appropriate media for

determination of colony forming units (cfu) after overnight growth in 5% CO_2 at 37°C.

Alternatively, bacterial cultures for use in other experiments were prepared by reconstituting lyophilized strains in distilled water for 20 min and plating them on appropriate media. The cultures were kept overnight at 37°C in 5% CO_2. For storage, colonies were emulsified in Microbank beads (Pro-Lab Diagnostic, Ontario, Canada) and kept at -20°C to use within three months. A fresh bead was used to inoculate the above plates for each set of experiments. Overnight growths on plates were collected in PBS and washed twice by twice centrifugation at 2500 *g* for 10 min before use in the experiments. For each strain the bacterial concentration was determined by light microscopy with a Thoma counting chamber for each experiment. The bacterial suspensions were adjusted to provide a range of ratios of bacteria per cell for use in the assays. .

2. 3. Labelling of bacteria

2. 3. 1 Fluorescein isothiocyanate (FITC) labelling

FITC (Sigma, Poole, Dorset, UK) was used for labelling the bacteria in some assays. The solution of FITC (0.4% w/v) was prepared in a buffer containing sodium carbonate (0.05 M) and sodium chloride (0.1 M) (pH 9.2). FITC solution was prepared immediately before use in each experiment.

The washed bacterial pellet from culture plates was suspended in 2 ml of FITC solution by gentle shaking at 37 C for 20 min. The FITC-labelled bacteria were washed three times with DPBS and resuspended in MM (2.4.1.2) without antibiotics. The bacterial concentration was adjusted as described above.

2. 3. 2. Ethidium bromide (EB) labelling

In some experiments the bacteria were labelled with EB. Bacteria in suspension were washed with PBS and fixed with 1% (V/V) buffered paraformaldehyde (Sigma) for 30 min in a water bath at 37^{o}C. The bacteria were washed twice with PBS and incubated with 50 μg ml^{-1} EB (Sigma) for 20 min in a water bath at 37^{o}C. Samples were washed twice to remove unbound stain,

2. 4 Tissue culture cell lines and media used

All the cell lines used were tested for mycoplasma with direct fluorescent assay using fluorochrome Hoechst No. 33258, a nucleic acid stain [Hessling et al., 1980]. The cells were grown for 24-48 hours in antibiotic free medium on cover slips (about 2 x 10^4 cells) in shell-vials. The cells were fixed with acetone and incubated for 30 min with the stain (0.05 μg/ml in Hanks Medium without sodium bicarbonate). The monolayers were washed with PBS to remove the excess and examined at x 400

magnfication for cytoplasmic bacteria using fluorescent microscope (Fluorescence, Leitz, Ortholux).

2. 4. 1 Cell culture media

2. 4. 1. 1 Growth medium (GM)

GM consisted of RPMI-1640 medium (Gibco, Paisley, UK) supplemented with foetal calf serum (FCS) (Gibco) (10%, v/v), 100 U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin sulphate (Sigma, Poole, Dorset) and 1m M L-glutamine (Gibco). RPMI-1640 was replaced in some assays with Dulbecco's Modified Eagle's medium (DMEM) (Gibco, BRL, Glasgow, UK) containing D-glucose (0.45 % w/v) and pyridoxine (4 mg L^{-1}). The rest of the additives remained the same,

2. 4. 1. 2 Maintenance medium (MM)

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

2. 4. 2 Cell cultures

2. 4. 2. 1 HEp-2 cells

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

Confluent monolayers of HEp-2 cells were obtained in 25 cm^2 tissue culture flasks (Costar) by growth in GM (containing RPMI-1640). The monolayer was rinsed twice with phosphate buffered saline (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. For the experiments, the cells were treated with EDTA only to preserve surface molecules from the enzymatic degradation by trypsin. 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% (v/v) 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. The cells to use in experiment were adjusted to 1 X 10^6 ml^{-1} in MM.

2. 4. 2. 1 THP-1 cells

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

described above, continually grown in 25 cm^2 tissue culture flasks (Costar) in GM containing RPMI-1640 and mercaptoethanol (2×10^{-5} M) at 37°C in 5% CO_2. The cells were harvested in MM without antibiotics and adjusted at 1×10^6 ml^{-1} for use in the experiment.

2. 4. 2. 3 Buffy coat preparations

One-day-old buffy coats from blood donors of group O, Rh+ screened for presence of blood-borne viruses were obtained from the Scottish National Blood Transfusion Service (SNBTS), Royal Infirmary, Edinburgh. Buffy coats were diluted 1 in 4 with sterile phosphate buffered saline (PBS). The diluted preparations (40 ml) were layered over 12 ml of Histopaque 1077 (Sigma, Poole, Dorset, UK) in 50 ml polypropylene conical tubes (Greiner, Gloucestershire, UK). The tubes were centrifuged at 400 *g* for 30 min at 25°C. Mononuclear cells in the opaque band formed at the interface of plasma and Histopaque were aspirated carefully. The cells were washed twice in sterile PBS at 150 *g* for 10 min and the supernatant discarded. The cells were resuspended in 20 ml of GM containing DMEM, transferred to a 75 cm^2 tissue culture flask (Greiner) and incubated at 37°C for 30 min to separate monocytes from non-adherent cells. The medium containing non-adherent cells was poured off and the monocytes harvested by gentle scraping with a cell scraper in 20 ml of fresh GM. A viable count was performed using the Trypan blue dye

exclusion method and the concentration of monocytes adjusted to $1X10^6$ ml^{-1} in GM. The cells (1 ml) were distributed in 24-well tissue culture plates (Costar) with $1X10^6$ cells in each well. Viability of cells at the end of each experiment was also assessed by microscopic examination of cells in the wells for exclusion of trypan blue.

2. 5 Standardisation of respiratory syncytial virus (RSV)

2. 5. 1 RSV stock

The Edinburgh strain of RSV (subgroup A) was grown in HEp-2 cells. One day old monolayers of HEp-2 cells were infected with at multiplicity of infection (MOI) of 2-3 infectious particles per cell for one h. The monolayer was maintained for 48 to 72 h at which time the cells started fusing due to virus infection. The flask was frozen at -70°C and thawed to lyse the cells to release the viruses. The virus suspension was adjusted to $2x10^6$ plaque forming units ml^{-1} using plaque assay described below. Aliquots of the suspension were stored at -70°C for use in the experiments.

2. 5. 2 Plaque assay

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 h at 37°C. 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 was added to each; this consisted of (methyl cellulose (3% w/v) (Sigma) in Hank's buffered salt solution (Gibco) and $NaHCO_3$ (2 g l^{-1}) mixed with maintenance medium at a ratio of 1:3. 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 formaldehyde in 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 (Olympus CK2, Japan).

2. 6 Immunofluorescence reagents for detection of RSV-infected cells

Antibodies from three sources directed to RSV antigens were used to detect RSV-infected cells in different assays. The RSV reagent (Imagen, Dako Diagnostics Ltd, UK) contains monoclonal antibodies conjugated to FITC. These conjugated antibodies bind specifically to viral antigens conserved among RSV subgroups. The reagent was used in a one-step direct immunofluorescence technique. Coverslips of HEp-2 cell

preparations were fixed in acetone for 10 min then 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 min. The coverslips were mounted and examined using fluorescence microscopy.

Alternatively, the cells in suspension (HEp-2, THP-1 or human blood monocytes) were assessed for RSV infection with monoclonal anti-G glycoprotein of RSV (mouse), kindly provided by Professor PJ Watt, Southampton University, detected with FITC-conjugated rabbit anti-mouse immunoglobulin (Serotec) (1/100). In some assays RSV- infected cells were detected using a convalescent serum from a patient with RSV infection (*i.e.*, polyclonal human anti-RSV) previously absorbed with THP-1 cells and appropriately diluted in PBS. FITC-conjugated anti-human immunoglobulin antibodies (Sigma) were used to detect primary antibodies on cells. The fluorescent RSV-infected cells were analysed by flow cytometry (Section 4.2.4).

2. 7 Statistical methods

In comparative data analyses, two-tail, paired *t*-tests were employed to test the significance, and values obtained were compared with non-parametric tests. The data from measurements of cell surface antigens expression were first converted to logarithms before testing with paired *t*-test. Wilcoxon's tests were used to analyse intracellular survival of bacteria.

Chapter 3

The effect of infection with respiratory syncytial virus and water-soluble components of cigarette smoke on production of inflammatory mediators

3. 1 Introduction

The influence of bacterial and viral infections and non-infectious air pollutants such as cigarette smoke on the inflammatory and immune responses underlies the pathological processes in the respiratory tissues. A number of reports have examined the release of inflammatory mediators from alveolar macrophages and there have been clinical and experimental studies on smoking and virus infection as contributory factors to COPD [Silverman & Spezier, 1996; Monto, 1995; Wedzicha, 2001]. Both TNF-α and NO are important inflammatory mediators in COPD and asthma [Barnes & Belvisi, 1993; de Godoy *et al.*, 1996; Keating *et al.*, 1996; Gan *et al*,. 2004; Churg *et al*,. 2004: Shao *et al*,. 2004; Yildiz *et al*,. 2003].

Infection with RSV induces release of TNF-α and NO from human alveolar macrophages, bovine peripheral blood mononuclear cells and a murine monocyte cell line [Panuska *et al.*, 1994; Frank *et al.*, 1994; Dietzscold, 1995]. There are, however, conflicting reports on the effect of smoking on TNF-α release [Tappia *et al.*, 1995; Sauty *et al.*, 1994; Kharitonov *et al.*, 1995; Laan *et al,*. 2004; drannik *et al,*. 2004] and NO release [Kharitonov *et al.*, 1995; Alving *et al.*, 1993; Marteus *et al,*. 2004; Warke *et al,*. 2003; Horvath *et al,*. 2004]. Both episodic and habitual smoking reduced NO exhalation [Kharitonov *et al.*, 1995]; but, in pigs challenged with cigarette smoke, a vasodilator response due to NO release was recorded [Alving *et al.*, 1993].

Both blood monocytes and alveolar macrophages can be infected with RSV (Becker *et al.*, 1992; Adair *et al*, 1992) and both cell types are expected to be exposed to water-soluble components of cigarette smoke absorbed across mucous surfaces. The aim of the present study was to assess the effect of a water-soluble cigarette smoke extract (CSE) on release of TNF-α and NO from peripheral blood monocytes infected with RSV. Because there is evidence that TNF-α responses are under genetic control, which can influence severity or fatal outcome of infection, [Westendorp *et al.*, 1995, 1997; Gander *et al,*. 2004; D'Aiuto *et al,*. 2004], the study examined monocytes from different blood donors to assess individual variations in TNF-α and NO responses to CSE and RSV infection.

In this chapter release of TNF-α and NO from human blood monocytes challenged with either RSV, a water-soluble cigarette smoke extract (CSE) or both were evaluated. Since many virus infections stimulate release of interferonγ (IFN-γ) [Roberts *et al.*, 1992] that might in turn mediate other secretory functions, the effect of IFN-γ on release of TNF-α and NO in this system was also analysed. Nicotine is metabolised in the liver to cotinine, which is secreted in body fluids including those of the respiratory tract [Berkman *et al.*, 1995]; therefore, the effects of nicotine and cotinine on TNF-α and NO release were also examined.

3. 2 Methods

3. 2. 1 Preparation of cigarette smoke extract

A water-soluble extract of cigarette smoke (CSE) was prepared (Fig 3.1) by the use of a vacuum pump to pass the smoke of 10 filter-tipped cigarettes (Benson & Hedges) through 100 ml of Dulbecco's Modified Eagle's medium (DMEM) (Gibco) containing 0.45 % w/v D-glucose and 4 mg L^{-1} pyridoxin (Higashimoto, *et al.*, 1992). To reduce LPS contamination, the glass bottles used were heated at 134°C for 1 hr. The CSE was filtered with a 0.2 μm filter (Millipore) and aliquots stored at -20°C for a maximum duration of 2 weeks.

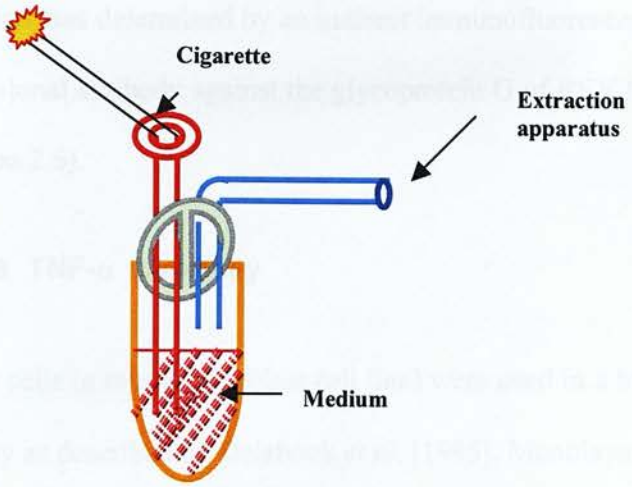


Figure 3.1. Apparatus used to prepare water-soluble extract of cigarette smoke.

3. 2. 2 Stimulation of monocytes

RSV stock was prepared as described in section 2.5.1 and used at a multiplicity of infection of 2 (Panuska *et al.*, 1995). The monocytes (2.4.2.3) were cultured at 37°C in 5% CO_2 for 24 hr in 1 ml of GM, CSE, RSV or with both CSE and RSV. Samples were collected from each well after 48 hr for determination of TNF-α (100 μl) and after 72 hr for determination of NO (400 μl). Negative control samples to which no cells were added included culture medium alone, medium with CSE and/or RSV. The samples were kept at -20°C until analysed.

The proportion of monocytes infected with RSV in each sample 24 hr post-infection was determined by an indirect immunofluorescence technique with monoclonal antibody against the glycoprotein G of RSV (Raza, *et al.*, 1993) (section 2.6).

3. 2. 3 TNF-α bioassay

L-929 cells (a mouse fibroblast cell line) were used in a bioassay for TNF-α activity as described by Delahook *et al.* [1995]. Monolayers were cultured in GM and maintained by splitting 1:10 twice weekly. Cells were dislodged by a mixture of trypsin (0.005%, w/v) and EDTA (0.02%, v/v) and resuspended in GM to 3.3 X 10^5 cells ml^{-1}. Cells were dispensed in flat-bottom microplates (Greiner) at 100 µl per well and incubated at 37°C in 5%CO_2 for 20 h. GM in each well was then aspirated, discarded, and replaced with 100 µl of MM with 2 µg of actinomycin D ml^{-1} to stop further cell growth without killing the cells.

The supernatant (100 µl) from stimulated monocytes was diluted 1:5 in MM and added to wells in triplicate. The positive control consisted of ten fold serial dilutions of a standard of recombinant TNF-α (National Institute for Biological Standards and Control, Potters Bar, United Kingdom) at a starting concentration of 1,000 IU ml^{-1}. Negative controls consisted of wells without TNF-α. Plates were covered and incubated at 37°C in 5%CO_2 for 24 h. The medium was aspirated. To each well the surviving cells were stained

with 100 µl of crystal violet solution containing crystal violet (0.5% w/v) in 20% in distilled water with methanol (20% v/v), filtered through a filter of 0.22 µm pore size before use. After 2 min, the plates were washed gently under tap water to remove the excessive stain and dried with a hair dryer. Each well received 100 µl of acetic acid (20%v/v), and the plates were read at 585 nm on a plate reader. The content of TNF-α bioactivity was calculated as percent of cells killed compared with controls.

3. 2. 4 Detection of NO

NO was detected as nitrite (ng ml^{-1}) by the spectrophotometric assay described by Zhang *et al* (1994). The samples were clarified by centrifugation at 12,000 rpm with a microcentrifuge (Sorval MC 12C, Dupont) for 5 min. Supernatants (400 µl) were reacted with equal volumes of Greiss reagent which contained naphthylethylenediamine dihydrochloride (0.3%, w/v) (Sigma), and sulphanilamide (1% w/v) (Sigma) in orthophosphoric acid (5%, v/v) (BDH, Poole, UK), mixed 1:1 immediately before use. After incubation for 10 min at room temperature, the absorbance at 540 nm was determined with a spectrophotometer (Jeway 6100). Concentrations of nitrites in the samples were derived from a standard curve for sodium nitrite concentrations ranging from 0.01 to 1.0 nM prepared for each experiment.

3. 2. 5 Statistical methods

The results obtained with buffy coats from 24 donors were assessed. The results from some samples for some treatments could not be included due to contamination of individual wells; therefore, the mean control values corresponding to different experiments were not all the same. The data from monocytes incubated with different agents were compared with those from monocytes incubated with medium only. Comparisons were made also between different treatment groups. The significance values obtained with paired *t* tests of the data were similar to values obtained with a non-parametric test (Wilcoxon's). The values obtained from *t* tests are presented here.

3. 3 Results

3. 3. 1 RSV infection of cells

On average, at 24 h post-infection more than 40% of monocytes from each individual tested were infected with RSV. One way 'analysis of variance' indicated no significant differences in the proportion of RSV-infected cells among the donors.

3. 3. 2 Assay standardisation

Ten-fold dilutions of CSE ranging from 0.1 to 0.0001 cigarette ml^{-1} were tested with monocytes from 4 donors, and a dilution of 0.001 cigarette ml^{-1} was selected for the assays on the basis of maximum effects on the production of TNF-α and NO without killing the monocytes (Figure 3.2). Doubling dilutions of IFN-γ, nicotine and cotinine ranging from 400 ng ml^{-1} to 6.25 ng ml^{-1} were tested [Geng *et al*, 1995]. A dose of 25 ng ml^{-1} for these reagents was used for further study (Table 3.1). More than 90% of monocytes survived until the end of the experiments under the conditions selected for the assays.

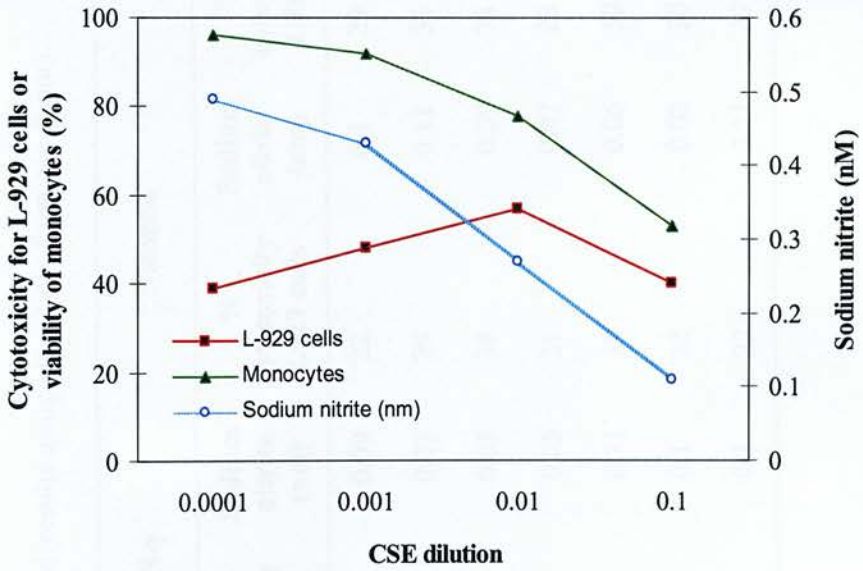


Figure 3.2. Viability of monocytes, cytotoxicity of L-9292 cells due to TNF-α and levels of sodium nitrite produced by monocytes incubated with various dilutions of CSE. (The values cell viability obtained with tests substances were compared with those from test cells incubated with medium only taken as control).

Table 3.1. TNF-α bioactivity (% cytotoxicity for L929 cells) and nitrite accumulation in response to IFN-γ, nicotine and cotinine (results using monocytes from one donor).

Quantity in ng ml^{-1}	IFN-γ		Nicotine		Cotinine	
	% cytotoxicity L929 cells	Sodium nitrite (nm)	% cytotoxicity L929 cells	Sodium nitrite (nm)	% cytotoxicity L929 cells	Sodium nitrite (nm)
6.25	27	0.09	22	0.1	29	0.08
12.5	25	0.07	29	0.11	33	0.09
25	38	0.05	38	0.09	38	0.28
50	40	0.05	21	0.07	23	0.39
100	55	0.11	39	0.06	50	0.25
200	30	0.1	35	0.08	30	0.33
400	37	0.1	22	0.11	37	0.38

Time course experiments with monocytes from 4 donors (6-72 h) found the maximal TNF-α bioactivity occurred at 48 hours and nitrite accumulation at 72 hours in response to CSE (Figure 3.3).

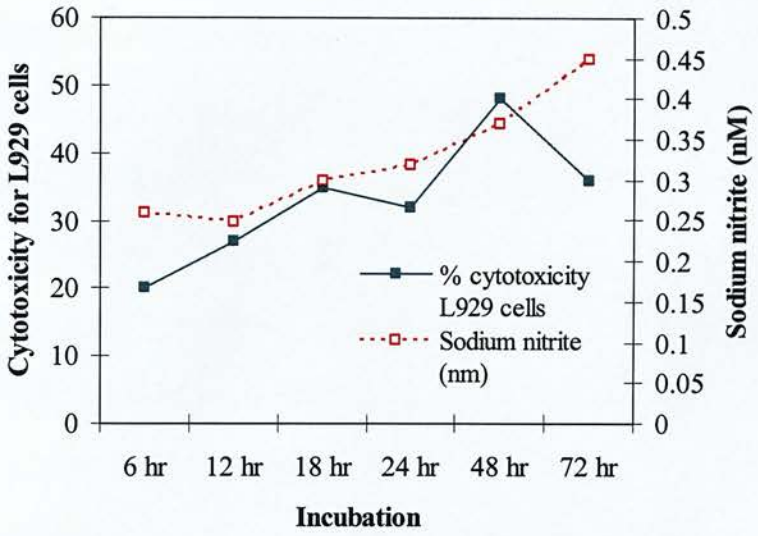


Figure 3.3. Time course experiments with monocytes from donors showing maximal TNF-α bioactivity and nitrite accumulation in response to CSE.

TNF-α activity was not detected in control samples without cells containing culture medium or medium with CSE, RSV, CSE and RSV, IFN-γ, nicotine or cotinine. For detection of nitrites, the spectrophotometer was blanked on these individual controls for assessment of their respective test samples. No

effect due to presence of these agents was recorded at the optical density used to detect sodium nitrite.

3. 3. 3 The effect of CSE and RSV infection on TNF-α bioactivity

The TNF-α bioactivities expressed as percent cytotoxicity of L-929 cells observed in experiments with monocytes from a total of 24 donors were compared. Figure 3.4 represents paired differences in L-929 cytotoxicity due to TNF-α bioactivities caused by different treatments of monocytes compared with monocytes incubated with medium only. Compared with supernatants from cells incubated with medium alone (mean cytotoxicity 48%, SE 5.2), supernatants from cells incubated with CSE (mean cytotoxicity 60%, SE 5.1) had significantly increased TNF-α bioactivities (95% CI of paired differences 3.7,19.7, t = 3.05, P = 0.006) as did the supernatants from RSV-infected cells (mean cytotoxicity 68%, SE 4.6) (95% CI of paired differences 16.4,34.15, t = 5.9, P = 0.000). There was no correlation between the % cytotoxicity for L-929 cells and proportions of virus-infected cells in RSV-infected samples.

Compared with TNF-α bioactivities detected in supernatants from cells (15 donors) exposed to medium alone (mean cytotoxicity 38%, SE 5.4), a significant increase was observed in supernatants from cells incubated with both CSE and RSV (mean cytotoxicity 71%, SE 6.6) (95% CI of paired differences 15.4,49.5, t = 4.08, P = 0.002).

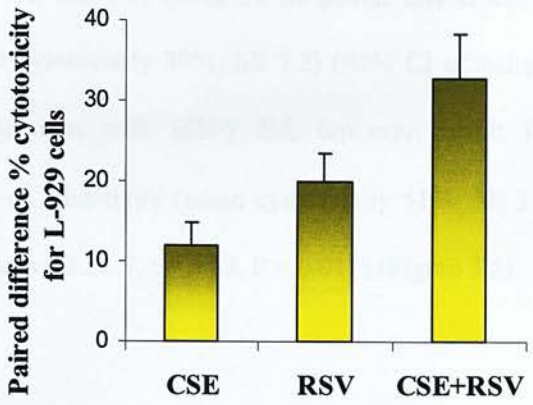


Figure 3.4. Paired difference in TNF-α bioactivity expressed as % cytotoxicity for L-929 cells from monocytes of individual donors exposed to medium only or to CSE and/or RSV. Error bar represent standard errors.

Compared with TNF-α bioactivities of monocytes from 15 donors exposed to CSE alone, the bioactivities observed with the combination of CSE and RSV infection were significantly higher (paired differences 26%, 95% CI 10.4,41.3, t = 3.58, P = 0.003). Compared with levels of TNF-α found for cells incubated only with RSV, addition of CSE did not significantly increase TNF-α bioactivities (95% CI -12.9,21.5). This indicates that the main contribution to increased levels of TNF-α was due to the virus infection.

Compared with TNF-α bioactivity of monocytes from 6 donors exposed to medium only (mean cytotoxicity 32%, SE 7.5), there was no significant increase observed in supernatants of cells incubated with nicotine (mean

cytotoxicity 34%, SE 8.7) (95% CI of paired differences -14.2,15.4) or cotinine (mean cytotoxicity 39%, SE 7.2) (95% CI of paired differences -1.6,15.2). Incubation with IFN-γ did, however, result in significantly increased TNF-α bioactivity (mean cytotoxicity 51%, SE 3.0) (95% CI of paired differences 6.2,31.7, t = 3.83, P = 0.012) (Figure 3.5).

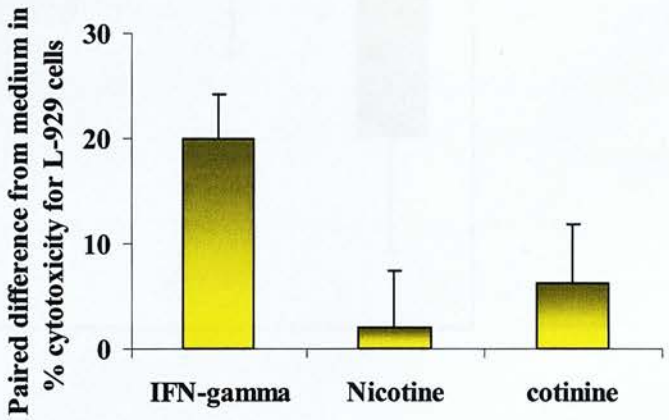


Figure 3.5. Paired difference in TNF-α bioactivity expressed as % cytotoxicity for L-929 cells from monocytes of individual donors exposed to medium only or IFN-γ, nicotine or cotinine.

3. 3. 4 The effect of CSE and RSV infection on NO release from monocytes

Since exposure to cigarette smoke can be considered to be a common or constant risk factor for smokers, the interaction between RSV was assessed

in relation to CSE as control. The supernatants from cells in the same experiments were examined for nitrite levels. Paired differences between nitrite levels resulting from different treatments of monocytes compared with those incubated with medium alone are given in Figure 3.6.

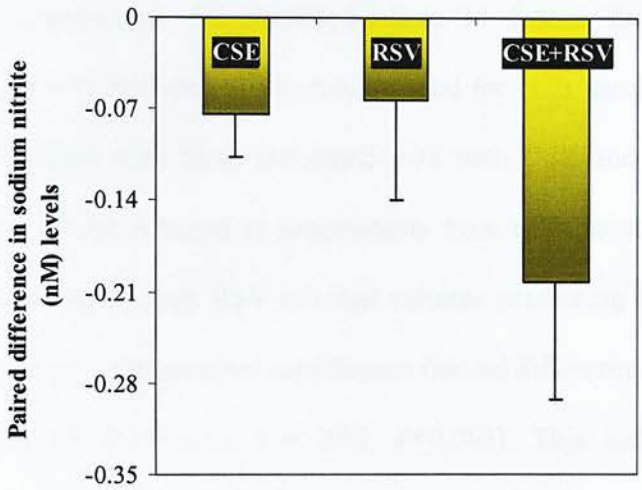


Figure 3.6. Paired difference sodium nitrite production from monocytes of individual donors exposed to medium only or CSE and/or RSV.

Compared with supernatants from cells incubated with medium only (mean 0.41 nM, SE 0.09), supernatants from cells incubated with CSE (mean 0.34 nM, SE 0.08) had significantly lower levels of nitrite (95% CI of paired differences -0.14,-0.008, $t = 2.3$, $P = 0.031$) as did RSV-infected cells (mean 0.35 nM, SE 0.05), but the results were not significant (95% CI of paired differences -0.023,0.099). There was no correlation between the levels of

nitrite detected and ratios of RSV-infected cells in the samples. Compared with cells incubated with medium only, cells incubated with both CSE and RSV showed a significant decrease in nitrite production (mean 0.25 nM, SE 0.06) (95% CI of paired differences -0.4,-0.01, t = 2.26, P = 0.04).

In experiments with monocytes from 15 donors, there was no significant difference between nitrite levels found for cells incubated with CSE alone compared with those incubated with both CSE and RSV. In comparison with nitrite detected in supernatants from cells incubated with RSV only, supernatants from RSV-infected cultures containing CSE had lower levels of nitrite with marginal significance (paired difference -0.098 nM, SE 0.048, 95% CI -0.006,-0.2, t = 2.02, P=0.063). This indicated that the main decrease in nitrite levels was caused by CSE.

Experiments with cells from 6 individual donors showed that, compared with nitrite levels observed with cells incubated with medium only (mean 0.10 nM, SE 0.03), addition of INF-γ (mean 0.15 nM, SE 0.03) (95% CI 0,0.057) or nicotine (mean 0.13 nM, SE 0.03) (95% CI 0,0.03) to cells did not result in significant changes in nitrite release, but addition of cotinine significantly increased release of nitrite from cells (mean 0.26 nM, SE 0.03) (95%CI of paired differences 0.03,0.15, t = 3.48, P = 0.018) (Figure 3.9).

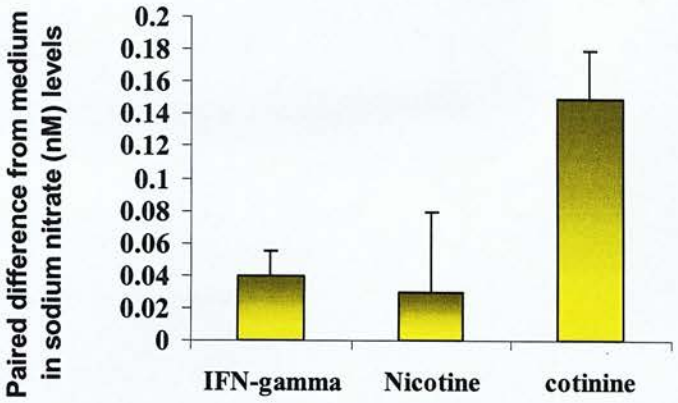


Figure 3.9. Paired difference sodium nitrite production from monocytes of individual donors exposed to medium only or IFN-γ, nicotine or cotinine.

3. 3. 5 Variability of TNF-α and NO responses of individual donors

Each set of experiments was carried out with cells from different donors. Individual TNF-α and NO responses to CSE, RSV infection, separately and in combination, are summarised in Table 3.2. TNF-α and NO responses of the test samples were arbitrarily classified as very high if the levels of cytotoxicity for L-929 cells or levels of nitrites were more than twice the value for the controls in which monocytes were cultured with medium alone. Responses were classified as very low if the levels of test samples were less than half the control. The most common pattern observed was increased TNF-α bioactivities and decreased NO production compared with

controls in which cells were incubated with medium only. The proportions of extreme responses in the presence of CSE (4%) or RSV (4%) increased in the presence of CSE and RSV (20%).

Cells from two individuals showed extreme responses (very high TNF-α and very low NO) to RSV or RSV+CSE and one individual showed very high response to RSV+CSE only.

Table 3.2. Individual responses to CSE and/or RSV.

		Percent			Percent with extreme response*		
Response to	**Measured**	**High**	**Low**	**↑TNF + ↓Nitrite**	**High**	**Low**	**↑TNF + ↓Nitrite**
CSE	**TNF**	75	25	42	12	0	4
(n = 24)	**Nitrite**	33	63		0	12	
RSV	**TNF**	92	8	33	25	4	4
(n = 24)	**Nitrite**	50	46		21	25	
CSE+RSV	**TNF**	87	13	67	48	7	20
(n = 15)	**Nitrite**	13	87		7	48	

*high ≥ 2 x control; low ≤ ½ control.

3. 4 Discussion

3. 4. 1 The model system

RSV is a common pathogen affecting infants and the elderly [Hall, 1980]. Complete immunity does not follow exposure to RSV, hence reinfections are not uncommon [Hall *et al.*, 1991]. Significant numbers of patients with COPD or bronchial asthma suffer bouts of exacerbation and possible residual effects due to RSV infection in the course of their disease [Philit *et al.*, 1992]. Cigarette smoking is a major cause of COPD [Silverman & Spezier, 1996]. Both viral infection and cigarette smoking enhance bacterial binding to epithelial cells in model systems [Raza *et al.*, 1993; El-Ahmer *et al.*, 1994; Saadi *et al.*, 1993, 1997] and cause immunosuppression [Babiuk *et al.*, 1988; Philips *et al.*, 1985]. Since TNF-α and NO are important mediators of inflammation in the respiratory tract, TNF-α and NO responses of blood monocytes to RSV infection and CSE were assessed.

Peripheral blood monocytes were used in the study for four reasons, 1) their ready availability in sufficient numbers; 2) alveolar macrophages in the lungs are derived from monocytes; 3) they were less likely to have been exposed to respiratory viruses or air pollutants; 4) they were more likely to

be in an unstimulated state. Some stimulation of monocytes due to the use of histopaque for isolation of monocytes could not be ruled out. There is evidence that genetic factors contribute to levels of TNF-α in response to endotoxins and the toxic shock syndrome toxin [Westendorp *et al.*, 1997; Blackwell et al., 2002;D'Aiuto *et al*,. 2004]. The variable spontaneous TNF-α and NO release from monocytes could be attributed to the individual donor's genetic make up or condition at the time of blood donation, recent virus infection, active smoking, passive exposure to cigarette smoke, or variable responses of monocytes to histopaque. The assays examined the responses to various agents in relation to background levels of each individual donor.

The dose of CSE (0.001 cigarettes ml^{-1}) used in the assays was similar to dilutions of smoke extract used in experiments with alveolar macrophages [Higashomoto *et al.*, 1992]. It was based on the range of numbers of cigarettes an average person can smoke and the water soluble components of the inhaled smoke that cross the mucosal lining and are diluted in the body fluids. .

Bioactivity of TNF-α in samples can differ from total TNF-α detected by ELISA because of the soluble TNF-α receptors produced by monocytes that block the functions of the cytokine. We found no correlation between the data from the bioassay and the ELISA with 200 samples (data not shown). This could partly be due to variable levels of TNF-α receptors in the

supernatant and partly to variable rate of degradation of TNF-α by the monocytes. The bioassay was selected for this study because it measures the levels of TNF-α activity in the solution at a given time.

3. 4. 2 TNF-α responses

RSV infection elicited variable TNF-α responses from alveolar macrophages from individual donors and in different patients [Midulla *et al.*, 1989; Rutigliano & Grham 2004; McNamara *et al*,. 2004; Gentile *et al*,. 2003]. Cells from most of the donors in the present study showed increased TNF-α responses to RSV infection or exposure to CSE. A minority exhibited very high responses to either of the agents. TNF-α is thought to play a protective role in RSV infection. Prior incubation with TNF-α has been shown to reduce the replication of RSV in alveolar macrophages by about half [Girino *et al.*, 1993]. Cells from 8% of donors in this study exhibited a decreased TNF-α response to RSV infection. Individuals with this response might be particularly susceptible to severe infection RSV. Persistence of RSV infection rendered murine macrophages dysfunctional and low in TNF-α [Guerrero *et al*,. 2001].

3. 4. 3 NO responses

The beneficial pulmonary vasodilatory, possible bronchodilatory, and bactericidal effects of NO in patients with COPD or asthma might be offset

by its induction of exudate formation, inflammation, DNA-toxicity and cytotoxicity. It is generally agreed that mild NO induction is protective in the respiratory tract while higher levels might be associated with deleterious consequences [Barnes & Bevisi, 1993; Nussler & Billiar, 1993]. Two distinct populations of donors based on the level of NO production have been recognised [Mautino *et al.*, 1994]. The assay for nitrite used in this study was sensitive down to $NaNO_2$ concentrations of approximately 1 pM. Results presented here indicate that increased responses of TNF-α are not, in most donors, accompanied by increased NO responses to RSV or CSE. TNF-α reduced the half life of mRNA encoding NO synthase in human umblical vein endothelial cells [Yoshizumi *et al.*, 1993]. The results indicate that TNF-α activity might also affect the production of this enzyme in monocytes.

3. 4. 4 The role of IFN-γ, cotinine or nicotine

Some of the effects of virus infections are mediated through release of TNF-α from monocytes [Sodhi & Basu, 1992]. The present data found that stimulatory effects of RSV infection on TNF-α bioactivity could be due in part to IFN-γ. Significant increases in TNF-α and significant decreases in NO response in the presence of CSE did not match with the observed slight increase in both responses mediated by nicotine alone. This suggests other factors in CSE are responsible for the effects observed in these assays. The

significant increase of NO from cells treated with cotinine indicated that some of the effects of cigarette smoking on inflammatory mediators *in vivo* might be mediated by this metabolite of nicotine. Cotinine was not expected to be present in the CSE as it is produced from metabolism of nicotine in the liver.

The results presented here examined some of the effects of two environmental factors that exacerbate COPD and asthma. Smoking or passive exposure to cigarette smoke and virus infections of the respiratory tract do not always lead to similar degrees of acute or chronic illness. This could reflect the individual differences in responses observed in the model system examined in this study. In addition to enhancing bacterial colonisation of the respiratory mucosa and immunosupression, these agents appear generally to enhance TNF-α response and reduce NO levels. The extreme responses noted with cells from a minority of subjects might contribute to increased susceptibility to chronic inflammatory disease of the respiratory tract or exacerbations. Comparison of monocytes from healthy donors with those from patients with these conditions for production of inflammatory cytokines is needed to obtain evidence for this hypothesis.

CHAPTER 4

Effect of infection with respiratory syncytial virus on expression of potential bacterial receptors native to THP-1 and HEp-2 cells

4. 1 Introduction

Viral infections are known to increase susceptibility of host to bacterial infections [Babiuk *et al.,* 1988; Stock *et al.*, 1999; Levine *et al.*, 2004; Peltola & McCullers, 2004; Beadling & Slifka, 2004; Ogra, 2004]. Host antibacterial mechanisms including phagocytic activity of monocytes provide defence against bacterial infections. Bacterial binding to the surface of monocytes is an essential pre-requisite to phagocytosis. Viral infections might alter proficiency of monocytes to bind bacteria, among other factors involved in monocyte-related bactericidal mechanisms (Chapter 5).

Native cell surface molecules such as integrins play a fundamental role in adhesion, differentiation and inter-cellular interactions; however, they might also be used by microbial pathogens, such as CD18 used by meningococci, [Rozdzinski & Tuomanen 1995; Virji et al., 1994] and other bacteria

[Miyamoto *et al.*, 2003; Weineisen *et al.*, 2004] as a means of entry to the host's cells. Numerous studies have examined these surface antigens on cells of myeloid origin. Lymphocyte function-associated antigen-1 (LFA-1) expression was increased on monocytes infected with rhinovirus that resulted in their increased adherence to endothelial cells [Hummel *et al.*, 1998]. Expression of surface antigens, *e.g.*, CD11c, CD14, CD15, on monocyte cells lines including THP-1 cells was altered due to acute or chronic infection with Human Immunodeficiency virus-1 [Ushijima et al., 1993].

RSV is an important agent of respiratory tract infections in older age groups [Hall et al., 1991; Walsh & Falsey, 2004]. Alveolar macrophages play an important role in killing and inhibition of replication of inhaled microorganisms and in inflammation in the respiratory tract due to these agents [Nelson & Summer, 1998; Van Reeth & Adair 1997]. A human monocytic leukaemia cell line, THP-1 [Tsuchiya et al., 1980], possesses the properties of alveolar macrophages [Chen *et al.*, 1996; Kurosaka *et al.*, 2001; Li *et al.*, 2001] and was adopted as a relevant model to study interaction between RSV and bacterial species commonly isolated from patients with COPD.

Density of colonisation of epithelial surfaces is an important factor in the pathogenesis of many infectious diseases [Beachey 1988; Beachey et al., 1988]. In a previous study of binding of *N. meningitidis* to epithelial cells, it

was shown that one of the surface proteins, glycoprotein G, of RSV expressed on the surface of infected cells contributed to enhanced binding of bacteria to cells [Raza *et al.*, 1994]. Reports on expression of surface antigens native to epithelial cells are emerging. Murine CD14 gene expression was demonstrated on epithelial cells in response to TNF secreted in the presence of bacterial lipopolysaccharide (LPS) [Fearns, 1997]. CD11a (α chain of LFA-1) was demonstrated on epithelial cells of rat lungs exposed to high oxygen pressure [Barquin et al., 1996]. CD11b/CD18, components of complement receptor 3 (CR3) were detected on rectal and cervico-vaginal epithelial cells in patients with HIV [Hussain et al., 1995]. HEp-2 cells provided a suitable model to perform studies on the effect of RSV-infection on expression of native surface antigens on epithelial cells.

The objective of this part of the study was to compare the effects of RSV infection on binding of monoclonal antibodies (MAbs) to the following antigens on THP-1, human peripheral blood monocytes (PBM) and HEp-2 cells: the two components of LFA-1, CD11a and CD18; the Lewis^x antigen, CD15; CD14, the cell surface receptor for LPS; and CD29, an antigen common to the β chains of β1 integrins.

4. 2 Materials and methods

4. 2. 1 Cells cultures and RSV-infected cell cultures

4. 2. 1. 1 RSV

The flow cytometry method described previously to detect host cell antigens on buccal epithelial cells [Ziegler & Ulevitch, 1993] was used in these experiments to measure cells surface antigens. The Edinburgh strain of RSV (subgroup A) harvested from HEp-2 cells maintained as described in Section 2.5 was used to infect the cells.

4. 2. 1. 2 THP-1 cells

THP-1 cells were grown as described in section 2.5.2. Fresh cultures of cells were infected with RSV at a multiplicity of infection of two. Overnight cultures of uninfected and RSV-infected cells were washed with MM without antibiotics by centrifugation at 300 x *g* for 7 min and the counts adjusted to 1 x 10^6 ml^{-1} in this medium for use in the experiments.

4. 2. 1. 3 HEp-2 cells

HEp-2 cells (Flow Lab) were grown in 25 cm^2 tissue culture flasks (Costar) in GM. Monolayers grown for 24 hrs were infected with RSV at a multiplicity of infection of 1.0 and cultured overnight in MM. RSV-infected and uninfected HEp-2 monolayers were rinsed twice with DPBS, and 0.05%

(w/v) ethylenediaminetetraactetic disodium acid (EDTA) (Sigma) was applied, 1 ml per $25cm^2$ flask at 37°C for 5-10 min, to suspend the cells. MM (10 ml) was added to the cells to terminate EDTA activity. After centrifugation at 460 *g* for 7 min, the cells were resuspended in MM without antibiotics, counted and adjusted to 1×10^6 cells ml^{-1} for use in the assay.

4. 2. 1. 4 Peripheral blood monocytes (PBM)

PBM obtained using methods described in section 3.2.2 were infected with RSV at a multiplicity of infection of two. Overnight cultures of uninfected and RSV-infected cells from two donors were washed with MM without antibiotics by centrifugation at 300 x *g* for 7 min. The cells were adjusted to 1×10^6 ml^{-1} in this medium for use in the experiments.

4. 2. 1. 5 RSV-infected cells

The proportions of monocytes infected with RSV in the samples were determined by flow cytometry and by indirect immunofluorescence (Section 2.6). Fluorescent cells were detected by flow cytometry (See below). Viability of RSV-infected and uninfected THP-1 cells was determined by trypan blue exclusion.

4. 2. 2 Binding of anti-CD monoclonals to cells

Uninfected and RSV-infected cell suspensions (Section 2.5) at $I \times I0^6$ ml^{-1} (200 µl) were mixed with equal volumes of the monoclonal antibodies

against the following human cell surface antigens: CD11a, CD18, CD15, CD14 and CD29 (Table 4.1).

Table 4.1. Anti-CD monoclonals used in the assays with THP-1 and HEp-2 cells.

	THP-1 cells			HEP-2 cells		
Antibody	**Source**	**Isotype**	**Dilution**	**Source**	**Isotype**	**Dilution**
CD11a	DAKO	IgG1	1/20	Serotec	IgG1	1/20
CD18	DAKO	IgG2b	1/20	Serotec	IgG2b	1/20
CD14	DAKO	IgM	1/10	SAPU	IgM	1/2
CD15	SAPU*	IgG1	1/20	SAPU	IgG1	1/20
CD29	Sertoec	IgG1	1/20	Serotec	IgG1	1/20
Antibody control	Serotec	IgG2b	1/20	Serotec	IgG2b	1/20
FITC-Anti-mouse Ig	Sigma		1/100			
FITC-Anti-rat Ig	Sigma		1/100			

*Scottish Antibody Production Unit

All the antibodies were produced in mice except anti-CD18 and IgG2b which were of rat origin and FITC-labelled antibodies of rabbit origin.

The samples were incubated for 30 min with gentle rotation (60 rpm) in an orbital incubator (Gallenkamp) and were washed 3 times with PBS. The appropriate fluorescein isothiocyanate (FITC)-labelled immunoglobulin antibody was mixed with the cells and incubated with gentle rotation for 30 min to detect binding of the primary antibodies to cells. The cells were washed as above to remove unbound FITC-labelled material. Background

binding of the FITC-labelled antibodies was determined in parallel samples to which the first antibody was not added. Cells with fluorescence greater than the control were assessed for mean fluorescence and binding index calculated. The assays with THP-1 were performed at 4°C to prevent ingestion of the antibodies and those with HEp-2 cells at 37°C.

Non-specific binding of antibodies to the cells was assessed by isotype control antibodies directed to irrelevant antigens. Cell samples were incubated as above with mouse isotype control (IgG2a) MAb to *Aspergillus niger* (DAKO) or rat isotype control (IgG2b) MAb to kappa chain myeloma protein (Serotec) (both diluted 1 in 20). Binding of the isotype controls was detected with FITC-labelled rabbit anti-mouse Ig (Sigma) or FITC-labelled rabbit anti-rat Ig (Serotec). As both uninfected and infected cells appeared to bind the isotype controls at low levels, a second experiment was carried out to determine if these control antibodies had any effect on binding of the anti-CD antibodies. After incubation with the isotype control, the cells were washed 3 times and incubated with unlabelled rabbit anti-mouse or rabbit anti-rat Ig (Sigma) (both diluted 1 in 20) to block the isotype control antibodies. The experiment to assess binding of anti-CD18, anti-CD14 and anti-CD29 to cells was then carried out as described above.

The samples were washed 3 times with PBS, suspended in 200 µl volumes of PBS and fixed with 100 µl of 1% (v/v) buffered paraformaldehyde

(Sigma). Dulbecco's phosphate buffered saline solution A (DPBS) was used for washing.

4. 2. 3 Flow cytometric analysis

The samples containing THP-1 cells and PBM were analysed with an XL flow cytometer (Coulter Electronics, Luton, UK) and samples with HEp-2 cells with EPICS-C flow cytometer (Coulter Electronics, Luton, UK) for the percentage and the mean fluorescence above the background. The main cell population was gated by forward and side light scatter to exclude debris and cell aggregates. The percentage of cells with fluorescence greater than the background was determined on a histogram produced by log-amplified fluorescence signals and mean fluorescence of the positive population on a histogram produced by linear signals. The values were multiplied to obtain a binding index (B_{Ind}) for each sample.

4. 2. 4 Statistical analysis

Paired *t* tests were employed to analyse the logarithms of B_{Ind} of replicate experiments for the flow cytometry studies to detect binding of antibodies or bacteria to the cells.

4. 3 Results

4. 3. 1 RSV-infected cells

At 24 hours post-infection 40-50 % of THP-1 cells, 20-30% of PBM and more than 80% of HEp-2 cells were infected with RSV. RSV infection did not affect the viability of cells at 24 hr post-infection.

4. 3. 2 Effect of RSV infection on binding of anti-CD MAbs to cells

4. 3. 2. 1 THP-1 cells

Background binding of FITC-labelled rabbit anti-mouse/rat Ig (second antibody) to HEp-2 cells was negligible. Mean B_{Ind} for the MAbs directed towards the cell surface antigens of THP-1 cells are summarised in Table 4.2. In 7 experiments, RSV infection resulted in significant decreases in binding anti-CD11a by 35%, anti-CD18 by 24%, anti-CD14 by 28% and anti-CD15 by 30%. There was an increase of 9% in binding of anti-CD29 to RSV-infected THP-1 cells but it was not statistically significant.

4. 3. 2. 2 HEp-2 cells

Background binding of FITC-labelled rabbit anti-mouse/rat Ig (second antibody) to HEp-2 cells was negligible. Data from 7 experiments to assess binding of MAbs to HEp-2 cells and the effect of RSV infection on their binding are summarised in Table 4.3. Traces of the histograms from flow cytometric analysis of fluorescence obtained with MAbs to CD14, CD 18 and CD29 are compared in Fig 4.1.

Antibodies to CD11a, CD14, CD 15, CD 18 and CD29 bound to uninfected HEp-2 cells. Approximately half the cells bound anti-CD18 and anti-CD11a, 40% bound anti-CD15, 15-25% bound anti-CD14 and over 90% bound anti-CD29. Infection of cells with RSV increased BIs significantly for anti-CD18, anti-CD14 and anti-CD15. The BIs for anti-CD11a and anti-CD29 were also increased for RSV-infected cells by a mean of 62% and 41% respectively above that observed for uninfected cells, but these varied between experiments and the effects were not statistically significant. Binding of the antibodies to cells was similar with slightly older cells (28 hr postinfection) or when the assay was performed at 4°C (data not given).

Table 4.2. Mean binding indices (B_{Ind}) of monoclonal antibodies to THP-1 cells and RSV-infected THP-1 cells. (Standard error of mean in the parenthesis) (Results from 7 experiments)

Anti-CD	HEp-2 cells								RSV+/ RSV-	95% CI decrease		P value
	RSV-				RSV+							
	%	Mean	BI	SE	%	Mean	BI	SE				
CD11a	40	99.4	3975	418	37	69.6	2576	521	65	-20	-70	<0.005
CD18	38	152.9	5810	830	35	126.2	4417	861	76	-16	-32	<0.001
CD15	82	428	35108	5272	73	336.6	24575	4235	70	-3	-57	<0.05
CD14	27	111.5	3010	743	25	81.8	2046	315	72	-3	-53	<0.05
CD29	72	75.5	5513	534	75	80.2	6018	764	109			NS

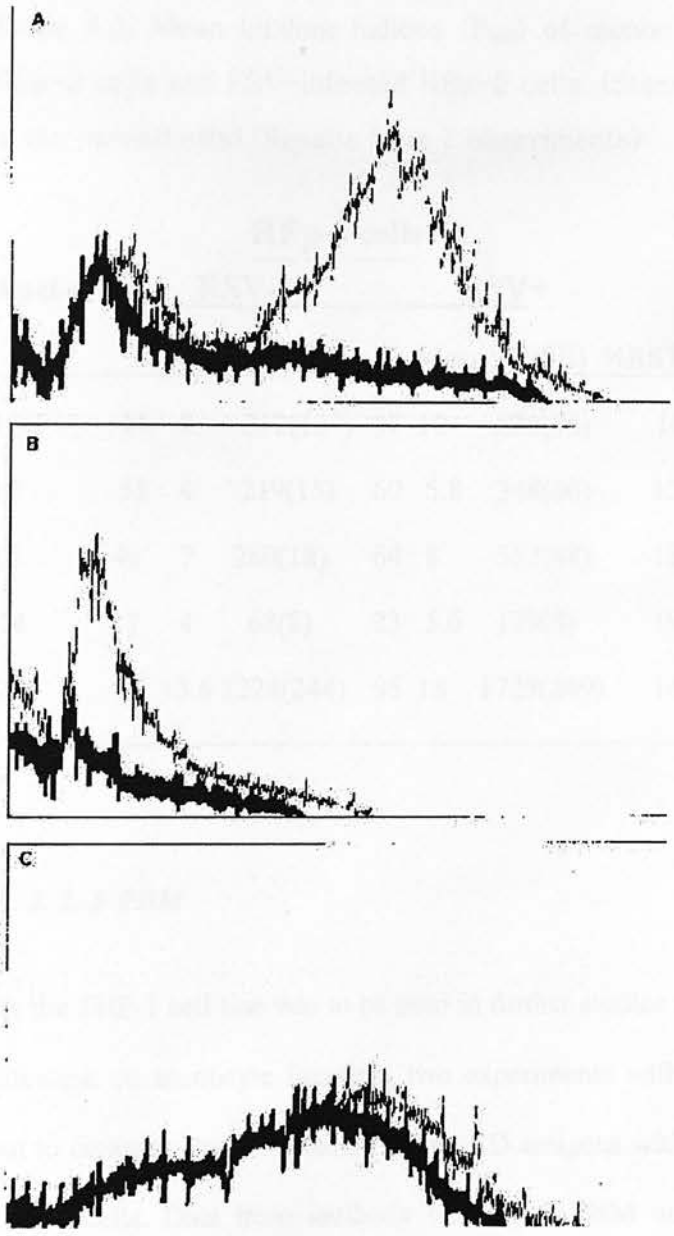


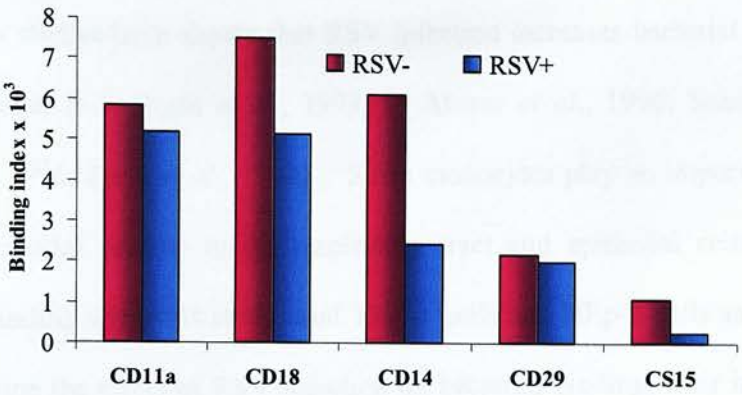
Figure 4.3. Traces of histograms from flow cytometeric analysis of green fluorescence on cells treated with antibodies to (A) CD18, (B) CD14 and (3) CD29. The darker curves in the tracings represent fluorescence obtained with uninfected HEp-2 cells and the lighter curves with RSV-infected HEp-2 cells. The peaks represent number of positive cells detected.

Table 4.3. Mean binding indices (B_{Ind}) of monoclonal antibodies to HEp-2 cells and RSV-infected HEp-2 cells. (Standard error of mean in the parenthesis) (Results from 7 experiments)

	HEp-2 cells								
Anti-CD	**RSV-**			**RSV+**					
	%	Mean	BI(SE)	%	Mean	BI(SE)	%RSV+/RSV-	95%CI	P value
11a	44	8	352(127)	57	10	570(86)	162	73-365	NS
18	55	4	219(15)	60	5.8	348(60)	159	123-204	<0.01
15	40	7	280(18)	64	8	512(88)	183	111-302	<0.05
14	17	4	68(8)	23	5.6	129(8)	190	152-238	<0.001
29	90	13.6	1224(244)	96	18	1725(249)	141	92-214	NS

4. 3. 2. 3 PBM

As the THP-1 cell line was to be used in further studies on the effect of RSV infection on monocyte function, two experiments with PBM were carried out to compare their expression of the CD antigens with those identified on THP-1 cells. Data from antibody binding to PBM were similar to those obtained for THP-1 cells except the expression of CD15 on the PBM was lower compared with THP-1 cells (Figure 4.2). There were insufficient experiments for statistical analyses with PBM.



4.2. Binding of anti-CD monoclonals to uninfected and RSV-infected PBM. (Means of two experiments)

4. 3. 3 Binding of control MAbs to cells

Since MAbs used to detect CD11b and CD11c were of IgG1 isotype, and as these antigens were not detected on HEp-2 cells, a further control for this isotype was not included in the assays. In two experiments, the two isotype control antibodies directed against irrelevant antigens bound to HEp-2 cells: IgG2a on average bound to 20% uninfected and 23% RSV-infected cells; and IgG2b to 26% uninfected cells and 32% RSV-infected cells. These antibodies, however, did not alter the binding of the same subclass isotype specific anti-CD antibodies to uninfected and RSV-infected cells.

4. 4 Discussion

Previous studies have shown that RSV infection increases bacterial binding to epithelial cells [Raza *et al.*, 1993; El Ahmer *et al.*, 1996; Saadi *et al.*, 1993 & 1996; Jiang *et al.*, 1999]. Since monocytes play an important role in bactericidal activity in the respiratory tract and epithelial cells act as initial binding sites, this study used THP-1 cells and HEp-2 cells as models to examine the effect of RSV infection on bacterial binding, their ingestion and killing (presented in Chapter 5). Changes in the surface receptors due to RSV-infection, some of which could be involved in bacterial binding, were examined.

Flow cytometry has recently been used extensively to detect fluorochrome-labelled particles. Because large populations can be examined, it is consistently more accurate and sensitive than direct microscopy.

The objective of this study was to determine if RSV infection enhanced the native surface components THP-1 and HEp-2 cells that might act as receptors for bacteria. Binding of antibodies to CD11a, CD14, CD18 and CD15 to THP-1 cells was decreased as a result of RSV-infection. In contrast, antibodies to CD14, CD15, CD18 and CD29 showed increased binding to RSV-infected HEp-2 cells. Prior treatment of cells with isotype control MAbs did not block the binding of specific antibodies of bacteria

indicating that the isotype control MAbs were binding to epitopes different from CD molecules and/or bacterial receptors.

The enhancement of surface antigens on HEp-2 cells infected with RSV observed in the present study might be due to a direct effect of virus on cells or mediated through cytokines secreted by the infected cells. Virus infections have been shown to alter surface expression of molecules.

The results for the THP-1 cells agreed with previously published results for human mononuclear cells. RSV infection of human mononuclear leukocytes has been shown to suppress LFA-1 (CD 18/CD11a) [Salkind et al., 1991; Koga et al., 2000]. Rhinovirus decreased expression of CD14 on peripheral blood mononuclear cells [Papadopoulos et al., 2002].

The results obtained with HEp-2 cells agreed with the previous studies on resected human tissue. RSV infection of the middle ear was shown to induce or enhance mRNAs for ICAM-1, VCAM-1, and ELAM (a selectin molecule). Cultures of resected tissue from the middle ear infected in vitro with RSV were positive for the mRNAs for ELAM and for the cytokines IL6 and TNF [Okamoto et al., 1993].

The changes in expression of cell surface antigens might be mediated by cytokines produced in response to the RSV infection. Cytokines have been shown to alter expression of CD14 on blood monocytes: IL-4 decreases its expression, while TNF and IL-6 induce a moderate increase in the

expression [Ziegler & Ulevitch, 1993]. Cytokine production from PBM in the presence or absence of RSV infection was examined in experiments summarised in Chapter 3. The effects of RSV infection on cytokine production from THP-1 cells were examined in Chapter 5 as a part of the study of their bactericidal functions.

CD14, CD11a, CD18 and CD15 have been identified as receptors for several bacterial species [van t'Wout et al., 1992; Wright and Jong, 1986; Wright et al., 1989; Espinoza et al., 2002; El-Azami et al., 2003; Weineisen et al., 2004]. Enhanced production of these antigens on HEp-2 cells might result in increased bacterial binding to epithelial cells which would contribute to increased density of colonisation by potential pathogens. Lower levels of these antigens on THP-1 cells associated with RSV infection might contribute to decreased bacterial binding and consequently ingestion and killing. These two hypotheses were further tested in Chapter 5.

CHAPTER 5

The effect of RSV infection on binding of *H. influenzae, S. pneumoniae* and *M. catarrhalis* to HEP-2 and THP-1 cells

5. 1 Introduction

Non-typeable *H. influenzae, S. pneumoniae* and *M. catarrhalis* are bacterial species commonly isolated from patients during episodes of COPD and asthma [Calder & Schonel, 1971; Smith *et al.*, 1976; Nicotra *et al.*, 1986; Seddon *et al.*, 1992]. RSV is an important viral pathogen in older age groups because complete immunity does not follow RSV disease and reinfections are not uncommon [Hall *et al.*, 1991; Hashem & Hall, 2003].

Results presented in Chapter 4 indicated that infection with RSV might alter cells surface antigens involved in bacterial binding to THP-1 and HEp-2 cells.

The objective of this part of study was to examine the changes associated with RSV infection in binding of strains of *H. influenzae, S. pneumoniae* and *M. catarrhalis* to HEp-2 cells and THP-1 cells. Uptake of bacteria into phagocytic cells and non-professional phagocytic cells, *e.g.*, cultured epithelial cells, could be mechanistically similar [Falkow, 1991]. The HEp-2 epithelial cell line model was used to compare the effects of RSV-infection on bacterial binding to these two types of cells. Alveolar macrophages play an important role in bacterial binding and further antibacterial functions. THP-1 cells that possess the properties of alveolar macrophages [Tsuchiya et al., 1980; Chen et al., 1996] were used to study the effect of RSV on intracellular bactericidal activity and cytokine release.

5. 2 Materials and methods

5. 2. 1 Respiratory Syncytial Virus

The Edinburgh strain of RSV (subgroup A) (Section 2.5) was used in these experiments.

5. 2. 2 THP-1 cells

THP-1 cells were grown as described in section 2.5.2. Fresh cultures of cells were infected with RSV at a multiplicity of infection of 2. Overnight cultures of uninfected and RSV-infected cells were washed with MM without antibiotics by centrifugation at 300 x *g* for 7 min and the cell

population adjusted to 1×10^6 ml^{-1} in this medium for use in the experiments.

The proportions of monocytes infected with RSV in the samples were determined by flow cytometry by indirect immunofluorescence (Section 2.6) with a convalescent serum from a patient with RSV infection previously absorbed with THP-1 cells and appropriately diluted in PBS (Section 2.6). Viability of RSV-infected and uninfected THP-1 cells was determined by trypan blue exclusion.

5. 2. 3 HEp-2 cells

HEp-2 cells were grown, maintained and infected with RSV for use in assays with bacterial binding as described in section 4.2.1.3.

5. 2. 4 Flow cytometric analysis

The main cell population was gated by forward and side light scatter to exclude debris and cell aggregates and analysed as described in section 4.3. A binding index (B_{Ind}) was obtained by multiplying the percentage of fluorescent cells and the mean fluorescence.

5. 2. 5 Bacteria

The method described previously for bacterial binding [Raza *et al.*, 1993] was used in this study. Dulbecco's phosphate buffered saline solution A (DPBS) was used for washing.

Isolates of non-typeable *H. influenzae* (HI1 and HI2), *M. catarrhalis* (MC1 and MC2) and *S. pneumoniae* (serotype 3, 6 and 23) were used in the assays (Section 2.2.1). The bacterial preparations were made as described in Section 2.2.3. The bacteria were washed twice with PBS and fixed with 1% buffered paraformaldehyde (Sigma) for 30 min in a water bath at 37°C. The bacteria were labelled with ethidium bromide (EB) according to the methods described in section 2.3.2. The bacteria were washed twice and the total count was adjusted to 4×10^8 m^{-1} in PBS by direct microscopy. Aliquots of the labelled bacteria were kept at -20°C for up to 3 months for use in experiments.

In some of the assays with HEp-2 cells, bacteria were labelled with fluorescein isothiocyanate (FITC) according to the method described in 2.3.1.

5. 2. 6 Bacterial binding

5. 2. 6. 1 THP-1 cells

Uninfected and RSV-infected THP-1 cells (2 x 10^5) in capped plastic tubes were mixed with EB-labelled bacteria to provide a ratio of 10 bacteria:cell and incubated at 37°C in an orbital incubator (40 rpm). Samples were removed after 0 or 30 min incubation and washed 3 times with ice-cold PBS by centrifugation at 300 x *g* for 7 min to remove unbound bacteria from the pellet. The cells were suspended in 1 ml of PBS and stored in the dark at 4°C until analysed within 24 hours by flow cytometry.

5. 2. 6. 2 HEp-2 cells

Samples (200 µl) of HEp-2 cells, RSV-infected HEp-2 cells were suspended in maintenance medium MM. An equal volume of FITC-labelled bacterial suspension was added to the cells to provide a ratio of 200 bacteria:cell. After incubation for 30 min at 37°C with gentle rotation in an orbital incubator, the samples were washed 3 times in PBS by centrifugation at 480 x g for 7 min, resuspended in 200 µl PBS. The cells were fixed with 100 µl of 1% (v/v) buffered paraformaldehyde and stored in the dark at 4°C until analysed by flow cytometry within 3 days of the experiment.

5. 2. 7 Statistical analysis

Paired *t* tests were on the logarithms of the binding indices were used to analyse data obtained from binding and ingestion of bacteria.

5. 3 Results

At 24 hours post-infection 40-50 % of THP-1 cells and more than 80% of HEp-2 cells were infected with RSV. RSV infection did not affect the viability of cells at 24 hr post-infection.

5. 3. 1 Bacterial binding to THP-1 cells

Figure 5.1 summarises data from bacterial binding to THP-1 cells. The binding observed for isolate HI1 to uninfected THP-1 cells was about half of that observed for isolate HI2 ($P < 0.05$). HI1 also bound significantly less to RSV-infected cells compared with HI2 ($P < 0.01$). Compared with uninfected cells, binding of both the isolates to cells infected with RSV was significantly reduced: HI1, $P < 0.01$, 95%CI -5520,-18141; HI2, $P < 0.02$, 95%CI -1588,-25237.

Compared with MC2, MC1 bound in higher numbers to uninfected THP-1 cells ($P < 0.001$) and to RSV-infected cells ($P < 0.005$). Both MC1 and MC2 isolates bound significantly less to RSV-infected cells compared with uninfected cells: MC1, $P < 0.01$, 95%CI -6796,-11840; and MC2, $P < 0.002$, 95%CI -12258,-28233.

Compared with SP6, SP3 bound significantly more to uninfected cells ($P < 0.05$) and to RSV-infected cells ($P < 0.05$). Compared with uninfected cells, fewer SP3 bound to RSV-infected cells ($P = 0.051$, 95%CI 55,-23737). There was no significant difference in binding of SP6 to uninfected or RSV-infected cells. A decrease in ingestion of SP3 by RSV infected cells was observed ($P = 0.059$, 95%CI 1212,-45717).

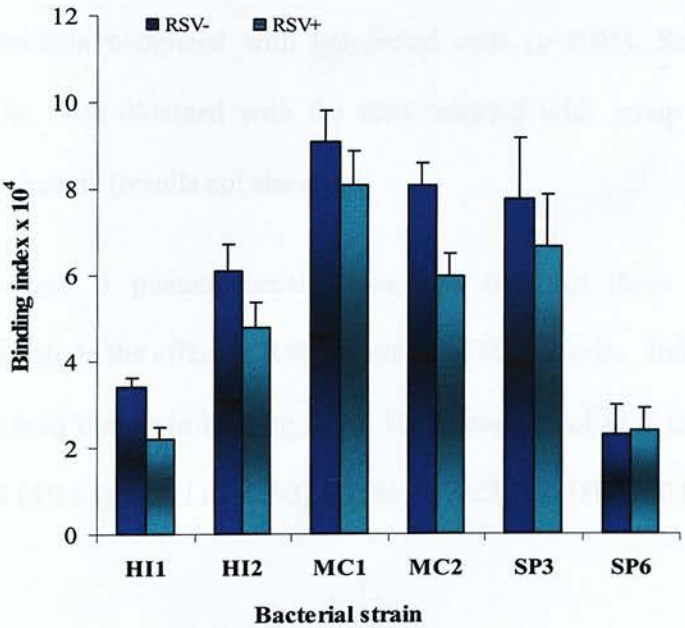


Figure 5.1. Binding indices of *H. influenzae* isolates (H1 and H2), *M. catarrhalis* (M1and M2) and *S. pneumoniae* type 3 and 6 (SP3 and SP6) to THP-1 and RSV-infected THP-1 cells (mean of nine experiments). (Error bars represented standard errors from seven experiments).

5. 3. 2 Bacterial binding to HEp-2 cells

Figure 5.2 summarises the data from binding of FITC-labelled bacteria to HEp-2 cells. Binding of all the strains except one of the two strains of *Moraxella catarrhalis* was increased due to infection of cells with RSV. Compared with uninfected cells, RSV-infected cells bound about twice as many bacteria giving the following *p* values: HI1, < 0.001; MC1, <0.001, SP6, <0.001; and SP23, <0.05. The effect of RSV infection on binding to MC2 was opposite: RSV-infected cells bound significantly lower numbers of bacteria compared with uninfected cells ($p<0.05$). Similar pattern of results were obtained with the cells infected with group B RSV in few experiments (results not shown).

The type 6 pneumococcal strain was used in these experiments to demonstrate the effect of RSV-infection of HEp-2 cells. Infection with RSV increased the mean binding index for pneumococci in 7 experiments from 1834 (SE 65) to 3211 (SE 93) (175%, 95%CI 168-180, P<0.001).

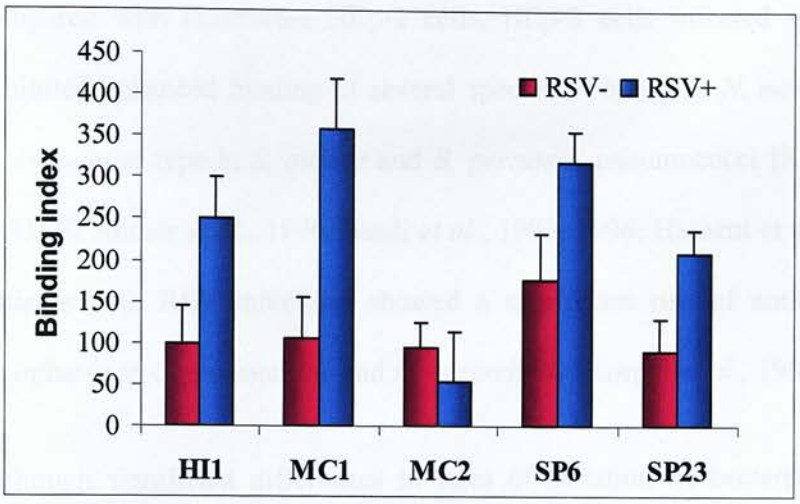


Figure 5.2. Binding indices of *H. influenzae* isolates (H1 and H2), *M. catarrhalis* (M1and M2) and *S. pneumoniae* type 3 and 6 (SP6 and SP23) to HEp-2 and RSV-infected HEp-2 cells.

5. 4 Discussion

Exacerbations in the course of COPD and asthma are usually attributed to infection. Non-typeable *H. influenzae, S. pneumoniae* and *M. catarrhalis* are common bacterial species isolated from these patients [Calder & Schonel, 1971; Smith *et al.*, 1976; Nicotra *et al.*, 1986; Seddon *et al.*, 1999]. Fagon and Chastre [1996] reviewed studies comparing the tracheobronchial microflora during acute exacerbations and stable periods in the course of COPD; significant differences in isolation rates in these studies were found only for viruses, influenza virus and RSV. These studies did not measure the bacterial flora of the respiratory tract. Previous studies found that

compared with uninfected HEp-2 cells, HEp-2 cells infected with RSV exhibited enhanced binding of several species of bacteria: *N. meningitidis*; *H. influenzae* type b; *S. aureus* and *B. pertussis*, pneumococci [Raza *et al,* 1993; El Ahmer *et al.*, 1996; Saadi *et al.*, 1993, 1996; Hament et al., 2004]. Patients with RSV infections showed a significant rise of antibodies to *H. influenzae, S. pneumoniae* and *M. catarrhalis* [Korppi *et al.*, 1989].

Although significant differences in rates of isolation of bacterial species during exacerbation and stable periods were not found in patients with COPD, the density of bacterial colonisation of the respiratory tract might contribute to exacerbation. Secondary bacterial infections following virus infections are thought to be associated with enhanced bacterial binding, and suppression of host immune responses and bactericidal functions associated with viral infections [de Graaf-Miltenburg *et al.*, 1994].

Virus infections might predispose individuals to secondary bacterial infections due to suppression of host immune responses and impairment of bactericidal functions among other factors [de Graaf-Miltenburg *et al.*, 1994]. Influenza virus modified monocytic antigen presentation, and cytokine production [Louie *et al.*, 1995]. RSV infection of a murine macrophage cell line P388D1 resulted in an increase in their phagocytic activity and a decrease TNF-α production [Guerrero-Plata et al., 2001]. Murine alveolar macrophages when infected with RSV, however, produced more cytokine and exhibited decreased bactericidal functions [Franke-

Ullmann et al., 1995]. RSV infection induced production of pro-inflammatory cytokines in human neonatal macrophages and milk macrophages [Matsuda et al., 1996; Sone et al., 1997]. RSV replication triggered chemokine production in epithelial cells and macrophages *in vitro* and in murine model [Miller et al., 2004]. Since monocytes play an important role in bactericidal activity in the respiratory tract, this study used THP-1 cells as model to compare the differences in binding of *H. influenzae*, *M. catarrhalis,* and *S. pneumoniae*.

5. 4. 1 Bacterial staining and use of flow cytometry

Flow cytometry has recently been used extensively to detect fluorochrome-labelled particles. Because large populations can be examined, it is consistently more accurate and sensitive than direct microscopy [Cunningham, 1994[a&b]]. Flow cytometric analysis in bacterial binding studies cannot provide an absolute measure of the mean number of bacteria attached to individual cells, but the mean fluorescence values obtained by this method can reliably compare the binding between samples [Loken and Stall, 1982; Rohde & Dyer, 2004].

Direct labelling of bacteria with fluorescein derivatives can affect the protein moieties on the surface. Compared with unlabelled bacteria, FITC-labelled *E. coli* and *Salmonella typhimurium* bound in higher numbers to mouse peritoneal monocytes (A. Uryet, personal communication). In this study,

bacteria were labelled with EB that binds to nucleic acid within the cells. EB enters through the pores in bacterial cells wall produced by fixation with paraformaldehyde. Any possible effects of bacterial fixation on surface molecules and on binding were not studied in these experiments but were not expected to alter the results of experiments that compared the binding between uninfected and RSV-infected cells.

An attempt was made to differentiate between FITC-labelled antibodies and EB-labelled bacteria in two-coloured histograms in flow cytometeric analysis. The analysis, however, did not produce conclusive results due to technical difficulties with the flow cytometer, which had settings for only low discrimination between the two colours. As a result, any direct evidence to associate the cellular surface antigens studies with bacterial binding could not be provided in this study.

5. 4. 2 Effect of RSV infection of cells on bacterial binding to THP-1 cells

It has been suggested that cellular antigens normally involved in cell-to-cell recognition might be 'hijacked' by bacteria [Isberg et al., 1994; Rozdzinski & Tuomanen, 1995]. There are reports that complement receptors, CR3 (CD 1 lb/CD18) and CR4 (CD11c/CD18), are receptors for *E. coli* [Wright & Jong, 1986] and for *Mycobacterium leprae* [Schlesinger *et al.*, 1991] on human monocytes. Binding of erythrocytes coated with pertussis toxin to

macrophages was inhibited by capping with anti-Lewis^a and anti-Lewis^x (CD15) [van't Wout *et al.*, 1992]. The antibodies to Lewis^a and Lewis^x antigens also inhibited binding of *S. aureus* and *B. pertussis* to buccal epithelial cells [Saadi *et al.*, 1993, 1996]. *Helicobacter pylori* bound to Leweis^a and Leweis^b antigens on cells [Alkout et al., 1997]. CD14 and LFA-l function as receptors for bacterial lipopolysaccharides [Schumann *et al.*, 1994; Wright *et al.*, 1989]. Collagen receptors on CD4+ cells, of which CD29 is a common β chain, are involved in binding of *Yersinia pseudotuberculosis* [Ennis et al., 1993]. Reduction in meningococcal binding and reduction in attachment of meningococcal LOS-coated sheep erythrocytes to HEp-2 cells pretreated with anti-CD14 or anti-CDl8 indicate that these antigens are involved in meningococcal binding [Raza *et al.*, 1999].

Decreased bacterial binding to RSV-infected THP-1 cells might be explained by decreased expression of certain surface antigens on these cells (Chapter 4).

5. 4. 3 Effect of RSV infection on binding of bacteria to HEp-2 cells

Increase in binding *H. influenzae, S. pneumoniae* and *M. catarrhalis* to HEp-2 cells due to RSV infection found in this work was consistent with previous studies by our group and others [Raza et al., 1993; Patel *et al.*, 1992; Jiang *et al.*, 1999; Ogra, 2004] (Section 1.5.3). The increased binding also matched

up with increased expression of surface antigens measured on HEp-2 cells that are considered as potential receptors on epithelial cells for bacteria (Chapter 4).

5. 4. 4 Comparison of effects of RSV infection in relation to host defence

RSV infection of the respiratory tract might increase density of colonisation due to increase in number of potential receptors for bacterial binding, among other factors promoting increased bacterial load, *e.g.,* local oedema formation, tissue injury, decreased bacterial clearance associated with loss of mucocilliary function and formation of exudate that increases bacterial growth (Section 1.5.3).

It might also increase frequency of colonisation due to spread of bacteria in aerosols due to hyperirritability of the respiratory tract and heavy bacterial load. 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].

RSV infected phagocytic cells in the lungs might have decreased capacity to deal with the bacteria due to decreased capacity to bind, which can become more important on relation to increased bacterial load in the respiratory tract.

The next part of the study examined phagocytic and bactericidal functions of THP-1 cells when challenged with *H. influenzae, S. pneumoniae* and *M. catarrhalis* and the effect of RSV infection on these functions.

CHAPTER 6

Bactericidal activity of a monocytic cell line (THP-1) against *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* is depressed after infection with respiratory syncytial virus

6. 1 Introduction

Previous results indicated that infection with RSV might decrease expression of potential bacterial receptors on monocytes (Chapter 4) and, compared with uninfected monocytes, RSV-infected monocytes bound fewer numbers of *H. influenzae, S. pneumoniae* and *M. catarrhalis* (Chapter 5). Chapters 4 and 5 also showed that RSV-infected HE-p2 cells might bind more of these bacterial species compared with uninfected cells. The decreased capacity of monocytes to deal with bacterial load might not be limited to decreased binding but other antibacterial functions might also be deranged due to virus infection.

THP-1 possesses the properties of alveolar macrophages that play an essential role in bactericidal functions against inhaled and ingested microorganisms and in inflammation in the respiratory tract due to these

agents. [Tsuchiya et al., 1980; Chen et al., 1996] were used for this part of the study. The first objective was to examine the effect of RSV infection on phagocytic and intracellular bactericidal activities of THP-1 cells.

Tumour necrosis factoro (TNF-α) plays a role in defence against viral [Neuzil et al., 1996; Cirino, et al., 1993; Minagawas et al., 2004] and bacterial infection [von der Mohlen et al., 1996; Westendorp et al., 1997; Ehlers, 2003], but has been considered responsible for full-blown infection in rhinovirus infection by down-regulating IFN-γ response [Berg et al., 2004]. Its release, however, also results in inflammation in the respiratory tract and contributes to the systemic symptoms in patients with COPD [Keatings et al., 1996; de Godoy et al, 1996]. The second objective was to investigate the effect of RSV infection and the presence of intracellular bacteria on TNF-α release from THP-1 cells.

6. 2 Materials and methods

The Edinburgh strain of RSV (subgroup A) (Section 2.5) was used to infect THP-1 cells (5.2.2). Two isolates each of non-typeable *H. influenzae* (HI1 and HI2), *M. catarrhalis* (MC1 and MC2) and *S. pneumoniae* (serotype 3 and 6) were used in the assays (Section 2.2.1) and the preparations made as described in Section 2.2.3.

6. 2. 1 Ingestion of bacteria

Bacterial preparations were incubated with cells at appropriate ratios as described for bacterial binding studies (5.2.7). To measure the fluorescence from bacteria ingested by cells, fluorescence from extracellular bacteria was quenched in each sample (1 ml) with 20 µl of 0.05% crystal violet (BDH) in 0.15 N sodium chloride. Since the optical density of the soluble dye and its quenching effect increased with time, the flow cytometric analysis was carried at 500-600 nm. In this range more than 90% of the EB-labelled bacteria outside the cells were completely quenched. Crystal violet at higher optical densities is membrane permeable and can quench intracellular bacteria. The laser power for these samples was adjusted to compensate for the decrease in background autofluorescence of the control samples due to quenching and the percentage of fluorescent cells and the mean fluorescence were measured to obtain an I_{Ind}.

6. 2. 2 Intracellular survival of bacteria

Uninfected and RSV-infected THP-1 cells were incubated with live unlabelled bacteria under the above conditions for 30 min. Extracellular bacteria were killed by adding gentamicin (30 µg ml^{-1}) and ampicillin (50 µg ml^{-1}) for 15 min at 37°C. After 3 washes with PBS, the cells were resuspended in 100 µl of PBS and lysed with an equal volume of 0.05% sodium lauryl sulphate in sterile distilled water. Samples were

immediately plated in triplicate for determination of cfu as described above. For a time course study of intracellular survival and growth of bacteria, cells were incubated for different periods before lysing and plating.

6. 2. 3 TNF-α bioactivity

Uninfected and RSV-infected THP-1 cells were incubated in 5% CO_2 at 37^{o}C with unlabelled live or EB-labelled fixed bacteria at a ratio of 10 bacteria:cell in 24-well tissue culture plates (Costar). After 24 hr of incubation, supernatants from individual wells were collected for determination of TNF-α. The bioassay described in section 3.2.4 was used to determine TNF-α activity.

6. 2. 4 Statistical analysis

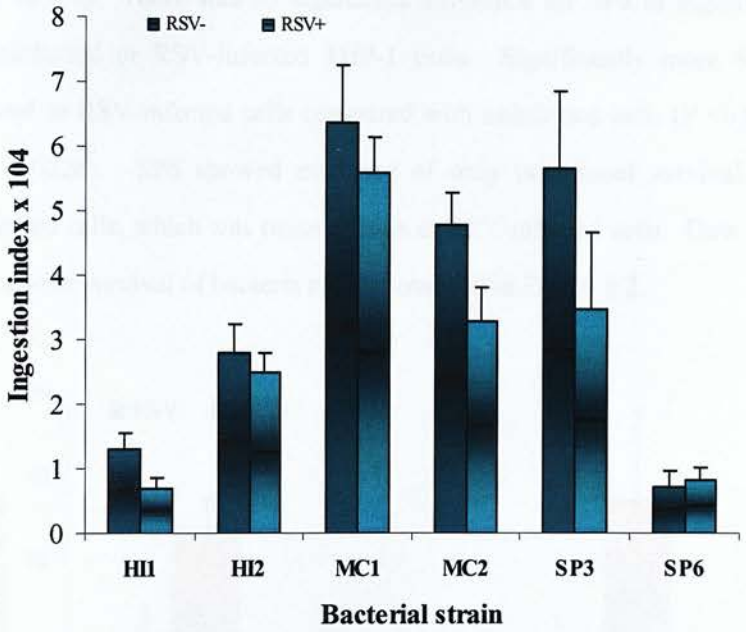
Paired *t* tests were used to analyse data from ingestion of bacteria. Wilcoxon's test for matched-pairs was applied to the data on experiments measuring intracellular survival of bacteria. The data for TNF-α production by uninfected or RSV-infected THP-1 cells under various conditions were analysed also by paired *t* tests.

6. 3 Results

6. 3. 1 Bacterial ingestion and survival

As described previously, at 24 hours post-infection 40-50 % of THP-1 cells were infected with RSV. RSV infection did not affect the viability of cells at 24 hr post-infection.

Bacteria bound to cells at 0 min (data not shown), but intracellular bacteria were not detected at this time either by quenching the external bacteria or by determination of intracellular survival. Data from 9 experiments on ingestion of *H. influenzae*, *M. catarrhalis* and pneumococci are presented in Figure 6.1.



6.1. Ingestion indices (SE) of *H. influenzae* isolates (H1 and H2), *M. catarrhalis* (M1and M2) and *S. pneumoniae* type 3 and 6 (SP3 and SP6) to THP-1 and RSV-infected THP-1 cells (mean of nine experiments). Error bars represent SEM.

RSV infection of cells reduced the ingestion of both the *H. influenzae* strains, but this was significant only for HI1 ($P < 0.05$, 95%CI −791,-12793). Both the isolates survived better in RSV-infected cells compared

with uninfected cells (P <0.02, HI1, Z = -2.366; P <0.05, HI2, Z = -2.1974). RSV infection of cells reduced the ingestion of both the *M. catarrhalis* strains (MC1, NS; MC2, P <0.05, 95%CI -335,-31328). Neither of the isolates survived in uninfected or RSV-infected THP-1 cells in the conditions used in the study. A decrease in ingestion of *S. pneumoniae* SP3 by RSV infected cells was observed (P = 0.059, 95%CI 1212,-45717). There was no significant difference for SP6 in ingestion by uninfected or RSV-infected THP-1 cells. Significantly more SP3 survived in RSV-infected cells compared with uninfected cells (P <0.05, Z = -2.0226). SP6 showed evidence of only occasional survival in uninfected cells, which was twice as high in RSV-infected cells. Data for intracellular survival of bacteria are summarised in Figure 6.2.

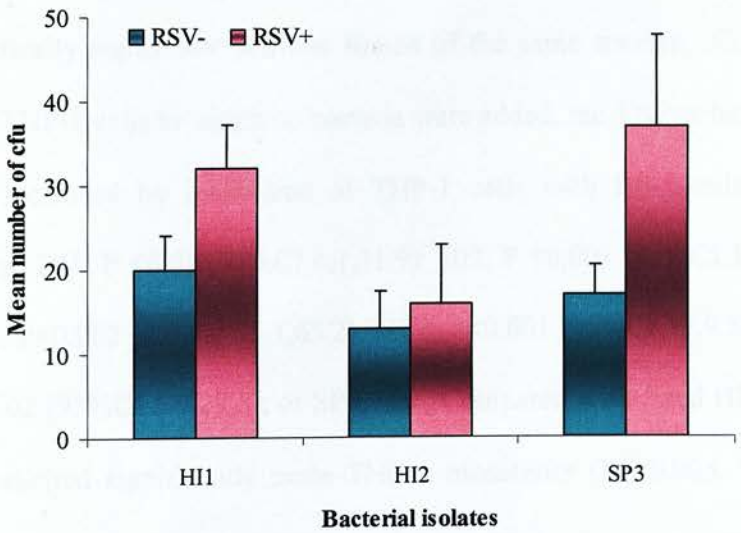


Figure 6.2. Survival of *H. influenzae* isolates (HI1 and HI2) and *S. pneumoniae* type 3 (SP3) in uninfected and RSV-infected THP-1 cells (mean of 7 experiments).

6. 3. 2 Effect of RSV infection and bacteria on TNF-α bioactivity of cells

TNF-α bioactivity in supernatants of uninfected or RSV-infected THP-1 cells incubated with either live unlabelled or fixed EB-labelled bacteria was determined (Figure 6.3).

In 7 experiments, compared with THP-1 cells to which no bacteria were added, the bioactivity was increased by incubation of THP-1 cells with live strains: HI1, P <0.01 (95%CI 11.5,45.9); HI2, P <0.001 (95%CI 24.3,50.6); MC1 P <0.05 (95%CI 8.5,59.2); MC2, P <0.05 (95%CI 5.1,47.2); SP3, P <0.001 (95%CI 48.6,65.9); or SP6, P <0.005 (95%CI 15.5,44.5). Differences in the levels of TNF-α induced were not statistically significant between strains of the same species. Compared with THP-1 cells to which no bacteria were added, the TNF-α bioactivity was increased by incubation of THP-1 cells with EB-labelled fixed strains: HI1, P <0.05 (95%CI 4.1,31.9); HI2, P <0.001 (95%CI 19,42.4); MC1, P<0.002 (95%CI 23.1,63.2); MC2, P<0.001 (95%CI 38.9,59); SP3, P <0.02 (95%CI 5.9,29.5); or SP6, NS. Compared with fixed HI1, fixed HI2 elicited significantly more TNF-α bioactivity (P <0.005, 95% CI 6,19.3). Fixed MC2 and SP3 elicited higher TNF-α bioactivity compared with fixed MC1 and SP6 respectively, but the differences were not statistically significant.

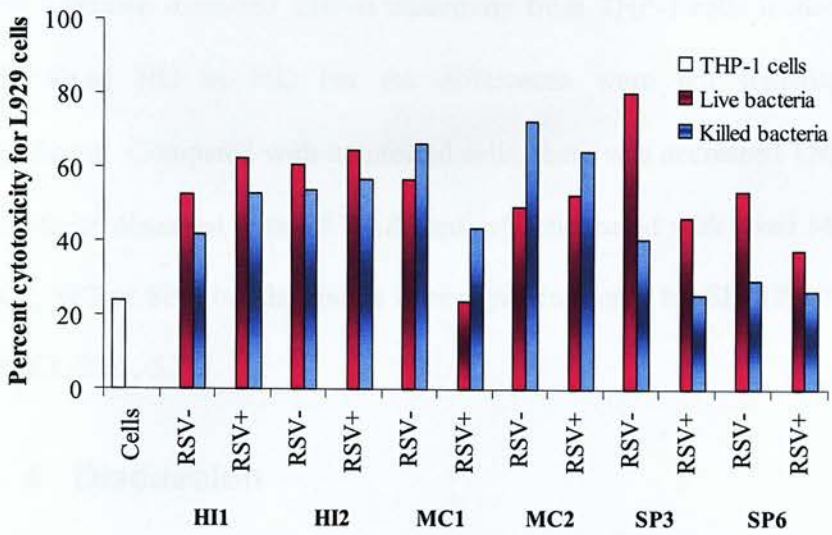


Figure 6.3. Percentage of cytotoxicity for L-929 cells caused by TNF-$_{\Gamma}$ in supernates from uninfected or RSV-infected THP-1 cells incubated with live or fixed *H. influenzae* (HI1 & 2), *M. catarrhalis* (MC1 & 2) and *S. pneumoniae* (SP3 & 6) obtained from 7 Experiments.

RSV infection increased TNF-α bioactivity from THP-1 cells incubated with live HI1, HI2 or MC2 but the differences were not statistically significant. Compared with uninfected cells, TNF-α bioactivity was decreased for RSV-infected cells incubated with live MC1, P <0.002 (95%CI -49.3-18.4), SP3, P <0.001 (95%CI –47.6, -20.4) or SP6, P <0.005 (95%CI -23.2,-8.2).

RSV infection increased TNF-α bioactivity from THP-1 cells incubated with fixed HI1 or HI2 but the differences were not statistically significant. Compared with uninfected cells, there was decreased TNF-α bioactivity observed with RSV-infected cells incubated with fixed MC1, MC2, SP3 or SP6, but the results were significant only for SP3 ($P < 0.01$, 95%CI -25.5,-5.2).

6. 4 Discussion

Decreased bacterial binding to and ingestion by THP-1 monocytes and their impaired ability to kill bacteria suggest a role of virus infection in pathogenesis of bacterial disease at multiple levels. While persistent RSV infection did not derange antigen presenting function of a monocyte cell line [Guerrero-Plata et al., 2004], it increased phagocytic activity and expression of Fc receptors and decreased TNF-α production from a murine macrophage cell line, P388D1 [Guerrero-Plata et al., 2001]. Infection with RSV, however, induces release of TNF-α from human alveolar macrophages and a murine monocyte cell line [Panuska *et al.*, 1994; Frank *et al.*, 1994]. Decreased ingestion of bacteria by THP-1 cells infected with RSV might be due to the lower levels of initial bacterial binding observed with these cells.

The differences in binding of different isolates of each strain of bacteria (Chapter 5) and their killing by the phagocytes found in this work might influence bacterial virulence and chronicity of disease. Isolates that bound

less to uninfected THP-1 cells, were ingested at lower levels and survived better in the cells might be more virulent than the others.

Heavy growths of bacteria over prolonged periods in the respiratory tract in COPD due to inefficient bactericidal mechanisms might cause further damage to the tract due to release of inflammatory cytokines. Increased TNF-α bioactivity contributes to inflammation while a marked decrease can jeopardise mucosal protection against bacteria and bactericidal activity [Degre et al., 1989; von der Mohlen et al,. 1996]. With this in view, this study examined the levels of TNF-α elicited from THP-1 cells in response to bacteria. TNF-α produced by uninfected and RSV-infected THP-1 cells in response to bacteria were also compared. While various degrees of increased TNF-α bioactivity were observed with all the bacterial isolates tested, the responses were reduced by concurrent RSV infection in some cases indicating different isolates can elicit different responses. RSV infection in human alveolar macrophages can persist for at least 25 days after *in vitro* infection [Panuska et al., 1990] and in a significant number of patients with COPD [Mikhalchenkova et al., 1987]. Persistently lower TNF-α responses to the bacteria observed with RSV infection combined with decreased intracellular bactericidal activity might prolong bacterial disease in patients with COPD and cause chronic damage to the respiratory tract.

In conclusion, the differences observed between uninfected and RSV-infected THP-1 cells in the pattern of binding, intracellular killing of bacteria and TNF-α production in response to bacteria can help to explain increased susceptibility of virus-infected patients to secondary bacterial infections. Bacteria that escape virus-infected monocytes might have a greater opportunity to grow in the milieu of the respiratory tract and cause disease.

Chapter 7

Discussion

7. 1 Introduction

Although the precise sequence of events in COPD leading to airway obstruction is not completely understood, inflammation in the airways has been unanimously seen by researchers as a pivotal factor. Understanding the role and pathophysiological and molecular mechanisms of inflammation in the lungs holds the key to the development of effective intervention strategies. The studies on inflammation in COPD might also enhance the insight into proposed mechanisms of cytokine upsurge in infants with sudden infant death syndrome (SIDS) [Raza & Blackwell, 1999] and of chronic damage to the respiratory tract due to presence of exceptional microflora in the respiratory tract in patients with cystic fibrosis.

7. 2 Role of microorganisms in COPD

Only a small proportion of smokers progress to COPD (Section 1.5.1). The effect of cigarette smoke on responses associated with different genetic polymorphisms has been regarded as the most important

determinant of the outcome. Cessation of smoking is the chief measure that can slow down the decline in lung functions in COPD [Anthonisen *et al.*, 1994]. Microbial pathogens in COPD as agents boosting inflammation and inflicting tissue damage have been considered as additional important deleterious factors (1.5.5.1).

Chronic lower respiratory tract bacterial colonisation is a hallmark of COPD (Section 1.5.2) [Hill et al., 2000]. Persistence of bacteria *e.g.*, *H. influenzae* in the respiratory tract, which is kept relatively sterile in healthy individuals, can be facilitated by intracellular niches in the mucosal epithelial cells [Wilson et al., 1986]. The evidence that latent [Hogg, 2001] or persistent [Krivitskaia *et al.*, 1996] viral infections can establish in the respiratory tract in these patients also supports the hypothesis for a role of microbial pathogens in COPD.

7. 3 Exacerbations in COPD

Acute exacerbation in the course of COPD entails significant morbidity and mortality. In addition to the role of microbial pathogens in the chronic setting, the course of illness is marked with frequent exacerbations in the symptoms due to acute bacterial infections. Compared with 'mucoid' non-infectious exacerbations, 'purulent' exacerbations exhibit increased levels of myeloperoxidase, neutrophil elastase IL-8 and C-reactive proteins (CRP), and are considered to result from infections [Gompertz *et al.*,

2001]. Antibiotics are typically prescribed to treat purulent exacerbations and usually this treatment benefit the patient. Pathophysiologic studies of exacerbation reveal the role of infection in COPD more clearly. Recurrent infections in patients with COPD enhance the progression of the underlying process. There is evidence that not all COPD exacerbation recover to baseline with respect to symptoms and lung functions [Seemungal et al., 2000]. A proposed course of COPD highlighting the role of respiratory tract infections in pathogenesis is illustrated in Figure 7.1.

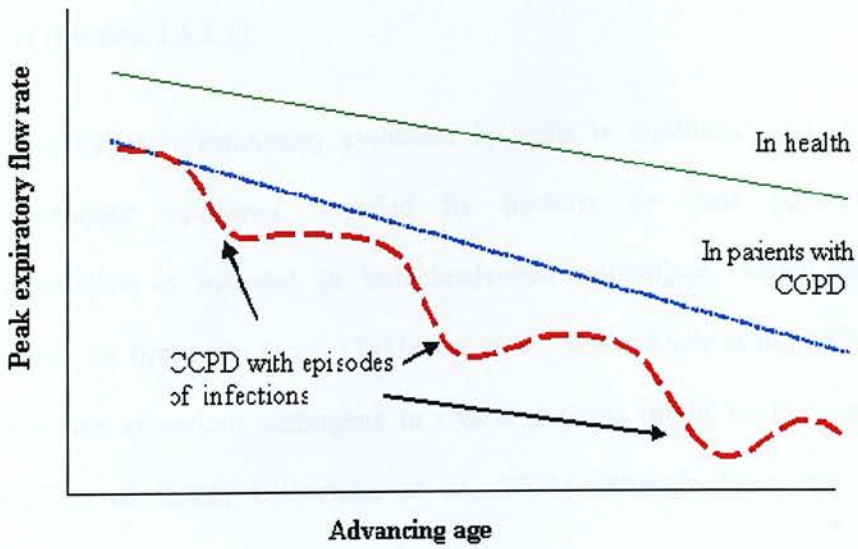


Figure 7.1. Decline in respiratory functions over the years expected in healthy individuals or patients with COPD with or without episodes of infections. (non-proportional representation)

7. 4 Combined effects of cigarette smoke and infectious agents

Combining the deleterious agents in *in vitro* assays could be useful method in depicting the *in vivo* scheme of events. Infectious agents and cigarette smoke incite numerous responses in the respiratory tract: cellular infiltration, *e.g.*, neutorphils [Aaron et al., 2001], lymphocytes [Hogg, 2001] or monocytes [Ofulue et al., 1998]; alter intercellular interactions resulting from change in the expression of surface antigens and/or cellular functions, *e.g.*, phagocytosis, or defective natural killer cells [Priet et al., 2001] (Section 1.5.1.1).

Release of proinflammatory cytokines by cells is facilitated and anti-inflammatory cytokines impeded by bacteria or their products. Inflammation is initiated in bronchoalveolar epithelium, which also becomes its first main target [Takizawa et al., 1998; Khair et al., 1994]. Antibodies to various pathogens in COPD patients might be increased [Bakri *et al*, 2002; Lieberman *et al.,* 2001] although their role in inflammation is less well explained.

Remodelling, parenchymal destruction, mucosal changes occur due to inflammatory cytokines and direct effects of deleterious agents [Jeffery, 2001; Maestrelli et al., 2001; McDonald, 2001].

7. 5 Models used in the present study

Commonly described pathogens in COPD, *H. influenzae*, *S. pneumoniae* and *M. catarrhalis,* and RSV were tested in these studies. The system using human peripheral blood monocytes [Gordon & Read, 2002] obtained from blood donors indicate individual differences in inflammatory responses to these common bacterial pathogens and cigarette smoke (Chapter3). Use of the monocyte cell line provided consistent genetic background to assess the effects of the individual bacterial strains and the effects of cigarette smoke [Guerrero-Plata et al., 2001]. It provided a model to examine the difference in expression of cell surface antigens due to viral infection (Chapter 4) and inflammatory and other responses to these agents (Chapter 6). Comparison of bacterial binding to monocytes and epithelial cells not only informed a more inclusive understanding of the effects of concurrent viral infection, but also improved the validity of the model due to the contrasting results of binding obtained with the two cell types (Chapter 5).

7. 6 Main conclusions

There is ample epidemiologic and clinical evidence to indicate the role of microbial pathogens in COPD (Section 1.5.2.1). Numerous experimental studies have examined their role using *in vitro* models [Inoue et al., 2003; O'Rielly, 1995]. The major conclusions of the current study can be summarised as: 1) RSV infection boosted the inflammatory response of

peripheral blood monocytes exposed to cigarette smoke extract. There was evidence in the data pointing to extreme responses in some (blood) donor of monocytes; 2) RSV infection increased expression of potential bacterial receptor molecules on epithelial cells, while decreased their expression on monocytes. This finding implied that RSV infection resulted in higher bacterial load whereas reduced the proficiency of the system to deal with that load; 3) Not only that monocytes did not bind bacteria as well due to RSV-infection but also they did not perform as well in internalising and killing them.

7. 6. 1 Immunomodulatory effects of Virus infection

Viruses influence the host in various ways. They modify immune system with consequential higher susceptibility to infections and immune-mediated diseases. Human immunodeficiency virus, Epstein Bar virus [Hillary et al., 1999; Porcu & Caligiuri, 2000] and cytomegalovirus (CMV) [Mocarski, 2004; Boeckh & Nichols, 2003] are well recognized immunomodulatory agents predisposing individuals to secondary bacterial, fungal and protozoal infections. Myxoma viruses release immunologically active proteins that simulate complement and chemokines [Barrett et al., 2001; Kotwal, 2000]. Varicella Zoster Virus demonstrates immunomodulatory effects during the primary infection, and during persistence and reactivation phases through interference with CD4 and CD8 T cell functions [Abendroth & Arvin, 2001]. Human herpes

virus-6 and -7 might predispose the individuals to infection with CMV [Singh, 2000]. Genomic sequences for immunomodulatory proteins, *e.g.*, complement control proteins, have been mapped in herpesviruses and poxvirus [McFadden & Murphy, 2000].

Respiratory tract viruses usually cause acute infection involving cell surface changes, cytokine production and tissue damage; this phase is followed by clearance of pathogens and repair of tissue. Their persistence in the respiratory tract in certain patients groups might provide an ongoing source of injury and alteration in inflammatory and immune mechanisms. ` mechanism leading to persistent infection with adenovirus are mediated by interference with antigen presentation on the surface of cells. Endogenous virus proteins resist presentation with MHC-1 and impede apoptosis of infected cells by TNF [Burgert & Blusch, 2000]. Persistence of RSV [Krivitskaia, et al., 1996] compounded by chronic colonisation with respiratory tract bacterial pathogens in COPD might provide sufficient stimulus to cause injury and alteration in the inflammatory and immune mechanisms.

Immunomodulatory effects of respiratory viruses are not, however, well described except for influenza virus. Secondary pneumonia with *S. aureus* following influenza virus infection in the elderly is a widely documented complication. This effect results from numerous factors including lung macrophages exhibiting transiently dysfunctional phagocytosis. The

findings of the present study identify RSV as disabling monocytes in the multitude of their functions: bacterial binding, internalisation, bacterial killing and inadequate production of cytokines. These findings might be more significant in the light of evidence of persistence of RSV infection in the lungs in patients with COPD and colonisation with bacteria.

7. 6. 2 Smoking and infection

Sexually transmitted human papillomavirus is more likely to cause cervical carcinoma in female patients who also smoke cigarettes [Severson et al., 2001; Castellsague & Munoz, 2003], although the pathogenesis is not clear. Epidemiological studies indicate smoking a risk factor for severe pneumonitis and admission to ITU due to Varicella Zoster virus infection [Frangides & Pneumatikos, 2004; Harger et al., 2002; Jones et al., 2001]. Interaction of effects of smoking and infections has been described in Section 1.5.4.

7. 7 Implications of the conclusions

7. 7. 1 Individual variations in inflammations response

Individual genetic polymorphisms are a primary source of variations among individuals' inflammatory responses to cigarette smoke and infections (Section 1.1.5). Cytokine profiles are also determined by inter-species variations and those between the members of the same species of pathogens. Complexity is further increased by the presence of both virus and bacterial pathogens in the lungs in COPD. Not only those pathogens

might induce inflammatory responses with qualitative and quantitative differences, but also they might also be differently affected by antibacterial or antiviral activity of cytokines induced. Some of the cytokines have simultaneous anti-pathogen activity and potential to cause tissue injury. On experimental level, there were contrasting differences among various monocyte cell lines in their cytokine profiles and phagocytic functions [Guerrero-Plata et al., 2001; Panuska et al., 1994; Frank *et al.*, 1994]. These factors make difficult any attempt of generalising the pathophysiological changes in the respiratory tract during cigarette smoking and concurrent infections. In spite of these complexities, the course of clinical illness in COPD is relatively well defined, and might allow selection of appropriate management strategies. The following areas of interest could be a focus for further development.

7. 7. 2 Further research

There are three areas of investigation into the role of infection in COPD need further work:

1) Isolation of bacterial species from routine respiratory specimens from patients with COPD does not necessarily indicate infection. This necessitates investigation into methods to determine densities and extents of colonisation by bacterial flora during exacerbations and stable periods in the course of COPD. Results of such studies might explain episodes of exacerbation in a proportion of cases.

2) The presence of bacteria in the normally sterile lower respiratory tract whether during stable COPD or acute exacerbation warrants investigation into their role in inflammation;

3) Possible changes in the growth patterns of bacteria might be due to the appearance of as yet unrecognised host factor(s) in the respiratory tract, changes in the bacterial phenotype {van Alphen, Jansen, et al. 1995 194 /id} or virus infections. Viral infection can increase bacterial load and possibly the extent of bacterial colonisation in the respiratory tract. The changes in the milieu of the lungs during exacerbation, modified by the genetic factors, disturb the fine-tuning of the cytokine production contributing to chronic inflammation (Figure 7.2). Interactions between virus and bacterial pathogens, cytokines and chemokines leading to inflammation and tissue damage are illustrated in Figure 7.3;

4) The model used in these studies could be extended to examine the effects of adenovirus, influenza virus and other respiratory tract viruses;

5) A population-based longitudinal study to examine the combined effects of smoking and infectious agents on cytokine production, lung functions and clinical features would be complementary to the observations made by this study;

6) It might be also pertinent to compare model of pathogenesis in COPD with other models of chronic respiratory inflammatory illness, e.g., cystic fibrosis, bronchial asthma and chronic bronchiectasis.

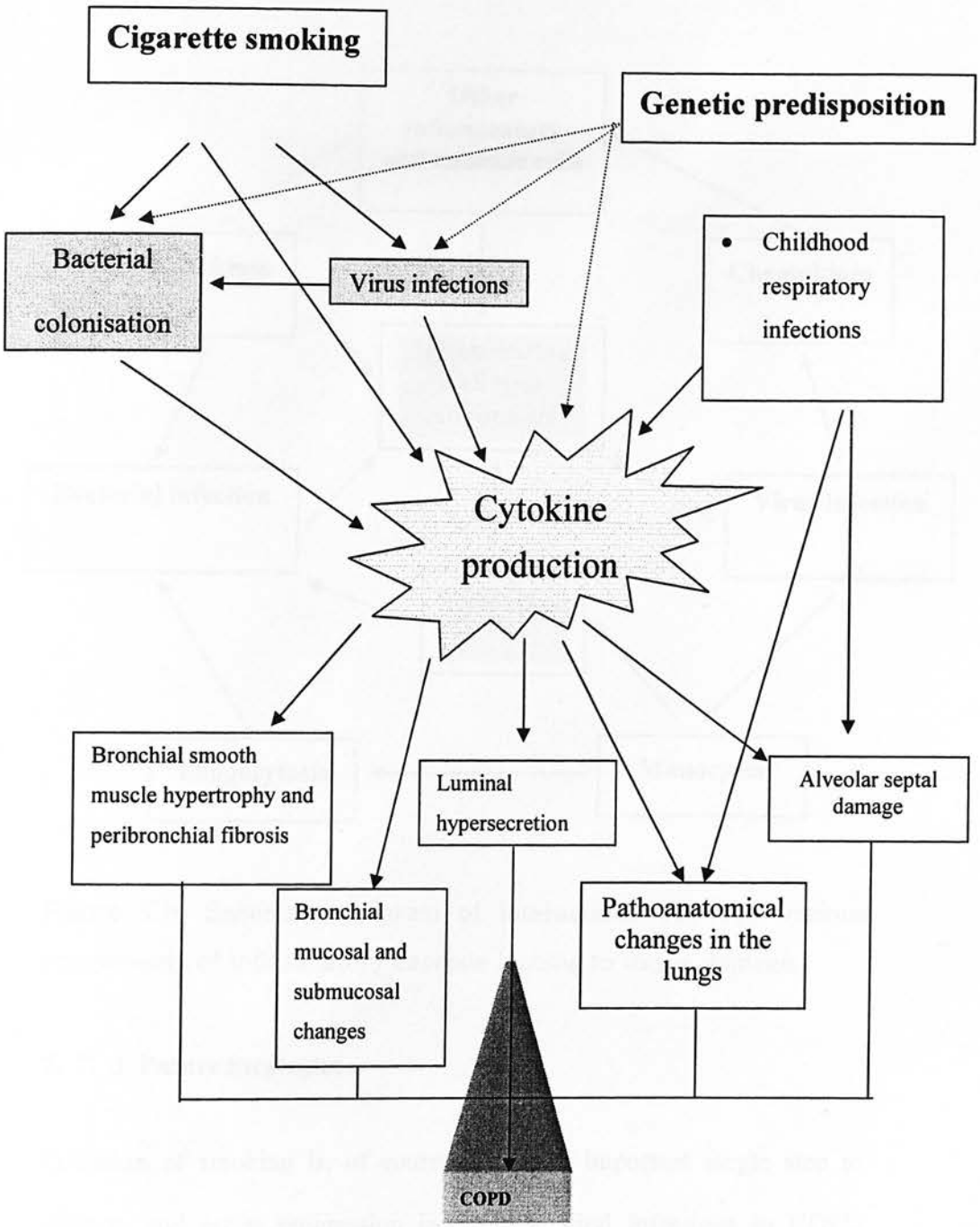


Figure 7.2. Schematic representation of events leading to chronic obstructive pulmonary disease (COPD) and episodes of exacerbation in its course.

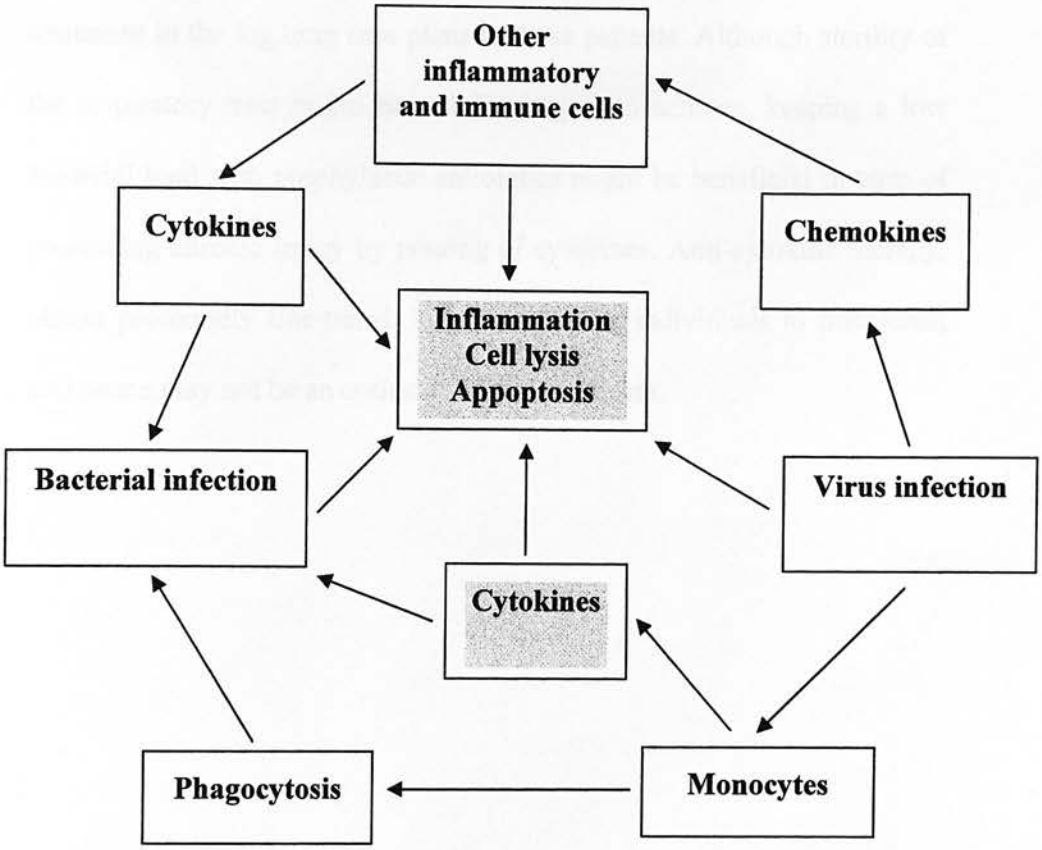


Figure 7.3. Schematic diagram of interactions between various components of inflammatory cascade leading to tissue damage.

7. 7. 3 Future Strategies

Cessation of smoking is, of course, the most important single step to prevent, and arrest progression in, COPD. Viral infections in COPD usually do not get treated. Their persistence makes appropriate treatment even more important to prevent secondary bacterial disease, cytokine over-production and substantial lung injury. It might be useful to be more

consistent in application of policies of using available vaccinations and treatment in the log term care plans in these patients. Although sterility of the respiratory tract might be a difficult goal to achieve, keeping a low bacterial load with prophylactic antibiotics might be beneficial in term of preventing chronic injury by pouring of cytokines. Anti-cytokine therapy, unless profoundly fine-tuned, might predispose individuals to infections, and hence may not be an option available at present.

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Chapter 1:

- **Raza MW**, Bint A, Blackwell CC (2001). Role of infection and cytokines in chronic obstructive airway disease. *Rev Med Microbiol;* 12: 109-119.
- **Raza MW**, Blackwell CC (1999). Sudden infant death syndrome, virus infections and cytokines and (Review). *FEMS Immunol Med Microbiol*; 25: 85-96.

Chapter 3:

- **Raza MW**, Essery SD, Weir DM, Ogilvie MM, Elton RA, Blackwell CC (1999). Infection with respiratory syncytial virus and water soluble components of cigarette smoke alter production of tumour necrosis factor-α and nitric oxide by human blood monocytes. *FEMS Immunol Med Microbiol*; 24: 387-94.
- **Raza MW**, Essery SD, Elton RA, Weir DM, Busuttil A, Blackwell CC, (1999). Exposure to cigarette smoke, a major risk factor for sudden infant death syndrome: effects of cigarette smoke on inflammatory responses to viral infection and bacterial toxins. *FEMS Immunol Med Microbiol*; 25:145-54.

Chapter 4:

- **Raza MW**, Ogilvie MM, Blackwell CC, El-Ahmer O, Saadi AT, Elton RA, Weir DM (1999). Infection with respiratory syncytial virus enhances expression of native receptors for non-pilate *Neisseria meningitidis* on HEp-2 cells. *FEMS Immunol Med Microbiol*; 23: 115-24.

Chapters 5 and 6:

- **Raza MW**, Blackwell CC, Ogilvie MM, Elton RA, Weir DM (2000). Bactericidal activity of monocyte cell line (THP-1) against common respiratory tract bacterial pathogens is depressed after infection with respiratory syncytial virus. *J Med Microbiol*; 49: 227-33.

Role of infection and cytokines in the pathogenesis of chronic obstructive pulmonary disease

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Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality world-wide. The precise sequence of events in COPD is not completely understood. Inflammation in the airways has been unanimously seen by researchers as a pivotal factor, and cigarette smoking is, without doubt, the main cause. A large proportion of heavy smokers, however, does not suffer with COPD, suggesting a role of additional risk factors in pathogenesis. The inflammatory response to cigarette smoke and infectious agents is determined by the host's genetic composition. Cigarette smoking, by altering the surface milieu of respiratory mucosa and by causing immunosuppression, increases the susceptibility of individuals to infection with respiratory viral and bacterial pathogens. Virus infection has also been recognised as a susceptibility factor for secondary bacterial infection. An investigation into the role of individual genetic variations in inflammatory cell and cytokine production and non-host factors involved in COPD forms the basis of the development of more effective strategies to intervene in pathogenesis, progression and exacerbation of COPD. The aims of this article are to review the evidence for predisposing factors for COPD, with a particular emphasis on respiratory tract infections, and to examine those findings in relation to individual genetic variations and their interactions for induction of pro-inflammatory cytokine production in the respiratory tract.

Keywords: chronic obstructive pulmonary disease, COPD, infection, cigarette smoking, genetic polymorphism, pathogenesis.

CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD): DEFINITIONS

COPD affects 16 million people in the USA alone [1], and is the fourth leading cause of death [2]. Clinically, COPD presents heterogeneously with features of emphysema and chronic bronchitis. It is confirmed by abnormal tests of expiratory flow that do not improve markedly over several months of observation. Emphysema is a form of restrictive lung disease caused by an irreversible distension of lung acini distal to the terminal bronchioles. There is destruction of alveolar septa but no fibrosis [3]. Chronic bronchitis is characterised by inflammation of large airways, and clinically it is characterised by a chronic productive cough for more than 3 months for at

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least 2 consecutive years in patients in whom other causes of chronic cough have been excluded [4]. Changes of varying severity in the peripheral airways determine the degree of airflow obstruction which often accompanies chronic bronchitis [5]. Airflow obstruction is common between emphysema, chronic bronchitis and bronchial asthma, and their relationship based on this feature is illustrated in Fig. 1.

INFLAMMATION AND COPD

Inflammatory cells

COPD shares many histopathological features with bronchial asthma [6] but differs from asthma by the presence of infiltration of inflammatory cells and cytokines [7,8]. Compared with healthy smokers or non-smokers, smokers with chronic bronchitis have increased numbers of macrophages, neutrophils and T cells. Non-smokers with asthmatic bronchitis show more eosinophils and mast cells in their bronchoalveolar lavage (BAL) [9]. BAL specimens and collagenase-dissociated lung tissue from rats exposed for 6 months to cigarette smoke exhibited at 1 month a wave of infiltration with neutrophils which were replaced by macrophages that persisted until the end of observation period [10].

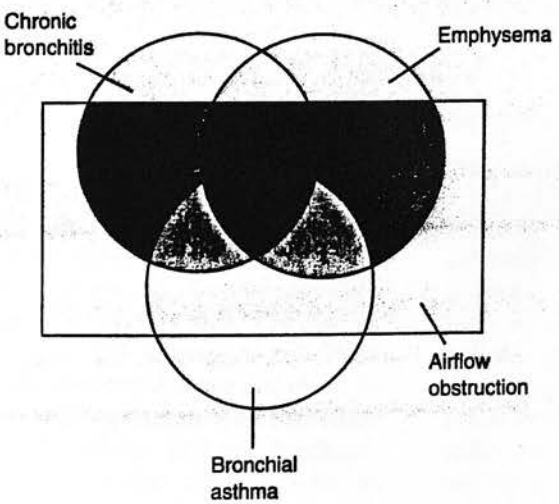

Fig. 1. Schematic non-proportional representation showing subsets of chronic obstructive pulmonary disease (COPD) patients with emphysema, chronic bronchitis and/or bronchial asthma with features of airflow obstruction (shaded areas in the box). The patients without airflow obstruction are not classified as COPD (areas of circles outside the box). Some bronchial asthmatic patients have reversible airflow obstruction and are not classified as COPD (represented by non-shaded area of circle within the box). Airflow obstruction can be due to causes other than COPD, e.g. cystic fibrosis (represented by the area in the box outside the circles). (Adapted from *Am J Respir Crit Care Med* 1995, **152**:578–583 with permission from American Thoracic Society).

Cytokines

Cytokines are present as an intricate network of soluble signalling substances that elicit and control inflammation, immune responses, cellular growth and fibrosis. Cytokines in the lungs can be divided into three categories: pro-inflammatory; anti-inflammatory; and growth-stimulatory (Table 1).

Pro-inflammatory cytokines are essential to combat infections [11–13], but their secretion in excessive quantities for prolonged periods can become detrimental to the tissue. Overproduction of anti-inflammatory cytokines may hamper the protective role played by pro-inflammatory cytokines against noxious agents [14]. Overproduction of tissue growth factors in the lungs is often associated with chronic inflammation and fibrosis.

Tumour necrosis factor (TNF), interleukin (IL)-6 and IL-8 are involved in inflammation in COPD [15]. Patients who have asthmatic bronchitis but do not smoke show IL-5 in their BAL specimens, whereas patients who smoke and have chronic bronchitis show IL-2, TNF-α and IL-8 in their BAL specimens [9]. There are conflicting reports on IL-8 production in COPD [16,17]. TNF-α has a central role in inflammation; it is a potent inducer of endothelial cells for the production of intercellular adhesion molecule-1, which is critical for the recruitment of phagocytes [18]. It activates macrophages and neutrophils, increases cytotoxicity, releases oxygen and nitrogen radicals [19], IL-6 and IL-8 [20,21] from inflammatory cells.

Nitric oxide (NO)

NO in human airway tissue is localised to the airway epithelium, sensory nerves, endothelium, vascular and airway smooth muscles, and inflammatory cells [22]. It is thought that, at moderate concentrations, NO is protective and

Table 1. Cytokines and tissue growth factors in the lungs

Group	Cytokines	Functions
Pro-inflammatory cytokines	Tumour necrosis factor	Combat infectious agents
	Interleukin-1	Chemotactic for inflammatory cells
	Interleukin-6	Cause inflammation
	Interferon α, β and γ	
	Nitric oxide	
Anti-inflammatory cytokines	Interleukin-4	Suppress production of pro-inflammatory cytokines
	Interleukin-10	Suppress inflammation
Growth stimulatory cytokines and factors	Nitric oxide	Mucus hypertrophy
	Interleukin-13	Fibroblast hyperplasia
	Interleukin-9	Subepithelial fibrosis
	Interleukin-4	

regulatory in function; at higher concentrations, it acts as a toxic factor. The beneficial pulmonary vasodilatory, bronchodilatory, and bactericidal effects are lost in cases where NO production is low. Higher levels might induce exudate formation, DNA-toxicity and cytotoxicity [23].

NO production from alveolar macrophages is enhanced by virus infection [24] whereas it is reduced by episodic or habitual smoking [25], and returns to normal on cessation of smoking [26].

RISK FACTORS

Cigarette smoking

COPD is associated with the total numbers of cigarettes smoked per year, current smoking status, smoking at an early age and duration of smoking [27]. More than 80% of smokers, however, do not suffer with COPD [28]. The factors that determine whether the disease is mild and short-lived or severe and chronic are not clear. In some individuals, smoking causes well described histopathological [29], cellular [30] and biochemical [31] abnormalities in the airways without causing COPD. These observations indicate the existence of additional host and environmental factors that might determine the outcome of the injury caused by smoking.

Smoking causes mucus gland hypertrophy with mucus hypersecretion in the proximal airways; mucous hyperplasia, metaplasia, smooth muscle hypertrophy and fibrosis in the bronchioles; and damage to respiratory bronchioles resulting in emphysema [32]. Differences in inhaling habits, smoking style, presence and type of the filter and the kind of the tobacco smoked might have effects on the lung pathology in response to smoking [33]. Shallow inhalation of smoke resulting in high nicotine uptake and smoke particle deposition in the airways might be associated with chronic bronchitis. High carbon monoxide absorption from alveoli exposed to smoke in deep inhalation might result in emphysema [34]. Cigarette smoke *in vivo* and *in vitro* has been shown to affect protective mechanisms, immune function and inflammatory balance in the respiratory tract (Table 2).

Infection

Respiratory infection has been postulated to have a role in pathogenesis and progression of COPD [45–47]. Treating exacerbations of COPD with antibiotics when bacteria are isolated is a common practice. There are, however, two difficulties with this approach to treating exacerbations: (i) although symptoms such as productive cough, purulent sputum and dyspnoea could be due to infection, a non-infectious cause might be responsible for the underlying inflammatory process in COPD; (ii) the incidence of microbial isolates from the respiratory tract during exacerbation is not usually different from that during remission.

The airways are constantly bombarded with microbial agents and there are normally efficient antimicrobial mechanisms keeping the lungs relatively sterile. It is likely that more bacteria colonise the lower airways and cause acute-on-chronic disease in patients with COPD due to structural derangement and possibly due to

Table 2. Effect of cigarette smoking, or cigarette smoke extracts in *in-vitro* models, on protecti mechanisms and immune responses against respiratory tract infection

Mechanism	Function	Effect	Reference
Surfactant covering alveolar epithelium	Protection against bacterial toxins and microorganisms	Thinning	35
Ciliary movement	Clearance of particles and microorganisms	Slowed down, cilia lost	36
Altered cell surface antigens on macrophage cell line		Increased bacterial binding	37
Cigarette smoke deposits on the epithelium		Increased bacterial binding	38
Natural killer cells, neutrophils, alveolar macrophages	Non-specific bactericidal activity	Bactericidal and bacterial uptake functions deranged	39–42
Immune and inflammatory response	Bactericidal and antiviral mechanisms	Altered expression of surface antigens on immune cells	43
Cytokines and nitric oxide	Bactericidal and pro-inflammatory	Increased tumour necrosis factor and decreased nitric oxide	44

interference with the local anti-bacterial and clearance mechanisms in areas of the lungs.

Non-typeable *Haemophilus influenzae, Streptococcus pneumoniae* and *Moraxella catarrhalis* are common bacterial species isolated from patients during episodes of exacerbation in the course of COPD and asthma [48–50]. Some recent reviews [47,51] did not find differences in isolation of bacterial flora, using routine respiratory specimens, between patients with exacerbation or with stable COPD. Studies in which samples were taken from the lower respiratory tract have, however, demonstrated differences between the two groups [52].

Studies comparing the tracheobronchial microflora during acute exacerbation and stable periods in the course of COPD showed significant differences in isolation rates of influenza virus, respiratory syncytial virus (RSV) [53] and rhinovirus [54]. Evidence of persistent infection with RSV was found in patients with COPD [55].

Compared with uninfected human peripheral blood monocytes (PBM), PBM infected with RSV exhibited a significant increase in the production of TNF and a significant decrease in production of NO [44].

Recurrent respiratory virus infections during childhood

Significant associations between childhood respiratory infections and the later development of chronic bronchitis have been demonstrated Repeated childhood respiratory infections were shown to have a greater influence than cigarette smoking in the later life on the subsequent development of COPD in later life [56]. Because passive smoking predisposes to respiratory infection, passive smoking at an earlier stage combined with repeated infections might be the underlying factor predisposing to COPD in their study.

Other risk factors

The incidence and mortality rates of COPD are higher in heavily industrialised urban areas [57]. Associations have been found between the amount of sulphur dioxide and particulate matter in the air and exacerbation of bronchitis [58].

Men suffer more than women with respiratory symptoms [27]. Compared with male smokers, female smokers, however, suffer a higher degree of morbidity based on degree of airflow obstruction [59]. Prevalence of airflow obstruction among grain workers was greater in groups of British origin than those of German or Eastern European origin [60]. Lower socioeconomic status is associated with higher morbidity and mortality due to COPD [27].

Smoking predisposes to infection

Smoking affects the respiratory tract in many ways: increased frequency and/or density of bacterial colonisation; symptomatic viral and

bacterial infection. Compared with non-smokers, buccal epithelial cells of smokers bound higher numbers of *S. pneumoniae*, non-typeable *H. influenzae* and *M. catarrhalis* [61,62]. Materials in a water-soluble extract of cigarette smoke were shown to enhance binding of major bacterial respiratory pathogens to cells of non-smokers [61].

Various mechanisms, including immunosuppression and cell surface alteration have been described (Table 2).

Virus infection of the respiratory tract predisposes to bacterial disease

Clinical, epidemiological and experimental evidence indicates that virus infection of the respiratory tract can be a predisposing factor for bacterial disease [63,64]. Infection of epithelial cells with RSV increases binding of respiratory bacterial pathogens to epithelial cells or respiratory mucosa [65,66]. Two mechanisms by which virus infection could increase binding of bacteria to epithelial cells are: (i) expression of new virus-induced cell surface antigens; and (ii) upregulation of host cell antigens that act as receptors for bacteria [67–69]. Patients with RSV infections showed a significant rise in antibodies to *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* [70], and invasive disease due to pneumococci followed infection with RSV [71]. Invasive infections due to pneumococci or *H. influenzae* were identified in 26% of children suffering with RSV [72]. Similarly, 54% of cases of invasive infection with *H. influenzae* and 47% with pneumococci also had serological evidence of infections with respiratory syncytial, parainfluenza or influenza viruses [73]. The ways in which viral infection can predispose individuals to secondary bacterial disease are given in Table 3.

GENETIC FACTORS IN INFLAMMATION

Damage to the respiratory tract might be associated with the magnitude of the inflammatory response to bacterial infection. Susceptibility to and progression of inflammatory conditions have been associated with polymorphisms in genes encoding cytokines and major histocompatibility antigens [78,79]. Associations have been found between certain TNF gene polymorphisms and rheumatoid disease [80], myasthenia gravis [81], cystic fibrosis [82], primary biliary cirrhosis [83], and ulcerative colitis [84]. Predominant pro- or anti-inflammatory cytokine responses to infectious agents based on the host genetic make up have been reported [85].

Gene polymorphism in cytokine production and susceptibility to infections

Variations in individual susceptibility to infection have aroused interest in the investigation of polymorphism of genes coding for cytokine production. Immune responses and inflammatory cytokines in response to infectious agents have been associated with a number of polymorphisms in genes encoding TNF, IL-6 and IL-10 (Table 4).

Host genetic factors for vulnerability to cigarette smoking and COPD

The finding that many cigarette smokers do not progress into COPD might suggest genetic polymorphism. Alpha$_1$-antitrypsin is an acute phase protein with anti-protease activities and is produced in many inflammatory conditions [94]. Alpha$_1$-antitrypsin is genetically determined and low levels have been associated with early onset

Table 3. Factors in viral infection considered to contribute to secondary bacterial disease

Effect	References
Immune suppression	74,75
Local oedema formation and tissue injury. Loss of mucociliary function and decreased bacterial clearance	76
Formation of exudate that enhances bacterial growth	74
Increased bacterial binding to virus infected cells	65,67,69,77
Decreased phagocytosis	37

Table 4. Polymorphism of genes encoding for TNF, IL-6 and IL-10 associated with increased susceptibility to infection and inflammation

Cytokine	Polymorphism	Comments	Reference
TNF	G to A transition at position −238	Higher inflammatory response in meningitis	85
	G to A transition at position −308	Patients more susceptible to cerebral malaria; higher production of TNF; differences in ethnic groups	86,87
	Point mutations in TNF gene	Decrease of TNF expression from cell lines transfected with the altered gene; patients with sepsis exhibited point mutations more than control group; resistance to human cytomegalovirus infection	87–89 90
	Alleles exhibiting *Ncol*-digestion	More patients with fatal sepsis exhibited these alleles than patients with non-fatal sepsis; finding more significant in homozygous individuals than the heterozygous; chronicity of hepatitis B and C infection; can affect the binding of nuclear factors, e.g. kappa B, to the promoters regions of the gene	91, 92
IL-6	G to C transition at position −174	Decreased production; altered binding site for NF-1; −174 C rare in Afro-Caribbean compared with UK Caucasians	93
IL-10	Substitution at −1082, −819 and −592 positions	−1082 G protects against Epstein–Barr virus infection; differential production in response to endotoxins	77

panacinar emphysema [95]. Compared with normal controls, lung function abnormalities were found more commonly in individuals heterozygous for the α_1-antitrypsin gene; 5–14% of the population were producing subnormal quantities of this factor [95,96]. Cigarette smoking has been found particularly deleterious to individuals homozygous (and possibly heterozygous) for α_1-antitrypsin gene [96].

Analyses of the incidence of COPD in affected families, twins and first-degree relatives of patients with COPD indicate the presence of additional unknown genetic factors [97,98]. Genetic rather than environmental factors correlated with lung function in families with members affected by COPD [98]. PBM from blood donors exhibited various responses of TNF and NO production when challenged with cigarette smoke extract, RSV or both; monocytes from some individuals demonstrated extreme responses compared with others tested under the same conditions [37].

CONCLUSION

Although the precise sequence of events in COPD leading to airway obstruction is not completely understood, inflammation in the airways has been seen unanimously by researchers as a pivotal factor. Understanding the pathophysiological and molecular mechanisms of inflammation in the lungs holds the key to the development of effective intervention strategies. Inflammation is initiated in bronchoalveolar epithelium which also becomes its first main target [99]; it is initiated by cigarette smoking but other genetic and environmental factors seem needed to promote it. Recurrent infections, to which patients with COPD are generally highly susceptible, enhance the progression of the underlying process and result in exacerbations. A proposed course of COPD highlighting the role of respiratory tract infections in pathogenesis is illustrated in Fig. 2.

There are five areas of investigation into the role of infection in COPD that require examination.

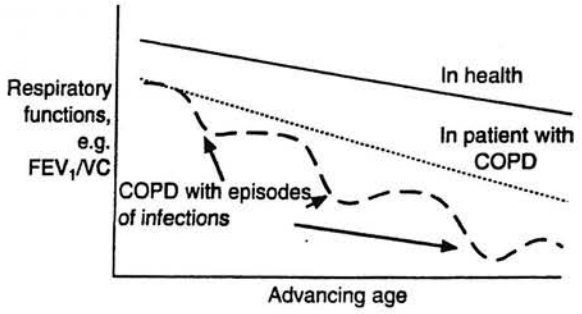

Fig. 2. Decline in respiratory functions over the years expected in healthy individuals or patients with chronic obstructive pulmonary disease (COPD) with or without episodes of infection (non-proportional representation).

(i) Isolation of bacterial species from routine respiratory specimens from patients with COPD does not necessarily indicate disease. This necessitates investigation into methods to determine density of colonisation and the areas of the respiratory tract colonised by bacteria during exacerbations and stable periods in the course of COPD. Results of such studies might explain episodes of exacerbation in a proportion of cases. (ii) The presence of bacteria in the normally sterile lower respiratory tract, whether during stable COPD or acute exacerbation, warrants investigation into their role in inflammation. (iii) Possible changes in the growth patterns of bacteria might be due to the appearance of as yet unrecognised host factor(s) in the respiratory tract, changes in the bacterial phenotype [100] or viral infections. Viral infection can increase bacterial load and possibly the extent of bacterial colonisation in the respiratory tract. (iv) The role of virus infection in priming or enhancing the inflammatory response to bacterial components or their products also needs to be investigated in relation to host genetic factors and cigarette smoking. (v) Gene polymorphisms and their effects on different levels of pro- and anti-inflammatory cytokine production in response to infection or cigarette smoke might provide additional insights into the genetic influence in development of COPD. These changes in the milieu of the lungs during exacerbation are expected to disturb the fine-tuning of the cytokine production contributing to chronic inflammation (Fig. 3).

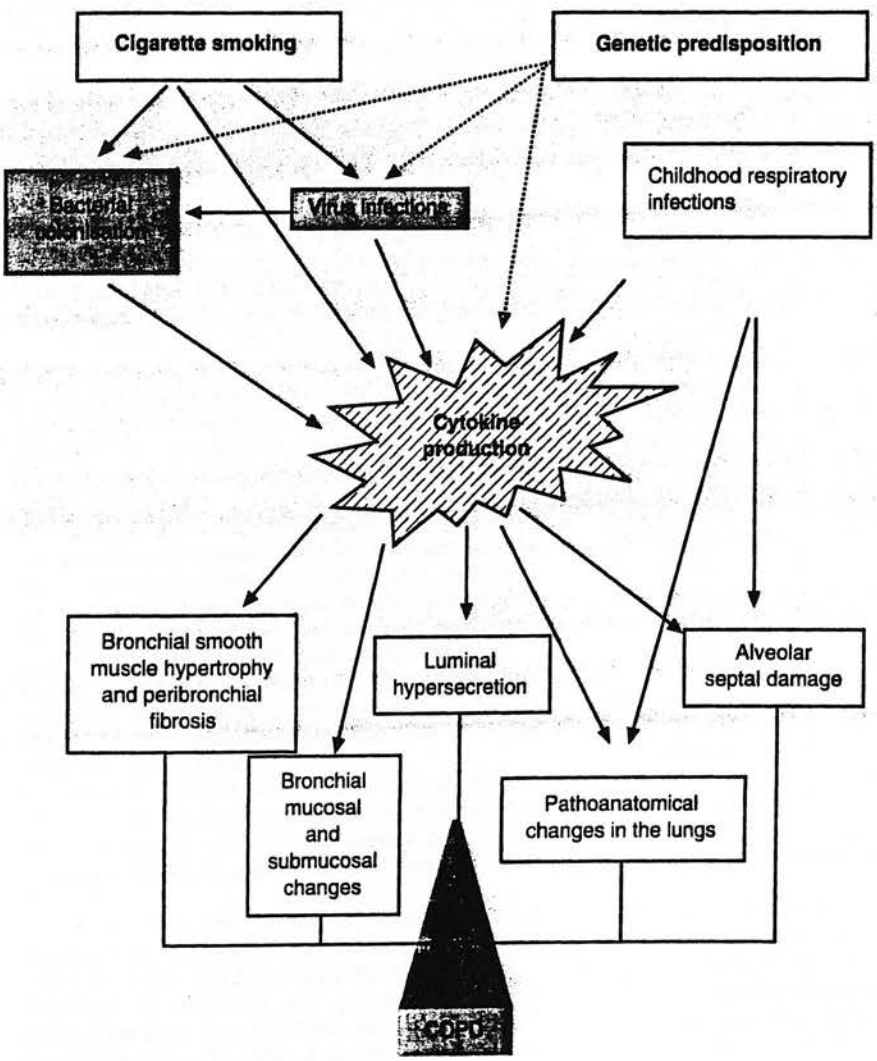

Fig. 3. Schematic representation of events leading to chronic obstructive pulmonary disease (COPD) and episodes of exacerbation in its course.

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Sudden infant death syndrome, virus infections and cytokines

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Abstract

Many epidemiological risk factors identified for sudden infant death syndrome (SIDS) suggest a viral aetiology, e.g. exposure to cigarette smoke and winter peak, mild respiratory symptoms. Virus infections and bacterial toxins induce cytokine activity and it has been suggested that uncontrolled inflammatory mediators could be involved in some cases of SIDS. The aim of this review was to assess the evidence for virus infection in SIDS and to examine those findings in relation to individual variations in cytokine responses and various pathophysiological mechanisms proposed for SIDS such as sleep derangement, hypoxia, cardiac arrhythmia, vascular hypotonicity and hypoglycaemia.

Keywords: Virus infection; Cytokine; Sudden infant death syndrome; Sleep; Cardiac arrhythmia; Hypoxia; Apnea; Hypoglycemia; Circadian rhythm; Cortisol

1. Virus infections and sudden infant death syndrome (SIDS)

Extensive epidemiological studies in several countries have identified the major risk factors for SIDS. Many developmental and environmental factors significantly associated with these deaths parallel those associated with susceptibility of infants and young children to infectious agents, particularly infections of the respiratory tract. These include the age range affected, a winter peak of SIDS in many countries, exposure to cigarette smoke and poorer socioeconomic backgrounds. Case histories of SIDS infants often contain references to mild upper respiratory symptoms prior to death. Major signs of respiratory illness (wheezing, drowsiness, vomiting and bouts of coughing) in these infants during the 2 weeks before death were not significantly different from infants matched for age and sex who died from other causes. There were, however, significant differences in incidence of minor symptoms, snuffles and occasional cough, in these groups [1]. More deaths due to SIDS occur in winter months (Table 1) when virus infections are also prevalent [14–17]. Outbreaks of influenza A virus in children were significantly associated with SIDS, and the association was independent of effects of lower atmospheric temperature [18]. SIDS mostly affects the poor in prosperous countries [19–21] in whom infectious diseases are also relatively more common. Forsyth et al. [22] found higher levels of IgG and IgM, but not IgA, in the lungs at

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Table 1
Association of SIDS with winter months

Country	Reference
Multinational	[2]
UK	[3–6]
France	[7]
New Zealand	[8]
Australia	[3]
USA	[9]
Sweden	[10,11][a]
China	[12]
Japan	[13]

[a]No association was found.

necropsy of infants who died of SIDS compared with infants who died of non-respiratory causes.

While no single agent has been clearly identified as causing SIDS, many different viruses affecting both respiratory and gastrointestinal tracts have been identified in these infants [19] (Table 2). Exposure to cigarette smoke enhances susceptibility to respiratory virus infections [14], possibly by affecting various arms of the host defences against infection: non-specific immune responses [41]; humoral [42] and cellular immunity [43]; and macrophage functions [44]. Maternal cigarette smoking during pregnancy and passive exposure to cigarette smoke are significantly associated with SIDS [5,8,45–51]. Cigarette smoke might also alter pathophysiological sequelae of virus infections [52].

Early infancy (2–4 months), when most deaths from SIDS occur, coincides with a period of declining levels of maternal antibodies and immature immune responses in infants. Breast-feeding in many studies has been shown to be protective against SIDS [45,53,54]. The effect of breast-feeding in relation to SIDS was significant in infants of mothers who were non-smokers [55]. If infectious agents are involved in SIDS, the protection afforded by breast-feeding could be due to the anti-viral and anti-bacterial activities of secretory IgG and oligosaccharides present in human milk. Oligosaccharides in human milk have been shown to have antiviral activity [56–58].

While significant necropsy findings are essentially absent in SIDS, mild inflammatory changes are commonly reported [59]. Epidemiological and autopsy studies of SIDS or epidemiological studies of near-miss infants have provided evidence of virus infections (Table 2). Some negative reports on association between virus infections and SIDS might be attributed to early virus infection with symptoms not yet noticed or not taken as significant by the parents. Failure to isolate or detect viruses might be due to late or inappropriate microbiological sampling or lack of facilities to identify viruses. Newer molecular techniques have been used to screen for viruses, but they have not significantly increased the identification rate [29,31].

2. Cytokine levels in virus infections and SIDS

Various hypotheses to explain SIDS have been postulated. Negative findings at necropsy in infants who died of SIDS suggest a serious physiological derangement: hypoxia and apnoea; extreme alterations in body temperature; hypoglycaemia; hypoten-

Table 2
Reports on respiratory symptoms and virus isolates in cases of SIDS

	Associated with SIDS	Not associated with SIDS
Upper respiratory symptoms	[8,23,24]	[6,25]
Population mixing	[26]	
Influenza virus	[18,27,28]	
RSV	[29,30]	[31]
Rhinovirus	[32]	
Adenovirus	[29,33–35]	
Cytomegalovirus	[33]	
Echovirus		[34]
Virus isolation	[36,37]	[1,27,38]
Enterovirus	[34,39]	
Rotavirus	[35,40]	

Table 3
Significant increase in cytokines in early phase (2–6 h) of virus infection in different models

Virus	Cytokine	Model	Reference
Influenza	IL-1β, TNF-α	Human peripheral blood leukocytes	[68]
RSV	IL-2	RSV-specific peripheral blood T cells	[69]
		Cord blood monocyte-derived macrophages	[70]
	IL-6	Cord blood monocyte-derived macrophages	[70]
		Nasal secretion	[71]
	IL-8	Human bronchial epithelial cell line	[72]
	IL-10, IL-12	Peripheral blood mononuclear cells	[69,73]
	IFN-γ	RSV-specific peripheral blood T cells	[69]
		Murine bronchial alveolar lavage	[74]
		Cord blood monocyte-derived macrophages	[70]
	TNF-α	Peripheral blood mononuclear cells	[73]
		Nasal secretion	[71]
Rhinovirus	IL-8	Respiratory epithelial cells	[75]
		Nasal secretion	[76]

sion; cardiac arrhythmia; or combinations of these factors. It has been suggested that in extreme cases interleukin 1 (IL-1), interferon (IFN) and tumour necrosis factor α (TNF-α) elicited by infections cause somnolence and hypoxia leading to death [60,61]. Blackwell et al. [62–64] suggested uncontrolled cytokine responses elicited by combinations of bacterial toxins, virus infection and/or cigarette smoke might precipitate a series of events leading to some of these unexplained deaths. Very high temperatures observed in some SIDS infants was thought to be the cause of death [65]; these extreme temperatures could have been induced by cytokines such as TNF-α and IL-1. A slight initial derangement of a few cytokines in response to an apparently trivial challenge is capable of triggering various cytokine cascades which might prove lethal. In septic shock, it is not the number of bacteria present but the body's response to the bacteria that determines the severity of illness [66].

Viruses might cause minimal clinical symptoms in infants but trigger cytokine cascades culminating in sudden death. Bacterial pathogens or their products, cigarette smoke, or any combination of these might similarly amplify the production of proinflammatory cytokines. Whatever the identity of the agents, a final common pathway in the pathogenesis of SIDS is suggested by a combination of factors unique to SIDS: (1) a higher prevalence in the early hours of the morning; (2) an association with presumed sleep; (3) peak incidence of SIDS at 2–4 months; (4) and an absence of gross necropsy abnormalities.

Studies of cytokines in SIDS babies are scarce. Howat et al. [67] used cells obtained from the lungs to assess IL-4, IL-5 and IL-10. Significantly higher numbers of cells stained for the cytokines were found in SIDS babies compared with controls. Table 3 summarises studies of cytokines in virus infections for both in vivo and in vitro models.

3. Genetic factors: race and gender

The incidence of SIDS varies significantly between different ethnic groups [77,78]. In Britain, the incidence of SIDS among Indian, Pakistani and Bangladeshi families is lower than in the white population. Infant deaths due to respiratory infections are also lower than in white families [79]. Although low socioeconomic standards have been significantly associated with SIDS in Britain [80], in Hong Kong, where many families live in suboptimal circumstances, there is also a very low incidence of SIDS [81].

On the other hand, some indigenous populations have high incidences of SIDS, e.g., American Indians, Alaskan natives and Australian Aborigines [82,83]. The criticism that the higher incidence of SIDS in the Aboriginal infants was due to differences in diagnosis was addressed by re-examination of all Aboriginal cases of SIDS and sudden unexpected death in infancy between 1980 and 1988 and a corresponding random sample of non-Aboriginal cases. There was no evidence of differences in diagnosis of SIDS in the two populations [84]. Among Native

Americans, Eskimos and Australian Aborigines, the incidence of serious respiratory tract and ear infections is also higher [85,86].

Environmental and cultural factors are thought to contribute to some of these differences. Epidemiological studies indicate that in groups in which smoking is less prevalent among women, deaths due to SIDS are lower. Studies on American Indians and Alaskan natives examined the prevalence of risk factors on populations in which there was a significant difference in incidence of SIDS. From 1984 to 1986 the incidence of SIDS was 4.6 per 1000 live births among Indians and Alaskan natives in the northern region of the USA. In contrast, the incidence among southwestern Indians was 1.4 per 1000 live births. There was no significant difference between the incidence of SIDS in white populations in the two regions with 2.1 and 1.6 per 1000 live births in the north and southwest regions respectively. Socioeconomic status, maternal age, birth weight or prenatal care were not significantly different among the Indian populations in the two areas. The prevalence of maternal smoking during pregnancy was exceptionally high among northern Indians and Alaskan natives but low among the Southwest Indians [82].

In Britain smoking is more prevalent among lower socioeconomic groups [87], and both smoking and poorer socioeconomic conditions were found to be significant risk factors for SIDS [51,80]. Among Asian women of all social classes, smoking is very rare [77], and we have suggested that this might contribute to the lower levels of both SIDS and respiratory deaths in these populations. This could be related to reduced frequency or density of colonisation by potential pathogens or to a lower level of absorption of water-soluble components of cigarette smoke that could enhance inflammatory responses to infection.

Polymorphism in individual susceptibility to infections is expected. Immune responses and proinflammatory cytokines have been reported to differ in several ethnic groups. In white subjects, a TNF2 variant at locus TNF-308 was shown to be significantly associated with HLA-DR3, which also showed a strong association with white subjects compared with black populations [88]. Important ethnic differences were found in the genotype of TNF-α and its linking to MHC alleles [89].

There appears to be a significant genetic component associated with induction of both pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines [90,91]. Fatal outcome of meningococcal disease was significantly associated with low TNF-α responses and/or high IL-10 responses of first-degree relatives of the patient [91]. Studies on differences in pro- and anti-inflammatory responses to virus infections have not been carried out in different ethnic groups.

SIDS affects more male than female infants [7,49,83]. RSV infection was more common in hospitalised male infants; the ratio of males to females was 1.44:1 [92]. Compared with female infants, significantly more males suffered with RSV, influenza and parainfluenza viruses, rhinovirus and adenovirus [93].

4. SIDS and sleep

Sleep is physiologically very different from wakefulness. Higher neuronal disinhibition in sleep is associated with changes in cardiovascular and respiratory systems, as witnessed in adult sleep apnoea syndrome. Most SIDS cases have been reported in infants during presumed sleep in the early hours of the morning [94]. IL-1, TNF-α and IFN-γ have been shown to be somnogenic in physiological conditions and during infections [95]. Since hypoxia has been proposed as a possible cause of SIDS, the effects of virus infections and cytokines on hypoxia are reviewed below (Section 8).

Circadian rhythm can affect the numbers of immune cells in circulation. Compared with wakefulness, there was an acute reduction of the number of natural killer cells, monocytes and all subsets of lymphocytes during nocturnal sleep in healthy men [96].

5. Cytokines, cortisol and circadian rhythm

Circadian rhythm is a characteristic of neuroendocrine pathways. Two important neuroendocrine hormones, cortisol and melatonin, were suggested to affect diurnal variations in the levels of IFN-γ and IL-10 observed with an in vitro model in which

whole blood was challenged with bacterial LPS or tetanus toxoid. INF-γ was highest and IL-10 was lowest during the early morning hours and correlated negatively with plasma cortisol and positively with melatonin [97]. Urinary free cortisol levels in subjects between 1.8 and 17 years were found to be positively correlated with age [98]. Impairment in the ability to control inflammatory mediators resulting from low night-time cortisol levels associated with changes accompanying development of adult-type night-time temperature rhythm was proposed as a 'window of vulnerability' to SIDS [63]. Viral infections might hinder cortisol release from the adrenals in response to stimulation by corticotrophin releasing hormone (CRH) from the pituitary gland. Stimulation by CRH resulted in a reduced or a blunted cortisol response in some men with HIV infection [99].

Sleep was associated with enhanced production of IL-2 by CD3+ T cells but not of IL-1β, TNF-α or IL-6, and the effects were independent of cortisol levels [96,100]. Uthgenannt et al. [101] found similar effects of sleep on IL-2 production. Monocytes from subjects obtained during nocturnal sleep were stimulated in vitro by LPS from *Escherichia coli*; they showed significantly higher TNF-α and IL-1β compared with monocytes obtained when the subjects were awake. Association of cytokines and sleep was further substantiated in patients with obstructive sleep apnoea syndrome. These patients experience disturbed sleep patterns, less sleep at night and spells of sleep during the day. Nocturnal peaks of TNF-α disappeared in these patients and a daytime peak had developed [102]. Cortisol was shown to have damping effect on the somnogenic cytokines, IL-1, IL-2, TNF-α and IFN. While cortisol is induced by virus infections, its production in response to virus infections is not as efficient compared with the response to bacterial infections [98].

6. Infections, cytokines and sleep regulation

Sleep, like fever, is a common manifestation of infection. Most deaths attributed to SIDS occur during presumed sleep between midnight and 8.00 a.m. when many somnogenic cytokines (IL-1, IL-2, Il-6, TNF-α, and IFN) are at a peak. Human recombinant TNF-α and IL-1 were shown to cause or prolong slow-wave sleep and suppress the rapid eye movement (REM) phase of sleep [103,104]. Similar effects were observed in rabbits with human recombinant IFN [105]. Immunisation and strain difference in mice were associated with dissimilar sleep pattern after challenge with influenza virus; some mice had deeper, more prolonged sleep than others [106].

Virus-associated double-stranded RNA extracted from mice infected with influenza virus and a synthetic double-stranded RNA were shown to cause flu-like symptoms and non-REM sleep in rabbits [107]. Serum anti-viral activity, probably due to IFN, was associated with sleep [107,108]. Bacteria and their products have similar somnogenic effects [109,110].

7. Hypoxia, reflex apnoea and SIDS

Airway obstructions, other than suffocation, leading to chronic and acute hypoxia have been postulated as a cause of death in SIDS infants. Profound hypoxia and infection were necessary experimental conditions to produce intrathoracic petechiae in rats, a characteristic of the autopsy changes observed in SIDS infants [59]. Levels of cortisol in infants who died of SIDS without petechiae (9 μg per 100 ml) were lower than that of SIDS infants showing intrathoracic petechiae at necropsy (25 μg per 100 ml). SIDS infants with petechiae also showed 20% more muscle mass in pulmonary arteries compared SIDS infants without petechiae, indicating the existence of chronic hypoxia/hypoventilation in the first group [59]. Rognum and Saugstad [111] suggested tissue hypoxia was a cause of death from comparison of hypoxanthine levels in vitreous humor from SIDS and comparison groups included in their study. Multiple brief apnoeic attacks were noticed in infants who eventually died of SIDS [112]. Some workers have, however, argued against hypoxia as a possible cause of SIDS [113,114].

8. Effects of virus infections and cytokines on hypoxia and reflex apnoea

Reinforced reflex apnoea was observed in infants

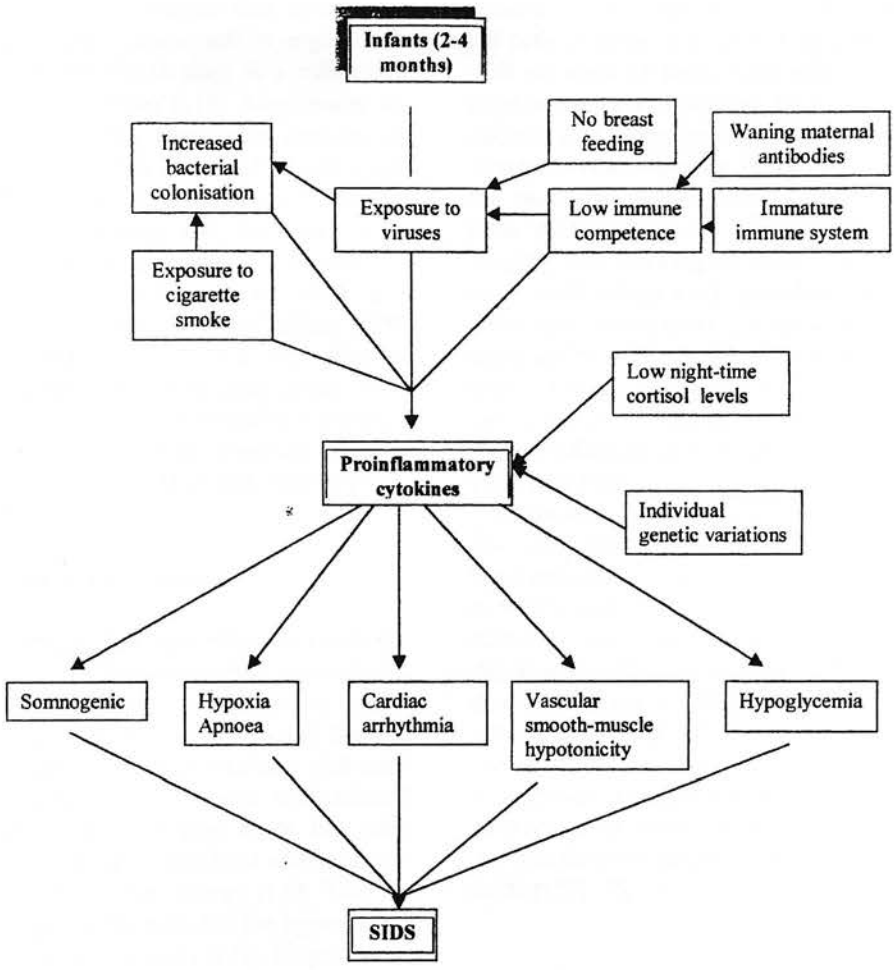

Fig. 1. Schematic representation of events leading to a final common pathway of physiological derangement and sudden death.

with infection due to RSV compared with uninfected infants [115], and this was suggested to be one of the mechanisms of sudden death in some infants who suffered mild respiratory symptoms before death [116]. Apnoeic attacks can cause near-miss SIDS in infants with RSV infection [117].

9. Cytokines and regulation of vascular smooth muscle cell contractility and vascular tone

Proinflammatory cytokines (TNF-α, IL-1, IFN-γ) released in response to viral infections modulate vascular contractility primarily through regulation of nitric oxide (NO), a potent vasodilatory factor [118]. Vascular endothelial NO production is constitutively controlled and modulated by bradykinin, acetylcholine and epinephrine. Baseline vascular tone is maintained in partial relaxation due to NO [118,119]. Vascular smooth muscle cells can also release vast amounts of NO when stimulated by the proinflammatory cytokines [120].

10. Cardiac arrhythmia, SIDS and cytokines

Gunteroth reviewed studies on SIDS relating to possible cardiac causes of death and concluded a

cardiac theory of SIDS was not sustainable [121]. There is, however, some evidence that arrhythmia without structural cardiac abnormalities might cause sudden death in SIDS. REM sleep is a vulnerable phase for cardiac arrhythmia [113]. Abnormally increased heart rates during sleep were reported in subsequent siblings of SIDS infants [122] and near-miss infants [123]. Long QT syndrome is characterised by ventricular fibrillation and, sometimes, fatal syncopal attacks [124]. Long QT interval was considered to be an important risk factor for SIDS in a prospective study of a large group of infants [125]. Arrhythmia has been reported as a side effect of treatment of patients with metastatic cancer with TNF, IL-2 and IFN-γ [126,127]. Negative ionotropic and arrhythmogenic effects were observed in myocytes cultured in IL-1, IL-2, IL-3 and TNF-α [128].

11. Hypoglycaemia and cytokines

Acute hypoglycaemia has been associated with deranged cytokine levels. Hypoglycaemia was induced in rats with TNF-α without changes in the insulin levels; it was ameliorated with corticosteroid therapy [129]. Hypoglycaemia in an elderly patient with non-Hodgkin's lymphoma was associated with normal insulin and insulin-like hormone levels but with high TNF-α levels. It was normalised after correcting TNF-α by cytoreductive therapy [130]. TNF-α-like molecules might be responsible for hypoglycaemia observed in cerebral malaria [131]. Staphylococcal enterotoxin B caused weight loss and hypoglycaemia in rats. This was prevented by antibodies against IFN-γ; levels of TNF-α and IL-6 remained unchanged [132].

12. Conclusion

Animal models indicate that the inflammatory response to viral infections could have a priming effect via INF for induction of high levels of mediators such as TNF-α or nitric oxide [133]. We proposed the hypothesis that virus infection, alone or in conjunction with bacteria, their toxins or cigarette smoke, might induce an uncontrolled cytokine cascade which could contribute to the events leading to sudden deaths in infants. Virus infection has been demonstrated to enhance bacterial binding to epithelial cells in vitro [134–136]. Compared with infants with no signs of respiratory infection, infants with respiratory viral infections have significantly more bacteria and more species of bacteria in their nasal secretions when sleeping in the prone position; and, the species of bacteria are similar to those isolated from SIDS infants at autopsy [137]. Fever, prone sleeping and blocking of nasal passages with secretions could induce a micro-environment in which the permissive temperature for induction of potent bacterial toxins is obtained. Virus infections have been demonstrated to enhance the lethality of bacterial toxins in animal models [138] and to enhance induction of inflammatory mediators from human cells in vitro [139,140].

Physiological systems regulating cytokines, sleep and body temperature are closely interrelated. Infants develop circadian rhythm during the age range in which most SIDS cases occur. During the period following the switch to adult-like temperature rhythms, the physiological changes that occur in endocrine responses and hormone levels might result in infants producing lethal amounts of proinflammatory cytokines in response to infectious agents and/or exposure to cigarette smoke. The uncontrolled production of these cytokines could affect any of the mechanisms proposed as possible causes for SIDS [133] (Fig. 1).

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FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY

Infection with respiratory syncytial virus and water-soluble components of cigarette smoke alter production of tumour necrosis factor α and nitric oxide by human blood monocytes

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Abstract

Cigarette smoke and virus infections contribute to the pathogenesis and exacerbation of chronic obstructive pulmonary disease and asthma. The objective of this study was to examine the effects of a water-soluble cigarette smoke extract (CSE) and/or respiratory syncytial virus (RSV) infection on release from monocytes of the blood from donors of tumour necrosis factor α (TNF-α) and nitric oxide (NO). Both RSV infection and CSE stimulated TNF-α release from monocytes and there was an additive effect if both the agents were present. There was a decrease in NO release, but the effect was significant only with CSE or a combination of CSE and RSV infection. Interferon γ significantly increased TNF-α release and cotinine significantly increased NO release. Nicotine decreased both TNF-α and NO responses. The general pattern observed for individual donors was increased TNF-α and decreased NO. The proportion of extreme responses with very high TNF-α and very low NO in the presence of both RSV and CSE increased to 20% compared with 5% observed with CSE or RSV alone. The results show that RSV infection and components of cigarette smoke elicit inflammatory responses that could contribute to damage to the respiratory tract and these individual factors could be more harmful in combination.

Keywords: Cigarette smoke; Respiratory syncytial virus; Tumor necrosis factor; Monocyte; Inflammation

1. Introduction

Bacterial and viral infections and non-infectious air pollutants such as cigarette smoke are important determinants in the pathogenesis of respiratory disease. Their influence on the inflammatory and immune responses underlies the pathological processes in the respiratory tissues. The factors that determine whether the disease is mild and short-lived or severe and chronic are not clear. A number of reports have examined the release of inflammatory mediators from alveolar macrophages and there have been clinical and experimental studies on smoking and virus

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infection as contributory factors to chronic obstructive pulmonary disease (COPD) [1,2]. Both tumour necrosis factor α (TNF-α) and nitric oxide (NO) are important inflammatory mediators in COPD and asthma [3–5]. Infection with respiratory syncytial virus (RSV) induces release of TNF-α and NO from human alveolar macrophages, bovine peripheral blood mononuclear cells and a murine monocyte cell line [6–8]. There are, however, conflicting reports on the effect of smoking on TNF-α release [5,9,10]. Both episodic and habitual smoking reduced NO exhalation [11]; but, in pigs challenged with cigarette smoke, a vasodilator response due to NO release was recorded [12].

Both blood monocytes and alveolar macrophages can be infected with respiratory syncytial virus (RSV) [13,14], and both cell types are expected to be exposed to water-soluble components of cigarette smoke absorbed across mucosal surfaces. The objective of the present study was to evaluate release of TNF-α and NO from human blood monocytes challenged with either RSV, a water-soluble cigarette smoke extract (CSE) or both. Since many virus infections stimulate release of interferon γ (IFN-γ) [15] that might in turn mediate other secretory functions, the effect of IFN-γ on release of TNF-α and NO in this system was also analysed. Nicotine is metabolised in the liver to cotinine which is secreted in body fluids including those of the respiratory tract [16]; therefore, the effects of nicotine and cotinine on TNF-α and NO release were also examined.

2. Materials and methods

2.1. Preparation of cigarette smoke extract

A water-soluble extract of cigarette smoke (CSE) was prepared by the use of a vacuum pump to pass the smoke of 10 filter-tipped cigarettes (Benson and Hedges) through 100 ml of Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 0.45% (w/v) D-glucose and 4 mg l^{-1} pyridoxin [17]. To reduce lipopolysaccharide contamination, the glass bottles were heated at 134°C for 1 h. The CSE was sterilised by filtration and aliquots were stored at −20°C.

2.2. Preparation of RSV stock

The Edinburgh strain of RSV (subgroup A) was grown in HEp-2 cells (an epithelial cell line) as described previously [18] except that the virus was harvested in growth medium (GM) containing DMEM supplemented with 10% (v/v) foetal calf serum (Gibco), 2 mm l^{-1} L-glutamine (Gibco), 200 μg ml^{-1} streptomycin (Gibco) and 100 IU ml^{-1} penicillin (Gibco). The virus suspension was adjusted to 2×10^6 plaque-forming units ml^{-1}.

2.3. Separation and stimulation of monocytes from blood

One-day-old buffy coats from the blood of group O, Rh+ donors were obtained from The Scottish National Blood Transfusion Service (SNBTS), Royal Infirmary, Edinburgh. Buffy coats were diluted 1 in 4 with sterile phosphate-buffered saline (PBS). The diluted preparations (40 ml) were layered over 12 ml of Histopaque 1077 (Sigma) in 50-ml polypropylene conical tubes (Greiner, Gloucestershire, UK). The tubes were centrifuged at $400\times g$ for 30 min at 25°C. Mononuclear cells, in the opaque band formed at the interface of serum and Histopaque, were aspirated carefully. The cells were washed twice in sterile PBS at $150\times g$ for 10 min and the supernatant fluid was discarded. The cells were resuspended in 20 ml of GM, transferred to a 75-cm^2 tissue culture flask (Greiner) and incubated at 37°C for 30 min to separate monocytes from non-adherent cells. The medium containing non-adherent cells was poured off and the monocytes harvested by gentle scraping with a cell scraper in 20 ml of fresh GM. A viable count was performed by the trypan blue dye exclusion method and the concentration of monocytes adjusted to 1×10^8 ml^{-1} in GM. The cells (1 ml) were distributed in 24-well tissue culture plates (Costar) with 1×10^6 cells in each well. Viability of cells at the end of each experiment was also assessed by microscopic examination of cells in the wells for exclusion of trypan blue.

The cells were cultured at 37°C in 5% CO_2 in 1 ml of GM, GM with CSE at various dilutions, RSV at a multiplicity of infection (MOI) of 2.0 [6], or with a combination of RSV and CSE. Some cells were also exposed to IFN-γ (Sigma), nicotine (Sigma) or coti-

nine (Sigma) ranging from 25 to 400 ng ml^{-1}. Samples were collected from each well after 48 h for determination of TNF-α (100 μl) and after 72 h for determination of NO (400 μl). Negative control samples to which no cells were added included culture medium alone, medium with CSE and/or RSV, or with IFN-γ, nicotine or cotinine. The samples were stored at −20°C until analysed.

The proportion of monocytes infected with RSV in each sample 24 h post-infection was determined by an indirect immunofluorescence technique with monoclonal antibody to glycoprotein G of RSV [18].

2.4. TNF bioassay and detection of NO

L-929 cells (a mouse fibroblast cell line) were used in a bioassay for TNF-α activity [19] and the results expressed as per cent cytotoxicity [20]. NO was detected as nitrites by the spectrophotometric assay described by Zhang et al. [21]. The samples were clarified by centrifugation at 12 600 × *g* with a microcentrifuge (Sorval MC 12C, Dupont) for 5 min. Supernatant fluids (400 μl) were reacted with equal volumes of Greiss reagent which contained 0.3% (w/v) naphthylethylenediamine dihydrochloride (Sigma), and 1% (w/v) sulfanilamide (Sigma) in 5% (v/v) orthophosphoric acid (BDH), mixed 1:1 immediately before use. After incubation for 10 min at room temperature, the absorbance at 540 nm was determined in a spectrophotometer (Jeway 6100). Concentrations of nitrites in the samples were derived from a standard curve for sodium nitrite prepared for each experiment.

2.5. Statistical methods

The results obtained with buffy coats from 24 donors are presented here. The results from some samples for some treatments could not be presented because of contamination in individual wells; therefore, the mean control values corresponding to different experiments were not the same. The data from monocytes treated with different agents were compared with those from the monocytes incubated with medium only. Comparisons were made also between different treatment groups. The significance values obtained with paired *t*-tests of the data were similar to values obtained with a non-parametric test (Wilcoxon's). The values obtained from *t*-tests are presented here.

3. Results

3.1. RSV infection of cells

On average, more than 40% of monocytes from each individual tested were infected with RSV at MOI of 2.0 in these assays. One-way 'analysis of variance' indicated no significant differences in the proportion of RSV-infected cells among the donors.

3.2. Standardisation of the assay

Ten-fold dilutions of CSE ranging from smoke of 0.1 to 0.0001 cigarette ml^{-1} were tested with monocytes from four donors, and a dilution of 0.001 cigarette ml^{-1} was selected for the assays on the basis of maximum effects on the production of TNF-α and NO without killing the monocytes (Fig. 1). Doubling dilutions of IFN-γ, nicotine and cotinine ranging from 400 ng ml^{-1} to 6.25 ng ml^{-1} were tested [22]. A dose of 25 ng ml^{-1} for these reagents was used for further study (data not shown). More than 90% of monocytes survived until the end of the ex-

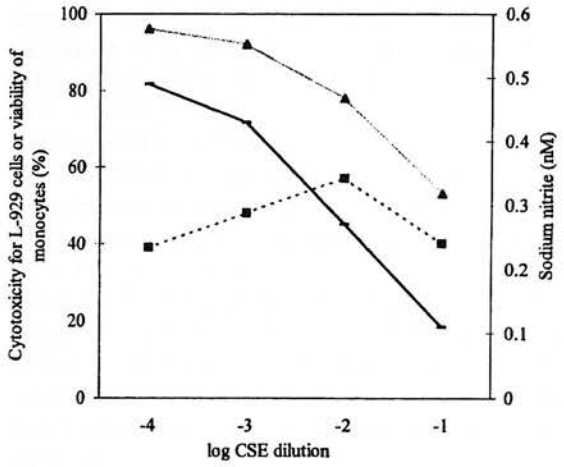

Fig. 1. Per cent viability of monocytes (▲), per cent cytotoxicity of L-929 cells due to TNF-α (■) and levels of sodium nitrite (solid line) produced by monocytes incubated for 24 h with various dilutions of cigarette smoke extract (CSE).

periments under the conditions selected for the assays.

Time course experiments with monocytes from four donors (6–72 h) found the maximal TNF-α bioactivity occurred at 48 h and nitrite accumulation at 72 h in response to CSE or RSV (data not shown). TNF-α bioactivity was not detected in control samples without cells containing culture medium or medium with CSE, RSV, CSE and RSV, IFN-γ, nicotine or cotinine. For the detection of nitrites, the spectrophotometer was blanked on these individual controls for assessment of their respective test samples. No effect due to presence of these agents was recorded at the absorbance used to detect sodium nitrite.

3.3. *The effect of CSE and RSV infection on TNF-α bioactivity*

The TNF-α bioactivities expressed as per cent cytotoxicity of L-929 cells observed in experiments with monocytes from a total of 24 donors were compared. Fig. 2 represents paired differences in L-929 cytotoxicity due to TNF-α bioactivities caused by different treatments of monocytes compared with monocytes incubated with medium only. Compared with cell culture fluids from cells (24 donors) incubated with medium alone (mean 48%, S.E.M. 5.2), cell culture fluids from cells incubated with CSE

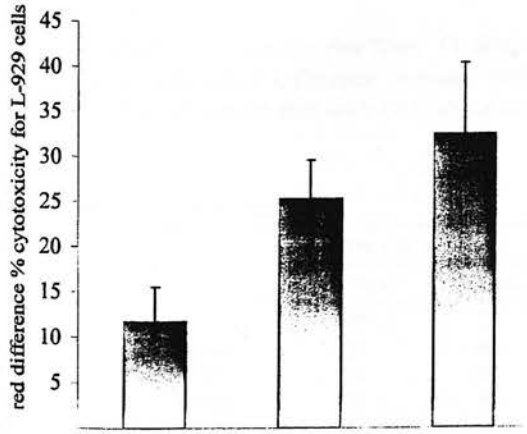

Fig. 2. Paired difference in TNF-α bioactivity expressed as % cytotoxicity for L-929 cells from monocytes of individual donors exposed to medium only or to CSE and/or RSV. Error bars represent S.E.M.

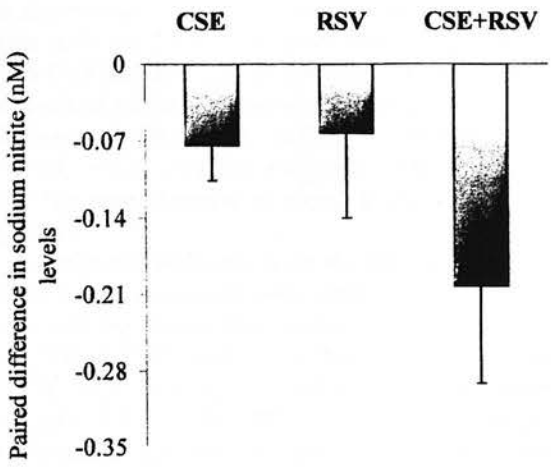

Fig. 3. Paired difference in sodium nitrite production from monocytes of individual donors exposed to medium only or to CSE and/or RSV. Error bars represent S.E.M.

(mean 60%, S.E.M. 5.1) had significantly increased TNF-α bioactivities (95% CI of paired differences 3.7, 19.7, $t=3.05$, $P=0.006$) as did the cell culture fluids from RSV-infected cells (mean 68%, S.E.M. 4.6) (95% CI of paired differences 16.4, 34.15, $t=5.9$, $P=0.000$). There was no correlation between the per cent cytotoxicity for L-929 cells and proportions of RSV-infected cells in RSV-infected samples. Compared with TNF-α bioactivities detected in cell culture fluids from cells (15 donors) exposed to medium alone (mean 38%, S.E.M. 5.4), a significant increase was observed in cell culture fluids from cells incubated with both CSE and RSV (mean 71%, S.E.M. 6.6) (95% CI of paired differences 15.4, 49.5, $t=4.08$, $P=0.002$).

Compared with TNF-α bioactivities of monocytes from 15 donors exposed to CSE alone, the bioactivities observed with the combination of CSE and RSV infection were significantly higher (paired differences 26%, 95% CI 10.4, 41.3, $t=3.58$, $P=0.003$). Compared with TNF-α bioactivity found for cells incubated only with RSV, addition of CSE did not significantly increase TNF-α bioactivities (95% CI −12.9, 21.5). This indicates that the main contribution to increased levels of TNF-α was due to the virus infection.

Compared with TNF-α bioactivity of monocytes from six donors exposed to medium only (mean 32%, S.E.M. 7.5), there was no significant increase

observed in cell culture fluids of cells incubated with nicotine (mean 34%, S.E.M. 8.7) (95% CI of paired differences −14.2, 15.4) or cotinine (mean 39%, S.E.M. 7.2) (95% CI of paired differences −1.6, 15.2). Incubation with IFN-γ did, however, result in significantly increased TNF-α bioactivity (mean 51%, S.E.M. 3.0) (95% CI of paired differences 6.2, 31.7, $t=3.83$, $P=0.012$).

3.4. The effect of CSE and RSV infection on NO release from monocytes

The supernatant fluids from cells in the same experiments were examined for nitrite levels. Paired differences between nitrite levels resulting from different treatments of monocytes compared with those incubated with medium alone are given in Fig. 3. Compared with supernatant fluids from cells incubated with medium only (mean 0.41 nM, S.E.M. 0.09), supernatant fluids from cells incubated with CSE (mean 0.34 nM, S.E.M. 0.08) had significantly lower levels of nitrite (95% CI of paired differences −0.14 ,−0.008, $t=2.3$, $P=0.031$) as did RSV-infected cells (mean 0.35 nM, S.E.M. 0.05), but the results were not significant (95% CI of paired differences −0.023, 0.099). There was no correlation between the levels of nitrite detected and ratios of RSV-infected cells in the samples. Compared with cells incubated with medium only, cells incubated with both CSE and RSV showed a significant decrease in nitrite production (mean 0.25 nM, S.E.M. 0.06) (95% CI of paired differences −0.4, −0.01, $t=2.26$, $P=0.04$).

In experiments with monocytes from 15 donors, there was no significant difference between nitrite levels found for cells incubated with CSE alone compared with those incubated with both CSE and RSV. In comparison with nitrite detected in supernatants from cells incubated with RSV only, supernatants from RSV-infected cultures containing CSE had lower levels of nitrite with marginal significance (paired difference −0.098 nM, S.E.M. 0.048, 95% CI −0.006, −0.2, $t=2.02$, $P=0.063$). This indicated that the main decrease in nitrite levels was caused by CSE.

Experiments with cells from six individual donors showed that, compared with nitrite levels observed with cells incubated with medium only (mean 0.10 nM, S.E.M. 0.03), addition of INF-γ (mean 0.15 nM, S.E.M. 0.03) (95% CI 0, 0.057) or nicotine (mean 0.13 nM, S.E.M. 0.03) (95% CI 0, 0.03) to cells did not result in significant changes in nitrite release, but addition of cotinine significantly increased release of nitrite from cells (mean 0.26 nM, S.E.M. 0.03) (95% CI of paired differences 0.03, 0.15, $t=3.48$, $P=0.018$).

3.5. Variability of TNF-α and NO response of individual donors

Each set of experiments was carried out with cells from different donors. Individual TNF-α and NO responses to CSE, RSV infection and combinations of both are summarised in Table 1. TNF-α and NO responses of the test samples were arbitrarily classified as very high if the levels of killing of L-929 cells or levels of nitrite were more than twice the value of the controls in which monocytes were cultured with medium alone. Responses were classified as very low if the levels of test samples were less than half the control. The most common pattern observed was increased TNF-α bioactivity and decreased NO pro-

Table 1
Individual responses to CSE and/or RSV

Response to		Per cent of donors			Per cent of donors with extreme response[a]		
		High	Low	↑TNF+↓Nitrite	High	Low	↑TNF+↓Nitrite
CSE	TNF	75	25	42	12	0	4
($n=24$)	Nitrite	33	63		0	12	
RSV	TNF	92	8	33	25	4	4
($n=24$)	Nitrite	50	46		21	25	
CSE+RSV	TNF	87	13	67	48	7	20
($n=15$)	Nitrite	13	87		7	48	

[a]High ≥2 ×control; low ≤ 1/2 control.

duction compared with controls incubated with medium only. In the presence of CSE or RSV, 4% of donors exhibited very high TNF-α bioactivity and very low nitrite levels. In the presence of both CSE and RSV, this rose to 20%.

4. Discussion

RSV is a common pathogen affecting infants and the elderly [23]. Complete immunity does not follow exposure to RSV, hence reinfections are not uncommon [24]. Significant numbers of patients with COPD or bronchial asthma suffer bouts of exacerbation and possible residual effects due to RSV infection in the course of their disease [25 (abstract)]. Cigarette smoking is a major cause of COPD [1]. Both viral infection and cigarette smoking enhance bacterial binding to epithelial cells in model systems [18,26,27] and cause immunosuppression [28,29]. Since TNF-α and NO are important mediators of inflammation in the respiratory tract, TNF-α and NO responses of blood monocytes to RSV infection and CSE were assessed.

Peripheral blood monocytes were used in the study for four reasons: (1) their ready availability in sufficient numbers; (2) alveolar macrophages in the lungs are derived from monocytes; (3) they were less likely to have been exposed to respiratory viruses and air pollutants; (4) they were more likely to be in an unstimulated state. Some stimulation of monocytes due to the use of Histopaque for isolation of monocytes could not be ruled out. There is evidence that genetic factors contribute to levels of TNF-α in response to endotoxins [30]. The variable spontaneous TNF-α and NO release from monocytes could be attributed to the individual donor's genetic make up or condition at the time of blood donation, their smoking habits, or variable responses of monocytes to Histopaque. The assays examined the responses to various agents in relation to background levels of each individual donor.

The dose of CSE (0.001 cigarette ml^{-1}) used in the assays was similar to dilutions of smoke extract used in experiments with alveolar macrophages by Higashimoto et al. [17]. It was based on the range of numbers of cigarettes an average person can smoke and the water-soluble components of the inhaled smoke that cross the mucosal lining and are diluted in the body fluids.

Bioactivity of TNF-α in samples can differ from total TNF-α detected by ELISA because of the soluble TNF-α receptors produced by monocytes that block the functions of the cytokine. There was no correlation between the data from the bioassay and the ELISA with 200 samples (data not shown). This could partly be due to variable levels of TNF-α receptors in the cell culture fluid and partly to variable rate of degradation of TNF-α by the monocytes. The bioassay was selected for this study because it measures the levels of TNF-α activity in the solution at a given time.

Midulla et al. found RSV infection elicited variable TNF-α responses from alveolar macrophages from individual donors [31]. Cells from most of the donors in the present study showed increased TNF-α responses to RSV infection or exposure to CSE. A minority exhibited very high responses to either of the agents. TNF-α is thought to play a protective role in RSV infection. Prior incubation with TNF-α reduced the replication of RSV in alveolar macrophages by about half [32]. Cells from 8% of donors in this study exhibited a decreased TNF-α response to RSV infection. Individuals with this response might be particularly susceptible to severe infection RSV.

The beneficial pulmonary vasodilatory, possible bronchodilatory, and bactericidal effects of NO in patients with COPD or asthma might be offset by its induction of exudate formation, inflammation, DNA toxicity and cytotoxicity. It is generally agreed that mild NO induction is protective in the respiratory tract while higher levels might be associated with deleterious consequences [3,33]. Two distinct populations of donors based on the level of NO production have been recognised [34]. The assay for nitrite used in this study was sensitive down to $NaNO_2$ concentrations of approximately 1 pM. Results presented here indicate that increased responses of TNF-α are not, in most donors, accompanied by increased NO responses to RSV or CSE. TNF-α reduced the half-life of mRNA encoding NO synthase in human umbilical vein endothelial cells [35]. The results indicate that TNF-α activity might also affect the production of this enzyme in monocytes.

Some of the effects of virus infections are mediated

through release of IFN-γ from monocytes [36]. The present data indicated that stimulatory effects of RSV infection on TNF-α bioactivity could be due in part to IFN-γ. Significant increases in TNF-α and significant decreases in NO response in the presence of CSE did not match with the observed slight increase in both responses mediated by nicotine alone. This suggests other factors in CSE are responsible for the effects observed in these assays. The significant increase of NO from cells treated with cotinine indicated that some of the effects of cigarette smoking on inflammatory mediators in vivo might be mediated by this metabolite of nicotine.

The results presented here examined some of the effects of two environmental factors that exacerbate COPD and asthma. Smoking or passive exposure to cigarette smoke and virus infections of the respiratory tract do not always lead to similar degrees of acute or chronic illness. This could reflect the individual differences in responses observed in this study. In addition to enhancing bacterial colonisation of the respiratory mucosa and immunosuppression, these agents appear generally to enhance TNF-α response and reduce NO levels. The extreme responses noted with cells from a minority of subjects might contribute to increased susceptibility to chronic inflammatory disease of the respiratory tract or exacerbations. Comparison of monocytes from healthy donors with those from patients with these conditions for production of inflammatory cytokines is needed to obtain further evidence for this hypothesis.

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Exposure to cigarette smoke, a major risk factor for sudden infant death syndrome: effects of cigarette smoke on inflammatory responses to viral infection and bacterial toxins

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Abstract

Exposure to cigarette smoke is a major risk factor for sudden infant death syndrome and also for respiratory infections in children. It has been suggested that toxigenic bacteria colonizing the respiratory tract might play a role in some cases of sudden infant death syndrome and nicotine has been demonstrated to enhance the lethality of bacterial toxins in a model system. Pyrogenic toxins of *Staphylococcus aureus* have been identified in tissues of infants who died of sudden infant death syndrome. It has been suggested that some of these deaths were due to induction of inflammatory mediators by infectious agents during a period when infants are less able to control these responses. The aim of this study was to assess the effects of a water-soluble cigarette smoke extract on the production of tumor necrosis factor α and nitric oxide from human monocytes in response to staphylococcal toxic shock syndrome toxin 1 or infection of the monocytes with respiratory syncytial virus. Cell culture supernatants were examined by a bioassay using mouse fibroblasts (L-929 cell line) for tumor necrosis factor α activity and by a spectrophotometric method for nitrite. Compared with monocytes incubated with medium only, monocytes incubated with any of the factors or their combinations tested in the study released higher levels of tumor necrosis factor α and lower levels of nitric oxide. Incubation with cigarette smoke extract increased tumor necrosis factor α from respiratory syncytial virus-infected cells while it decreased tumor necrosis factor α from cells incubated with toxic shock syndrome toxin. Incubation with cigarette smoke extract decreased the nitric oxide production from respiratory syncytial virus-infected cells while it increased the nitric oxide production from cells incubated with toxic shock syndrome toxin. Monocytes from a minority of individuals demonstrated extreme tumor necrosis factor α responses and/or very high or very low nitric oxide. The proportion of samples in which extreme responses with a very high tumor necrosis factor α and very low nitric oxide were detected was increased in the presence of the three agents to 20% compared with 0% observed with toxic shock syndrome toxin 1 or 4% observed with cigarette smoke extract or respiratory syncytial virus.

Keywords: Toxic shock syndrome toxin; Cigarette smoke; Respiratory syncytial virus; Monocytes; Sudden infant death syndrome

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1. Introduction

Exposure to cigarette smoke has been identified as one of the major risk factors for sudden infant death syndrome (SIDS) in the majority of epidemiological studies [1–4]. Parental smoking, especially that of the mother is also a major risk factor for respiratory infections in children [5]. It has been suggested that exposure to cigarette smoke and subclinical co-infection with viral and/or bacterial pathogens could trigger the series of events leading to SIDS and that inflammatory mediators elicited by infection contribute to the fatal outcome [6].

Several studies identified respiratory syncytial virus (RSV) in a substantial proportion of SIDS cases [7,8]. Staphylococcal toxic shock syndrome toxin 1 (TSST-1) and staphylococcal enterotoxin C (SEC) were identified in SIDS infants by immunohistochemical methods [9,10] and more recently in over half the SIDS cases examined by flow cytometric and enzyme-linked immunosorbent assay (ELISA) methods for the toxins [11]. Synergy between nicotine and bacterial toxins has been demonstrated in the chick embryo model [12]; however, there are many other toxic substances in cigarette smoke in addition to nicotine.

Tumor necrosis factor α (TNF-α) and nitric oxide (NO) are two important mediators of inflammation and shock in response to microbes and their components or products. Uncontrolled production of these mediators can lead to death [13,14]. TNF-α and NO were chosen for investigation in this study because experiments with mice demonstrated that a combined challenge with an inapparent virus infection and a well-tolerated dose of staphylococcal enterotoxin B (SEB) caused fatal shock. Interferon (INF) γ produced by the viral infection activated macrophages and subsequent exposure to the toxin induced TNF-α and NO radicals. The mice were protected from the effects of the toxin by TNF receptor-Fc fusion protein or NO synthase inhibitor [15].

Animal models have been used to examine the effect of staphylococcal toxins and virus infections [15,16] but there were no human models in which the effects of these combinations on the inflammatory responses were investigated. RSV infection has been reported to induce release of TNF-α and NO from human alveolar macrophages [17,18]. There are, however, conflicting reports on the effect of smoking on TNF-α [19–21] and NO release [22,23]. Blood monocytes and alveolar macrophages can be infected with RSV [24,25] and both cell types are expected to be exposed to water-soluble components of cigarette smoke or TSST-1 absorbed across mucous surfaces.

The aim of the present study was to assess the effect of a water-soluble cigarette smoke extract (CSE) on the release of TNF-α and NO from peripheral blood monocytes incubated with TSST-1 and/or infected with RSV. Because there is evidence that TNF-α responses are under genetic control which can influence the severity or fatal outcome of infection [26,27], the study examined monocytes from different blood donors to assess individual variations in TNF-α and NO responses to CSE, TSST-1 and RSV infection.

2. Materials and methods

2.1. Preparation of CSE

A water-soluble CSE was prepared by using a vacuum pump to pass the smoke of 10 filter-tipped cigarettes (Benson and Hedges) through 100 ml of Dulbecco's modified Eagle's medium (DMEM) (Gibco, BRL, Glasgow, UK) containing 0.45% D-glucose and 4 mg l^{-1} pyridoxin [28]. To reduce LPS contamination, the glass bottles used were heated at 134°C for 1 h. The CSE was filtered with a 0.2 μ filter and aliquots were stored at −20°C.

2.2. RSV stock

The Edinburgh strain of RSV (subgroup A) was grown in HEp-2 cells (an epithelial cell line) as described previously. The virus was harvested in growth medium (GM) containing DMEM supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco), 2 μM L-glutamine (Gibco), streptomycin (Gibco) (200 mg ml^{-1}) and penicillin (Gibco) (100 IU ml^{-1}). The virus suspension was adjusted to 2×10^6 plaque forming units ml^{-1}.

2.3. Preparation of monocytes

One-day old buffy coats from blood donors of

group O, Rh^+ were obtained from The Scottish National Blood Transfusion Service (SNBTS), Royal Infirmary, Edinburgh. Buffy coats were diluted 1:4 with sterile phosphate-buffered saline (PBS). The diluted preparations (40 ml) were layered over 12 ml of Histopaque 1077 (Sigma, Poole, Dorset, UK) in 50-ml polypropylene conical tubes (Greiner, Gloucestershire, UK). The tubes were centrifuged at $250 \times g$ for 30 min at 25°C. Mononuclear cells in the opaque band formed at the interface of serum and Histopaque were aspirated carefully. The cells were washed twice in sterile PBS at $150 \times g$ for 10 min and the supernatant was discarded. The cells were resuspended in 20 ml GM, transferred to a 75-cm^2 tissue culture flask (Greiner) and incubated at 37°C for 30 min to separate monocytes from non-adherent blood cells. The medium containing non-adherent cells was poured off and the monocytes were harvested by gentle scraping with a cell scraper in 20 ml fresh GM. A viable count was performed using the trypan blue dye exclusion method and the concentration of monocytes was adjusted to 1×10^8 ml^{-1} in GM. The cells were distributed in 24-well tissue culture plates (Costar) with 1×10^6 cells in each well. The viability of the cells at the end of each experiment was also assessed by microscopic examination of cells in the wells for exclusion of trypan blue.

2.4. Stimulation of monocytes

The cells were cultured at 37°C in 5% CO_2 for 24 h in 1 ml GM, CSE, RSV at a multiplicity of infection of 2.0 [30] or TSST-1 (Toxin Technology, Sarasota, FL, USA). TSST-1 was added to cells by first diluting the toxin in supernatant (200 μl) obtained from each well to avoid abrupt contact of concentrated toxin with the cells. Samples were collected from each well after 48 h for determination of TNF-α (100 μl) and after 72 h for determination of NO (400 μl). Negative control samples to which no cells were added included culture medium alone, medium with CSE, RSV and/or TSST-1. The samples were kept at −20°C until analyzed.

The proportion of monocytes infected with RSV in each sample 24 h post-infection was determined by an indirect immunofluorescence technique with monoclonal antibody against the glycoprotein G of RSV.

2.5. TNF bioassay and detection of NO

L-929 cells (a mouse fibroblast cell line) were used in a bioassay for TNF-α activity [31]. NO was detected as nitrites by the spectrophotometric assay described by Zhang et al. [32]. Concentrations of nitrites in the samples were derived from a standard curve for sodium nitrite prepared for each experiment.

2.6. Statistical methods

The results obtained with buffy coats from 31 donors were assessed. The results from some samples for some treatments could not be included due to contamination of individual wells. Therefore, the mean control values corresponding to different experiments were not all the same. The data from monocytes incubated with different agents were compared with those from monocytes incubated with medium only. Comparisons were made also between different treatment groups. The significance values obtained with paired t tests of the data were similar to values obtained with a non-parametric test (Wilcoxon's). The values obtained from t tests are presented here.

3. Results

3.1. RSV infection of cells

On average, at 24 h post-infection, more than 40% of the monocytes from each individual tested were infected with RSV. One way 'analysis of variance' indicated no significant differences among the donors in the proportion of RSV-infected cells.

3.2. Optimization of CSE and TSST-1 concentrations and time of incubation

Ten-fold dilutions of CSE ranging from 0.1 to 0.0001 cigarette ml^{-1} and TSST-1 ranging from 0.5 to 0.005 μg ml^{-1} were incubated for 24 h with monocytes from four donors. Dilutions of 0.001 ml^{-1} for

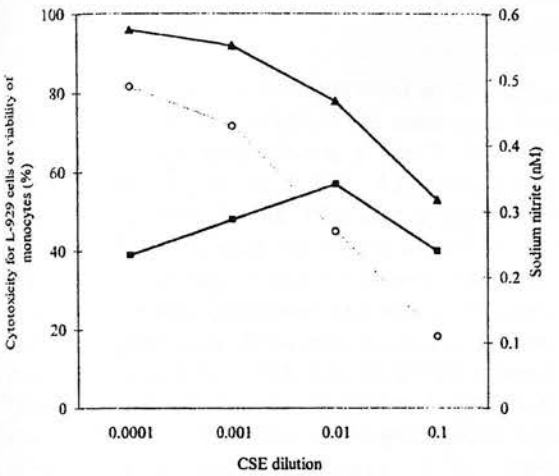

Fig. 1. Percent viability of monocytes (-▲-), cytotoxicity for L-929 (-■-) due to TNF-α bioactivity and nitrite (nM) (-O-) produced by monocytes from four donors incubated with various dilutions of CSE.

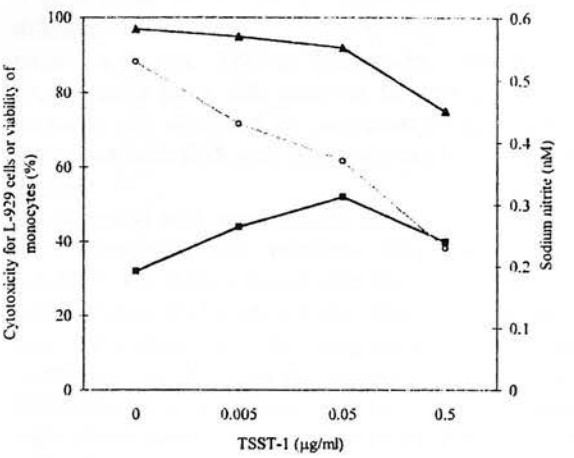

Fig. 2. Percent viability of monocytes (-▲-), cytotoxicity for L-929 cells (-■-) due to TNF-α bioactivity and nitrite (nM) (-O-) produced by monocytes from four donors incubated with various dilutions of TSST-1.

CSE and 0.05 μg ml^{-1} for TSST-1 were selected for the assays on the basis of maximum effects (increase or decrease) on the production of TNF-α or NO without killing the monocytes (Figs. 1 and 2). More than 90% of the monocytes survived until the end of the experiments with the treatments selected.

Time course experiments with monocytes from four donors ranging from 6 to 72 h found that the maximum TNF-α bioactivity was detected at 48 h and release of NO at 72 h in response to CSE, RSV or TSST-1 at the selected concentrations (data not shown). TNF-α bioactivity was not detected in culture medium or medium with CSE, RSV and/or TSST-1 used as negative controls. For detection of nitrites, the spectrophotometer was blanked on these controls for the assessment of their respective test samples. No effect of these agents was recorded on the optical density at which sodium nitrite is detected.

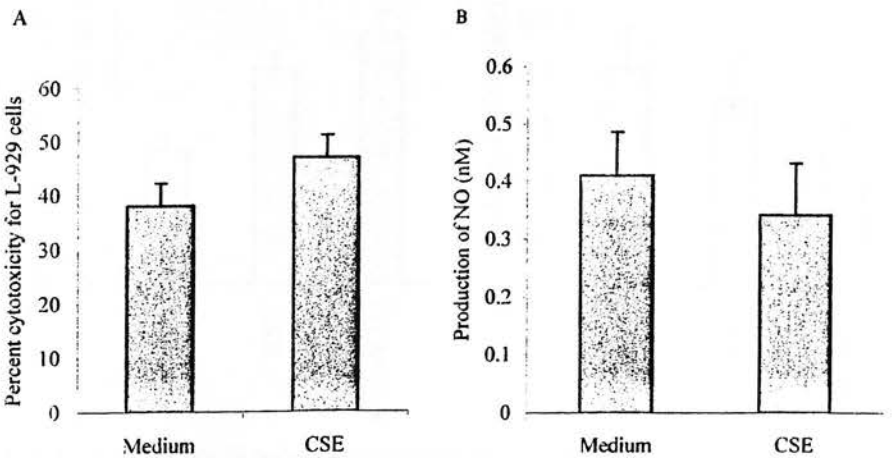

Fig. 3. Mean and S.E.M. of TNF-α bioactivities of monocytes expressed as % cytotoxicity for L-929 cells (A) and nitrite (nM) (B) of individual donors ($n = 31$) exposed to CSE.

3.3. The effect of CSE on TNF-α and NO production from monocytes

The TNF-α bioactivities expressed as percentage killing of L-929 cells observed for monocytes from a total of 31 donors are shown in Fig. 3. Compared with controls (mean 38, S.E.M. 4.2), supernatants from cells incubated with CSE had a significantly increased TNF-α bioactivity (mean 47, S.E.M. 4.2) (95% confidence interval (CI) of the paired difference 3, 15.7, $P<0.01$). Compared with nitrite concentrations of supernatants from cells incubated with medium only (mean 0.41 nM, S.E.M. 0.076), supernatants from cells incubated with CSE had significantly lower levels of nitrites (mean 0.34 nM, S.E.M. 0.09) (95% CI of the paired differences −0.14, −0.008, $P<0.05$) (Fig. 3A, B).

3.4. The effect of CSE on levels of TNF-α and NO produced by RSV-infected monocytes

Compared with supernatants from cells incubated with medium alone, supernatants from RSV-infected samples had significantly increased levels of TNF-α (mean 61, S.E.M. 4.1) (95% CI of the paired differences 14.8, 30.9, $P<0.005$) (Fig. 4A). Compared with the TNF-α bioactivity detected in supernatants from cells exposed to RSV alone, an increase was observed in supernatants from cells incubated with CSE and RSV, but this was not significant.

Compared with supernatants from cells of 20 donors incubated with medium only (mean 0.41, S.E.M. 0.09), RSV-infected cells had lower levels of nitrite (mean 0.35 nM, S.E.M. 0.05) but the effect was not significant. There was no correlation between the levels of nitrite detected and ratios of RSV-infected cells in individual samples. Compared with nitrite levels in supernatants of RSV-infected cells, there was a decrease in nitrite observed with cells incubated with CSE and RSV (mean 0.25 nM, S.E.M. 0.055) (95% CI of the paired differences −0.25, −0.07, $P<0.005$) (Fig. 4B). Compared with nitrite levels with cells incubated with RSV alone, a decrease was observed in supernatants from cells incubated with CSE and RSV, but this was not significant.

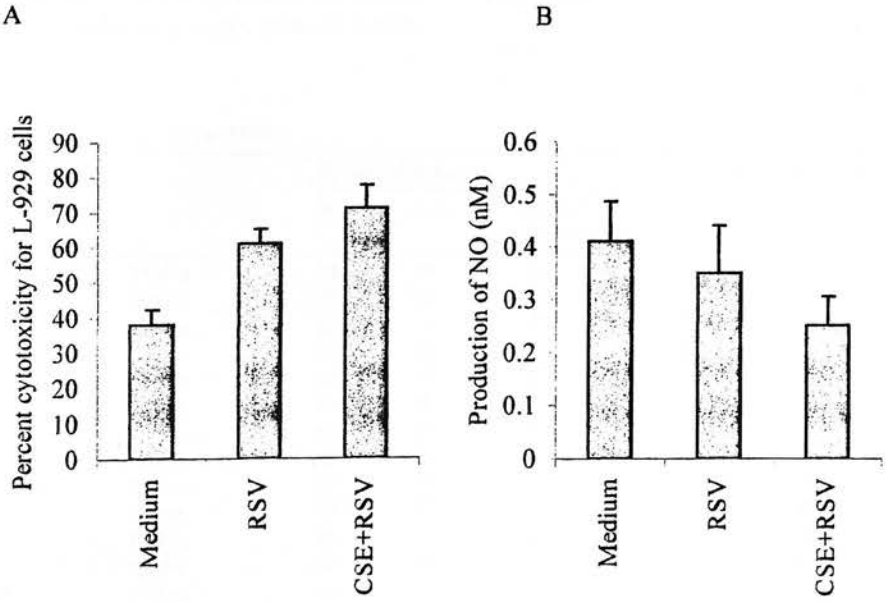

Fig. 4. Mean and S.E.M. of TNF-α bioactivities of monocytes expressed as % cytotoxicity for L-929 cells (A) and nitrite (nM) (B) of individual donors ($n=31$) exposed to CSE and RSV.

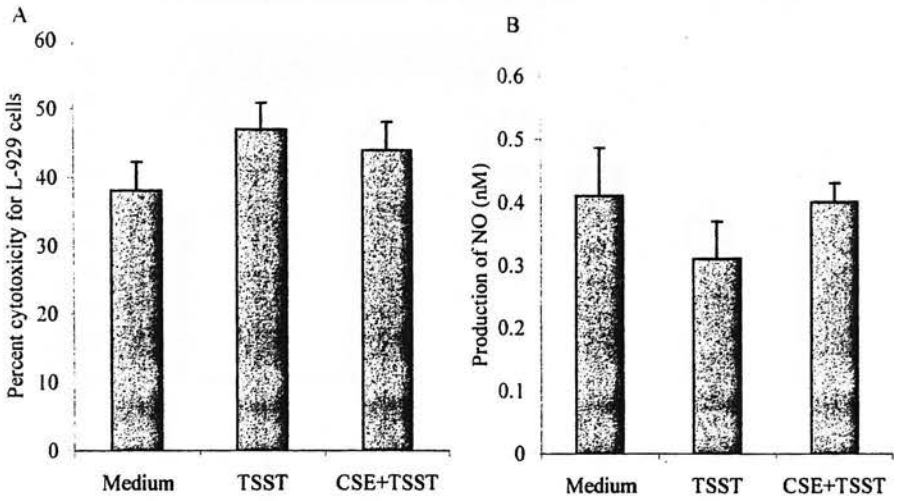

Fig. 5. Mean and S.E.M. of TNF-α bioactivities of monocytes expressed as % cytotoxicity for L-929 cells (A) and nitrite (nM) (B) of individual donors ($n = 31$) exposed to CSE and TSST-1.

3.5. *The effect of CSE on levels of TNF-α and NO produced by monocytes exposed to TSST-1*

Monocytes from 31 donors incubated with TSST-1 produced significantly higher levels of TNF-α bioactivity (mean 47, S.E.M. 3.9) (95% CI of the paired differences 4.9, 19.2, $P < 0.005$) compared with the controls incubated with GM only (mean 38, S.E.M. 5.4). In experiments with monocytes from 20 donors incubated with TSST-1 and CSE, the levels of TNF-α bioactivity were increased (mean 44, S.E.M. 6.4) but this was not significantly different from the effects observed for TSST-1 (Fig. 5A).

Supernatants from cells incubated with TSST-1 alone or in combination with CSE did not have nitrite levels significantly different from the controls (Fig. 5B).

Table 1
Individual responses to CSE, RSV and/or TSST-1

Response to		Percent of donors			Percent of donors with extreme response*		
		High	Low	↑TNF+ ↓Nitrite	High	Low	↑TNF-α+ ↓Nitrite
CSE	TNF-α	75	25	42	12	0	4
($n = 24$)	Nitrite	33	63		0	12	
RSV	TNF-α	92	8	33	25	4	8
($n = 24$)	Nitrite	50	46		21	25	
TSST	TNF-α	81	19	29	26	3	0
($n = 31$)	Nitrite	58	42		6	12	
CSE+TSST	TNF-α	65	35	40	30	5	0
($n = 20$)	Nitrite	45	50		0	20	
CSE+RSV	TNF-α	87	13	67	48	7	20
($n = 20$)	Nitrite	13	87		7	48	
RSV+TSST	TNF-α	95	5	50	40	0	20
($n = 20$)	Nitrite	30	60		20	30	
RSV+CSE+TSST	TNF-α	95	5	55	60	5	20
($n = 20$)	Nitrite	35	60		5	25	

*high ≥ 2×control; low ≤ 1/2 control.

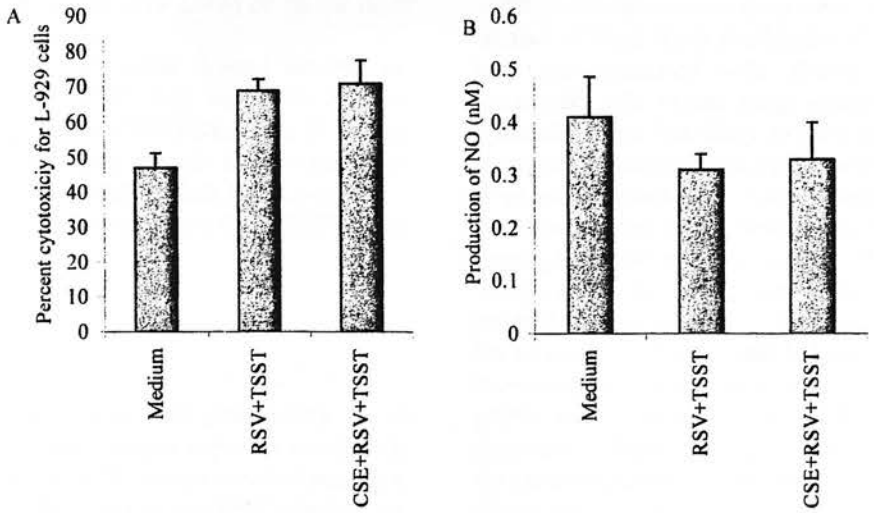

Fig. 6. Mean and S.E.M. of TNF-α bioactivities of monocytes expressed as % cytotoxicity for L-929 cells (A) and nitrite (nM) (B) of individual donors ($n = 31$) exposed to CSE, RSV and TSST-1.

3.6. *The effect of CSE on levels of TNF-α bioactivity production by RSV-infected cells stimulated by TSST-1*

Compared with controls, a significant increase was observed in supernatants from cells incubated with RSV and TSST-1 (mean 69, S.E.M. 3.2) (95% CI of the paired differences 23.4, 38.4, $P < 0.001$) and in supernatants incubated with RSV, CSE and TSST-1 (mean 71, S.E.M. 6.6, 95% CI 15.4, 49.5, $P = 0.000$) (Fig. 6A). The increase in TNF-α bioactivity caused by CSE with RSV-infected cells stimulated by TSST-1 was not significant. Compared with the control, a decrease in NO levels was observed but was not significant (Fig. 6B).

3.7. *Comparison of the TNF-α bioactivity in relation to CSE*

Since exposure to cigarette smoke can be considered to be a common or constant risk factor for children of smokers, the interactions between RSV or TSST were assessed in relation to CSE as the control. Compared with TNF-α bioactivities found in supernatants from cells exposed to CSE alone, the levels observed in supernatants of cells incubated with a combination of CSE and TSST-1 were not significantly different, but the bioactivities observed for a combination of CSE, RSV and TSST-1 were significantly higher ($P < 0.001$). Compared with the TNF-α bioactivity found in supernatants from cells incubated with CSE alone, supernatants from cells incubated with CSE and RSV had an increased TNF-α bioactivity ($P < 0.001$).

3.8. *Variability of TNF-α and NO responses of individual donors*

Each set of experiments was carried out with cells from different donors. Individual TNF-α and NO responses to CSE, RSV infection and TSST-1, separately and in combination, are summarized in Table 1. TNF-α and NO responses of the test samples were arbitrarily classified as very high if the levels of cytotoxicity for L-929 cells or levels of nitrite were more than twice the value for the controls in which monocytes were cultured with medium alone. Responses were classified as very low if the levels of test samples were less than half the control. The most common pattern observed was increased TNF-α bioactivities and a decreased NO production compared with controls in which cells were incubated with medium only. The proportions of extreme responses in the presence of CSE (4%), RSV (8%) or TSST-1 (0%) increased in the presence of CSE and

RSV (20%), TSST-1 and RSV (20%) or all the three agents (20%).

Cells from two individuals showed extreme responses (very high TNF and very low NO) to RSV, RSV+TSST and CSE+RSV. Cells from one of these individuals also showed these extreme responses to CSE+RSV+TSST. Cells from another donor showed extreme responses to CSE, RSV+TSST and CSE+RSV.

4. Discussion

Epidemiological studies have consistently shown an association between passive exposure to cigarette smoke and SIDS [1,4]. It has previously been demonstrated that both smoking and RSV infection enhance bacterial binding to epithelial cells by *Staphylococcus aureus* and some of the Gram-negative bacteria isolated from SIDS infants [30,33,34]. In this study, we examined the hypothesis that water-soluble components of cigarette smoke might significantly enhance the levels of potent mediators of inflammation and circulatory shock, TNF-α and NO [35] elicited by RSV and TSST-1.

4.1. The method for assessing the effects of CSE and other agents on inflammatory responses

Although animal models such as the chick embryo assay have been used successfully to examine the interactions between bacterial toxins and single components of cigarette smoke [12], we developed a model using human cells and CSE to attempt to simulate more closely the conditions in the human infant. We did not observe the synergistic effects noted for nicotine with the bacterial toxins in the chick embryo assay. This might be due to differences in the sensitivity of the chick embryo to the toxins compared with the human monocyte model. The current study used purified TSST-1 in contrast to the toxins used by Sayers et al. which were a crude extract from the bacteria and might have contained fragments of endotoxin or peptidoglycan which could enhance inflammatory reactions. A third factor could be that Sayers et al. used purified nicotine and the CSE used in these studies might have been more toxic to the cells due to the presence of other compounds.

Peripheral blood monocytes were used in the study because of their ready availability in sufficient numbers and compared with alveolar macrophages, phagocytic cells in the lungs derived from monocytes, they were less likely to have been exposed to pathogens or other agents and were more likely to be in an unstimulated state. Some stimulation of monocytes due to the use of Histopaque for isolation of monocytes could not be ruled out. There is evidence that genetic factors contribute to the levels of TNF-α produced in response to endotoxins [27]. The variable spontaneous TNF-α and NO release from monocytes could be attributed to the individual donor's genetic make up, conditions at the time of blood donation or their smoking habits. For each donor, the results obtained in the various experimental conditions were compared with the background levels of their unstimulated cells.

The dose of CSE (0.001 cigarette ml^{-1}) used in the assays was similar to dilutions of smoke extract used in the experiments with alveolar macrophages by Higashimoto et al. [28]. It was based on the average level of cigarette smoke to which an infant might be exposed and the water-soluble components of the inhaled smoke that cross the mucous membranes and diluted in the body fluids.

The bioavailability of TNF-α in samples can differ from the total TNF-α detected by ELISA because of soluble TNF-α receptors produced by monocytes that block its functions. We found no correlation between the data from the bioassays and ELISA of 200 samples (data not shown). This could partly be due to variable levels of TNF-α receptors in the supernatant and partly due to variable rates of degradation of TNF-α by the monocytes. The bioassay was selected in this study because it measures the effective levels of TNF-α bioactivity at any given time. The TNF-α bioactivity was expressed as measure of cytotoxicity for L-929 cells [36].

4.2. The effects of CSE on production of TNF-α

The increase in TNF-α production from RSV-infected monocytes [37] due to exposure from CSE indicated enhanced pro-inflammatory effects of these factors. This was further supported by the observation that monocytes from more donors showed a high level of TNF-α bioactivity with both CSE and RSV compared with either agent alone. An increased

TNF bioactivity in most cases was not accompanied by similar increases in the NO production. However, in a small subset of the donors tested, both TNF-α and NO were increased. In another small subset of subjects, a high TNF production associated with a very low NO production was observed. These subjects might have higher inflammatory responses with a reduced protection by optimum levels of NO [14].

4.3. The effects of CSE on production of TNF-α and NO by TSST-1-stimulated monocytes

CSE did not produce significant differences in TNF-α or NO from cells exposed to TSST-1. The concentration of TSST-1 used was optimal for eliciting inflammatory responses from the monocytes without killing the cells. Future studies in this area should examine the effects of cigarette smoke on suboptimal levels of the toxin.

4.4. The effects of CSE on production of TNF-α and NO by RSV-infected monocytes stimulated with TSST-1

Although CSE did not significantly affect the mean levels of TNF-α or NO production from RSV-infected monocytes incubated with TSST, the number of donors showing heightened TNF-α responses and very low NO responses due to CSE, RSV or TSST alone increased from 0–4% to 20% with the combination of the three agents. Our hypothesis suggested that it is a combination of infectious insults and exposure to cigarette smoke that leads to induction of exaggerated inflammatory responses that cannot be controlled by the infant's physiological reaction to these mediators [6,38]. Most SIDS deaths occur at night. It has been demonstrated that the night time cortisol levels (0.5–5 μg dl^{-1}) observed in infants following the development of circadian rhythm between 7 weeks and 4 months were not sufficient to control the production of TNF-α and IL-6 responses to TSST-1 [39]. Infants who have low levels of antibodies to viruses, bacteria or their products could be at an increased risk of SIDS if they are exposed to combinations of infectious agents and cigarette smoke during this period of vulnerability.

We conclude that products of cigarette smoke could influence inflammatory mediators which can cause fatal shock. However, the genetic and physiological background of the individual also plays an important part in these responses. There was a minority of individuals in which the experimental conditions tested in this study induced a very high TNF-α bioactivity and a marked decrease in NO levels. Studies on the genetic control of inflammatory responses in SIDS and non-SIDS families are the subject of our future investigations.

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Infection with respiratory syncytial virus enhances expression of native receptors for non-pilate *Neisseria meningitidis* on HEp-2 cells

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Abstract

Respiratory virus infections have been suggested to be predisposing factors for meningococcal disease. Respiratory syncytial virus (RSV) affects young children in the age range at greatest risk of disease caused by *Neisseria meningitidis*. It has been previously shown that glycoprotein G expressed on the surface of RSV-infected HEp-2 cells (a human epithelial cell line) contributed to higher levels of binding of meningococci compared with uninfected cells. The aim of the present study was to examine the effect of RSV infection on expression of surface molecules native to HEp-2 cells and their role in bacterial binding. Flow cytometry and fluorescence microscopy were used to assess bacterial binding and expression of host cell antigens. Some molecules analysed in this study have not been reported previously on epithelial cells. RSV infection significantly enhanced the expression of CD15 ($P < 0.05$), CD14 ($P < 0.001$) and CD18 ($P < 0.01$), and the latter two contributed to increased binding of meningococci to cells but not the Gram-positive *Streptococcus pneumoniae*.

Keywords: Respiratory syncytial virus; *Neisseria meningitidis*; Endotoxin CD15; CD11; CD18; CD14; CD29

1. Introduction

Density of colonisation of epithelial surfaces is an important factor in the pathogenesis of many infectious diseases [1,2]. Our previous studies found that compared with uninfected HEp-2 cells, HEp-2 cells infected with respiratory syncytial virus (RSV) exhibit enhanced binding of several species of bacteria that cause disease in young children: *Neisseria meningitidis*, *Haemophilus influenzae* type b [3], *Staphylococcus aureus* [4], *Bordetella pertussis* [5] and *Streptococcus pneumoniae* [6].

Two hypotheses were proposed to explain the findings: (1) RSV-infected cells bear glycoproteins of viral origin that might act as additional receptors for bacteria; (2) virus infection might enhance ex-

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pression of existing cellular molecules that act as receptors for bacteria. Among the viral glycoproteins, glycoprotein G (attachment) and glycoprotein F (fusion) are expressed on the surface of RSV-infected cells. In a previous study, we found that glycoprotein G contributed to enhanced binding of *N. meningitidis* to RSV-infected cells while glycoprotein F did not [7].

In the present study, we tested the second hypothesis. Surface molecules such as integrins play a fundamental role in adhesion and other functions of cells; however, they might also be used by microbial pathogens, such as CD18 used by meningococci, as a means of entry to the host's cells [8,9]. Studies on expression of these surface antigens on epithelial cells are emerging; most studies have, however, examined these antigens on cells of myeloid origin to analyse their adhesion and homing functions. Murine CD14 gene expression was demonstrated on epithelial cells elicited by tumour necrosis factor (TNF) secreted in response to bacterial lipopolysaccharide (LPS) [10]. CD11a (α chain of LFA-1) was demonstrated on epithelial cells of rat lungs exposed to high oxygen pressure [11]. CD11b/CD18, components of complement receptor 3 (CR3), were detected on rectal and cervico-vaginal epithelial cells in patients with HIV [12]. We investigated binding of monoclonal antibodies (mAbs) to the following antigens on an epithelial cell line (HEp-2) and RSV-infected HEp-2 cells: CD11a, CD11b, CD11c and CD18; the Lewis^x antigen (CD15); CD14; and CD29, an antigen common to the β chains of β1 integrins. Uptake of bacteria into phagocytic cells and non-professional phagocytic cells, e.g., cultured epithelial cells, could be mechanistically similar [13]. The role of these antigens in binding of *N. meningitidis* and *S. pneumoniae* was assessed by inhibition studies with the mAbs specific for the host cell antigens.

2. Materials and methods

2.1. HEp-2 cells and RSV

The methods described previously [3] for bacterial binding were used in this study. Dulbecco's phosphate-buffered saline solution A (DPBS) was used for washing. HEp-2 cells (Flow Lab) were grown in 25-cm^2 tissue culture flasks (Costar) containing growth medium (GM). GM was composed of Eagle's minimal essential medium (Gibco) supplemented with 5% (v/v) foetal calf serum (FCS) (Gibco), 0.85 g l^{-1} sodium bicarbonate, 2 mmol L-glutamine, 200 μg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin (pH 7.4). Monolayers grown for 24 h were infected with the Edinburgh strain of RSV (a subgroup A strain) at a multiplicity of infection (MOI) of 1.0. The infected cells were cultured overnight in maintenance medium (MM) which had the same constituents as GM except the quantity of FCS was reduced to 1% (v/v). RSV-infected monolayers and uninfected HEp-2 monolayers were rinsed twice with DPBS and 0.05% (w/v) ethylenediaminetetraacetic disodium acid (EDTA) (Sigma) was applied, 1 ml per 25-cm^2 flask at 37°C for 5–10 min to suspend the cells. MM (10 ml) was added to the cells to counteract EDTA activity. After centrifugation at $460\times g$ for 7 min, the cells were resuspended in MM without antibiotics, counted and adjusted to 1×10^6 cells ml^{-1} for use in the assay.

2.2. Bacteria

The meningococcal isolates used are listed in Table 1. C:2b:P1.2 and C:2a:NT were provided by Dr R.J. Fallon (Scottish Meningococcal Reference Laboratory, Glasgow). The standard immunotype strains L1–L12, which differed in their lipooligosaccharide (LOS) structure, and the information on serogroup, serotype and subtype were provided by Dr W.D. Zollinger (Walter Reed Army Medical Institute, USA) (Table 1). *S. pneumoniae* type 6, a serotype associated with meningitis, was used as the Gram-positive control.

Overnight cultures of meningococci grown on boiled blood agar and pneumococci grown on blood agar were used. Bacteria were harvested and washed twice with DPBS by centrifugation at $2500\times g$ for 10 min for use in the assay. The bacteria were labelled with fluorescein isothiocyanate (FITC) (Sigma) freshly prepared as a 0.4% (w/v) solution in NaH_2CO_3 (0.05 M) and NaCl (0.1 M). FITC-labelled bacteria were washed three times with DPBS and resuspended in MM without antibiotics. The bacterial concentration was determined by optical density (OD) at 540 nm [3].

Table 1
Strains of *N. meningitidis*

Strain	Serogroup	Serotype	Subtype	Immunotype
	C	2b	P1.2	L(3,7,9)
	C	2a	NT	L(3,7,9)
L1	C	NT	P1.2	L1,8
L2	C	2c	P1.1	L2
L3	B	2a	P1.5,2	L(3,7,9)
L4	C	11	P1.16	L4
L5	B	4	P1.NT	L5
L6	B	5	P1.7,1	L6
L7	B	9	P1.7,1	L(3,7,9)
L8	B	8,19	P1.7,1	L(3,7,9),8
L9	A	21	P1.10	L(3,7,9)
L10	A	21	P1.?	L10
L11	A	21	P1.10	L11
L12	A	21	P1.NT	L12

2.3. Antibody binding to cell surface antigens

2.3.1. Flow cytometry

Details of the mAbs, their isotype and working dilutions are listed in Table 2. Samples of cell suspensions at 1×10^6 ml^{-1} (200 μl) were incubated with each of the mAbs for 30 min at 37°C with gentle rotation (60 rpm) in an orbital incubator (Gallenkamp). After washing three times with DPBS at $480\times g$ for 7 min, the samples were incubated with FITC-labelled rabbit anti-mouse immunoglobulin (Ig) (Sigma) or FITC-labelled rabbit anti-rat Ig (Serotec) (both diluted 1 in 100) at 37°C for 30 min. Background binding of the fluorochrome-labelled antibodies was determined in parallel samples to which the first antibody was not added. The samples were washed three times with DPBS, suspended in 200 μl of DPBS and fixed with 100 μl of 1% (v/v) buffered paraformaldehyde. The samples were analysed with an EPICS-C flow cytometer (Coulter Electronics, Luton, UK) for the percentage of fluorescent cells on a log scale and the mean fluorescence on a linear scale. The percentages obtained were further analysed by the 'immunoanalysis programme' (Coulter) that compares the fluorescence of the test samples with that of the control samples at each of the corresponding channels of the two histograms. A binding index (BI) for each sample was calculated by multiplying the values of the percentage from immunoanalysis by the mean fluorescence of the positive cells converted from the log scale.

Non-specific binding of antibodies to the cells was assessed by isotype control antibodies directed to irrelevant antigens. Cell samples were incubated as

Table 2
Monoclonal antibodies

Antibody[a]	Source 1	Isotype	Source 2	Isotype	Dilution
CD11a	Serotec	IgG1	DAKO	IgG1	1/20
CD11b	Serotec	IgG1			1/20
CD11c	Serotec	IgG1			1/20
CD18	Serotec	IgG2b	DAKO	IgG1	1/20
CD15	SAPU[b]	IgM			1/20
CD14	SAPU	IgG1	DAKO	IgG2a	1/2, 1/5
CD29	Serotec	IgG1	DAKO	IgG2a	1/20
Control	DAKO	IgG2a			1/20
Control	Serotec	IgG2b			1/20

[a]All the monoclonals were produced in mice except anti-CD18 and IgG2b control which were of rat origin.
[b]Scottish Antibody Production Unit, Carluke, Lanarkshire, UK.

above with mouse isotype control (IgG2a) mAb to *Aspergillus niger* (DAKO) or rat isotype control (IgG2b) mAb to kappa chain myeloma protein (Serotec) (both diluted 1 in 20). Binding of the isotype controls was detected with FITC-labelled rabbit anti-mouse Ig (Sigma) or FITC-labelled rabbit anti-rat Ig (Serotec). As both uninfected and infected cells appeared to bind the isotype controls at low levels, a second experiment was carried out to determine if these control antibodies had any effect on binding of the anti-CD antibodies. After incubation with the isotype control, the cells were washed three times and incubated with unlabelled rabbit anti-mouse or rabbit anti-rat Ig (Sigma) (both diluted 1 in 20) to block the isotype control antibodies. The experiment to assess binding of anti-CD18, anti-CD14 and anti-CD29 to cells was then carried out as described above.

2.3.2. *Fluorescence microscopy*

The percentages of cells in monolayers, which bound the above antibodies, were also determined by fluorescence microscopy. Glass coverslips with monolayers of HEp-2 cells or HEp-2 cells 24 h post infection with RSV at an MOI of 1.0 were treated with chilled acetone for 7 min and mounted on glass slides. The monolayers were incubated with 20 μl of the first antibody at dilutions listed in Table 2 for 30 min at room temperature. The slides were washed three times with PBS and 20 μl of FITC-labelled second antibody (1/50) was added for 30 min at room temperature. The slides were washed three times with DPBS and the monolayers examined by ultraviolet microscopy (Leitz, Wetzler).

2.4. *Bacterial binding and inhibition of binding*

Bacterial binding studies were carried out as described previously [3]. Samples (200 μl) of HEp-2 cells, RSV-infected HEp-2 cells and aliquots of uninfected or RSV-infected cells treated with individual mAbs were suspended in MM without antibiotics. An equal volume of FITC-labelled bacteria was added to the cells to provide a ratio of 200 bacteria:cell. After incubation for 30 min at 37°C with gentle rotation in an orbital incubator, the samples were washed three times in DPBS by centrifugation at $480\times g$ for 7 min, resuspended in 200 μl DPBS. The cells were fixed with 100 μl of 1% (v/v) buffered paraformaldehyde and stored in the dark at 4°C until analysed by flow cytometry within 3 days of the experiment. The samples from the experiments for comparison of LOS-immunotype strains were analysed with an XL flow cytometer (Coulter Electronics, Luton, UK).

2.5. *Binding of LOS-coated erythrocytes to HEp-2 cells*

The method described by Wright and Jong [14] was used to coat sheep erythrocytes (Scottish Antibody Production Unit) (1×10^8 ml^{-1}) with 1 ml of freshly sonicated LOS (5 mg ml^{-1}) from the C:2a:NT strain of *N. meningitidis* (a gift from R. Brown of this department), which was of the same LOS-immunotype as the C:2b:P1.2 strain (Table 1). Erythrocytes were kept at 4°C until used in the assay within 24 h of coating with LOS.

Erythrocytes or LOS-coated erythrocytes (1×10^7 ml^{-1}) in 2 ml volumes were incubated for 2 h with 48 h old monolayers of HEp-2 cells or RSV-infected HEp-2 cells (5×10^5) on coverslips in 24-well tissue culture plates (Costar). Some monolayers were incubated with 100 μl volumes of monoclonal anti-CD14 (1/2) (SAPU), anti-CD18 (1/10) (Serotec) or anti-CD29 (1/10) (Serotec) for 30 min at 37°C and washed three times prior to incubation with uncoated or LOS-coated erythrocytes. The coverslips were retrieved from the wells and dipped in three samples of veronal-EDTA buffer [14], 25 times for each sample. The number of HEp-2 cells with attached erythrocytes in a total of 100 cells was counted microscopically. The numbers of erythrocytes attached to individual cells were recorded for each coverslip and an attachment index for each coverslip was calculated by multiplying the percentage of cells in a population with erythrocytes attached by the mean number of erythrocytes for 100 cells counted per coverslip.

2.6. *Statistical analysis*

Paired *t*-tests were employed to analyse the logarithms of BIs of replicate experiments for the flow cytometry studies to detect binding of antibodies or

bacteria to the cells and to analyse the data from experiments to compare the erythrocyte attachment to cells infected with RSV and/or treated with mAbs. The data from experiments with different LOS-immunotypes of *N. meningitidis* to compare the binding of strains were analysed with the Kruskal-Wallis and Mann-Whitney tests.

3. Results

3.1. RSV infection of HEp-2 cells

More than 80% of HEp-2 cells infected with RSV at MOI 1.0 had detectable glycoprotein G at 24 h post infection detected by flow cytometry or fluorescence microscopy.

3.2. Effect of RSV infection on expression of surface antigens

3.2.1. Flow cytometry

Background binding of fluorochrome-labelled rabbit anti-mouse/rat Ig (second antibody) to HEp-2 cells was negligible. Data from seven experiments to assess binding of mAbs to HEp-2 cells and the effect of RSV infection on their binding are summarised in Table 3. Traces of the histograms from flow cytometric analysis of fluorescence obtained with mAbs to CD14, CD18 and CD29 are compared in Fig. 1.

Binding of mAbs to CD11b or CD11c was not detected on HEp-2 cells or RSV-infected HEp-2 cells. Antibodies to CD11a, CD14, CD15, CD18 and CD29 bound to uninfected HEp-2 cells. Approximately half the cells bound anti-CD18 and anti-CD11a, 40% bound anti-CD15, 15–25% bound anti-CD14 and over 90% bound anti-CD29. Infection of cells with RSV increased BIs significantly for anti-CD18, anti-CD14 and anti-CD15 (Table 3). The BIs for anti-CD11a and anti-CD29 were also increased for RSV-infected cells by a mean of 62% and 41% respectively above that observed for uninfected cells, but these varied between experiments and the effects were not statistically significant. Binding of the antibodies to cells was similar with slightly older cells (28 h post infection) or when the assay was performed at 4°C (data not given). The

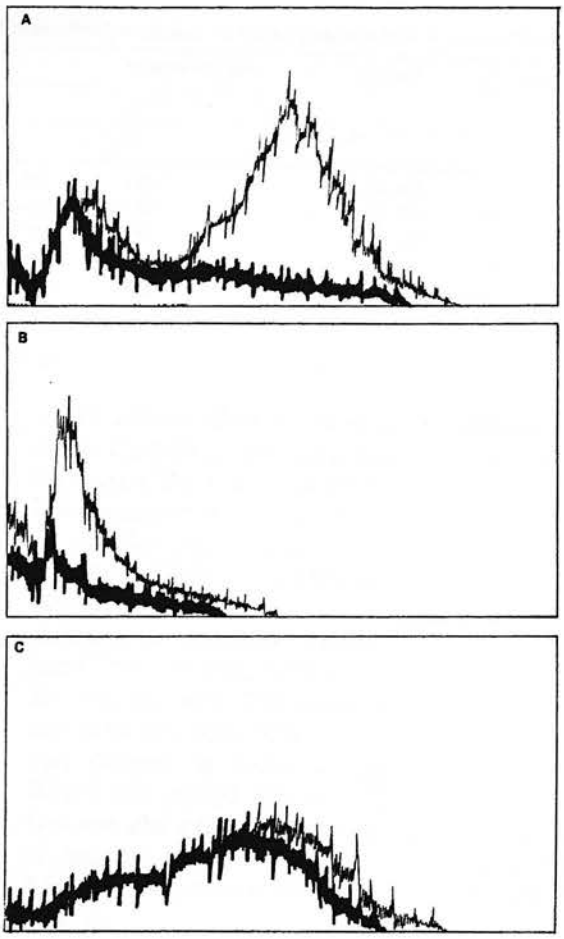

Fig. 1. Traces of histograms from flow cytometric analysis of green fluorescence on cells treated with antibodies to (A) CD18, (B) CD14 and (C) CD29. The darker curves in the tracings represent fluorescence obtained with uninfected HEp-2 cells and the lighter curves with RSV-infected HEp-2 cells.

results were confirmed in two experiments with mAbs from a second source (Table 2).

Since mAbs used to detect CD11b and CD11c were of the IgG1 isotype, and as these antigens were not detected on HEp-2 cells, a further control for this isotype was not included in the assays. In two experiments, the two isotype control antibodies directed against irrelevant antigens bound to HEp-2 cells: IgG2a on average bound to 20% uninfected and 23% RSV-infected cells; and IgG2b to 26% uninfected cells and 32% RSV-infected cells. These antibodies, however, did not alter the binding of the

Table 3
Mean binding indices (BI) from seven experiments on attachment of monoclonal antibodies to HEp-2 cells and RSV-infected HEp-2 cells

Anti-CD	HEp-2 cells						% RSV+/RSV−	95% CI	*P* value
	RSV−			RSV+					
	%	Mean	BI	%	Mean	BI			
11a	44	8	352 (127)	57	10	570 (86)	162	73–365	
18	55	4	219 (15)	60	5.8	348 (60)	159	123–204	**
15	40	7	280 (18)	64	8	512 (88)	183	111–302	*
14	17	4	68 (8)	23	5.6	129 (8)	190	152–238	***
29	90	13.6	1224 (244)	96	18	1725 (249)	141	92–214	

Figures in parentheses are S.E.M. $*P<0.05$, $**P<0.01$, $***P<0.001$.

same subclass isotype anti-CD antibodies to uninfected and RSV-infected cells.

3.2.2. *Fluorescence microscopy*

Binding of antibodies to uninfected and infected cells in monolayers was analysed by fluorescence microscopy in four experiments. The proportions of cells positive for these surface molecules were similar to those obtained with flow cytometry (data not shown).

3.3. *Inhibition of bacterial binding*

In seven experiments, meningococcal strain C:2b:P1.2 bound in higher numbers to RSV-infected cells compared with uninfected cells ($P=0.001$) (Fig. 2). Treatment of uninfected and RSV-infected HEp-2 cells with isotype controls IgG2a, IgG2b or anti-CD15 did not affect the binding of meningococcal strain C:2b:P1.2. The data from the experiments with anti-CD11a and anti-CD29 were variable and non-significant. A summary of the results for binding of meningococci to cells pretreated with antibodies to CD18, CD14 and CD29 is given in Fig. 2. A significant reduction in bacterial binding was observed with uninfected HEp-2 cells treated with anti-CD18 ($P=0.05$, 95% confidence interval (CI) 56–100) and with RSV-infected cells treated with this mAb ($P<0.01$, 95% CI 57–81). A non-significant decrease in bacterial binding to uninfected HEp-2 cells treated with anti-CD14 was observed; however, the effect of this antibody on inhibition of bacterial binding to HEp-2 cells infected with RSV was significant ($P<0.001$, 95% CI 48–73) (Fig. 2).

The type 6 pneumococcal strain was used as a

Table 4
Mean binding indices (BI) of six experiments on binding of FITC-labelled LOS-immunotype strains of *N. meningitidis* to HEp-2 cells and HEp-2 cells infected with RSV

Immunotype strain	BI uninfected cells	BI RSV-infected cells	Percent increase (95% CI)
L1	2835 (174)	4083 (165)	145 (133–156)***
L2	2598 (196)	3675 (149)	143 (124–164)**
L3	2684 (87)	3830 (123)	143 (125–164)***
L4	2596 (92)	3739 (78)	149 (114–164)***
L5	2375 (115)	3562 (72)	151 (136–167)***
L6	2823 (81)	3878 (98)	137 (127–149)***
L7	2835 (153)	3937 (50)	140 (123–159)**
L8	3157 (216)	4784 (186)	153 (132–177)***
L9	3627 (162)	4969 (150)	137 (119–159)**
L10	2948 (98)	3804 (111)	129 (123–136)***
L11	2958 (216)	4763 (173)	163 (138–192)***
L12	2806 (171)	3709 (88)	134 (117–152)**

Figures in parentheses are S.E.M. $**P<0.01$, $***P<0.001$.

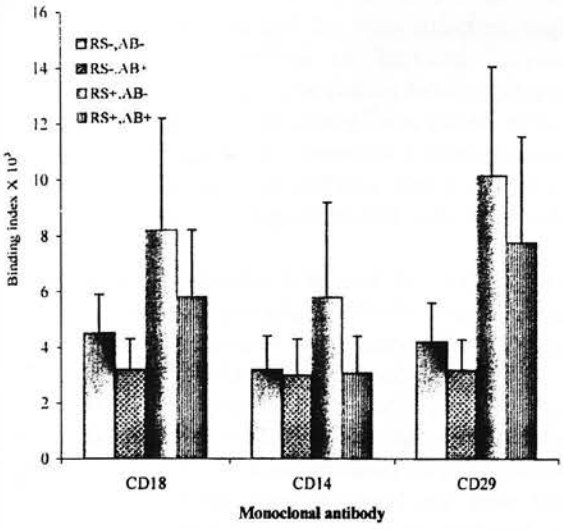

Fig. 2. Binding of FITC-labelled *N. meningitidis* C:2b:P1.2, to HEp-2 cells (RS−) and RSV-infected HEp-2 cells (RS+) treated with different mAbs (AB+).

Gram-positive control in these experiments. Infection with RSV increased the mean binding index for pneumococci in seven experiments from 2199 (S.E.M. 73) to 3035 (S.E.M. 102) (138%, 95% CI 129–147, $P<0.001$). Treatment of uninfected and RSV-infected cells with anti-CD18, anti-CD14 or anti-CD15 did not alter binding of pneumococci.

3.4. Binding of different LOS-immunotype meningococcal strains to cells

Because our previous studies had found no difference in binding to RSV-infected cells associated with serogroup, serotype or subtype [3], 12 immunotype strains of meningococci were used in bacterial binding studies with uninfected or RSV-infected HEp-2 cells to determine if there were differences in binding associated with LOS structures. Table 4 summarises the results of six experiments. The results showed that meningococci added at a ratio of 400 bacteria: cell bound in higher numbers to RSV-infected cells compared with uninfected cells ($P<0.001$) (Table 4).

3.5. Binding of LOS-coated sheep erythrocytes to HEp-2 cells

Sheep erythrocytes coated with meningococcal LOS were incubated with uninfected and RSV-infected HEp-2 cells to determine if LOS was a bacterial adhesin. In three experiments, untreated erythrocytes did not attach to cells but erythrocytes coated with LOS did. RSV-infected cells attached significantly more of the LOS-coated erythrocytes. HEp-2 cells were pre-incubated with the mAbs to determine if these altered the attachment of erythrocytes to cells. Pretreatment with anti-CD14 and anti-CD29 decreased significantly erythrocyte attachment to uninfected cells; attachment of erythrocytes to RSV-infected cells was inhibited significantly by anti-CD14, anti-CD18 and anti-CD29 (Table 5).

4. Discussion

Carriage of bacteria in general does not lead to

Table 5
Attachment of sheep erythrocytes coated with meningococcal (C:2a:NT) lipooligosaccharide to uninfected or RSV-infected HEp-2 cells or cells pre-treated with mAbs

	Untreated HEp-2 cells		HEp-2 cells treated with mAB					
	RSV−	RSV+	anti-CD14		anti-CD18		anti-CD29	
			RSV−	RSV+	RSV−	RSV+	RSV−	RSV+
Cells with erythrocytes (%)	64	72	35	55	58	48	62	65
Mean number of erythrocytes per cell	18	21	13	14	19	18	11	16
Attachment index (S.E.M.)	1152 (50)	1512 (112)	455 (60)	770 (46)	1102 (116)	864 (68)	682 (68)	1040 (77)
Paired difference		360	−697	−742	−50	−648	−470	−472
95% CI		204, 516	−667, −727	−1953, −431	−232, 132	−743, −553	−532, −403	−609, −335
P value		<0.05	<0.001	<0.01	NS	<0.01	<0.01	<0.01

Results are means of three experiments.

disease. Changes in the nature of the mucosal surfaces such as those caused by virus infection might predispose the individual to bacterial invasion. Beachey [1] suggested a correlation between in vitro adhesion and in vivo infectivity for a variety of bacterial pathogens. In our previous studies, in vitro assays with human cell cultures demonstrated enhanced bacterial binding associated with RSV infection [3–5].

Binding of capsulate strains of *N. meningitidis* to cells by means of non-pilate adhesins was examined throughout. The strains were potentially pilate but the preparative methods used involved washing the bacteria which removed the bacterial pili. There are numerous reports indicating that factors other than pili are important in meningococcal carriage and disease [15–17]. While meningococcal pili have been shown to mediate in vitro binding to epithelial and endothelial cells [18], pili had been shown not to be involved in binding of meningococci to monocytes [19], cells which express the surface antigens under examination in the present studies.

FITC used to label bacteria in this study is a monovalent molecule widely used to label biological reagents without affecting their activity or reactivity. Previous work found that FITC did not interfere with or mediate binding of meningococci [20], *S. aureus* or *B. pertussis* to epithelial or HEp-2 cells [5]. Flow cytometry has recently been used extensively to detect fluorochrome-labelled particles. Because large populations can be examined, it is consistently more accurate and sensitive than direct microscopy. Flow cytometric analysis in bacterial binding studies cannot provide an absolute measure of the mean number of bacteria attached to individual cells, but the mean fluorescence values obtained by this method can reliably compare the binding between samples [21].

The first objective of this study was to determine if RSV infection enhanced the native surface components on HEp-2 cells that might act as receptors for bacteria. Antibodies to CD14, CD15, CD18 or CD29 of different isotypes from different sources showed similar increased binding to RSV-infected cells. Prior treatment of cells with the isotype control mAbs did not block the binding of specific antibodies or bacteria indicating that the isotype control mAbs were binding to epitopes different from CD molecules and/or bacterial receptors.

The enhancement of surface antigens on HEp-2 cells infected with RSV observed in the present study might be due to a direct effect of virus on cells or mediated through cytokines secreted by the infected cells. Virus infections have been shown to alter surface expression of molecules. RSV infection of human mononuclear leukocytes has been shown to suppress LFA-1 (CD18/CD11a) [22]. RSV infection of the middle ear was shown to induce or enhance mRNAs for ICAM-1, VCAM-1, and ELAM (a selectin molecule). Cultures of resected tissue from the middle ear infected in vitro with RSV were positive for the mRNAs for ELAM and for the cytokines interleukin (IL) 6 and TNF [23]. Cytokines have been shown to alter expression of CD14 on blood monocytes: IL-4 decreases its expression, while TNF and IL-6 induce a moderate increase in the expression [24].

It has been suggested that cellular antigens normally involved in cell to cell recognition might be 'hijacked' by bacteria [8]. There are reports that complement receptors, CR3 (CD11b/CD18) and CR4 (CD11c/CD18), are receptors for *Escherichia coli* [14] and a number of intracellular microorganisms that infect myeloid cells [25]. Binding of erythrocytes coated with pertussis toxin to macrophages was inhibited by capping with anti-Lewis^a and anti-Lewis^x (CD15) [26]. The antibodies to Lewis^a and Lewis^x antigens also inhibited binding of *S. aureus* and *B. pertussis* to buccal epithelial cells [4,5]. CD14 and LFA-1 function as receptors for bacterial lipopolysaccharides [27,28]. Collagen receptors on CD4+ cells, of which CD29 is a common β chain, are involved in binding of *Yersinia pseudotuberculosis* [29]. In the present study, reduction in meningococcal binding and reduction in attachment of meningococcal LOS-coated sheep erythrocytes to HEp-2 cells pretreated with anti-CD14 or anti-CD18 indicate that these antigens are involved in meningococcal binding mediated through the LOS. Pneumococci which lack endotoxin bound in higher numbers to RSV-infected HEp-2 cells; however, their binding was not inhibited by pre-treatment of the uninfected or RSV-infected cells with anti-CD14 or anti-CD18. These results indicate that other changes in the sur-

face antigens of the RSV-infected HEp-2 cells contribute to increased binding of Gram-positive species.

In conclusion, the data presented here indicate that infection with RSV enhanced expression of antigens native to HEp-2 cells and that CD18 and CD14 are involved in binding of non-pilate *N. meningitidis* to these cells. The data also indicate the role for meningococcal LOS in binding to HEp-2 cells.

Acknowledgments

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MICROBIAL PATHOGENESIS

Bactericidal activity of a monocytic cell line (THP-1) against common respiratory tract bacterial pathogens is depressed after infection with respiratory syncytial virus

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Non-typable *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis* and respiratory syncytial virus (RSV) are commonly isolated from patients during the course of chronic obstructive pulmonary disease (COPD). Earlier studies found that virus infection enhanced binding of bacterial respiratory pathogens to epithelial cells *in vitro*. The objective of the present study was to assess the effect of RSV infection of a human monocytic cell line on bactericidal activity and cytokine production in response to these bacterial respiratory pathogens. The effect of RSV infection on binding, uptake and intracellular killing of bacteria by a human monocytic leukaemia cell line, THP-1, was assessed. Cell culture supernates were examined with a mouse fibroblast cell assay for tumour necrosis factor-α (TNF-α) bioactivity. Expression of CD14, CD11a, CD18, CD15 and CD29 on uninfected and RSV-infected THP-1 cells was assessed by flow cytometry in relation to differences in bacterial binding. RSV infection of THP-1 cells significantly decreased their ability to bind and kill bacteria. Compared with uninfected cells, fewer bacteria bound to RSV-infected THP-1 cells and the surface antigens that have been reported to bind bacteria were expressed at lower levels on RSV-infected cells. RSV-infected cells incubated with bacteria exhibited less TNF-α bioactivity than uninfected cell incubated with bacteria. The results elucidate some of the mechanisms involved in the increased susceptibility of virus-infected patients to secondary bacterial infection. Reduced bacterial killing by virus-infected monocytes might contribute to reduced clearance of bacteria from the respiratory tract and damage elicited by the bacteria or cytokine response in COPD patients.

Introduction

Chronic obstructive pulmonary disease (COPD), which includes chronic bronchitis and emphysema, is an important cause of ill health in older age groups. Whilst cigarette smoking is a major risk factor for the condition [1], the precise role of microbial infections in its development and progress has yet to be elucidated [2, 3].

Non-typable *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* are common terial species isolated from patients during episodes exacerbation in the course of COPD and asthma ·7]. Respiratory syncytial virus (RSV) is an important viral pathogen in older groups, because complete immunity does not follow RSV disease and re-infections are not uncommon [8]. Fagon and Chastre [9] reviewed studies comparing the tracheobronchial microflora of patients during acute exacerbations and stable periods in the course of COPD; significant differences in isolation rates in these studies were found only for viruses (influenza virus and RSV). These studies did not measure the bacterial flora of the respiratory tract or the effect of acute viral infections on inflammation due to the resident flora. Infection of epithelial cells with RSV increased binding of respiratory bacterial pathogens [10–12]. Patients with RSV infections showed a significant rise of antibodies to *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* [13].

Viral infections might also alter the bactericidal

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mechanisms in the respiratory tract. Alveolar macrophages play an important role in killing and inhibition of replication of inhaled micro-organisms and in inflammation in the respiratory tract due to these agents. A human monocytic leukaemia cell line, THP-1 [14], possesses the properties of alveolar macrophages [15] and was adopted as an appropriate model to study interaction between RSV and bacterial species isolated from patients with COPD.

The first objective of this study was to examine the effect of RSV infection of THP-1 cells on binding, ingestion and intracellular survival of strains of *H. influenzae*, *S. pneumoniae* and *M. catarrhalis*. Surface antigens on monocytes, CD14, CD11a, CD18, CD15 and CD29 – some of which act as bacterial receptors [16, 17] – can be modified during inflammation [18]. RSV infection suppressed the expression of LFA-1 (CD11a + CD18) on human monocytes [19]. The second objective of this study was to examine the changes in the cell-surface antigens associated with RSV infection in relation to the ability of monocytes to bind and ingest bacterial isolates. Tumour necrosis factor-α (TNF-α) plays a role in defence against viral [20, 21] and bacterial infection [22, 23], but its release also results in inflammation in the respiratory tract and contributes to the systemic symptoms in patients with COPD [24, 25]. The third objective was to examine the effect of RSV infection and different strains of bacteria, or both, on TNF-α release from THP-1 cells.

Materials and methods

RSV

The Edinburgh strain of RSV (subgroup A) was harvested from HEp-2 cells maintained in maintenance medium (MM) which consisted of RPMI-1640 supplemented with fetal calf serum (FCS) 1% and 2 mM L-glutamine, penicillin 100 IU/ml and streptomycin 200 μg/ml. The suspension was tested by immunofluorescence for presence of mycoplasma. The concentration was adjusted to 2×10^6 plaque forming units/ml [10].

Bacteria

There were two isolates each of nontypable *H. influenzae* (HI1 and HI2), *M. catarrhalis* (MC1 and MC2) and *S. pneumoniae* (types 3 and 6) from patients with exacerbation of COPD obtained from the Clinical Bacteriology Laboratory of the department. *H. influenzae* and pneumococcal isolates were sensitive to ampicillin. *M. catarrhalis* strain MC1 grew on New York City medium with antibiotics selective for the pathogenic neisseria: lincomycin 1 μg/ml, colistin 6 μg/ml, amphotericin 1 μg/ml and trimethoprim lactate 6.5 μg/ml. *M. catarrhalis* MC2 did not grow on this medium. Overnight growths of *H. influenzae* and *M. catarrhalis* on boiled blood agar and *S. pneumoniae* on blood agar were collected in phosphate-buffered saline (PBS) and washed twice by centrifugation at 2500 *g* for 10 min. Heavy bacterial suspensions in MM without antibiotics were stored in small volumes at −20°C for up to 3 months for use in the assays. Concentrations of live bacteria in the frozen samples were determined by plating triplicate samples (5 μl) of appropriate dilutions in PBS on appropriate media for determination of colony forming units (cfu) after overnight growth in air with CO_2 5% at 37°C.

Ethidium bromide-labelling of bacteria

The bacterial suspensions were washed with PBS, and fixed with buffered paraformaldehyde (Sigma) 1% for 30 min in a water bath at 37°C. The bacteria were washed twice with PBS and incubated with ethidium bromide (EB; Sigma) 50 μg/ml for 20 min in a water bath at 37°C. The bacteria were washed twice and the total count was adjusted to 4×10^8/ml in PBS by direct microscopy. The labelled bacteria were held in small volumes at −20°C for up to 3 months.

THP-1 cells

THP-1 cells (European Collection of Animal Cell Cultures, Salisbury, Wilts) were cultured in growth medium (GM) which contained the same components as MM except for the higher concentration of FCS (10%) and the presence of mercapto-ethanol (2×10^{-5} M). The suspension was tested as described earlier for presence of mycoplasma. The cells were kept at 37°C in CO_2 5%. Fresh cultures of cells were infected with RSV at a multiplicity of infection of 2. Overnight cultures of uninfected and RSV-infected cells were washed with MM without antibiotics by centrifugation at 300 *g* for 7 min and the counts were adjusted to 1×10^6/ml in this medium by microscopy, for use in the experiments.

The proportions of monocytes infected with RSV in the samples were determined by flow cytometry by indirect immunofluorescence. The proportions were compared in one experiment with a mouse monoclonal antibody (MAb) to a viral surface glycoprotein G [10] or a convalescent serum from a patient with RSV infection previously absorbed with THP-1 cells and appropriately diluted in PBS. FITC-conjugated anti-mouse immunoglobulin or anti-human immunoglobulin antibodies (Sigma) were used to detect primary antibodies on cells. The convalescent serum was used to detect RSV-infected cells in subsequent experiments. The viability of RSV-infected and uninfected THP-1 cells was determined by trypan blue exclusion.

Bacterial binding

Uninfected and RSV-infected THP-1 cells (2×10^5) in capped plastic tubes were mixed with EB-labelled bacteria to provide a ratio of 10 bacteria per cell and

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Bacterial binding

Uninfected and RSV-infected THP-1 cells (2×10^5) in capped plastic tubes were mixed with EB-labelled bacteria to provide a ratio of 10 bacteria per cell and

incubated at 37°C in an orbital incubator (40 rpm). Samples were removed after incubation for 0 or 30 min and washed three times with ice-cold PBS by centrifugation at 300 *g* for 7 min to remove unbound bacteria from the pellet. The cells were suspended in 1 ml of PBS and stored in the dark at 4°C until analysed within 24 h by flow cytometry.

Flow cytometric analysis

The main cell population was gated by forward and side light scatter to exclude debris and cell aggregates. The percentage of cells with fluorescence greater than the background was determined on a histogram produced by log-amplified red fluorescence signals and mean fluorescence of the positive population on a histogram produced by linear signals. The values were multiplied to obtain a binding index (B_{Ind}) for each sample.

Ingestion of bacteria

To measure the fluorescence from bacteria ingested by cells, fluorescence from extracellular bacteria was quenched in each sample (1 ml) with 20 μl of crystal violet (BDH) 0.05% in 0.15 N sodium chloride. As the optical density of the soluble dye and its quenching effect increases with time, flow cytometry was performed when the OD_{595} reading of the sample was 500–600. In this range >90% of the EB-labelled bacteria outside the cells were completely quenched. Crystal violet at higher OD values is membrane permeable and can quench intracellular bacteria. The laser power for these samples was adjusted to compensate for the decrease in background autofluorescence of the control samples due to quenching. An ingestion index (I_{Ind}) for each sample was calculated by multiplying the percentage of fluorescent cells and the mean fluorescence as described above.

Intracellular survival of bacteria

Uninfected and RSV-infected THP-1 cells were incubated with live unlabelled bacteria under the above conditions for 30 min. Extracellular bacteria were killed by adding gentamicin 30 μg/ml and ampicillin 50 μg/ml for 15 min at 37°C. After three washes with PBS, the cells were resuspended in 100 μl of PBS and lysed with an equal volume of sodium lauryl sulphate 0.05% in sterile distilled water. Samples were immediately plated in triplicate for determination of cfu as described above. For a time course study of intracellular survival and growth of bacteria, cells were incubated for different periods before lysing and plating.

Binding of anti-CD antibodies to cells

The flow cytometry method described previously to detect host cell antigens on buccal epithelial cells was used in these experiments [26]. Uninfected and RSV-infected THP-1 cells were incubated at 4°C for 30 min with the following mouse MAbs: CD11a (Dako) diluted 1 in 20, CD18 (Dako) diluted 1 in 20, CD15 (Scottish Antibody Production Unit, Carluke) diluted 1 in 20, CD14 (Dako) diluted 1 in 10 and CD29 (Serotec) diluted 1 in 20. The samples were washed three times with PBS. Fluorescein isothiocyanate (FITC)-labelled anti-mouse immunoglobulin antibody (Sigma) diluted 1 in 100 was used to detect binding of the primary antibodies to cells. The samples were washed three times and fixed with buffered paraformaldehyde (Sigma) 0.5%. Cells with fluorescence greater than the control (cells treated only with the FITC-labelled second antibody) were assessed for mean fluorescence and the binding index was calculated as described above.

TNF-α bioactivity

Uninfected and RSV-infected THP-1 cells were incubated in CO_2 5% at 37°C with unlabelled live or EB-labelled fixed bacteria at a ratio of 10 bacteria per cell in 24-well tissue culture plates (Costar). After incubation for 24 h, supernates from individual wells were collected for determination of TNF-α. A bioassay with L-929 cells (a mouse fibroblast cell line) was used to determine TNF-α activity [27].

Statistical analysis

Paired *t* tests were used to analyse data from binding and ingestion of bacteria, and for binding of anti-CD MAbs to uninfected or RSV-infected cells. Wilcoxon's test for matched pairs was applied to the data from experiments measuring intracellular survival of bacteria. The data for TNF-α production by uninfected or RSV-infected THP-1 cells under various conditions were also analysed by paired *t* tests.

Results

The proportions of RSV-infected cells were similar with both mouse anti-G MAb and absorbed human convalescent serum. At 24 h after infection, 40–50% of THP-1 cells were infected with RSV. RSV infection did not affect the viability of cells 24 h after infection.

Bacterial binding, ingestion and survival

Bacteria were able to bind to cells at 0 min (data not shown), but intracellular bacteria were not detected at this time either by quenching the external bacteria or by determination of intracellular survival. Data from nine experiments on binding of *H. influenzae*, *M. catarrhalis* and pneumococci are presented in Fig. 1 and on ingestion of these bacteria in Fig. 2.

H. influenzae. The binding observed for strain HI1 to

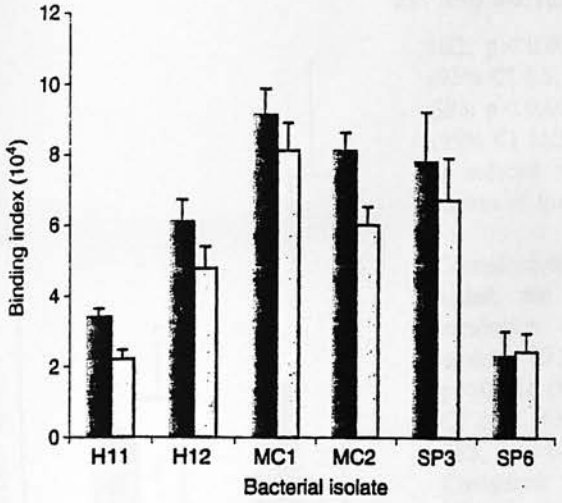

Fig. 1. Binding indices (SE) of *H. influenzae* isolates (HI1 and HI2), *M. catarrhalis* isolates (MC1 and MC2) and *S. pneumoniae* types 3 and type 6 (SP3 and SP6) to THP-1 and RSV-infected THP-1 cells (mean of nine experiments); ■, uninfected THP-1 cells; ▩, RSV-infected THP-1 cells.

uninfected THP-1 cells was 50% of that observed for strain HI2 ($p < 0.05$). Strain HI1 also bound significantly less to RSV-infected cells than strain HI2 ($p < 0.01$). Compared with uninfected cells, binding of both the strains to cells infected with RSV was significantly reduced: HI1, $p < 0.01$, 95% CI −5520, −18141; HI2, $p < 0.02$ 95% CI −1588, −25237. RSV infection of cells reduced the ingestion of both strains, but this was significant only for HI1 ($p < 0.05$, 95% CI −791, −12793). Both the isolates survived better in RSV-infected cells than in uninfected cells ($p < 0.02$, HI1, $Z = -2.366$; $p < 0.05$, HI2, $Z = -2.1974$) (Fig. 3).

M. catarrhalis. Compared with strain MC2, strain MC1 bound in greater numbers to uninfected THP-1 cells ($p < 0.001$) and to RSV-infected cells ($p < 0.005$). Both strains MC1 and MC2 bound significantly less t RSV-infected cells than to uninfected cells: MC1 $p < 0.01$, 95% CI −6796, −11840; MC2, $p < 0.002$ 95% CI −12258, −28233. RSV infection of cell reduced the ingestion of both strains (MC1, NS; MC2 $p < 0.05$, 95% CI −335, −31328). Neither of th isolates survived in uninfected or RSV-infected THP-1 cells in the conditions used in the study.

S. pneumoniae. Compared with strain SP6, strain SP3 bound significantly more to uninfected cells ($p < 0.05$) and to RSV-infected cells ($p < 0.05$). Compared with uninfected cells, lower numbers of strain SP3 bound to RSV-infected cells ($p = 0.051$, 95% CI 55, −23737). There was no significant difference in binding of strain SP6 to uninfected or RSV-infected cells. A decrease in ingestion of strain SP3 by RSV-infected cells was observed ($p = 0.059$, 95% CI 1212, −45717). There was no significant difference for strain SP6 in ingestion by uninfected or RSV-infected THP-1 cells. Significantly greater numbers of strain SP3 survived in RSV-infected cells than in uninfected cells ($p < 0.05$, $Z = -2.0226$) (Fig. 3). Strain SP6 showed evidence of only occasional survival in uninfected cells, which was twice as high in RSV-infected cells.

Effect of RSV infection on binding of anti-CD MAbs to cells

Mean B_{Ind} for the MAbs directed towards the cell surface antigens of THP-1 cells are summarised in Fig. 4a and b. In seven experiments, RSV infection resulted in significant decreases in binding of the following MAbs: anti-CD11a by 35% ($p < 0.05$, 95% CI −20, −50); anti-CD18 by 24% ($p < 0.001$, 95% CI −16, −32); anti-CD14 by 28% ($p < 0.05$, 95% CI −3, −53) and anti-CD15 by 30% ($p < 0.05$, 95% CI −3, −57). There was an increase of 9% in binding of anti-CD29 to RSV-infected THP-1 cells, but it was not statistically significant.

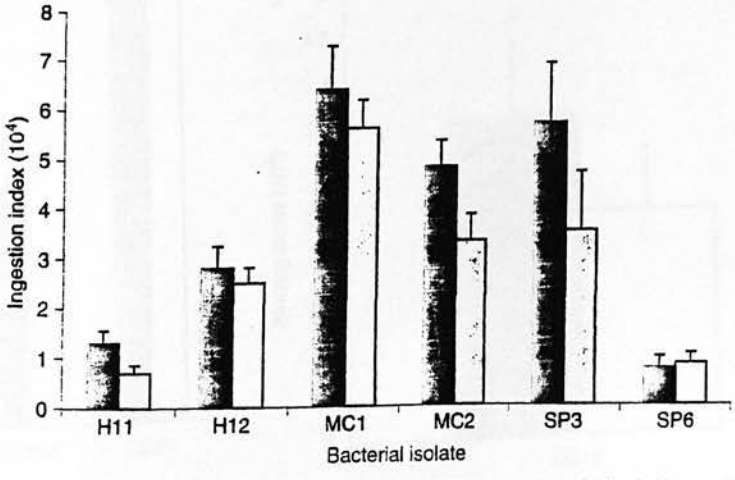

Fig. 2. Ingestion indices (SE) of *H. influenzae* isolates (HI1 and HI2), *M. catarrhalis* isolates (MC1 and MC2) and *S. pneumoniae* types 3 and type 6 (SP3 and SP6) to THP-1 and RSV-infected THP-1 cells (mean of nine experiments); ■, uninfected THP-1 cells; □, RSV-infected THP-1 cells.

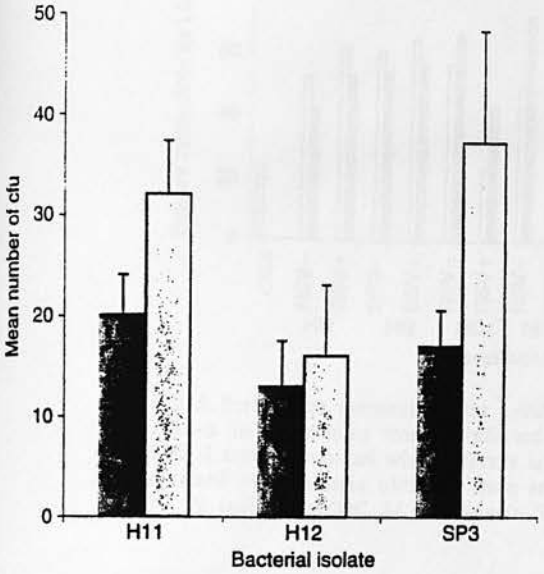

Fig. 3. Survival of *H. influenzae* isolates (HI1 and HI2) and *S. pneumoniae* type 3 (SP3) in uninfected and RSV-infected THP-1 cells (mean of seven experiments); ■, uninfected THP-1 cells; ▨ RSV-infected THP-1 cells.

Effect of RSV infection and bacteria on TNF-α bioactivity of cells

TNF-α bioactivity in supernates of uninfected or RSV-infected THP-1 cells incubated with either live unlabelled or fixed EB-labelled bacteria was determined (Fig. 5). In seven experiments, compared with THP-1 cells to which no bacteria were added, the bioactivity was increased by incubation of THP-1 cells with live strains: HI1, $p < 0.01$ (95% CI 11.5, 45.9); HI2, $p < 0.001$ (95% CI 24.3, 50.6); MC1, $p < 0.05$ (95% CI 8.5, 59.2); MC2, $p < 0.05$ (95% CI 5.1, 47.2); SP3, $p < 0.001$ (95% CI 48.6, 65.9) or SP6, $p < 0.005$ (95% CI 15.5, 44.5). Differences in the levels of TNF-α induced were not statistically significant between strains of the same species.

Compared with THP-1 cells to which no bacteria were added, the TNF-α bioactivity was increased by incubation of THP-1 cells with EB-labelled fixed strains: HI1, $p < 0.05$ (95% CI 4.1, 31.9); HI2, $p < 0.001$ (95% CI 19, 42.4); MC1, $p < 0.002$ (95% CI 23.1, 63.2); MC2, $p < 0.001$ (95% CI 38.9, 59); SP3, $p < 0.02$ (95% CI 5.9, 29.5) or SP6, NS. Compared with fixed strain HI1, fixed strain HI2 elicited significantly more TNF-α bioactivity ($p < 0.005$, 95% CI 6, 19.3). Fixed MC2 and SP3 strains elicited greater TNF-α bioactivity compared with fixed MC1 and SP6 strains, respectively, but the differences were not statistically significant.

RSV infection increased TNF-α bioactivity from THP-1 cells incubated with live HI1, HI2 or MC2 strains, but the differences were not statistically significant. Compared with uninfected cells, TNF-α bioactivity was decreased for RSV-infected cells incubated with live strain MC1, $p < 0.002$ (95% CI −49.3, −18.4); SP3, $p < 0.001$ (95% CI −47.6, −20.4) or SP6, $p < 0.005$ (95% CI −23.2, −8.2).

RSV infection increased TNF-α bioactivity from THP-1 cells incubated with fixed HI1 or HI2 strains, but the differences were not statistically significant. Compared with uninfected cells, there was decreased TNF-α

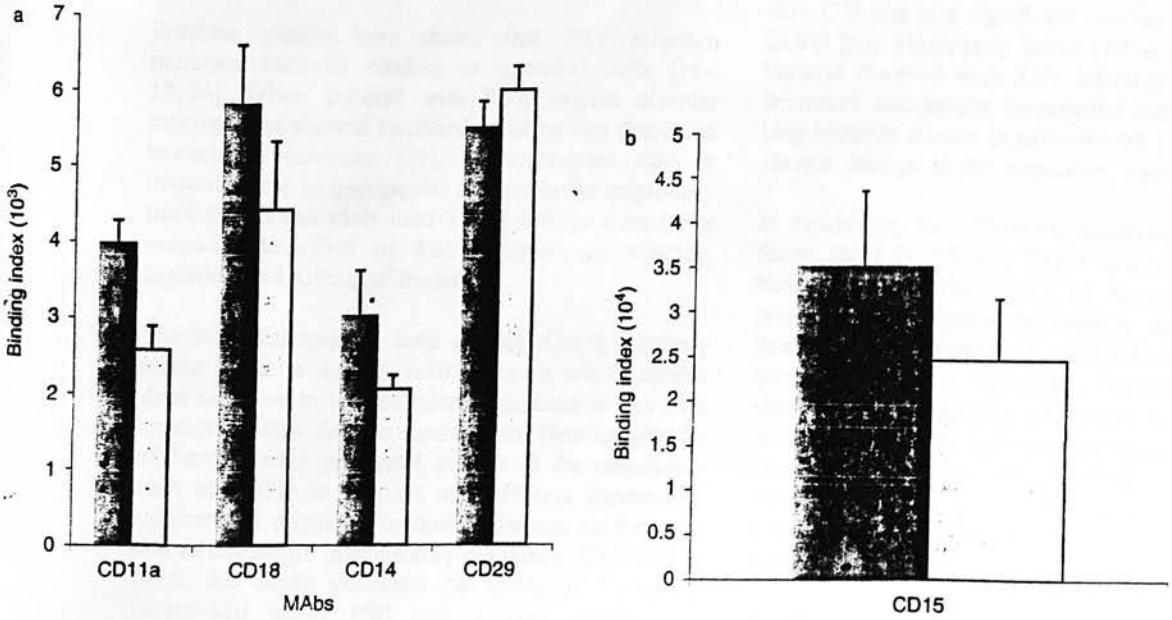

Fig. 4. (a) Binding of MAbs to CD11a, CD18, CD14 and CD29 to uninfected (■) and RSV-infected (□) THP-1 cells (mean of seven experiments). (b) Binding of MAb to CD15 to uninfected (■) and RSV-infected (□) THP-1 cells (mean of seven experiments).

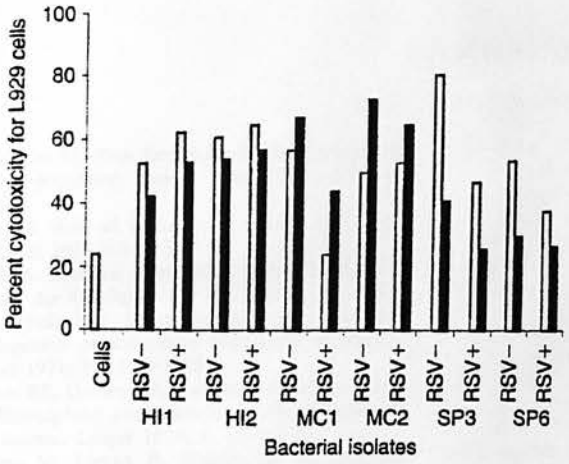

Fig. 5. Percentage cytotoxicity for L-929 cells caused by TNF-α in supernates from uninfected or RSV-infected THP-1 cells incubated with different isolates of live (▨) or fixed (■) bacteria obtained from seven experiments. HI, *H. influenzae*; MC, *M. catarrhalis*; SP, *S. pneumoniae*.

bioactivity with RSV-infected cells incubated with fixed MC1, MC2, SP3 or SP6 strains, but the results were significant only for strain SP3 ($p < 0.01$, 95% CI −25.5, −5.2).

Discussion

Although no significant differences in rates of isolation of bacterial species during exacerbation and stable periods were found in patients with COPD [9], the density of bacterial colonisation of the respiratory tract might contribute to exacerbation. Secondary bacterial infections following virus infections are thought to be associated with enhanced bacterial binding, suppression of host immune responses and bactericidal functions associated with viral infections [28].

Previous studies have shown that RSV infection increases bacterial binding to epithelial cells [10–12, 26]. When infected with RSV, murine alveolar macrophages showed increased cytokine and decreased bactericidal functions [29]. As monocytes play an important role in bactericidal activity in the respiratory tract, the present study used THP-1 cells as a model to examine the effect of RSV infection on binding, ingestion and killing of bacteria.

The bronchial tree has been envisaged as a relatively sterile organ in healthy individuals, in which continuous exposure to inhaled micro-organisms is met with appropriate host defence mechanisms. Heavier growths of bacteria over prolonged periods in the respiratory tract in COPD as a result of inefficient bactericidal mechanisms might cause further damage to the tract due to release of inflammatory cytokines. With this in mind, this study examined the levels of TNF-α, a bactericidal agent [22] and a very potent pro-inflammatory cytokine, from THP-1 cells in response to common microbial pathogens.

The first objective was to compare the differences in binding of individual isolates of each bacterial species and their killing by uninfected and RSV-infected THP-1 cells. RSV-infected cells demonstrated reduced levels of binding, ingestion and killing of most of the bacteria. The second objective was to assess the effects of RSV infection on surface antigens of the phagocytes that act as receptors for bacteria. Decreased ingestion of most of the bacteria by THP-1 cells infected with RSV might be due to the lower levels of initial bacterial binding observed with these cells. CD14, CD11a, CD18 and CD15 have been identified as receptors for several bacterial species [16, 17]. Lower levels of these antigens associated with RSV infection might contribute to the decreased bacterial binding observed in this study. However, a correlation test was not performed for BIs of the antibody and bacterial binding to cells, because the two types of experiments were performed on different preparations of cells. Previous studies have demonstrated a significant relationship between bacterial binding and expression of blood group antigens that act as receptors on epithelial cells [26, 30].

Increased TNF-α bioactivity contributes to inflammation, whereas a marked decrease can jeopardise mucosal protection against bacteria and bactericidal activity [31, 22]. The third objective of the present study was to compare TNF-α produced by uninfected and RSV-infected THP-1 cells in response to bacteria. Compared with cells not exposed to bacteria, various degrees of increased responses were observed with all the bacterial isolates. RSV infection reduced TNF-α bioactivity from cells incubated with strains MC1, SP3 and SP6. RSV infection in human alveolar macrophages can persist for at least 25 days after infection *in vitro* [32] and in a significant number of patients with COPD [33]. Persistently lower TNF-α responses to the bacteria observed with RSV infection combined with decreased intracellular bactericidal activity might prolong bacterial disease in patients with COPD and cause chronic damage to the respiratory tract.

In conclusion, the differences observed between uninfected and RSV-infected THP-1 cells in the pattern of binding, intracellular killing of bacteria and TNF-α production in response to bacteria may explain the increased susceptibility of virus-infected patients to secondary bacterial infections. Bacteria that escape virus-infected monocytes might have a greater opportunity to grow in the milieu of the respiratory tract and cause disease. Further work to examine the effects of specific antibodies to *S. pneumoniae* on intracellular bacterial survival and production of cytokines from monocytes is under way.

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