THE EFFECT OF PULMONARY SURFACTANT ON INNATE IMMUNE RESPONSES IN INFLUENZA VIRUS INFECTED HUMAN AIRWAY EPITHELIAL CELLS

BY

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THE EFFECT OF PULMONARY SURFACTANT ON INNATE IMMUNE RESPONSES IN INFLUENZA VIRUS INFECTED HUMAN AIRWAY EPITHELIAL CELLS

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DOCTOR OF PHILOSOPHY

BY

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ABSTRACT

Overwhelming inflammatory responses leading to neutrophil invasion are hypothesised to be the main cause of mortality in influenza virus induced acute respiratory distress syndrome (ARDS). Previously, pulmonary surfactant has been shown to modulate inflammatory responses to bacterial agents. The aim of the present study was to investigate the effect of pulmonary surfactant on innate immune responses in an *in vitro* model of influenza virus infected human airway epithelial cells. Human lung type II alveolar epithelial cells A549 and BEAS-2B human bronchial epithelial cells were infected with influenza A virus H1N1 strains A/Swine/1976/31, A/WSN/33 and A/PR/8/34. Poly I:C, *Escherichia coli* 0111:B4 LPS and measles virus strain Edmonston were used as cytokine stimulation controls. The effect of pulmonary surfactant was compared to that of dexamethasone. This *in vitro* study showed that physiological concentrations (up to 500 μg/ml) of clinically approved SP-A and SP-D depleted surfactant preparations (i) were non-toxic in BEAS-2B cells, (ii) had no effect on influenza virus infectivity, and (iii) reduced influenza virus induced cytokine production comparable to dexamethasone. Porcine Curosurf® significantly inhibited IL-8 and RANTES production in A/WSN/33 infected cells, by 30 and 35% respectively ($p<0.05$). Bovine Survanta® had a less pronounced effect. In luciferase reporter assays pulmonary surfactant, in contrast to dexamethasone, non-specifically inhibited both TLR3/RIG-I mediated NF-κB promoter activation and IFN-β promoter activation. Our results indicate that SP-A and SP-D depleted surfactant preparations attenuate pro-inflammatory responses in influenza A virus infected human airway epithelial cells, but inhibitory effects on IFN-β promoter activity were also observed. This suggests that pulmonary surfactant may be of clinical benefit in reducing pro-inflammatory responses in virus induced ARDS, however, a weakening of IFN-β mediated anti-viral responses can not be excluded.

ABBREVIATIONS

A549	human alveolar epithelial cell line
AAF	IFN-α activated factor
AGPT	agar gel precipitin test
amp^r	ampicillin resistant
AMs	alveolar macrophages
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATP	adenosine triphosphate
B95-8	B lymphoblastoid cell line
B95a	B lymphoblastoid cell line adherent
BEAS-2B	human bronchial epithelial cells
CARD	caspase recruitment domains
CARDIF	CARD adaptor inducing IFN-β
CD150	cluster differentiation 150
CD46	cluster differentiation 46
CMV	cytomegalovirus
COPD	chronic obstructive pulmonary disease
CPE	cytopathic effect
CpG	cytosine poly guanine
cRNA	complementary RNA
DIGs	detergent insoluble glycolipid enriched domains
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPC	dipalmitoylphosphatidylcholine
ds	double stranded
EBV	Epstein-Barr virus
ECACC	European Collection of Cell Cultures
ECE	embryonated chicken egg
eIF2	eukaryotic translation initiation factor 2
ELISA	enzyme linked immunosorbent assay
ERK1/2	extracellular signal regulated kinase 1/2

ETR	electron transfer reagent
F	fusion protein
FAPP	filtered-air-positive-pressure
FAT	fluorescent antibody technique
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
G418	geneticin
GAF	IFN-γ activated factor
GAS	IFN-γ activated sequence
gp120	glycoprotein 120
GRO	growth related gene product
H/HA	haemagglutinin
HAU	haemagglutination unit
HCV	hepatitis C virus
HEK293	human kidney epithelial cell line
HI	haemagglutination inhibition
HIV	human immunodeficiency virus
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSV	herpes simplex virus
IFNR	IFN receptor
IFNs	interferons
IFN-α	interferon-alpha
IFN-β	interferon-beta
IFN-γ	interferon-gamma
IKK	IκB kinase
IKKα	IκB kinase alpha
IKKβ	IκB kinase beta
IKKε	IκB kinase epsilon
IL-1R	interleukin-1 receptor
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IL-8	interleukin-8

ILR #	internal laboratory reference number
IP-10	interferon inducible protein-10
IPS-1	IFN-β promoter stimulator-1
IRAK1	IL-1R associated kinase 1
IRAK4	IL-1R associated kinase 4
IRDS	infant respiratory distress syndrome
IRF3	interferon regulatory factor 3
IRF7	interferon regulatory factor 7
IRF9	interferon regulatory factor 9
IRSE	IFN stimulated regulatory element
ISGF3	IFN stimulated gene factor 3
ISGs	IFN stimulated genes
IκBs	inhibitory κB proteins
Jak1	Janus associated kinase 1
JNK	c-jun-NH_2-terminal kinase
K	lysine
KQCL	kinetic quantitative chromogenetic LAL
L	large protein
LAL	Limulus amoebocyte lysate
LB	Luria Bertani (broth)
LC	liquid chromatography
LMB	leptomycin B
LPS	lipopolysaccharide
LRR	leucine rich repeat
LTC_4	leukotriene C_4
M	matrix protein
M1	matrix protein
M2	ion channel protein
MAL	MyD88 adaptor like protein
MAP	mitogen activating protein
MAVS	mitochondrial antiviral signalling protein
MCP-1	monocyte chemoattractant protein-1
MDA5	melanoma differentiation associated protein 5
MDCK	Madin Darby Canine Kidney

MIG	monokine induced by IFN-γ
MIP-1	macrophage inflammatory protein-1
MM6	Mono Mac 6
MMR	measles mumps rubella vaccine
MODS	multiple organ disfunction syndrome
MOI	multiplicity of infection
MoMLV	Moloney murine leukemia virus
Mon	monkey
MR	measles rubella vaccine
mRNA	messenger RNA
MS	mass spectrometry
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt]
MyD88	myeloid differentiation factor 88
N/NA	neuraminidase
N	nucleoprotein
NADH	nicotinamide adenine dinucleotide hydride
NADPH	nicotinamide adenine dinucleotide phosphate
NC	negative control
NDV	Newcastle disease virus
NEMO	NF-κB essential modulator
NF-κB	nuclear factor-kappa B
NK	natural killer
NLS	nuclear localization sequence
NP	nucleoprotein
NS1	non-structural protein 1
NS2	non-structural protein 2
OAS	2'5'-oligoadenylate synthetase
P	phosphoprotein
p.i.	post infection
p38	p38 MAP kinase
PAF	platelet activating factor
PAMPs	pathogen associated molecular patterns
PBMCs	peripheral blood mononuclear cells

PBS	phosphate buffer saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PGE_2	prostaglandin E_2
PHA	phytohaemagglutinin
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3 kinase
PIV-1	parainfluenza virus-1
PIV-2	parainfluenza virus-2
PIV-3	parainfluenza virus-3
PKR	protein kinase R
PMNs	polymorphonuclear lukocytes
PMS	phenazine methosulfate
Poly(I-C)	polyinosinic-polycytidylic acid
PP	phosphorylation
PRRs	pattern recognition receptors
PS	phosphatidylserine
P_{TAL}	TATA like promoter
rAAV	recombinant adeno associated virus
RANTES	regulated on activation normal T cell expressed and secreted
RBCs	red blood cells
RIG-I	retinoic acid inducible protein-I
RLRs	RIG-like receptors
RNA	ribonucleic acid
RNase L	ribonuclease L
RNF125	ring finger protein 125
RNPs	ribonucleoproteins
ROIs	reactive oxygen intermediates
RPAT	rapid plate agglutination test
RSV	respiratory syncytial virus
SAP	surface associated phase
SARM	sterile α and armadillo motif containing protein
SCAP	severe community acquired pneumonia

SIDS	sudden infant death syndrome
SLAM	signalling lymphocyte activation molecule
SM	sphingomyelin
SP-A	surfactant protein-A
SP-B	surfactant protein-B
SP-C	surfactant protein-C
SP-D	surfactant protein-D
SPF	specific pathogen free
ss	single stranded
Stat1	signal transducers and activators of transcription 1
Stat2	signal transducers and activators of transcription 2
SV40	simian virus 40
TAB2	TAK1 binding protein 2
TAK1	TGF-β activating kinase
TANK	TRAF family member associated NF-κB activator
TB	transcription blocker
TBK1	TANK binding kinase 1
TCID50	tissue culture infecting dose 50
TGF-β	transforming growth factor -beta
TGN	*trans* Golgi network
TICAM-1	TIR containing adaptor molecule-1
TIM	TRAF interaction motif
TIR	Toll/Interleukin-1 receptor
TK	thymidine kinase
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor-alpha
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated
TRAF	TNF receptor associated factor
TRAF3	TNF receptor associated factor 3
TRAF6	TNF receptor associated factor 6
TRAM	TRIF related adaptor molecule
TRIF	TIR domain containing adapter inducing IFN-β
TRIKA1	TRAF6 regulated IKK activator 1

TRIM25	tripartite motif protein 25
TxB_2	tromboxane B_2
Tyk2	tyrosine kinase 2
Ub	ubiquitination
VISA	virus induced signalling protein
vRNA	viral RNA
κB_4	κ enhancer element

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1 Introduction

1.1 General introduction

The respiratory tract, both upper tract and lower bronchial and alveolar surfaces, are highly susceptible to viral respiratory infections. A variety of respiratory syndromes including common cold, pharyngitis, tracheobronchitis, laryngotracheobronchitis (croup), bronchiolitis and pneumonia are caused by different respiratory viruses (Richman et al. 2002). Viral agents associated with these respiratory syndromes are summarised below (Table 1.1). Influenza A virus is the major cause of acute pneumonia in adults, accounting for more than one quarter of all reported cases (Table 1.1). In the United States alone, influenza virus infections result in 20 million respiratory illnesses, 114000 hospitalisations and 20000 deaths annually, and the great 1918 pandemic killed as many as 20-50 million people worldwide (Richman et al. 2002). More recently, in 1997, the first direct transmission of the highly pathogenic H5N1 avian influenza to humans occurred in Asia (Hong Kong) (Hsieh et al. 2006), raising the prospect of a deadly new global outbreak. In addition to the H5N1 virus, H9N2 and highly virulent H7N7 viruses were also reported to be directly transmit from birds to humans causing severe diseases (Doherty et al. 2006). Numerous studies suggest a direct correlation between massive inflammation during influenza virus infection and injuries of the lung tissue, non respiratory organ failure and disease severity (Van Reeth 2000; Julkunen et al. 2001; Le Goffic et al. 2006; Hsieh et al. 2006).

In normal as well as diseased lung tissues maintenance of pulmonary homeostasis strongly depends on the presence and function of pulmonary surfactant. Pulmonary surfactant is a surface reducing material consisting of lipids and proteins, particularly rich in phospholipids, produced by bronchial and alveolar cells (Chevalier & Collet 1972; Balis et al. 1985; Walker et al. 1986; Persson et al. 1988; Phelps & Floros 1988; Voorhout et al.

1992; Crouch et al. 1992). In addition to its surface tension lowering activity, pulmonary surfactant has been shown to modulate the inflammatory processes within the lung. In the past, the majority of work focused on the role of hydrophilic pulmonary proteins (SP-A and SP-D) during bacterial (Ferguson et al. 2006; Giannoni et al. 2006) and viral infection (Levine et al. 2002; Levine et al. 2004). However, SP-A and SP-D are not yet approved for clinical use. Therapeutic application of animal derived surfactant preparations, from which SP-A and SP-D are depleted by chemical purification processes (also know as exogenous replacement therapy) has been proven to be an effective treatment in infant respiratory distress syndrome (IRDS). This treatment has dramatically improved survival rates in premature infants *in vivo* (Berggren et al. 1984). *In vitro* studies on the biological activity of SP-A and SP-D depleted pulmonary surfactant preparations and surfactant specific lipids are scarce. Recent *in vitro* work from our group and others has shown high effectiveness and a wide spectrum of activity of the phospholipid fraction in reducing inflammatory responses in human cells, including primary peripheral blood mononuclear cells (PBMCs) and Mono Mac 6 (MM6) cell line (Thomassen et al. 1992). In these studies inflammation was induced by lipopolysaccharide (LPS) from gram negative bacteria as an inflammatory stimulant and surfactant phospholipids were found to attenuate production of inflammatory cytokines (Morris et al. 2000), lipid mediators (Tonks et al. 2003) and reactive oxygen intermediate (ROI) production (Tonks et al. 2001; Tonks et al. 2005). These reports highlighted the potential immunomodulatory function of commercially available pulmonary surfactants as adjuvant to current treatment of acute respiratory diseases, to potentially reduce the inflammatory damage associated with these conditions.

The most commonly used anti-inflammatory treatment in the clinical study are corticosteroid related compounds, which are systemically active, leave the patient more susceptible to secondary bacterial infections and are known to induce a large number of

adverse systemic side effects, including immunosupression and metabolic disturbance (Deshmukh 2007). Pulmonary surfactant therapy could offer a safe anti-inflammatory action at the local level within the lungs for patients with acute respiratory failure caused by viral and/or bacterial pathogens.

In the light of these studies, this project aimed to investigate the possible therapeutic potential of different preparations of exogenous natural pulmonary surfactant, which are currently in clinical use, in an *in vitro* model of influenza virus airway infection. The project will:

(i) assess toxicity of pulmonary surfactant in human airway epithelial cells, the principal site for influenza virus invasion and primary replication,

(ii) determine the effect of pulmonary surfactant on influenza virus entry and completion of the lytic cycle,

(iii) investigate the effect of pulmonary surfactant on the biosynthesis of cytokines and chemokines associated with influenza virus pathology and inflammatory damage,

(iv) provide more insight regarding the mechanisms involved in any modulatory action of pulmonary surfactant.

Virus	*Frequency of syndrome*							
	Colds	Pharyngitis	Tracheobronchitis	Croup	Bronchiolitis	Pneumonia Children	Adults	Immunocompromised individuals
RNA viruses								
Influenza A virus	+	++	+++	++	+	++	++++	+
Influenza B virus	+	++	++	+	+	+	++	+
PIV-1	+	++	+	++++	+			
PIV-2	+	++	+	++	+			
PIV-3	+	++	+	+++	++	+++	+	+
RSV	++	+		++	++++	++++	+	++
Measles virus			+	+		++	+	+
Rhinovirus	++++	++	+	+	+	+		
Enterovirus	++	++			+	+		
Coronavirus	++	+			+	+		
HIV		+						
DNA viruses								
Adenovirus		++	+	++	++	++	++	++
Herpes simplex virus		+			+	+		+
Varicella virus						+	+	+
EBV		++						
CMV		+				++		+++

+ 1-5% of cases, ++ 5-15% of cases, +++ 15-25% of cases, ++++ >25% of cases

Table 1.1: Frequency with which viral respiratory syndromes are caused by specific viral pathogens (Richman et al. 2002).

1.2 Pulmonary surfactant

1.2.1 Pulmonary surfactant

Pulmonary surfactant is a multicomponent complex fluid lining the alveolar and small airways, composed of lipids, proteins and carbohydrates. The major function of pulmonary surfactant is to reduce the surface tension in the lungs at the end of expiration. The surface tension acts at the air-water interface and tends to make alveoli smaller. By decreasing the surface tension pulmonary surfactant increases elasticity of the alveoli allowing the lung to inflate more easily (King & Clements 1972a, b and c).

1.2.1.1 Life cycle and recycling of pulmonary surfactant

Pulmonary surfactant is produced by alveolar type II cells (Chevalier & Collet 1972; Persson et al. 1988; Crouch et al. 1992) and bronchial Clara cells (Balis et al. 1985; Walker et al. 986; Phelps & Floros 1988; Crouch et al. 1991; Voorhout et al. 1992). Intracellular pulmonary surfactant is stored in multilayered membrane structures called lamellar bodies (Askin & Kuhn 1971). After release from lamellar bodies pulmonary surfactant forms an organised structure known as tubular myelin (Weibel et al. 1966; Gil & Weibel 1969) from which, during inspiration, a monolayer at the air-water interface is formed. During expiration, this monolayer is compressed therefore lowers surface tension and contributes to alveolar stability. After use, the pulmonary surfactant is taken up by alveolar type II cells (Geiger et al. 1975) or degraded by alveolar macrophages (AMs) (Nichols 1976; Williams & Benson 1981) (Figure 1.1).

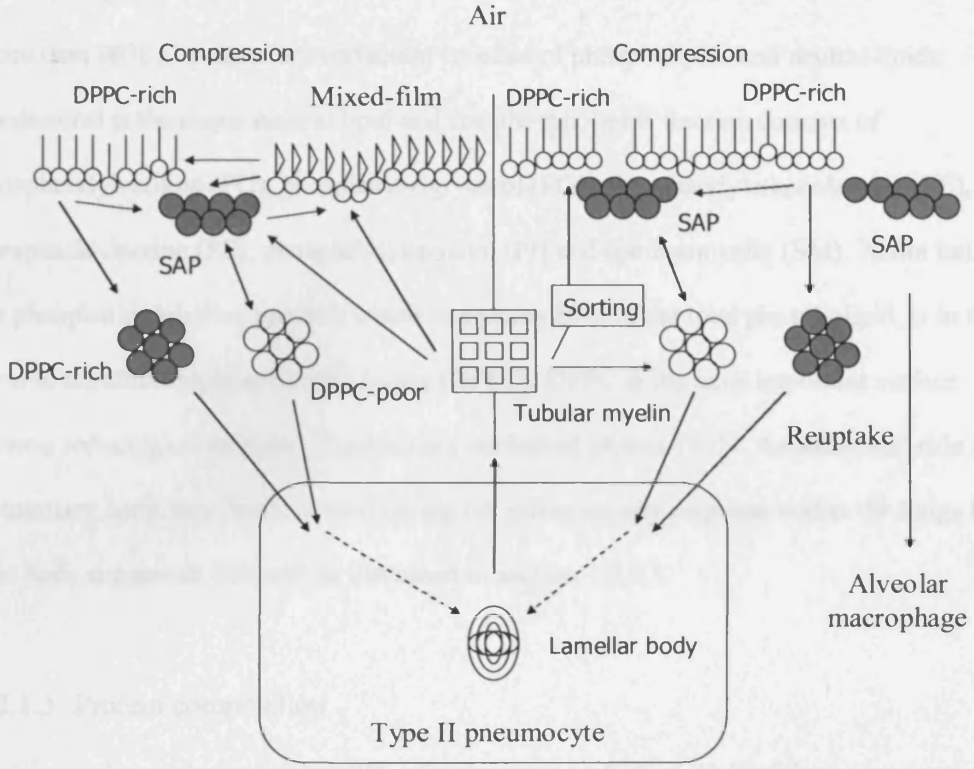


Figure 1.1: Life cycle and recycling of pulmonary surfactant (Goerke 1998). (SAP – surface associated phase, DPPC - dipalmitoylphosphatidylcholine)

1.2.1.2 Lipid composition

More than 90% of pulmonary surfactant consists of phospholipids and neutral lipids. Cholesterol is the major neutral lipid and the phospholipids fraction consists of phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (SM). About half of the phosphatidylcholine present, which comprises 80% of the total phospholipid, is in the form of dipalmitoylphosphatidylcholine (DPPC). DPPC is the most important surface tension reducing component of pulmonary surfactant (Klaus 1961). An additional role for pulmonary surfactant lipids in modulating the inflammatory response within the lungs has also been suggested, this will be discussed in section 1.2.2.1.

1.2.1.3 Protein composition

Proteins make up the remaining 10% of pulmonary surfactant. Half of these proteins are plasma proteins such as albumins, globulins, fibrinogens and regulatory proteins, the remaining are surfactant specific proteins SP-A, SP-B, SP-C and SP-D (Crouch & Wright 2001). Two hydrophilic proteins SP-A and SP-D are formed with subunits consisting of trimers of polypeptide chains (Weis & Drickamer 1994). Each peptide has two domains. The N-terminal portion contains repeating glycine triplets and hydroxyproline residues which are present in collagen and are responsible for the triple helical structure of this protein. The C-terminal portion shows resemblance to lectins (Crouch et al. 2000). SP-A is necessary for the formation of tubular myelin and regulates pulmonary surfactant uptake (Wright et al. 1986; Wright et al. 1987). SP-A together with SP-D participate in innate immune responses to various infectious agents, this will be discussed in section 1.2.2.2. Two strictly hydrophobic proteins SP-B (79 amino acids) and SP-C (33-35 amino acids) are essential for a rapid adsorption of phospholipids at the air-water interface (Curstedt et al.

1987). Surprisingly only two short hydrophobic sequences (valine-, leucine- and isoleucine rich) are found in SP-B at positions 37-42 and 54-58, and only one such sequence is found in SP-C between positions 13 and 28 (Johansson et al. 1994).

1.2.2 Pulmonary surfactant – evidence for a role in pulmonary defense

1.2.2.1 Lipid fractions

In addition to surface tension reductive effects, pulmonary surfactant has been reported to modulate innate immune responses to different bacterial (Speer et al. 1991) and viral agents (Benne et al. 1995). Commercial pharmaceutical surfactant preparations which lack specific surfactant proteins SP-A and SP-D, and also individual classes of pulmonary surfactant lipids affect *in vitro* the inflammatory activity of various immune cells, including monocytes, lymphocytes and natural killer (NK) cells. Among them, the most widely studied were human monocytes and macrophages (Speer et al. 1991).

In 1988, whole pulmonary surfactant and surfactant phospholipids were shown to suppress the lymphoproliferative response to phytohaemagglutinin (PHA) (Wilsher et al. 1988a). This immunosuppressive property of pulmonary surfactant was shown to be related to its phospholipid composition. Phosphatidylcholine, phosphatidylglycerol and phosphatidylinositol were the most potent inhibitors of this response (Wilsher et al. 1988b). The same group reported that cytotoxic activity of NK cells was affected by pulmonary surfactant lipids treatment (Wilsher et al. 1988c). In 1991 Speers and co-workers showed that phagocytosis of *Stapylococcus aureus* in human monocytes exposed to Curosurf® was impaired (Speer et al. 1991). Curosurf® is modified porcine pulmonary surfactant containing 99% phospholipids and 1% surfactant proteins SP-B and SP-C. One year later synthetic Exosurf Neonatal®, composed of 85% DPPC, 9% hexadecanol and 6% spreading agent tyloxapol, and modified bovine pulmonary surfactant Survanta® were reported to suppress LPS induced TNF-α, IL-1β and IL-6 production in alveolar macrophages and a monocytic cell line (Thomassen et al. 1992), by inhibition of NF-κB activation (Antal et al. 1996). Interestingly, the inhibitory effect of Exosurf Neonatal® was later associated with the spreading agent tyloxapol in this surfactant preparation (Thomassen et al. 1995). Walti and

others showed that release of superoxide anions, arachidonic acid metabolites (prostaglandin E_2 (PGE_2), tromboxane B_2 (TxB_2) and leukotriene C_4 (LTC_4)) and TNF-α induced by various bacterial components was impaired in peripheral human blood monocytes incubated with Curosurf® (Walti et al. 1997). Previously our group has shown that LPS induced reactive oxygen intermediates (ROIs) (Tonks et al. 2001; Tonks et al. 2005), lipid mediators (platelet activating factor (PAF) and PGE_2) (Tonks et al. 2003) and TNF-α (Morris et al. 2000) production is attenuated by treatment of monocytes with surfactant preparations or surfactant phospholipids. Moreover, DPPC was reported to reduce cell membrane fluidity (Tonks et al. 2001). Consequently, since enzymes responsible for PAF and ROIs generation affected by phospolipids are membrane associated (Tonks et al. 2003; Tonks et al. 2005), a possible mechanism of action of pulmonary surfactant lipids may be the disruption of cell membranes.

In contrast, little is known about the function of pulmonary surfactant lipids in viral infections, with a previous study indicating that entry of adenovirus was increased in the presence of DPPC (Balakireva et al. 2003).

1.2.2.2 Surfactant specific proteins

The role of pulmonary surfactant associated proteins SP-A and SP-D in innate immunity against various viruses, including influenza virus, is well characterised. In contrast, the role of SP-B and SP-C in viral infections is unknown. *In vitro* SP-A was shown to bind to influenza virus via its carbohydrate domain and to reduce influenza virus infectivity in alveolar macrophages (Benne et al. 1995). The observed reduction was due to an opsonic activity of SP-A and increased phagocytic clearance of the opsonised virus by these cells (Benne et al. 1997). SP-A was also found to bind to cytomegalovirus (CMV) and stimulate its binding and uptake by type II pneumocytes and alveolar macrophages, without having

any effect on CMV replication (Weyer et al. 2000). Unlike SP-A, SP-D binds influenza virus via its lectin domain (Hartshorn et al. 2000). Incubation of SP-D with influenza virus enhanced neutrophil binding of influenza virus and neutrophil respiratory burst to the virus (Hartshorn et al. 1994). Augmentation of respiratory burst was found to depend on the multimerisation level of SP-D molecule. Interestingly, when neutrophils were incubated with SP-D before influenza virus was added, the oxidative response to the virus was reduced (White et al. 2005). Both, SP-A and SP-D have been shown to inhibit haemagglutination of influenza virus (Malhotra et al. 1994; Hartshorn et al. 1994). Additionaly, SP-D was found to bind to envelope protein (gp120) of immunodeficiency virus (HIV), consequently inhibiting HIV infectivity and replication (Meschi et al. 2005). *In vivo* studies reported that SP-A and SP-D deficient mice had decreased influenza virus clearance, increased inflammation, greater epithelial injury and more cellular infiltrates within the lung (Levine et al. 2001; Levine et al. 2002; Li et al. 2002), indicating that pulmonary surfactant associated proteins SP-A and SP-D play an important role in innate defence responses to influenza virus. A similar *in vivo* outcome was observed in SP-A and SP-D deficient mice during respiratory syncytial virus (RSV) (Levine et al. 1999; Levine et al. 2004) and adenovirus infection (Harrod et al. 1999).

Details concerning the involvement of SP-A and SP-D in the pulmonary defence against bacteria go beyond the subject of this work. However, it is worth mentioning that these proteins are active against wide range of microbial patogens, including *Mycobacterium tuberculosis* (Tino & Wright 1996; Ferguson et al. 2006), *Pseudomonas aeruginosa* (Griese & Starosta 2005; Giannoni et al. 2006), *Streptococcus pneumoniae* (Hartshorn et al. 1998; Jounblat et al. 2004), *Klebsiella pnumoniae* (Kostina et al. 2005), *Staphylococcus aureus* (Hartshorn et al. 1998), *Escherichia coli* (Hartshorn et al. 1998), *Haemophilus influenzae* (Tino & Wright 1996) and many other microbial pathogens commonly infecting the

respiratory tract. SP-A and SP-D significantly contribute to microbial recognition, regulation of phagocytosis and modulation of inflammation within respiratory tract.

1.2.3 Pulmonary surfactant – role in pulmonary diseases

1.2.3.1 Infant respiratory distress syndrome (IRDS)

Pulmonary surfactant starts to be produced around 24 weeks of pregnancy but does not reach adequate level until 32 weeks of pregnancy. Insufficiency of pulmonary surfactant production in premature infants leads to IRDS (previously called hyaline membrane disease). Exogenous surfactant replacement therapy was shown to be an effective treatment option in this syndrome (Speer et al. 1995). Introduction of this treatment has significantly lowered mortality of premature babies (Speer et al. 1995).

Two types of pulmonary surfactants are used in exogenous surfactant therapy - natural surfactants derived from animals and synthetic surfactants (Table 1.2). Animal surfactants are obtained from either lung minces or lung lavages. Bovine (Survanta®, Surfacten®, Infasurf®, Alveofact® and BLES™) and porcine (Curosurf®) preparations contain small amounts of hydrophobic proteins SP-B and SP-C. Many of the components of natural surfactants, including pulmonary surfactant proteins, are not present in artificial preparations (Exosurf Neonatal® and ALEC) (Suresh & Soll 2002). Comparative studies showed that animal surfactant preparations are superior in exogenous surfactant replacement therapy to their synthetic analogues which lack surfactant specific proteins (Soll & Blanco 2001). As a result, Exosurf Neonatal® is no longer in use and novel artificial preparations (Surfaxin® and Venticute®) are enriched with recombinant human-like proteins (Suresh & Soll 2002).

Origin	*Pulmonary surfactant*	*Additional characteristic*	*Specific proteins*
Bovine lung mince	**Survanta® (beractant)** Surfacten® (surfactant TA)	Enriched with DPPC, tripalmitoylglycerol and palmitic acid	SP-B, SP-C
Bovine lung lavage	Infasurf® (calfactant) Alveofact® (SF-RI) BLES™	Chloroform/methanol extracted	
Porcine lung mince	**Curosurf® (poractant alfa)**	No neutral lipids Chloroform/methanol extracted Liquid gel chromatography purified	
Human amniotic fluid		Currently not used	
Artificial surfactant	Exosurf Neonatal® (colfosceril palmitate)	Composed of 85% DPPC, 9% hexadecanol, 6% tyloxapol (spreading agent)	
	ALEC (pumactant)	Composed of 7:3 mixture of DPPC and phosphatidylglycerol	
	Surfaxin® (lucinactant)	Composed of DPPC, palmitoyloleoylphosphatidyl-glycerol and palmitic acid	21-residue peptide called KL4 (sinapultide), a mimic of SP-B
	Venticute®	Composed of DPPC, palmitoyloleoylphosphatidyl-glycerol, palmitic acid and calcium chloride	Recombinant human SP-C

Table 1.2: Comparison of two types of pulmonary surfactant preparations (Suresh & Soll 2002).

Pulmonary surfactant preparations are derived from different sources and undergo different purification processes (Table 1.2). As a consequence some of them show higher performance *in vivo* than the others, porcine preparations seem to be more advantageous than bovine. As an example, a comparative study between bovine Survanta® and porcine Curosurf® shows that premature children treated with Curosurf® have more rapid improvement in oxygenation, reduced ventilatory requirements, fewer side effects and higher survival rate (Speer et al. 1995). Other comparative studies between these two pulmonary surfactant preparations show that Curosurf® treatment needs fewer additional doses of pulmonary surfactant (Ramanathan et al. 2004). A similar study reports that not only porcine Curosurf® but also bovine Alveofact® treatment is associated with shorter ventilation, oxygen administration and hospitalisation when compared to Survanta® (Baroutis et al. 2003). In addition, another bovine pulmonary surfactant Infasurf® has been shown to produce a longer duration of beneficial effects than Survanta® in IRDS (Bloom et al. 1997). Similar therapeutic effects are observed when comparing BLES™ and Survanta®. No differences in ventilator requirements, survival rate and occurrence of chronic lung disease are observed, however BLES™ treated infants achieve better oxygenation rate that Survanta® treated infants (Lam et al. 2005).

Pulmonary surfactant deficiency and alterations in pulmonary surfactant composition with regard to surfactant proteins and lipids can also develop secondarily in patients with acute respiratory distress syndrome (ARDS) (Nakos et al. 1998), pneumonia (Griese 1999), sudden infant death syndrome (SIDS) (Griese 1999), asthma (Heeley et al. 2000), chronic pulmonary diseases (Greene et al. 1999) and idiopathic pulmonary fibrosis (Gunther et al. 1999). These changes are affecting the quantity and/or the composition of human pulmonary surfactant.

1.3 Pulmonary surfactant and ARDS

Currently there is insufficient data whether surfactant replacement therapy in ARDS reduces morbidity and mortality, however, it has been shown to improve oxygenation, improve lung compliance and decrease need for ventilator support in patients with ARDS. In 1994 Spragg and co-workers performed studies on six patients with ARDS which were treated with a single dose of hydrophobic components of porcine surfactant. This treatment was well tolerated, had no pro-inflammatory activity and resulted in modest improvement in gas exchange. No significant changes in lung compliance were detected in this study (Spragg et al. 1994). One year later artificial surfactant replacement therapy was shown to bring stabilisation in gas exchange, reduced ventilatory support and improved pulmonary compliance in children (Perez-Benavides et al. 1995). In 1996 Anzueto and others analysed the administration of Exosurf Neonatal® in a patient group consisting of 725 adults with sepsis induced ARDS. They reported that synthetic surfactant therapy had no effect on survival rate, duration of stay in the intensive care unit and ventilatory requirements (Anzueto et al. 1996). In contrast, reduced mortality was observed in bovine Survanta treated ARDS patients, as evaluated in study group of fifty nine patients (Gregory et al. 1997). Some promising data were also obtained in severe burn patients. In this study bovine Alveofact® treatment significantly improved gas exchange and lung compliance. Of note is that all these patients survived in spite of extensive lung injuries (Pallua et al. 1998). Improvement in oxygenation was also achieved by administration of a new generation pulmonary surfactant preparation Venticute®, although no differences in survival rate were observed in this study involving 448 patients with ARDS from various causes (Spragg et al. 2004).

Surfactant replacement in the treatment of endotoxin induced ARDS was also studied in a pig model. Moderate improvement in lung function was preliminary observed in the

presence of bovine pulmonary surfactant by Nieman and co-workers (Nieman et al. 1996). In this study aerosolised Infasurf® failed to completely improve ventilation abnormalities and histological changes caused by ARDS. Nebuliser administration system improved oxygenation in this porcine model of endotoxin induced lung injury (Lutz et al. 1998).

1.4 Corticosteroids and ARDS

The administration of exogenous corticosteroids is the current anti-inflammatory treatment approach in ARDS and in other respiratory conditions associated with increased pro-inflammatory cytokine production, including sepsis and severe community acquired pneumonia (SCAP) (Thomas & Fraser 2007; Annane 2007). Although corticosteroids are potent inhibitors for various inflammatory cytokines *in vitro* (Mukaida et al. 1994), corticosteroid therapy in ARDS show variable results. Some studies show that corticosteroids treatment results in increased mortality (Steinberg et al. 2006) whereas others show that corticosteroid administration results in reduced mortality (Annane et al. 2006). Although patients treated with corticosteroids also have a shorter ventilation period, improved oxygenation, improved lung injury and reduced stay in the intensive care units (Steinberg et al. 2006; Annane et al. 2006; Meduri et al. 2007), oral or intramuscular administration of corticosteroids for more than a few days results in severe side effects, including immunosuppression, metabolic disturbance, fat redistribution, osteoporosis, growth suppression, myopathy, ophthalmic side effects and hypothalamic-pituitary-adrenal (HPA) axis suppression (Deshmukh 2007).

1.5 Influenza virus

1.5.1 Classification

The influenza virus is a negative stranded (-) RNA virus of the family *Orthomyxoviridae* with 5 genera (Influenza virus A, B and C, Thogotovirus, and Isavirus). Influenza types A and B cause seasonal epidemics whereas influenza virus type C infection causes mild respiratory illness. Influenza viruses A and C infect multiple species, whereas influenza virus type B only infects humans. Influenza type A viruses are divided into subtypes based on two surface glycoproteins called haemagglutinin (HA or H) and neuraminidase (NA or N), also known as sialidase. There are 16 known HA types and 9 NA types. Three types of HA (HA1, HA2 and HA3) and two types of NA (NA1 and NA2) are found in influenza viruses commonly circulating in humans, whereas all of types are present in avian influenza viruses (Richman et al. 2002). The first isolated human influenza virus H1N1 caused the greatest influenza pandemic in 1918, also known as "Spanish flu" killing 20-50 million people woldwide (Taubenberger 2006). Genetic rearrengement, known as antigenic shift, resulted in the emergence of two other human viruses: H2N2 and H3N2. These subtypes caused the "Asian" pandemic in 1957 and the "Hong Kong" pandemic in 1968, respectively (Kilbourne 2006). In 1997 the first direct transmission of highly pathogenic H5N1 avian influenza to humans occurred in Hong Kong, later in Vietnam, Thailand, Indonesia and Cambodia, however, human to human transmission of this subtype has not yet been reported (Hsieh et al. 2006). In addition to H5N1 virus, H9N2 and H7N7 avian influenza viruses were also shown to directly transmit from birds to human causing severe diseases and raising the possibility of new pandemic (Doherty et al. 2006). Only A type influenza viruses undergo antigenic shift, and consequently cause epidemic outbreaks in unprotected populations. Historically, influenza virus pandemics appeared at irregular intervals and

caused significant increases in mortality rates in all age groups (Richman et al. 2002) (Table 1.3).

Year of appearance	*Time of circulation (year)*	*Virus subtype*	*Common designation*	*Estimated mortality in the United States*	*Comments*
1889	?28	H3N?		High	Estimated mortality of 270000 – 360000 in Europe
1918	39	H1N1	Spanish, swine	548000	Avian ancestor
1957	11	H2N2	Asian	86000	Avian reassortant
1968	Present	H3N2	Hong Kong	34000	Avian reassortant
1976	<1	H1N1	Swine	1 death	Outbreak limited to one U.S. military base
1977	Present	H1N1	Russian	Negligible	Reappearance of earlier circulating virus
1997	<1	H5N1	Chicken or bird	Not applicable (6 deaths in Hong Kong)	Firs proven avian-human transmission

Table 1.3: Influenza virus pandemics and other important influenza events during the past century (Richman et al. 2002).

1.5.2 Structure

The influenza A virion is 80 – 120 nm in diameter and usually roughly spherical, although filamentous forms can occur (Sieczkarski & Whittaker 2005). The genome contains eight pieces of segmented negative-sense single stranded RNA, which encode 10 proteins. Three viral proteins are inserted into the lipid envelope: haemagglutinin, neuraminidase and the ion channel protein (M2). Between membrane and core is the location of the matrix protein (M1). The core is made up of ribonucleoproteins (RNPs) which are composed of the genomic RNA segments, a trimeric RNA polymerase (PB1, PB2 and PA subunits) and stoichiometric quantities of nucleoprotein (NP). Small quantities of two non-structural proteins (NS1 and NS2) are also found in the virion (Whittaker 2001) (Figure 1.2).

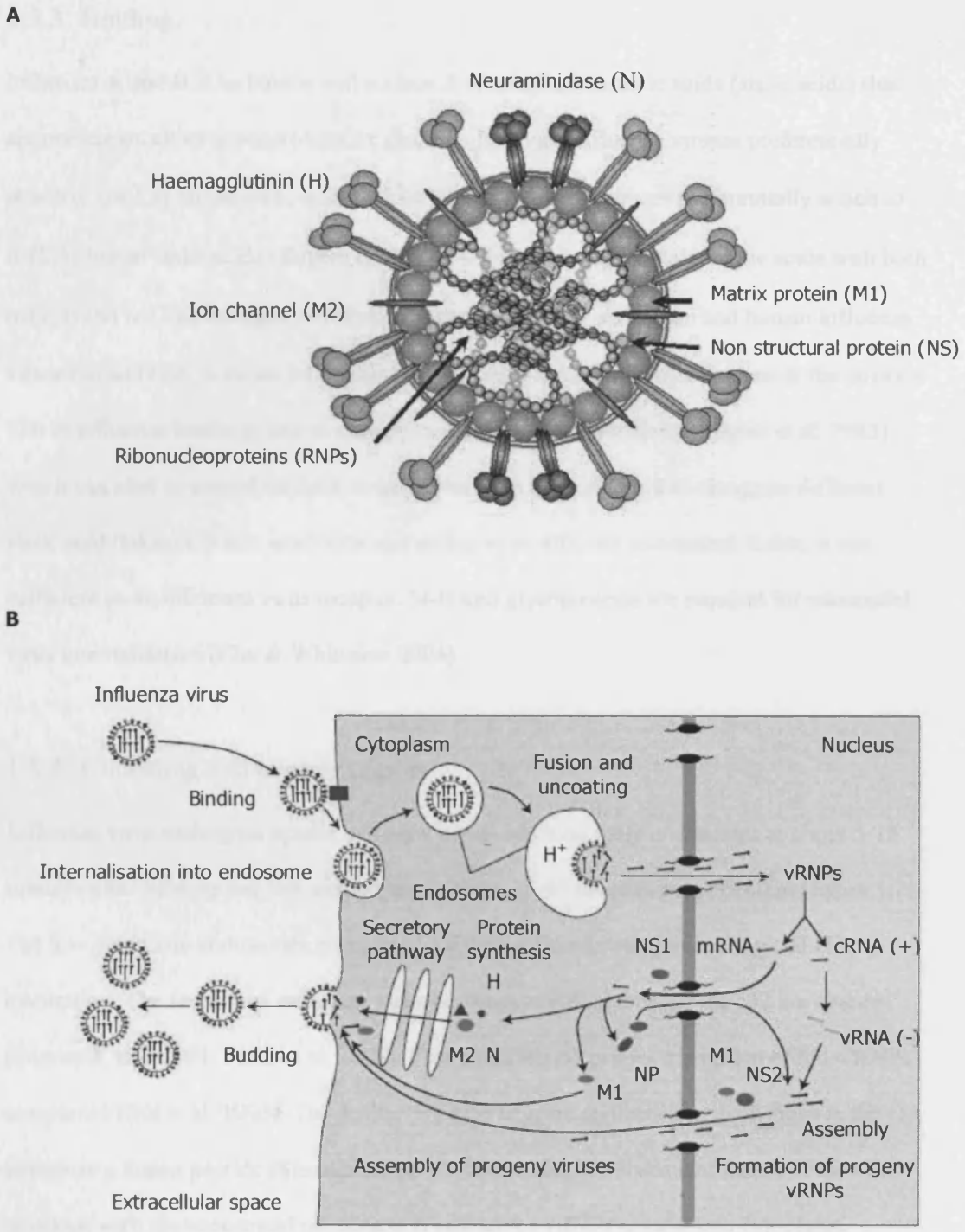


Figure 1.2: Structure (panel A) and life cycle (panel B) of influenza virus (Whittaker 2001).

1.5.3 Binding

Influenza A and B HAs bind to cell surface 5-*N*-acetyl neuraminic acids (sialic acids) that are present on either glycoproteins or glycolipids. Avian influenza viruses preferentially attach to α-(2,3)-linked sialic acids, while human influenza viruses preferentially attach to α-(2,6)-linked sialic acids (Rogers et al. 1983). Swine trachea contains sialic acids with both α-(2,3) and α-(2,6) linkages therefore pigs can be infected with avian and human influenza viruses in addition to swine influenza viruses. Single amino acid substitutions at the position 226 in influenza haemagglutinin change receptor binding specificity (Rogers et al. 1983) which can alter or extend the host range of the virus by enabling it to recognise different sialic acid linkages. Sialic acid, although acting as an efficient attachment factor, is not sufficient as an influenza virus receptor. N-linked glycoproteins are required for successful virus internalisation (Chu & Whittaker 2004).

1.5.4 Uncoating and nuclear import

Influenza virus undergoes uptake by endocytosis, reaching early endosomes at about 5-15 minutes after binding and late endosomes at about 35-45 minutes post infection (figure 1.2). The low pH in late endosomes is essential for both influenza virus uncoating and HA maturation. The interior of influenza virus becomes acidified through the M2 ion channel (Sugrue & Hay 1991; Pinto et al. 1992). This acidification primes disruption of M1-vRNPs complexes (Bui et al. 1996). The further pH drop triggers conformational changes in the HA to expose a fusion peptide (Sieczkarski & Whittaker 2003). Subsequent fusion of the viral envelope with the endosomal membrane is crucial for vRNPs release into cytoplasm. Nuclear import of vRNPs is mediated by a nuclear localization sequence (NLS) present on viral nucleoprotein (O'Neill et al. 1995) (Figure 1.2).

1.5.5 Replication, transcription and nuclear export

The influenza virus type A genome is transcribed and replicated by the viral RNA-dependent RNA polymerase within the cells nucleus. This polymerase consists of three subunits: PB1, PB2 and PA. During the initial phase of replication PB2 binds to the N^7-methyl guanine cap of host mRNAs (Fechter et al. 2003). This structure is cleaved from mRNA by PB1, remaining attached to PB2 (Shi et al. 1995). The cap serves as a primer for RNA synthesis and 11-15 nucleotides (complementary to the conserved sequence at 3' end of the vRNA) are added by PB1, after which PB2 dissociates from the growing strand. PB1 and PA then complete the synthesis (Braam et al. 1983).

In infected cells the negative-sense vRNA templates are:

- transcribed into gene lenght 3'polyadenylated messenger RNA (mRNA),
- replicated through full lenght non-polyadenylated complementary RNA

(cRNA) intermediates to produce negative-sense viral RNA (vRNA) molecules.

The nuclear export of viral mRNA is controlled by the viral non-structural protein NS1 (Chen & Krug 2000). The vRNPs assembly takes place in the nucleus and influenza virus NS2 protein mediates the nuclear export of vRNPs (O'Neill et al. 1998; Neumann et al. 2000) (Figure 1.2).

1.5.6 Glycoprotein processing and the role of HA

Haemagglutinin mediates binding to cell surface and fusion of the viral envelope with the endosomal membrane. Before the fusion of viral and host membranes can occur, the precursor HA molecule (HA0) must be cleaved into two disulfide-linked subunits (HA1 and HA2). The cleavability of the HA molecule is associated with the pathogenicity of influenza virus (Bosch et al. 1979). The HAs of some highly virulent avian strains have a polybasic cleavage site and are activated by proteases present in almost all cells. The most important

enzyme among these proteases is furin (Stieneke-Grober et al. 1992; Walker et al. 1994). Furin is located in the *trans* Golgi network (TGN) therefore HAs of these strains are mainly cleaved in the TGN while transported to the site of virus maturation. The HAs of apathogenic avian strains and the human influenza viruses have a single arginine at their cleavage site and are activated at the cell surface on budding virus or on released particles by secretory proteases, such as a factor X-like enzyme present in allantoic fluid of embryonated eggs (Gotoh et al. 1990) or tryptase Clara found in rat bronchiolar epithelial Clara cells (Kido et al. 1992).

1.5.7 Assembly and budding

For successful assembly all influenza constituents are trafficked to the apical surface of epithelial cells. Matrix protein plays a critical role in this process. M1 interacts with HA, NA (Ali et al. 2000), M2 (McCown & Pekosz 2006), vRNA (Ye et al. 1987) and host cell membrane (Ruigrok et al. 2000). Budding of the virus take place in specific regions of the plasma membrane which are known as detergent insoluble glycolipid enriched domains (DIGs) or lipid rafts (Scheiffele et al. 1999; Zhang et al. 2000) therefore influenza virions seem to have a lipid composition different from the host membrane. At the stage of virus release viral morphology is determined. HA, NA (Jin et al. 1997), M1, M2 (Roberts et al. 1998) but also actin microfilament networks and a polarised cell phenotype (Roberts & Compans 1998) are important determinants of viral spherical versus filamentous nature. The mature viruses are able to detach because neuraminidase cleaves sialic acid residues from the host cell. After the release of new influenza virus the host cell dies.

1.5.8 Clinical picture and pathology

The influenza disease is characterised by chills, fever, aches throughout the body, fatigue, sore throat and nasal congestion. Gastrointestinal symptoms may also occur, including vomiting, abdominal pain and diarrhoea. The influenza virus causes a sudden onset of illness, about 24 – 48 hours after infection. The infection starts when influenza virus reaches lung epithelial cells. After primary replication and innate immune responses in these cells, influenza virus infects alveolar macrophages. Subsequent activation of neutrophils, T cells and macrophages from the peripheral blood at the site of infection results in massive cytokine response, leading to severe local inflammation (Table 1.4). Highly virulent strains of influenza virus can also replicate in non-respiratory organs, leading to severe systemic inflammation (de Jong et al. 2006; Hsieh et al. 2006).

Influenza virus infected monocytes and macrophages produce mainly mononuclear cell attracting CC chemokines, including monocyte chemoattractant protein-1 (MCP-1) (CCL2), macrophage inflammatory protein-1 (MIP-1) (CCL4) and regulated on activation normal T cell expressed and secreted (RANTES) (CCL5) (Matikainen et al. 2000) whereas expression of polymorphonuclear lukocyte (PMNs) chemoattractants CXC, including growth related gene product (GRO) (CXCL2) and interleukin-8 (IL-8) (CXCL8) is limited in these cells (Sprenger et al. 1996). Human airway epithelial cells produce RANTES, MCP-1 and IL-8 in response to influenza virus infection (Matsukura et al. 1996; Adachi et al. 1997). Type I interferons (IFNs) are the key anti-viral cytokines produced by influenza virus infected monocytes, macrophages and lung epithelial cells (Ronni et al. 1995; Ronni et al. 1997). Human airway epithelial cells show rather poor production of interferon alpha/beta (IFNα/β) and pro-inflammatory cytokines, such as interleukin-1beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (Ronni et al. 1997) whereas influenza infected

monocytes and macrophages produce large amounts of IFNα/β, IL-1β, IL-6 and TNF-α (Ronni et al. 1995).

Cytokine/ chemokine	*Function*	*During influenza virus infection produced by*	*References*
IFN-α/β and IFN-γ	Inhibit of viral replication Stimulate of CLT mediated killing Increase MHC I expression Activate macrophages and neutrophils Promote T-cell proliferation	Respiratory epithelium T cells NK cells	(Zhang et al. 1996; Moskophidis & Kioussis 1998; To et al. 2001; Tumpey et al. 2005; Lipatov et al. 2005; de Jong et al. 2006)
TNF-α	Direct antiviral effect Neutrophil chemoattractant Stimulates macrophage phagocytosis and production of IL-1 Increases vascular preambility	T cells Monocytes/Macrophages Neutrophils DC	(Shaw et al. 1992; Peper & Van Campen 1995; Zhang et al. 1996; Liu et al. 1999; Zhao et al. 2001; Hussell et al. 2001; Cheung et al. 2002; Humphreys et al. 2003; Xu et al. 2004; Guan et al. 2004; Beigel et al. 2005; Tumpey et al. 2005)
IL-1β	Increases expression of adhesion factors on endothelium Increases vascular preambility Stimulates IL-6 production	Monocytes/Macrophages DC	(Kozak et al. 1995; Kobasa et al. 2004; Schmitz et al. 2005; Lipatov et al. 2005)
IL-6	Pro-inflammmatory cytokine Activates T-cells	Respiratory epithelium T cells Monocytes/Macrophages DC	(Kozak et al. 1997; Adachi et al. 1997; Hayden et al. 1998; Kaiser et al. 2001; Kobasa et al. 2004; Chan et al. 2005; Lipatov et al. 2005; de Jong et al. 2006)
MIP-1 (CCL4)	Monocyte and T-cell chemoattractant Activates neutrophils	T cells Monocytes/Macrophages Neutrophils DC	(Dawson et al. 2000; Cheung et al. 2002; Kobasa et al. 2004)
MIG (CXCL9)	Monocyte and T-cell chemoattractant	Respiratory epithelium Monocytes/Macrophages	(Peiris et al. 2004b;de Jong et al. 2006c)
IP-10 (CXCL10)	Monocyte and T-cell chemoattractant	Respiratory epithelium T cells Monocytes/Macrophages	(Peiris et al. 2004; Guan et al. 2004; Chan et al. 2005; Zhou et al. 2006; de Jong et al. 2006)
RANTES (CCL5)	Monocyte, T-cell and DC chemoattractant Activates T cells	Respiratory epithelium T cells Monocytes/Macrophages	(Dawson et al. 2000; Cheung et al. 2002; Chan et al. 2005; Zhou et al. 2006)
IL-8 (CXCL8)	Neutrophils and T-cell chemoattractant Activates neutrophils	Respiratory epithelium Monocytes/Macrophages Neutrophils	(de Jong et al. 2006)

Table 1.4: Links between influenza virus infections and cytokine/chemokine mediated pathology (La Gruta et al. 2007).

1.5.9 Influenza virus specific anti-viral treatment and vaccine

Some of the steps in the influenza virus life cycle can be inhibited by anti-viral drugs. Amantadine (Symmetrel®) and rimantadine (Flumadine®) target the pH-dependent uncoating of virus. The target of amantadine is the ion channel M2 (Hay et al. 1985; Pinto et al. 1992). Nuclear export of NP in influenza-infected cells is inhibited by the antibiotic leptomycin B (LMB) (Elton et al. 2001). Zanamivir (Relenza®) and oseltamivir (Tamiflu®), two analogues of sialic acid, bind and block sialic acid-binding sites of NA. Although these antiviral agents have prophylactic and therapeutic activity, amantadine, rimantadine and oseltamivir resistance has appeared recently (He et al. 2007; Aoki et al. 2007; Deyde et al. 2007).

Currently, the use of inactivated influenza vaccine is the most important tool to reduce influenza virus associated morbidity and mortality. However, vaccine immunity decreases gradually and annual re-immunisation is necessary even if the vaccine antigens remain unchanged. Specific groups such as elderly and those with respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) are encouraged to take up such vaccinations.

1.6 Measles virus

1.6.1 Classification

The measles virus is a negative stranded (-) RNA virus of the *Morbillivirus* genus within the *Paramyxoviridae* family. Although some antigenic drift was detected in the surface glycoproteins of measles virus, these variations did not reduce the effectiveness of the immunity induced by natural infection and measles vaccines. On the basis of the nucleotide sequences of haemagglutinin and nucleoprotein genes, wild type measles viruses were divided into at least 15 different genotypes (Richman et al. 2002).

1.6.2 Structure

The measles virus virion is enveloped and measures 100 – 300 nm in diameter. The viral genome is nonsegmented negative sense single stranded RNA, which encodes 8 proteins. Two viral polypeptides are inserted into lipid envelope: the haemagglutinin (H) and fusion protein (F). Under the lipid membrane are a matrix protein (M) and the nucleocapsid which encompasses the genomic RNA molecule in association with nucleoprotein (N), phosphoprotein (P) and large polymerase protein (L). The measles virus P and L proteins are components of the RNA-dependent RNA polymerase complex. Small quantities of two non-structural proteins (C and V) are also found in the infected cells but not in the measles virus virion. These proteins regulate replication of the genome (Richman et al. 2002) (Figure 1.3).

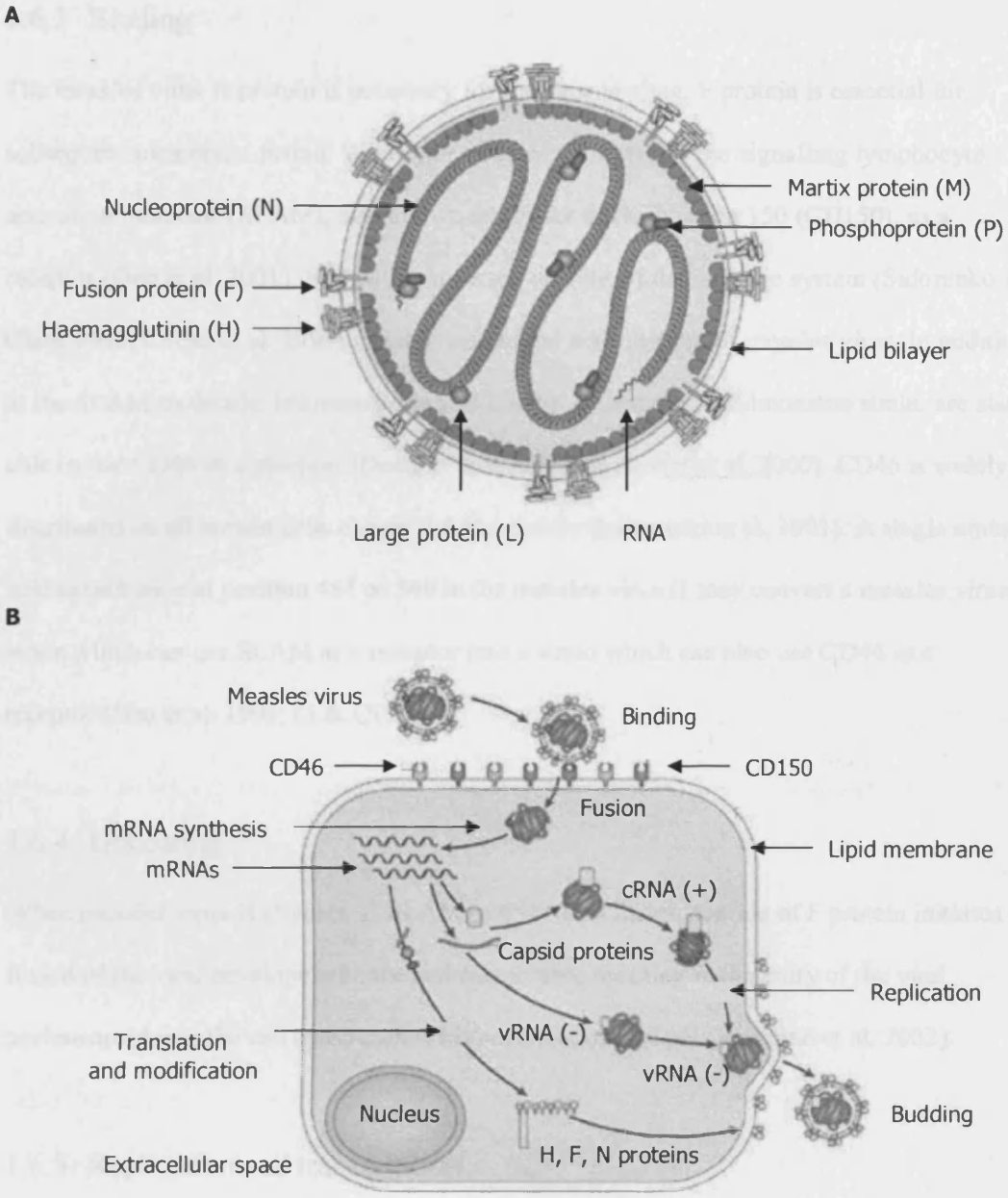


Figure 1.3: Structure (panel A) and life cycle (panel B) of measles virus (Moss & Griffin 2006).

1.6.3 Binding

The measles virus H protein is necessary for receptor binding. F protein is essential for subsequent membrane fusion. Wild-type measles virus strains use signalling lymphocyte activation molecule (SLAM), also known as cluster differentiation 150 (CD150), as a receptor (Ono et al. 2001). SLAM is expressed on cells of the immune system (Sidorenko & Clark 1993; Cocks et al. 1995) which is consistent with tropism of measles virus. In addition to the SLAM molecule, laboratory-adapted strains, including the Edmonston strain, are also able to use CD46 as a receptor (Dorig et al. 1993; Manchester et al. 2000). CD46 is widely distributed on all human cells except red blood cells (Liszewski et al. 1991). A single amino acid substitution at position 481 or 546 in the measles virus H may convert a measles virus strain which can use SLAM as a receptor into a strain which can also use CD46 as a receptor (Hsu et al. 1998; Li & Qi 2002).

1.6.4 Uncoating

When measles virus H attaches to SLAM or CD46 the fusion peptide of F protein initiates fusion of the viral envelope with the cell membrane, resulting in the entry of the viral nucleocapsid into the cell cytoplasm. This occurs at neutral pH (Richman et al. 2002).

1.6.5 Replication and transcription

The measles virus genome is transcribed by the viral RNA-dependent RNA polymerase in the cytoplasm. The polymerase recognises a consensus sequence at the beginning of each gene, terminates synthesis at the end of each gene and then reinitiates at the consensus sequence of the next gene. The viral mRNAs are capped at the 5' end and polyadenylated at the 3' end. The viral mRNAs are translated to produce viral proteins. Viral RNA replication involves full length positive-sense cRNA synthesis. This is used as a template for full length

negative-sense vRNA. The measles virus N protein shifts transcription to replication. The nucleocapsid, and not free genomic RNA, is the template for all measles RNA synthesis (Richman et al. 2002) (Figure 1.3).

1.6.6 Assembly and budding

Measles virus virions are produced by budding. M is the key protein during virus assembly. This protein interacts with the cytoplasmic domains of the integral membrane proteins (H and F protein) and the nucleocapsid and brings them into association with cell membrane. Cellular actin microfilaments are also required for measles virus maturation (Bohn et al. 1986).

1.6.7 Pathology

Measles is a disease characterised by fever, cough, coryza (runny nose), conjunctivitis and maculopapular rash (Richman et al. 2002). The infection starts when measles virus reaches epithelial cells of the respiratory tract. During the 8 to 12 days incubation period between exposure to the virus and the onset of clinical symptoms, the measles virus proliferates in epithelial cells and spreads to local lymph nodes (Richman et al. 2002). Replication in lymph tissues is followed by viraemia and dissemination of measles virus to many organs, including lungs, lymph nodes, skin, gastrointestinal tract, liver and kidney (Richman et al. 2002). Infection of epithelial cells, endothelial cells, lymphocytes, monocytes and macrophages as well as production of cytokines and chemokines in these target organs plays a central role in measles pathogenesis (Table 1.5). In developing countries, measles virus alone accounts for 6-21% of the morbidity and 8-93% of mortality due to acute lower respiratory infections (Markowitz & Nieburg 1991).

Cytokine/ chemokine	*Function*	*During measles virus infection produced by*	*References*
IFN-α/β and IFN-γ	See table 1.2	Respiratory epithelium T cells Glial cells	(Schneider-Schaulies et al. 1993; Finke et al. 1995; Helin et al. 2001)
TNF-α		T cells Monocytes/Macrophages Glial cells	(Schneider-Schaulies et al. 1993; Finke et al. 1995; Zilliox et al. 2007)
IL-1β		Monocytes/Macrophages Glial cells	(Schneider-Schaulies et al. 1993; Zilliox et al. 2007)
IL-6		Respiratory epithelium Glial cells	(Ghali & Schneider-Schaulies 1998; Helin et al. 2001)
MIP-1 (CCL4) MIG (CXCL9)		Glial cells	(Xiao et al. 1998)
IP-10 (CXCL10)		Neurons	(Patterson et al. 2003)
RANTES (CCL5)		Neurons Glial cells	(Xiao et al. 1998; Noe et al. 1999; Patterson et al. 2003)
IL-8 (CXCL8)		Respiratory epithelium Monocytes/Macrophages	(Sato et al. 2005; Zilliox et al. 2007)

Table 1.5: Links between measles virus infections and cytokine/chemokine mediated pathology (La Gruta et al. 2007).

1.6.8 Measles virus specific anti-viral treatment and vaccine

At the moment, live attenuated measles vaccine is the safest and the most effective tool to tackle virus transmission. Measles vaccine is available in a monovalent form, and with live attenuated rubella vaccine (MR), and with live attenuated rubella mumps vaccine (MMR) (Beck et al. 1986). In 1998 Wakefield and co-workers suggested an association between MMR vaccine and neurodevelopmental disorders leading to autism in children (Wakefield et al. 1998). However, further studies of the MMR and autism hypothesis did not support this causal association (Fombonne & Chakrabarti 2001; D'Souza et al. 2006; Richler et al. 2006). Moreover, Wakefield was charged with serious professional misconduct by the General Medical Council in 2006. In the United States and other developed countries MMR is the vaccine of choice for use in routine vaccination programme. Although a compulsory vaccination programme is imposed, measles virus is still resisting eradication, mainly due to some limitations of available measles vaccines, including low vaccine uptake (Fleck 2003) and light and heat inactivation (Hilleman 1989).

For the therapeutic treatment, a number of compounds have been investigated which could be used as inhibitors of measles virus. These include ribavirin, antisense molecules (Koschel et al. 1995), adenosine and guanosine nucleosides (Barnard et al. 2001), brassinosteroids (Wachsman et al. 2002), coumarines, pyranocoumarines (Barnard et al. 2002) and peptide inhibitors (Barnard 2004). None of them however was accepted for human clinical trials mainly because of high toxicity and low effectiveness.

1.7 Virus recognition and the host response

The human host is able to recognise and respond to the presence of a wide range of infectious agents. The innate immune response to viruses is regulated by different ***pattern recognition receptors*** (PRRs) and associated signalling pathways. Viral genomes are recognised by ***Toll-like receptors*** (TLRs) and ***RIG-like receptors*** (RLRs). Their signalling pathways lead to upregulation of pro-inflammatory and anti-viral responses. Amongst these receptors, ***Toll-like receptor 3*** (TLR3) (Alexopoulou et al. 2001), ***Toll-like receptor 7*** (TLR7) (human)/***Toll-like receptor 8*** (TLR8) (mice) (Lund et al. 2004) and ***retinoic acid inducible protein-I*** (RIG-I)/***melanoma differentiation associated protein 5*** (MDA5) (Pichlmair et al. 2006) are involved in the innate immune response to RNA viruses, their signalling pathways will be discussed in sections 1.7.1.1, 1.7.1.2 and 1.7.1.3. Whilst beyond scope of this study, it should be noted that the presence of virus is also detected by antibody dependent (Rothbarth et al. 1999) and independent mechanisms (McMichael 1994) associated with the adaptive immune response.

1.7.1 TLR-dependent recognition

TLRs are group of receptors first identified in *Drosophila* (Rosetto et al. 1995). These receptors have structural similarities to ***interleukine-1 receptor*** (IL-1R) and to date 10 TLRs have been described in man. All have been found to participate in recognition of ***pathogen associated molecular patterns*** (PAMPs). TLRs are transmembrane proteins with an intracellular ***Toll/interleukin-1 receptor*** (TIR) domain and extracellular ***leucine rich repeat*** (LRR) motifs (Sandor & Buc 2005). TLRs play a critical role in immunity against bacterial and viral pathogens. Different TLRs interact with specific ligands and lead to different cytokine responses. ***Toll-like receptor 1*** (TLR1) and ***Toll-like receptor 2*** (TLR2) heterodimers sense bacterial triacylated lipopeptides (Jin et al. 2007) whereas TLR2 and

Toll-like receptor 6 (TLR6) heterodimers recognise bacterial diacylated lipopeptides (Okusawa et al. 2004). ***Toll-like receptor 4*** (TLR4) detects LPS from gram negative bacteria (Chow et al. 1999). ***Toll-like receptor 5*** (TLR5) is a receptor for flagellin from bacteria (Hayashi et al. 2001). TLR3 senses synthetic and viral dsRNA (Alexopoulou et al. 2001) whereas TLR7 and TLR8 recognise viral ssRNA and synthetic imidazoquinolene based compounds (Diebold et al. 2004). ***Toll-like receptor 9*** (TLR9) is a receptor for unmethylated CpG-containing DNA motifs, which occur in bacterial and viral DNA (Hemmi et al. 2000). Signalling by TLRs involves five adaptor proteins, including ***myeloid differentiation factor 88*** (MyD88), ***MyD88 adaptor like protein*** (MAL), ***TIR domain containing adapter inducing IFN-β*** (TRIF), ***TRIF related adaptor molecule*** (TRAM) and ***sterile α and armadillo motif containing protein*** (SARM). MyD88 is used by all TLRs with the exception of TLR3 which uses only TRIF (Yamamoto et al. 2003). MAL is used by TLR2 and TLR4 and its function is recruitment of MyD88 (Yamamoto et al. 2002). TRAM is required to recruit TRIF to TLR4 (Fitzgerald et al. 2003). SARM negatively regulates TRIF (Carty et al. 2006), therefore modulates TLR3 and TLR4 signalling.

1.7.1.1 TLR3

TLR3 is a sensor for single and double stranded RNA (Alexopoulou et al. 2001; Marshall-Clarke et al. 2007). The localisation of TLR3 is cell type dependent. Human fibroblasts express TLR3 on the cell surface (Matsumoto et al. 2002) whereas dendritic cells (Matsumoto et al. 2003), macrophages (Nishiya et al. 2005), epithelial cells (Guillot et al. 2005) and TLR3 transfected HEK293 cells (Funami et al. 2004) express TLR3 in endosomal compartments. As shown in figure 1.4, TLR3 signalling leads to the activation of two transcription factors: ***nuclear factor-kappa B*** (NF-κB) and ***interferon regulatory factor 3*** (IRF3). This activation is mediated by the TLR3 associated molecule TRIF also known as

TIR containing adaptor molecule-1 (TICAM-1). Binding of ***TNF receptor associated factor 6*** (TRAF6) to the N-terminal region of TRIF activates NF-κB whereas association of ***TANK binding kinase 1*** (TBK1) with C-terminal domain of TRIF is essential for IRF3 activation (Yamamoto et al. 2002; Oshiumi et al. 2003; Sato et al. 2003; Jiang et al. 2004). Upon stimulation, TRAF6 forms a complex with ***TGF-β activating kinase*** (TAK1) and ***TAK1 binding protein 2*** (TAB2) which first co-localises with TLR3 and then translocates to the cytosol where TAK1 is activated (Jiang et al. 2004). Activation of TAK1 (Wang et al. 2001) and auto-ubiquitination of TRAF6 (Lamothe et al. 2007) are two steps required for subsequent ***IκB kinase*** (IKK) activation. TAK1 phosphorylates IKK and activated IKK complex (composed of ***IκB kinase alpha*** (IKKα), ***IκB kinase beta*** (IKKβ), and ***NF-κB essential modulator*** (NEMO) subunits) phosphorylates ***inhibitory κB proteins*** (IκBs) which are then degraded through the ubiquitin-dependent proteasome pathway. The role of IκBs is to sequester NF-κB in the cytoplasm of unstimulated cells. After proteasomal degradation of IκBs, NF-κB translocates to the nucleus and triggers the transcription of proinflammatory genes. Phosphorylation of IKK by TAK1 is regulated by TRAF6 and Ubc13-Uev1A complex also known as ***TRAF6 regulated IKK activator 1*** (TRIKA1) (Wang et al. 2001). TRAF6 together with Ubc13-Uev1A catalyse the synthesis of polyubiquitin chains linked through lysine 63 (K63) of ubiquitin (Deng et al. 2000) and this K63-linked polyubiquitination is necessary for TAK1 activation (Wang et al. 2001). Interestingly, whilst K63-linked ubiquitin chains are important for activation and interaction of proteins, K48-linked ubiquitin chains are important for proteasomal degradation of proteins. The same TRIKA1 complex is necessary for TRAF6 K63-linked autoubiquitination (Figure 1.4).

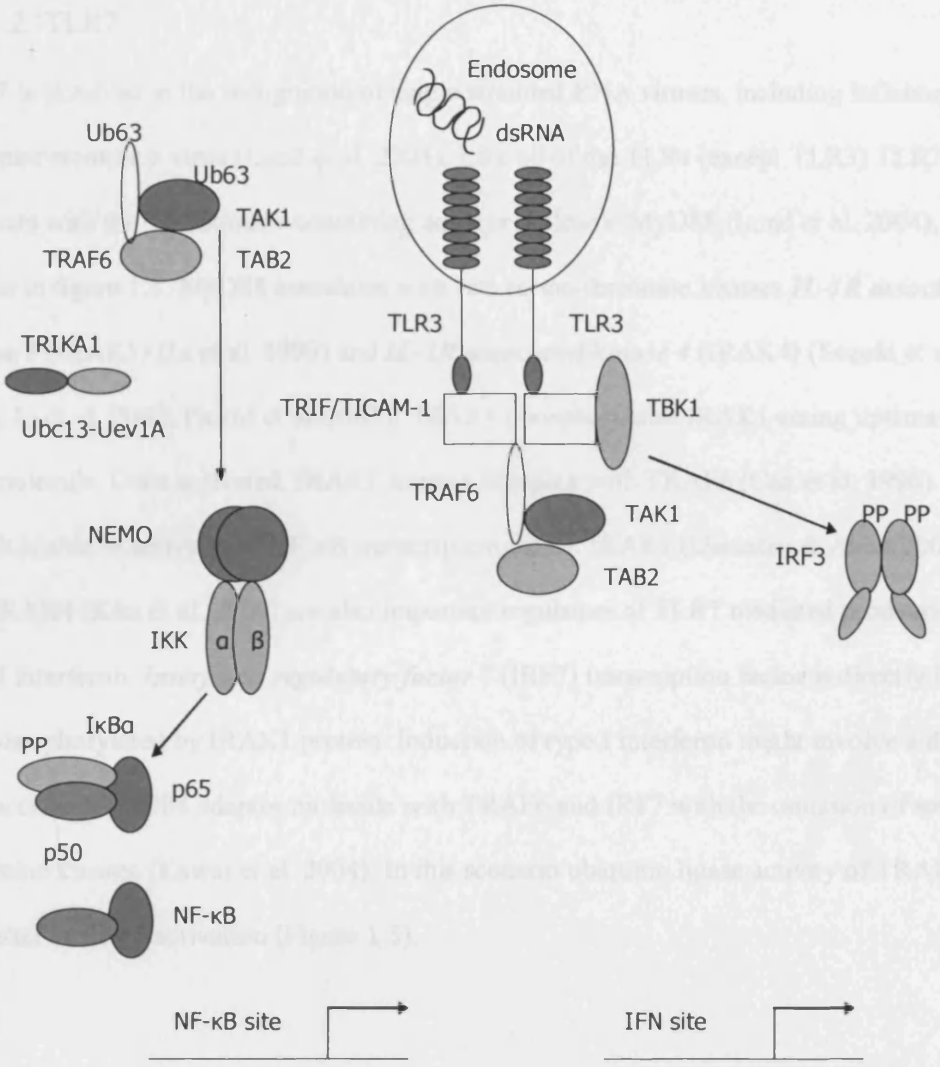


Figure 1.4: TLR3 signalling pathway (Hiscott et al. 2006; Hiscott 2007). (arrow – activation by phosphorylation)

1.7.1.2 TLR7

TLR7 is involved in the recognition of single stranded RNA viruses, including influenza and vesicular stomatitis virus (Lund et al. 2004). Like all of the TLRs (except TLR3) TLR7 interacts with the TIR domain-containing adaptor molecule MyD88 (Lund et al. 2004), as shown in figure 1.5. MyD88 associates with two serine-threonine kinases ***IL-1R associated kinase 1*** (IRAK1) (Li et al. 1999) and ***IL-1R associated kinase 4*** (IRAK4) (Suzuki et al. 2002; Li et al. 2002; Picard et al. 2003). IRAK4 phosphorylates IRAK1 acting upstream of this molecule. Once activated, IRAK1 forms a complex with TRAF6 (Cao et al. 1996) which is able to activate the NF-κB transcription factor. IRAK1 (Uematsu & Akira 2007) and IRAK4 (Kim et al. 2007) are also important regulators of TLR7 mediated production of type I interferon. ***Interferon regulatory factor 7*** (IRF7) transcription factor is directly bound and phosphorylated by IRAK1 protein. Induction of type I interferon might involve a direct interaction of MyD88 adaptor molecule with TRAF6 and IRF7 with the omission of serine-threonine kinases (Kawai et al. 2004). In this scenario ubiquitin ligase activity of TRAF6 is essential for IRF7 activation (Figure 1.5).

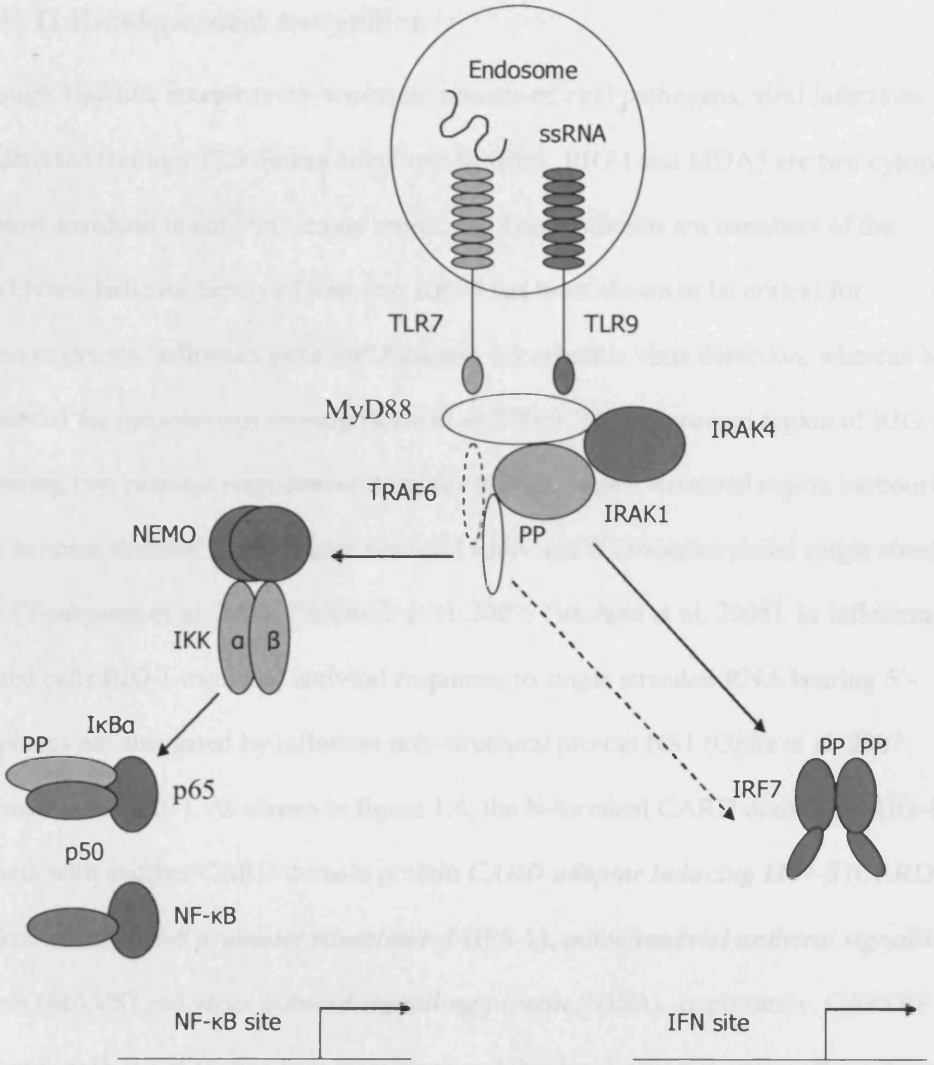


Figure 1.5: TLR7 signalling pathway (Hiscott et al. 2006; Hiscott 2007). (arrow – activation by phosphorylation)

1.7.2 TLR-independent recognition

Although Toll-like receptors are important sensors of viral pathogens, viral infections are also detected through TLR-independent mechanisms. RIG-I and MDA5 are two cytoplasmic receptors involved in antiviral innate immunity. These proteins are members of the DExD/Hbox helicase family of proteins. RIG-I has been shown to be critical for paramyxoviruses, influenza virus and Japanese encephalitis virus detection, whereas MDA5 is essential for picornavirus sensing (Kato et al. 2006). The N-terminal region of RIG-I, containing two ***caspase recruitment domains*** (CARD) and C-terminal region harbouring RNA helicase domain, binds double stranded RNA and 5'-phosphorylated single stranded RNA (Yoneyama et al. 2004; Pichlmair et al. 2007; Takahasi et al. 2008). In influenza virus infected cells RIG-I-mediated antiviral responses to single stranded RNA bearing 5'-phosphates are abrogated by influenza non-structural protein NS1 (Opitz et al. 2007; Pichlmair et al. 2007). As shown in figure 1.6, the N-terminal CARD domain of RIG-I interacts with another CARD domain protein ***CARD adaptor inducing IFN-β*** (CARDIF), also known as ***IFN-β promoter stimulator-1*** (IPS-1), ***mitochondrial antiviral signalling protein*** (MAVS) and ***virus induced signalling protein*** (VISA). Importantly, CARDIF associates with the mitochondrial membrane and this localization is crucial for its function. The major protease expressed by the hepatitis C virus (HCV) – NS3/4A releases CARDIF from the mitochondrial membrane by proteolytic cleavage. This cleavage results in cytoplasmic distribution of CARDIF, loss of interaction with RIG-I and inhibition of downstream signalling (Li et al. 2005; Lin et al. 2006; Loo et al. 2006). Stimulation of RIG-I/CARDIF leads to activation of transcription factors NF-κB, IRF3 and IRF7. ***TNF receptor associated factor 3*** (TRAF3), ***TRAF family member associated NF-κB activator*** (TANK), ***TANK binding kinase-1*** (TBK1) and ***inducible IκB kinase epsilon*** (IKKε) are responsible for type I IFN induction. TRAF3 binds CARDIF (Saha et al. 2006). TANK bridges

upstream mediators CARDIF and TRAF3 with downstream mediators TBK1, IKKϵ and IRF3 (Guo & Cheng 2007). In addition TANK links TBK1 and IKKϵ to NEMO. Upon RIG-I stimulation NEMO acts upstream of kinases TBK1 and IKKϵ and is essential for IRF3 phosphorylation, formation of IRF3 dimers, DNA binding and IRF3-dependent gene expression (Zhao et al. 2007). RIG-I is positively regulated by ***tripartite motif protein 25*** (TRIM25) (Gack et al. 2007) and negatively regulated by ***ring finger protein 125*** (RNF125) (Arimoto et al. 2007). The TRIM25 ubiquitin ligase induces lysine 63 (K63)-linked ubiquitination of the N-terminal CARD domains of RIG-I (Gack et al. 2007) which is crucial for antiviral activity. The RNF125 is also a ubiquitin ligase which is responsible for ubiquitin-dependent proteasomal degradation of RIG-I (Arimoto et al. 2007) (Figure 1.6).

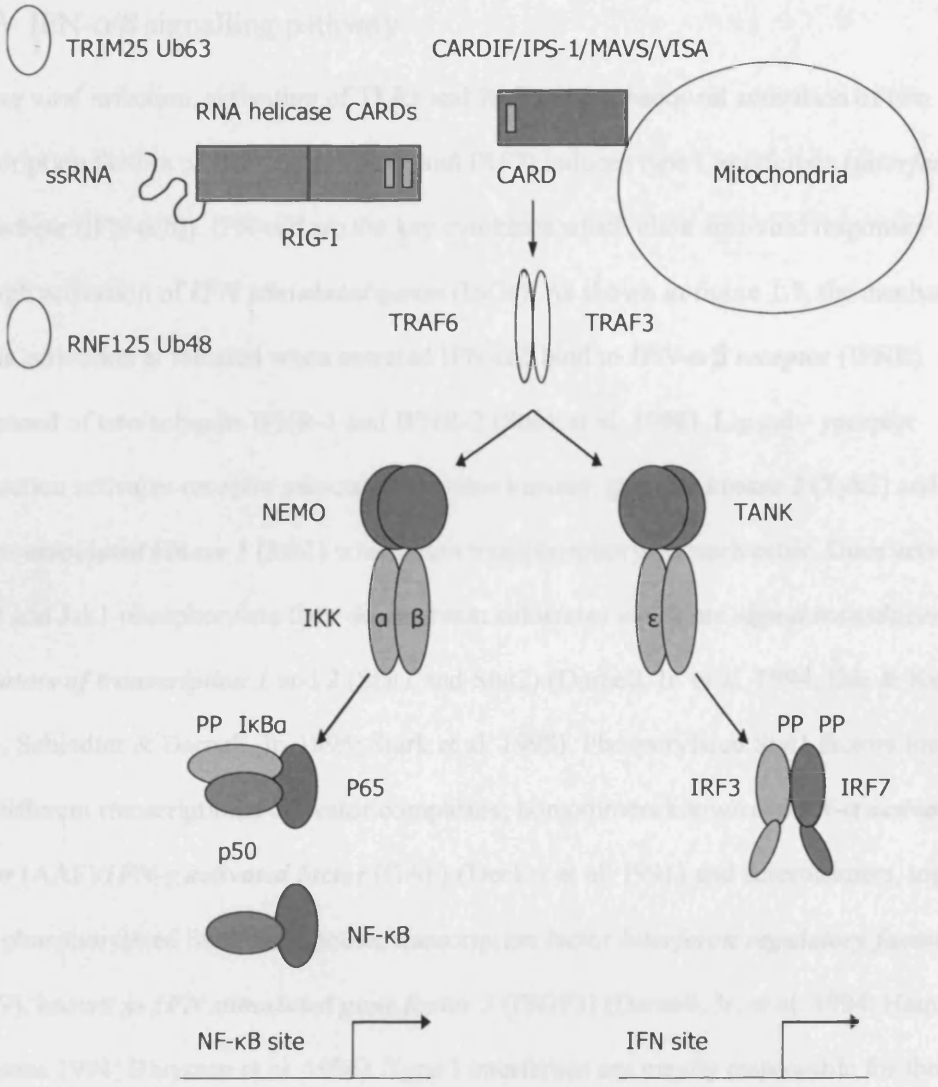


Figure 1.6: RIG-I signalling pathway (Hiscott et al. 2006; Hiscott 2007). (arrow – activation by phosphorylation)

1.7.3 IFN-α/β signalling pathway

During viral infection, activation of TLRs and RLRs and subsequent activation of two transcription factors of IRF family (IRF3 and IRF7) induces type I interferons (***interferon alpha/beta*** (IFN-α/β)). IFN-α/β are the key cytokines which elicit anti-viral responses through activation of ***IFN stimulated genes*** (ISGs). As shown in figure 1.7, the mechanism of this activation is initiated when secreted IFN-α/β bind to ***IFN-α/β receptor*** (IFNR) composed of two subunits IFNR-1 and IFNR-2 (Stark et al. 1998). Ligand – receptor interaction activates receptor associated tyrosine kinases; ***tyrosine kinase 2*** (Tyk2) and ***Janus associated kinase 1*** (Jak1) which then transphosphorylate each other. Once activated, Tyk2 and Jak1 phosphorylate their downstream substrates which are ***signal transducers and activators of transcription 1*** and ***2*** (Stat1 and Stat2) (Darnell, Jr. et al. 1994; Ihle & Kerr 1995; Schindler & Darnell, Jr. 1995; Stark et al. 1998). Phosporylated Stat1 factors form two different transcriptional activator complexes; homodimers known as ***IFN-α activated factor*** (AAF)/***IFN-γ activated factor*** (GAF) (Decker et al. 1991) and heterotrimers, together with phosphorylated Stat2 and another transcription factor ***interferon regulatory factor 9*** (IRF9), known as ***IFN stimulated gene factor 3*** (ISGF3) (Darnell, Jr. et al. 1994; Haque & Williams 1994; Bluyssen et al. 1996). Type I interferons are mainly responsible for the formation of ISGF3. These complexes translocate into the nucleus where AAF binds to the ***IFN-γ activated sequence*** (GAS) (Decker et al. 1991; Lew et al. 1991) and ISGF3 binds the ***IFN stimulated regulatory element*** (ISRE) (Kessler et al. 1990; Williams 1991) leading to transcriptional induction of large number of ISGs. Amongst the best known IFN induced proteins are the ***2'5'-oligoadenylate synthetase*** (OAS) and ***protein kinase R*** (PKR). OAS catalyses the formation of 2'5'-adenylic acid which in turn activates ***ribonuclease L*** (RNase L). RNase L is involved in degradation of RNA within the infected cell therefore creating the signal for apoptosis. Interestingly, activation of RNase L by 2'5'-adenylic acid, which

results in production of small RNA products from self RNA may also initiate IFN production. This signalling involves RIG-I, MDA-5 and CARDIF activation (Malathi et al. 2007). PKR phosphorylates ***eukaryotic translation initiation factor 2*** (eIF2). After phosphorylation, eIF2 has a reduced ability to initiate translation therefore PKR inhibits viral and cellular protein synthesis (Figure 1.7).

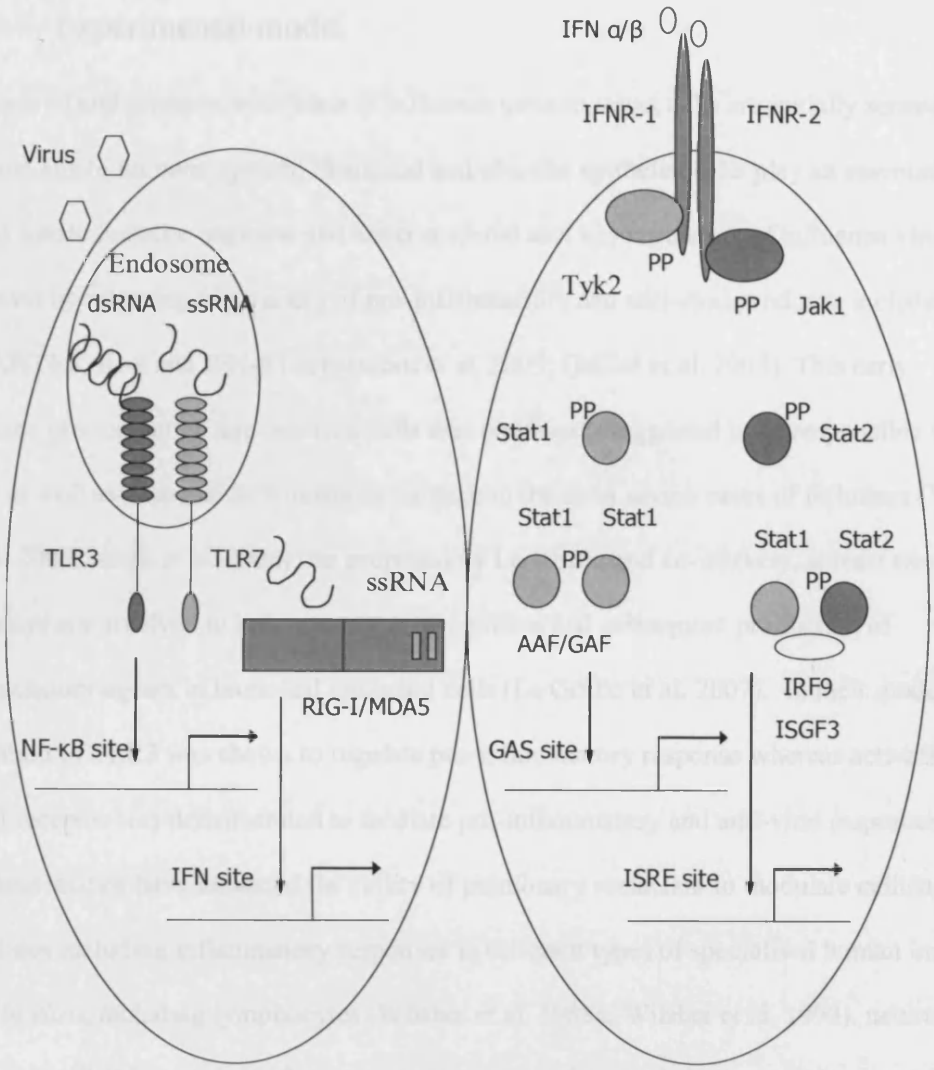


Figure 1.7: IFN signalling pathway (Taniguchi & Takaoka 2002).

1.8 Experimental model

Invasion of and primary replication of influenza virus in target cells are initially sensed by the host innate immune system. Bronchial and alveolar epithelial cells play an essential role in this innate immune response and are considered as a key regulators of influenza virus infection by releasing of a variety of pro-inflammatory and anti-viral products, including IL-8, RANTES, IL-6 and IFN-β (Bernasconi et al. 2005; Guillot et al. 2005). This early cytokine production by non-immune cells was previously suggested to be responsible for local as well as systemic inflammatory damage in the most severe cases of influenza (Van Reeth 2000; Hsieh et al. 2006). As proposed by Le Goffic and co-workers, at least two pathways are involved in influenza virus recognition and subsequent production of inflammatory agents in bronchial epithelial cells (Le Goffic et al. 2007). In their model, activation of TLR3 was shown to regulate pro-inflammatory response whereas activation of RIG-I receptor was demonstrated to mediate pro-inflammatory and anti-viral responses. Previous studies have indicated the ability of pulmonary surfactant to modulate cellular responses including inflammatory responses in different types of specialised human immune cells *in vitro*, including lymphocytes (Wilsher et al. 1988a; Wilsher et al. 1990), natural killer cells (Wilsher et al. 1988c) and monocytes upon bacterial challenge (Morris et al. 2000; Tonks et al. 2001; Tonks et al. 2003; Tonks et al. 2005). In light of these studies it was hypothesised that pulmonary surfactant may also play a role in the modulation of cytokine and chemokine produced by structural pulmonary epithelial cells in response to viral challenge.

The purpose of this study was therefore to investigate the effect of pulmonary surfactant on inflammatory responses induced by influenza virus infection in human airway epithelial cells, in an experimental *in vitro* model. Specifically the effect on IL-8, RANTES and IL-6 production, which promotes rapid inflammatory leukocytes extravasation, and IFN-β

production were examined (Guillot et al. 2005). Two types of cells were studied simultaneously, human bronchial epithelial cells (BEAS-2B) and alveolar epithelial cells (A549). These cell lines were infected with three different strains of influenza virus type A (two human strains A/WSN/33 and A/PR/8/34, and one swine A/Swine/1976/31). Influenza A virus is the most pathogenic in man and the importance of parallel investigation of strains from different species is supported by evidence of interspecies transmissions of this virus (Myers et al. 2007). LPS stimulation was used in this study as a positive control for cells ability to upregulate NF-κB mediated innate responses (Rittig et al. 2003). The effect of natural pulmonary surfactant preparations, derived either from porcine (Curosurf®) or bovine (Survanta®) sources, was compared to that achieved by dexamethasone, one of the most potent synthetic anti-inflammatory corticosteroid hormones and a direct inhibitor of NF-κB.

We further investigated the role of pulmonary surfactant and dexamethasone on TLR3 and RIG-I dependent signalling in influenza infected BEAS-2B and HEK293 cells transfected with expression vectors encoding these proteins. The introduction of HEK293 cells to our study facilitated the clear dissection of pathways involved in the observed effect by the receptor of interest. We were interested specifically in NF-κB dependent pro-inflammatory responses and IFN-β dependent anti-viral responses. In order to demonstrate the validity of our test system, controls, consisting of the cells infected with measles virus or transfected with synthetic dsRNA, were also included in these experiments.

1.9 Aims

The aim of this study was to investigate the biological effect of commercially available SP-A and SP-D depleted preparations of pulmonary surfactant on influenza virus infected human airway epithelial cells.

The main aims of the study were:

1. to assess toxicity of commercially available pulmonary surfactant preparations in human bronchial and alveolar epithelial cells,
2. to determine the effect of pulmonary surfactant on influenza virus entry in lung epithelial cells,
3. to investigate the effect of pulmonary surfactant on the production of inflammatory mediators during *in vitro* influenza virus infection,
4. to determine the effect of pulmonary surfactant on innate signalling pathways which regulate pro-inflammatory and anti-viral responses during *in vitro* influenza virus infection.

2 Materials and Methods

2.1 General methods

2.1.1 Cell culture techniques

2.1.1.1 Cell lines

2.1.1.1.1 BEAS-2B

The human bronchial epithelial cell line (BEAS-2B) was obtained from the European Collection of Cell Cultures (ECACC, UK – ECACC No. 95102433). BEAS-2B cells were derived from an autopsy of a non-cancerous individual. These cells were infected with a replication-defective SV40/adenovirus 12 hybrid and cloned (Reddel et al. 1989). Bronchial and alveolar epithelial cells were previously shown to be the primary entry sites for respiratory viruses within the lung and to play a major role in virus induced inflammation. Influenza virus infected BEAS-2B cells have been reported to produce significant amounts of IL-8, RANTES, IL-6 and IFN-β. In addition, using dual luciferase reporter assays, influenza virus treated BEAS-2B cells have been found to be easily transfected and a suitable model for studying revevant signalling pathways (Guillot et al. 2005).

2.1.1.1.2 A549

The human alveolar epithelial cell line (A549) was obtained from ECACC (ECACC No. 86012804). A549 cells were initiated in 1972 by Giard through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male (Giard et al. 1973). Similar to BEAS-2B cells, influenza virus infected A549 cells were observed to express many different cytokines and chemokines, including IL-8, RANTES, IL-6 and IFN-β and therefore this cell type may play an important role in influenza pathology. Previous data also indicated that NF-κB activation was essential for IL-8 production in influenza virus treated A549 cells (Bernasconi et al. 2005).

2.1.1.1.3 MDCK

The dog kidney epithelial cell line (MDCK) was obtained from ECACC (ECACC No. 85011435). MDCK cells were isolated from the kidney of a normal female adult Cocker Spaniel in 1958 by Madin and Darby (Madin Darby Canine Kidney). These cells were demonstrated to be permissive for a range of viruses including influenza virus (Meguro et al. 1979). Although embryonated chicken eggs are used for influenza vaccine production, MDCK cells are an alternative host for influenza virus propagation for laboratory purposes. In addition, these cells proved to be highly sensitive for plaque assay of influenza virus (Gaush & Smith 1968) and this method enabled the accurate and sensitive quantitation of infectious virus.

2.1.1.1.4 HEK293

The human kidney epithelial cell line (HEK293) was a kind gift from Dr Mario Labéta, Infection and Immunity, Department of Medical Biochemistry and Immunology, School of Medicine, Cardiff University, Cardiff, UK. HEK293 cells were previously shown to be easily transfectable are therefore routinely used in reporter assay experiments (Bowie et al. 2000). These cells do not express TLRs therefore the TLR of interest can be introduced and the observed effect can be associated with the receptor transfected in a clean system.

2.1.1.1.5 HEK293-TLR3

The human kidney epithelial cell line (HEK293) stably transfected with human TLR3 (HEK293-TLR3) was a kind gift from Dr Andrew Bowie, School of Biochemistry and Immunology, Trinity College, Dublin, Ireland. These cells were recently used to identify ligands for TLR3 (Marshall-Clarke et al. 2007). Since influenza virus infected BEAS-2B cells were found to activate NF-κB and IFN-β promoters in a TLR3 dependent manner

(Guillot et al. 2005), HEK293-TLR3 cells were chosen as a model to investigate the mechanisms of action of pulmonary surfactant in our experimental model.

2.1.1.1.6 HEK293-RIG-I

HEK293-RIG-I cells were generated as part of this thesis. This is described in section 2.1.1.7. Cytoplasmic receptor RIG-I has previously been demonstrated to mediate antiviral responses in influenza virus (Le Goffic et al. 2007) and measles virus (Berghall et al. 2006; Shingai et al. 2007; Plumet et al. 2007) infected cells. Consequently, HEK293-RIG-I cells were used as a model to investigate the mechanisms underlying the immunomodulatory actions of pulmonary surfactant.

2.1.1.1.7 B95a

The B lymphoblastoid cell line (B95a), transformed with Epstein-Barr virus (EBV), was obtained from ECACC (ECACC No. 01092505). The parent line B95-8 was derived from a cotton-top marmoset (*Saguinus oedipus*). These cells express SLAM the receptor for wild-type measles virus present in clinical specimens (Tatsuo et al. 2000). Therefore they are used as a host cell line for the isolation of measles virus. In addition, these cells are also a useful tool for measles virus propagation and quantitation since B95a cells infected with measles virus show syncytium formation and giant cell cytopathic effect (CPE) (Figure 2.3).

All cell lines were routinely tested for mycoplasma contamination using the Venor®GeM (Minerva, UK) PCR screening method.

2.1.1.2 Preparation of supplemented media

F-12K Nutrient Mixture, Kaighn's Modification Medium with L-glutamine (Gibco, UK) was supplemented with 10% (*v/v*) foetal bovine serum (FBS) (Gibco, UK). This medium was routinely used for the culture of BEAS-2B cells. Dulbecco's Modified Eagle's Medium (DMEM) with 4500 mg/L glucose and L-glutamine without sodium pyruvate (Gibco, UK) was supplemented with 5% (*v/v*) FBS and used for the culture of A549, MDCK and B95a cells. DMEM with 4500 mg/L glucose and L-glutamine without sodium pyruvate (Gibco, UK) supplemented with 10% FBS was used for the culture of HEK293 cells. G-418 was added to this medium at the final concentration 500 μg/ml for selection of stably transfected HEK293-RIG-I cells. G418 disulfate salt, also known as geneticin, is an aminoglycoside antibiotic which is toxic to mammalian cells. This salt blocks polypeptide synthesis by inhibiting the elongation step in eukaryotic cells. The toxic effect of G418 can be neutralised by the product of the neomycin resistant gene of bacterial origin which can be expressed in eukaryotic cells and which is present in RIG-I expression vector used in this study.

2.1.1.3 Sub-culturing of cells

All cell lines were sub-cultured before reaching confluency, on average every 2 to 3 days. In order to propagate adherent cells culture medium was removed and discarded and the cell monolayer was briefly rinsed with trypsin solution TrypLE^{TM} Express (Gibco, UK) (1 ml per T75 flask) to remove all traces of serum that contains trypsin inhibitors. Fresh trypsin solution was added to flask (1 ml per T75 flask) and the cells were incubated at 37^{0}C until the cell monolayer was dispersed (usually within 5 minutes - inverted microscope observation). The cells were aspirated by gentle pipetting. Harvested cells were centrifuged at room temperature for 5 minutes at 500 x g. Appropriate aliquots of the cell suspension were added to culture vessels in supplemented media.

2.1.1.4 Determination of cell number

Cell density was determined using a counting chamber (also known as haemocytometer). The entire counting grid of this chamber consists of 9 large squares (Figure 2.1). Each square has a surface area of 1 mm^2, and the depth of the chamber is 0.1 mm. To determine the final cell density in cells/ml, 15 μl of an aliquot of evenly suspended cells was applied to chamber and the cells in one large square were counted (using a light microscope). Then the total cell count was divided by 0.1 (chamber depth) and divided by 1 mm^2 (the total surface area counted). The count obtained in cells/mm^3 was multiplied by 1000 because there are 1000 mm^3 per ml.

Cell count calculation example

Each large square has surface area of 1mm^2 and a depth of 0.1 mm, giving it a volume of 0.1 mm^3. If the total cell count in one large square is 80 (number of cells should be statistically significant, about 100 cells), then the cell count is 80 cells per 0.1 mm^3, which equals 800 cells per mm^3. This result needs to be multiplied by 1000 to determine cell count per ml. In this example, the final count is 800000 cells per ml.

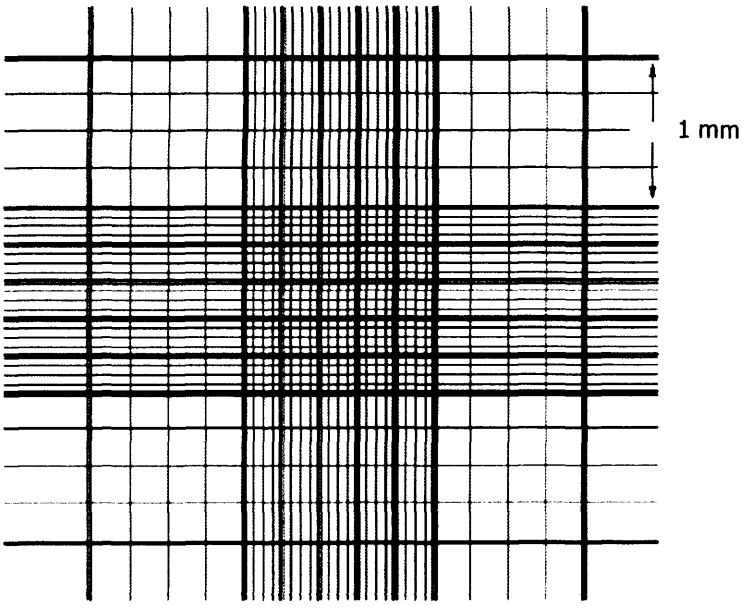


Figure 2.1: Counting grid of haemocytometer.

2.1.1.5 Freezing of cells

For long term storage and to maintain laboratory stocks, cells are routinely stored in liquid nitrogen. Before placing the cells in liquid nitrogen, the cells are exposed to a gradual freezing process, an optimum cooling rate is an important factor during this process. When the freezing is slow extracellular ice is produced before intracellular ice crystal start to form leading to osmotic imbalance. As a result water migrates out of the cell preventing intracellular organelles damage due to ice crystal formation and recrystallization during the thawing process. During fast freezing less osmotic imbalance is observed however more intracellular ice is produced. Eukaryotic cells are therefore frozen at a slow rate with an addition of cryoprotectants like dimethyl sulfoxide (DMSO) or glycerol. DMSO acts at two levels; it interferes with the lattice structure of the ice reducing the formation of ice crystals and partially solubilises the membrane so that it is more resistant to damage. Freezing medium contains also high percentage of FBS to dilute DMSO and to reduce its toxic effect (Mazur 1970).

The cells (at 50 – 80% confluency) were suspended in freezing solution containing: 10% dimethyl sulfoxide (DMSO) (*v/v*) (Sigma-Aldrich, UK), 40% FBS (*v/v*) and 50% supplemented medium (*v/v*). The cells were transferred to a cryogenic vial (Nunc, UK). Cells were initially frozen at -70^0C for 24 hours in isopropanol containing vessel (for a gradual freezing) before transfer to the vapour phase of liquid nitrogen at -196^0C for long-term storage.

2.1.1.6 Thawing of cells

Cryogenic vials containing cells were removed from liquid nitrogen storage and thawed rapidly at 37^0C. Thawed cells were transferred to pre-warmed supplemented medium with

FBS and incubated for 1 hour at 37^0C till cells have settled. After incubation medium was changed to avoid the toxic effect of DMSO on cells.

2.1.1.7 Generation of stably transfected HEK293-RIG-I cells

Stable transfection is achieved by integration of the plasmid DNA into the chromosomal DNA of target cells. While transient transfection is beneficial for fast analysis, stable transfection enables long-term gene expression. The mechanism of integration of the plasmid DNA, delivered by non-viral methods, is not clear and depends probably on DNA repair and recombination enzymes present in cells (Haber 1999).

HEK293 cells were transfected with 0.3 μg of RIG-I expression vector in a 96 well plate as described in section 2.2.8.6. Following transfection, cells were incubated for 48 hours at 37^0C prior to re-seeding into 12 wells plate in DMEM with 10% FBS and 500 μg/ml of G-418 (selection medium). The cells were re-seeded (since they were not reaching confluence) three more times into new 12 wells plates every second day, using selection medium. This time was required for the G-418 to act on the nontransfected cells, which detached and were washed away during the medium exchange. During this process all cells died in the negative control well (nontransfected HEK293 cells). After one week from the last transfer, when cells formed eleven relatively large and separated colonies, cells were sub-cultured at the ratio of 1:10 into T25 flask in selection medium. Again after one week, the cells were sub-cultured at the ratio of 1:10 into new T25 flask in DMEM with 10% FBS and frozen when they reached confluence as described in section 2.1.1.5. A mixed population of resistant cells was used in this study since single cell culture of resistant cells could contain resistant mutant cells.

2.1.2 Virus cultivation

2.1.2.1 Strains of viruses

2.1.2.1.1 Influenza virus strain A/WSN/33

Influenza A (H1N1) A/WSN/33 was obtained from the ECACC - the National Collection of Pathogenic Viruses (ECACC-NCPV – NCPV No. 402). The strain was isolated from throat washings of a patient with influenza in London in 1933 (Burnet 1951).

2.1.2.1.2 Influenza virus strain A/PR/8/34

Influenza A (H1N1) A/PR/8/34 was also obtained from the ECACC-NCPV (NCPV No. 235). The strain was isolated from a patient in Puerto Rico in 1934 (Francis, Jr. & Magill 1935).

2.1.2.1.3 Influenza virus strain A/Swine/1976/31

Influenza A (H1N1) A/Swine/1976/31 was obtained from the American Type Culture Collection (ATCC – ATCC No. VR-99). The strain was isolated from a hog in Iowa in 1976 (Jensen & Peterson, Jr. 1957).

2.1.2.1.4 Measles virus strain Edmonston

Measles virus Edmonston was obtained from the ATCC (ATCC No. VR-24). The strain was isolated from the blood of patient in acute phase of typical measles in 1954 in Massachusetts (Enders & Peebles 1954).

2.1.2.2 Propagation of viruses

2.1.2.2.1 Propagation of influenza virus in cell culture

MDCK cells support influenza virus replication therefore these cells were initially selected for virus propagation and testing of virus entry and cytopathogenicity. Since replication of influenza virus requires proteolytic activation (Klenk et al. 1975) MDCK cells were infected with influenza virus strain A/PR/8/34 and A/WSN/33 in the presence of a high purity TrypLE or in the presence of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated (TPCK) trypsin (Sigma, UK). TPCK treatment inhibits contaminating chymotrypsin activity without affecting trypsin activity (Schoellmann & Shaw 1963). For virus propagation MDCK cells (harvested from confluent T75 flasks) were incubated with influenza virus (original stock) (MOI 0.5) in 1900 µl of DMEM with 2 µg/ml TrypLE or with 2 µg/ml trypsin TPCK treated at 37^{0}C for 30 minutes. After the adsorption period, 10 ml of DMEM were added to each cell suspension. The cells were seeded into T75 flasks and incubated for 3 days at 37^{0}C. Harvested supermatants were centrifuged for 5 minutes at 500 x g to remove cell debris and FBS was added as a stabilizer at a final concentration of 0.5%. The influenza virus was quantified by haemagglutination test as described in section 2.1.2.4.1. Influenza stocks were stored at -70^{0}C. Cytopathogenicity tests and entry assays were conducted similarly in a reduced scale, in 96 and 12 well plates, without or with cover slips, respectively, as described in sections 2.1.2.4.2 and 2.1.2.4.3.

2.1.2.2.2 Inoculation of embryonated chicken eggs with influenza virus

Embryonated chicken eggs (ECEs) may be used for the isolation of a number of different viruses. Influenza virus was propagated for the first time using this laboratory technique in 1940 by Frank Macfarlane Burnet (Pearson 1944). Embryonated chicken eggs are bacteriologically sterile. All the three germinal layers (mesoderm, ectoderm and endoderm)

are present in the embryo so that its tissues and covering membranes provide useful sites for the propagation of viruses. Although tissue culture methods have to some extent replaced the use of eggs for culture of most viruses, the chicken embryo still offers the most sensitive host system for influenza and poxviruses (Norpoth et al. 1975; Clavijo et al. 2002). Eggs are used at various stages of the embryo's development according to the tissue preference of the virus to be cultivated. The inoculation is made in such a way that the virus is introduced as close as possible to the most susceptible tissue. The amniotic cavity, allantoic cavity, yolk sac and chorioallantoic membrane are used for isolation of different viruses (Figure 2.2).

Intra-amniotic inoculation

Inoculation of the amniotic sac allows for the propagation of influenza A, B and C, parainfluenza 1, 2, 3, 4 and mumps viruses. The embryo is located in this compartment, therefore viruses can efficiently replicate within the respiratory track of the chicken. This method uses 10 to 15 day old embryos. The disadvantages are small amount of yield (about 1 ml per egg) and high possibility of damage to the embryo during inoculation (Sommerville 1983).

Intra-allantoic inoculation

Inoculation of the allantoic cavity is used for influenza A, B and parainfluenza 1, 2, 3, 4 viruses. This compartment is used for propagation of the viruses which are already adapted to the chicken eggs by being primary isolated in the amniotic sac. This method, widely used by the vaccine manufacturers, uses 9 to 12 day old embryos. In comparison to the intra-amniotic route of inoculation, the yield is large (5 to 10 ml per egg) however allantoic fluids usually contain urate and phosphate and the virus may be adsorbed to these and can be removed by centrifugation (Sommerville 1983).

Yolk sac inoculation

Inoculation of the yolk sac is used for various Chlamydia strains which multiply in the primitive haemopoietic cells lining the membrane of the yolk sac. This method uses 5 to 8 day old embryos (Sommerville 1983).

Chorioallantoic membrane inoculation

Inoculation of the chorioallantoic membrane is used for the propagation of poxvirus or herpes simplex viruses, using embryos 10 to 12 days old (Sommerville 1983).

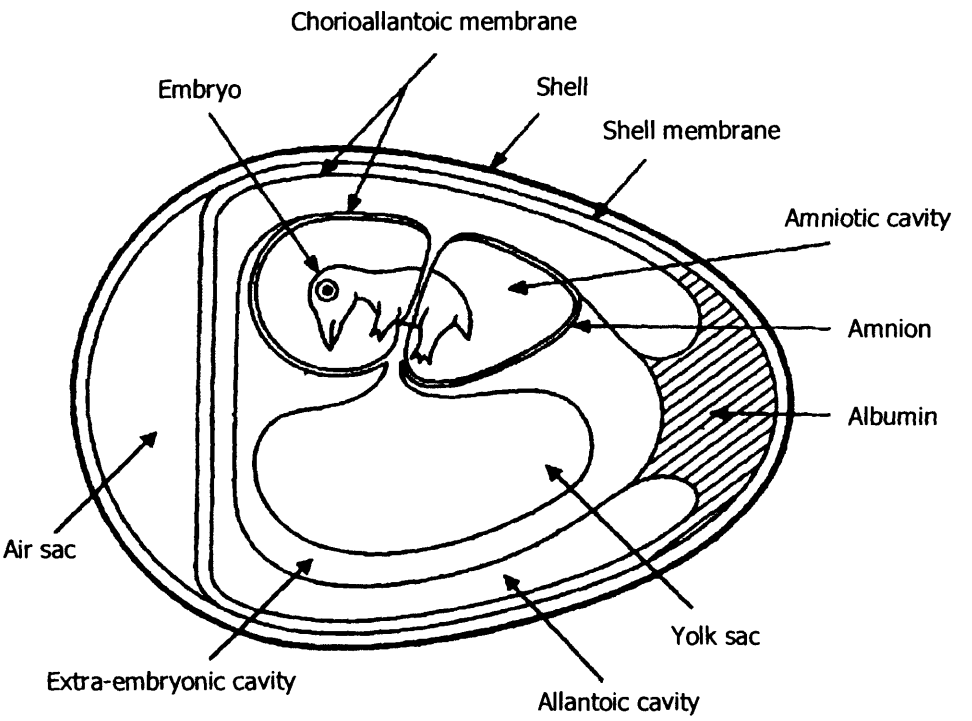


Figure 2.2: Diagram of the embryonated chicken egg indicating the regions used for propagation of viruses (Jeffries 1987).

Freshly layed, fertilised specific pathogen free (SPF) eggs were obtained from the Institute for Animal Health (Compton, Newbury, UK). SPF eggs are produced in filtered-air-positive-pressure (FAPP) poultry housing and are proven to be free from specific pathogens.

The list of these specific pathogens includes fourteen viruses, two bacteria, two mycoplasma, two protozoa, one coccidian and helminth (Table 2.1).

No.	Disease	Test
1	Infectious bronchitis	AGPT
2	Infectious bursal disease	AGPT
3	Avian encephalomyelitis	AGPT
4	Chicken embryo lethal orphan	AGPT
5	Avian influenza	AGPT
6	Reovirus infection	AGPT
7	Reticuloendotheliosis	AGPT
8	Fowl pox	AGPT
9	ıryngotracheitis	AGPT
10	Marek's disease	AGPT
11	Newcastle disease	HI
12	Egg drop syndrome	HI
13	Avian leucosis	ELISA
14	Swollen head syndrome	ELISA
15	Chicken anaemia	FAT
16	*Mycoplasma gallisepticum*	RPAT
17	*Mycoplasma synoviae*	RPAT
18	*Salmonella pullorum*	RPAT
19	Infectious coryza	HI
20	Lecocytozoonosis	AGPT
21	Blood protozoa	Giemsa stain
22	Coccidiosis	Salt floatation
23	Helminths	Salt floatation

Table 2.1: Pathogen monitoring for SPF poultry (adapted from Veterinary Research Institute). (AGPT – agar gel precipitin test, HI – haemagglutination inhibition, ELISA - enzyme-linked immunosorbent assay, FAT – fluorescent antibody technique, RPAT – rapid plate agglutination test)

After 2-3 hours in transit at room temperature, the eggs were kept in the incubator at 37^0C at relative humidity of 30-40%. They were turned twice daily by hand for 7 days. The eggs were examined daily in a darkened room over a bright trans-illuminating lamp to make sure embryo was present alive and mobile, by looking for "eye" complex. A pencil mark was made on the shell to indicate the air sac position. Before inoculation the egg shell was cleaned with 80% ethanol and small hole was made with sterile drill over the air sac. Holding the egg up to the candler, the embryo was located. The needle was inserted into the hole of the egg and the chorioallantoic membrane was pierced. Using a short stabbing

motion, the needle was pushed down further and when the embryo was moved with the tip of the needle 100 µl of influenza virus stock solution was injected into the amniotic cavity. The needle was removed and the hole was sealed with a drop of candle wax. Avian influenza grows well at 35^{0}C and at 37^{0}C. The recommended optimal temperature for mammalian influenza is 35^{0}C. The inoculated eggs were incubated at this temperature for 3 days. Before harvesting virus, the eggs were chilled at 4^{0}C for 18 hours to constrict blood vessels and to make harvesting easier. The shell over the air sac, wiped with 80% ethanol, was broken with sterile forceps and the chorioallantoic membrane was pushed aside. Both amniotic and allantoic fluids were collected using a syringe and needle. Chicken embryos were killed by decapitation. Harvested fluids were centrifuged at 4^{0}C for 5 minutes at 500 x g to remove excess blood and tissues. The isolates were titrated by haemagglutination test, tissue culture infecting dose 50 and immunofluorescence. Influenza stocks were stored at -70^{0}C. Non-infected ECE fluids were shown to be non-toxic for A549 and BEAS-2B cells. The embryo was inoculated with influenza virus at day 7 of incubation and collection was performed at day 10. The gestation period of chicken (*Gallus domesticus*) varies between 20 and 22 days. According to The Animals (Scientific Procedures) Act 1986 these embryos are not in the group of protected animals.

"Protection extends to certain immature forms from the following stages of development:

- mammals, birds and reptiles - from halfway through the gestation or incubation period;
- fish, amphibia and *Octopi vulgaris* - from the time at which they become capable of independent feeding. " (Paragraph 2.7)

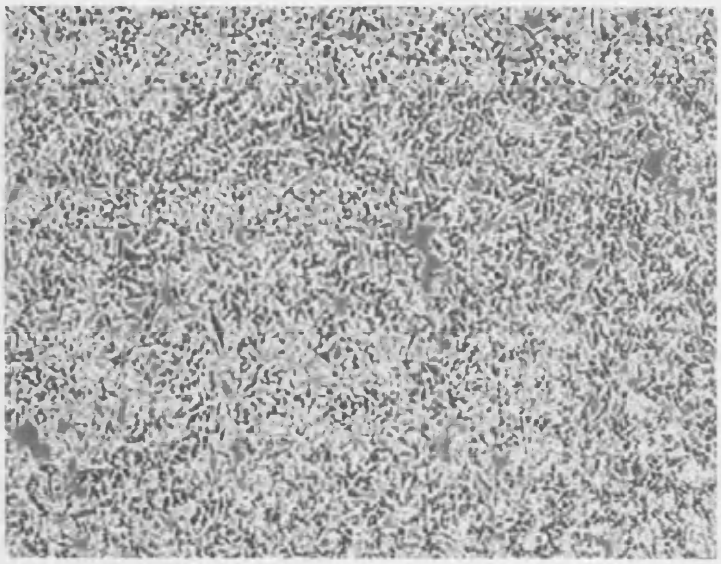
Since the influenza virus inoculation and collection were completed before the halfway point of gestation, these experiments were outside the act and therefore did not require a Home Office Personal or Project licence.

2.1.2.2.3 Propagation of measles virus in cell culture

Wild-type measles virus strain require different numbers of passages in various cells, including primary monkey kidney cells, continuous monkey kidney cell lines (VERO and CV-1) and B95a cells, before appearance of cytopathic effects (CPE) characteristic for measles virus (Kobune et al. 1990). For that reason it is recommended to passage the virus form one cell culture to another over extended period of time and to check each passage for CPE. Here, as early as 1 day after infection, fused cells (syncytia) were visible (Figure 2.3). These multinucleated giant cells are a typical CPE of measles virus infection.

Measles virus strain Edmonston was grown in B95a cells. B95a cells (confluent T75 flask) were infected with 2 ml of measles virus stock (MOI 0.5). After a 15 minutes adsorption period, during which the flask was moved from side to side to allow the virus to adsorb, 8 ml of DMEM were added to the cells. The cells were incubated for 2 days at 37^0C. Using a cell scraper the cells were collected together with the supernatant, frozen at -70^0C and then thawed at 37^0C. The sample was centrifuged for 5 minutes at 500 x g and the collected supernatant (10 ml) was used for the next infection. B95a cells (confluent T150 flask) were infected with prepared supernatant. After 15 minutes of adsorption period 10 ml of DMEM were added to the cells. The cells were incubated for 2 days at 37^0C. Again using cell scraper the cells were collected together with the supernatant (20 ml), frozen at -70^0C and the thawed at 37^0C. The process was repeated with additional four T150 flasks of B95a cells. 40 ml of supernatant were frozen in 1 ml aliquots after FBS was added to the harvested fluids as a stabilizer at a final concentration of 20%. Measles stocks were stored at -70^0C.

A



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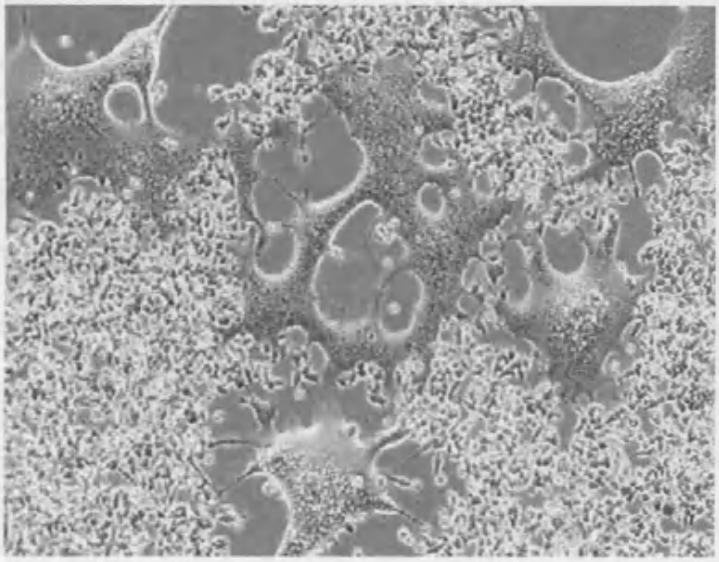


Figure 2.3: Uninfected B95a cells (X20) (A) and B95a cells infected with measles virus strain Edmonston (X20) (B).

2.1.2.3 Purification and concentration of influenza virus

Density gradient centrifugation is a widely used method for purification of many different biological particles. The purpose of influenza virus gradient centrifugation was to purify and concentrate the virus without affecting viral infectivity. The conventional influenza virus purification protocol uses sucrose gradient centrifugation. Recently, a new iodixanol centrifugation medium was described which was claimed to be superior to sucrose and other conventional gradient media (Ford et al. 1994). Sucrose solutions are both highly viscous and hyperosmotic therefore the rate of sedimentation for small particles is slow and water migrates out of the centrifuged specimens (dehydratation). Iodixanol is highly soluble in water and a 60% (*w/v*) solution has low density (about 1.32 g/ml). In addition iodixanol solutions are iso-osmotic up to 60% (*w/v*) (Ford et al. 1994). Above all *in vitro* (Ford et al. 1994) and *in vivo* (Nossen et al. 1990; Svaland et al. 1992) data show that this novel density gradient medium has very low toxicity for cells in culture.

A/Swine/1976/31 was purified in Optiprep^{TM} (iodixanol) (Nycomed Pharma AS, Norway) gradient. 10 ml of influenza-containing fluid from embryonated chicken eggs was transferred to a 33 ml quick-seal polyallomer tube (Beckman Coulter, UK). The virus solution was underlaid with 10 ml of 15% (*w/v*) iodixanol containing 1 M NaCl in PBS-MK (PBS containing 1 mM $MgCl_2 \cdot 6H_2O$ and 2.5 mM KCl), 8 ml of 25% (*w/v*) iodixanol in PBS-MK and 7 ml of 40% (*w/v*) iodixanol in PBS-MK. To locate the 25% (*w/v*) density area after centrifugation phenol red solution (Sigma-Aldrich, UK) was included in the upper 15% (*w/v*) and lower 40% (*w/v*) density volumes at a concentration of 0.1 μg/ml. The tube was sealed, placed into SW 28 - swinging-bucket rotor (Beckman Coulter, UK) and centrifuged at 4^0C for 1 hour at 96500 x g (Hermens et al. 1999; Zolotukhin et al. 1999; Locker et al. 2000). Fractions of 1ml were collected from the bottom of the tube and the influenza virus titer was determined by haemagglutination test. Influenza stocks were stored at -70^0C.

2.1.2.4 Titration of viruses

2.1.2.4.1 Haemagglutination test – titration of influenza virus

Many viruses contain proteins that can agglutinate the red blood cells of different species. This property is called haemagglutination. The viruses most commonly showing haemagglutination are the ortho- and paramyxoviruses. These viruses agglutinate erythrocytes of human (group 0), chicken, monkey and guinea-pig origin. Animal species of red blood cells (RBCs), temperature, diluent and pH are critical parameters of the haemagglutination assay (Table 2.2).

Influenza virus contains an envelope glycoprotein called haemagglutinin, which binds to N-acetyl-neuraminic acid-containing glycoprotein on erythrocytes.

Virus	*Animal Species Erythrocytes Showing Haemagglutination (0.5%)*				*Optimal Condition for Erythrocyte Suspension*		
	GP	HO	Chick	Mon	pH	Diluent	Temp.(^{0}C)
Influenza A, B, C	+	+	+	+	7.0	PBS	4-22
Parainfluenza 1-3, mumps, NDV	+	+	+	+	7.0	PBS	4-22
Measles		-	-	+	7.0	PBS	37
Respiratory syncytial virus	-	-	-	-	7.0	PBS	

Table 2.2: Haemagglutination characteristics of Orthomyxoviridae and Paramyxoviridae (Sommerville 1983). (+ virus-induced agglutination of RBCs, - no agglutination of RBCs, GP – guinea pig, HO – human type O, Chick – day old chicken, Mon – monkey, preferably African green)

The haemagglutination test is a non-specialised method to determine the amount of virus present in a sample. This is achieved by making serial dilutions of the virus and adding the same volume of dilute RBCs suspension to each dilution. In wells where the concentration of influenza virus is high, RBCs are agglutinated and linked together in a 3-dimensional matrix. In wells where the concentration of influenza virus is below a critical point (1 HAU), the RBCs settle out at the bottom of the well to form "button" (Figure 2.4).

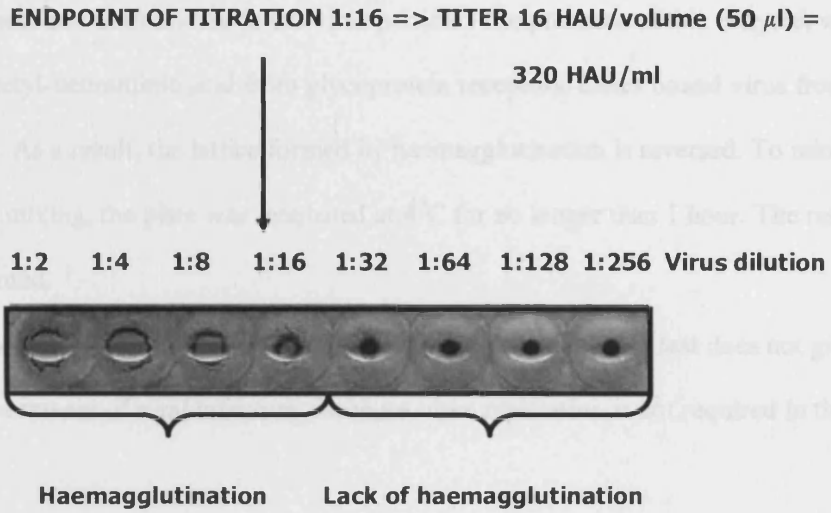


Figure 2.4: Haemagglutination assay of influenza virus strain A/WSN/33 grown in SPF-16 egg. Virus stock was two fold diluted from 1:2 to 1:256. A suspension of chicken red blood cells was added and the result was recorded.

The end point of the titration is the highest dilution of the virus, which causes complete agglutination. The titre of the virus is expressed as the reciprocal of the endpoint dilution in haemagglutination units (HAUs).

Before use the chicken red blood cells (RBCs) (Fiebig Nahrstofftechnik, Germany) were washed twice in 0.85% saline by centrifugation and a 5% chicken RBC suspension was prepared in 0.85% saline. This solution was kept at 4^0C for no longer than 7 days. Longer storage results in lysis of RBCs (haemolysis). 50 μl of 0.85% saline were added to each well of round bottom 96 well plate (Nunc, Denmark). The last row H1-H12 was used as a negative controls. 50 μl of undiluted influenza-containing fluid was added to the following wells of the first column A1-G1 and 50 μl of 0.85% saline were added to the last well of this column H1. 50 μl of well mixed fluid was transferred from well A1 to well A2. This procedure was repeated for the whole row and 50 μl was discarded from the last well in the series A1-A12. The other rows were set up in the same manner. Finally, 50 μl of 0.5%

chicken RBCs in 0.85% saline suspension was added to each well. Influenza virus also contains a neuraminidase in the virus particle. The presence of this enzyme, which cleaves N-acetyl-neuraminic acid from glycoprotein receptors, elutes bound virus from red blood cells. As a result, the lattice formed by haemagglutination is reversed. To minimize elution, after mixing, the plate was incubated at 4^0C for no longer than 1 hour. The results were recorded.

In contrast to plaque assay or TCID50, the haemagglutination test does not give any measurement of viral infectivity because virus replication is not required in this assay.

2.1.2.4.2 Tissue culture infecting dose 50 – titration of influenza virus

Influenza virus was titrated by tissue culture infecting dose 50 (TCID50). TCID50 is the quantity of virus in a specified volume that will infect 50% of a number (n) of cell culture microplate wells. TCID50 and plaque assays are used to determine viral CPE whereas counts of fluorescent foci are used to quantify virus infectivity.

MDCK and A549 cells (10^4 cells/well) were plated into a 96 well plates (Nunc, Denmark) in DMEM with 5% FBS one day before infection. BEAS-2B cells (10^4 cells/well) were plated into 96 well plate in F-12K with 10% FBS one day before infection. Virus stocks were serially diluted in DMEM/F-12K, and dilutions from 10^{-1} to 10^{-11} were plated across 8 wells (100 μl per well). At 6 days post infection, the wells were stained with crystal violet, and scored for the presence of cytopathic effect (CPE). Titers were calculated according to Karber's formula (Karber, G. 1931) (Figure 2.5).

I = (% of wells infected at dilution above 50% - 50%) / (% of wells infected at dilution above 50% - % of wells infected at dilution below 50%)

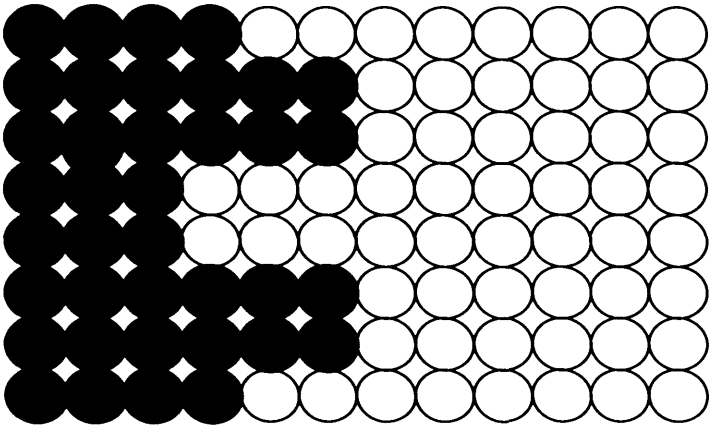
50% endpoint titer = $10^{\text{log dilution above 50\% - (I x log h)}}$

I = interpolated value of the 50% endpoint (also known as the proportional distance)

h = dilution factor

A

10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8} 10^{-9} 10^{-10} 10^{-11} NC



B

Dilutions	*Positives*	*Wells inoculated*	*% Infected*
10^{-1}	8	8	100
10^{-2}	8	8	100
10^{-3}	8	8	100
10^{-4}	6	8	75
10^{-5}	4	8	50
10^{-6}	4	8	50
10^{-7}	0	8	0
10^{-8}	0	8	0
10^{-9}	0	8	0
10^{-10}	0	8	0
10^{-11}	0	8	0
NC	0	8	0

C

I = (75 – 50) / (75 – 0) = 0.5

50% endpoint titer = $10^{-4-(0.33 \times 1)} = 10^{-4.33}$ = 21 379.620 =

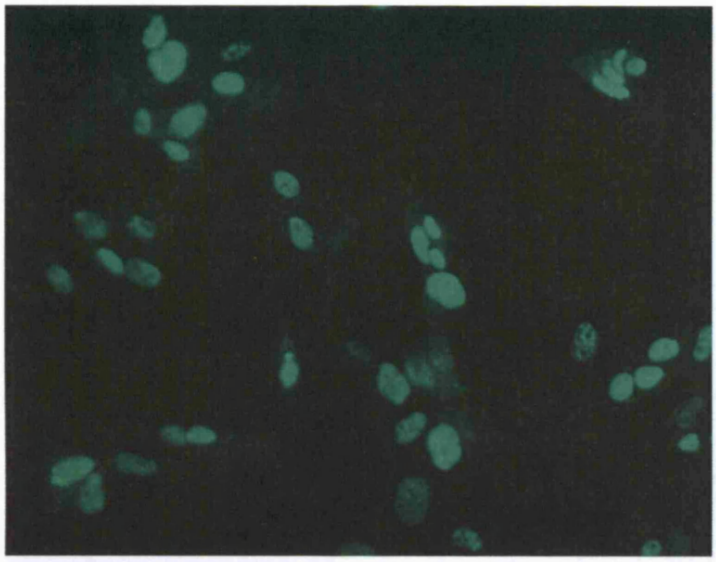
21.4 x 10^3 TCID50 / 100 µl = 21.4 x 10^4 TCID50 / ml = 2.14 x 10^5 TCID50 / ml

Figure 2.5: TCID50 calculation example - virus stock was serially diluted from 10^{-1} to 10^{-11}, and each dilution was plated across 8 wells. At 6 days post infection, the wells were stained with crystal violet, and scored for the presence of CPE (filled circles) – graphical presentation (panel A), tabular presentation (panel B) and calculation (panel C). (NC-negative control, I = (% of wells infected at dilution above 50% - 50%) / (% of wells infected at dilution above 50% - % of wells infected at dilution below 50%), 50% endpoint titer = $10^{\text{log dilution above 50\% - (I x log h)}}$, I = interpolated value of the 50% endpoint (also known as the proportional distance), h = dilution factor)

2.1.2.4.3 Immunofluorescence – titration of influenza virus

Infectivity/entry into target cells of influenza virus was determined by direct immunofluorescence using purified murine monoclonal antibodies. These antibodies were targeted against the matrix protein and nucleoprotein and were conjugated to fluorescein isothiocyanate (FITC). FITC has an absorption maximum at 490 nm and emission maximum at 520 nm (Figure 2.6A). Characteristic bright apple green fluorescence was observed within cytoplasm and nucleus of infected cells (Figure 2.7). Background staining was performed with Evans Blue. This unspecific fluorochrom has an absorption maximum at 550 nm and emission maximum at 610 nm therefore fluorescence was red-shifted (Figure 2.6B). MDCK and A549 cells ($1x10^5$ cells/well) were plated into 12 well plates (Nunc, Denmark) with 16 mm glass cover slips in DMEM with 5% FBS one day before infection. BEAS-2B cells ($1x10^5$ cells/well) were plated into 12 well plates with 16 mm glass cover slips in F-12K with 10% FBS one day before infection. Virus stocks were serially diluted in DMEM/F-12K, and each dilution plated across 4 wells (500 μl per well). After the adsorption period at 37^0C for 30 minutes, 1500 μl of DMEM/F-12K were added per each well. 24 hours later the cells were fixed with 3% paraformaldehyde for 5 minutes, permeabilized with 0.5% Triton X-100 for 30 seconds and washed with PBS prior to incubation with blocking buffer (PBS containing 50 mM Tris, 100 mM NaCl, 5mM EDTA, 0.05% Tween 20 pH 7 with 2% BSA and 0.25% gelatine) at 37^0C for 30 minutes. Monoclonal antibodies (DakoCytomation, UK) against the matrix protein and nucleoprotein of influenza virus A at 1:10 dilution in blocking buffer were added, followed by incubation at 37^0C for 30 minutes. Samples were then washed three times with PBS, and cover slips were mounted onto glass slides using mounting fluid (DakoCytomation, UK). Infection was examined by using a fluorescence microscope Olympus BX5I and AxioVision 4.4 software. Influenza-infected cells were counted.

A



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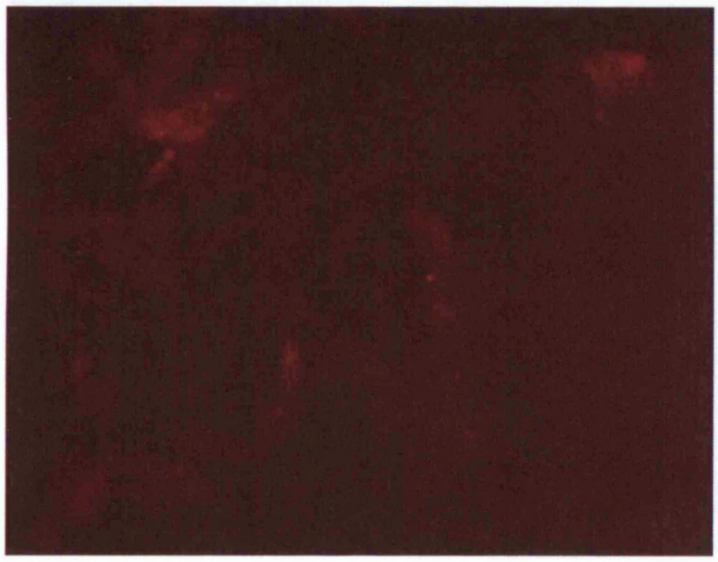
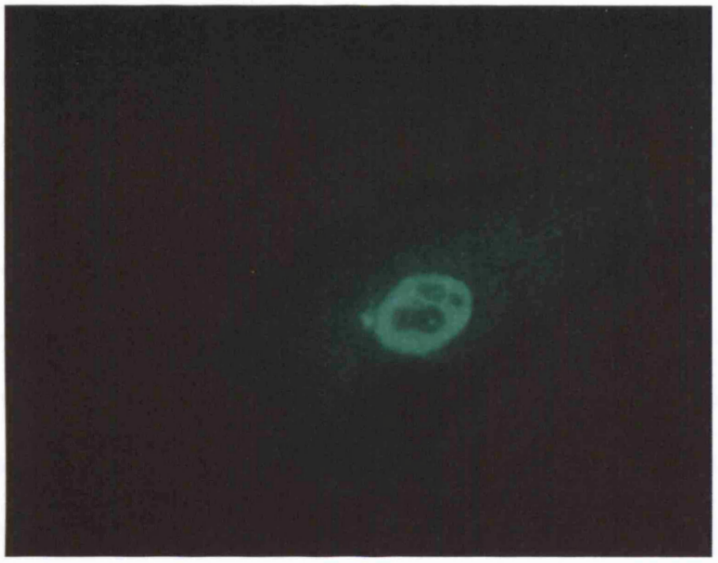


Figure 2.6: MDCK cells infected with influenza virus strain A/Swine/1976/31 – FITC staining (X20) (A) and Evans Blue staining (X20) (B).

A



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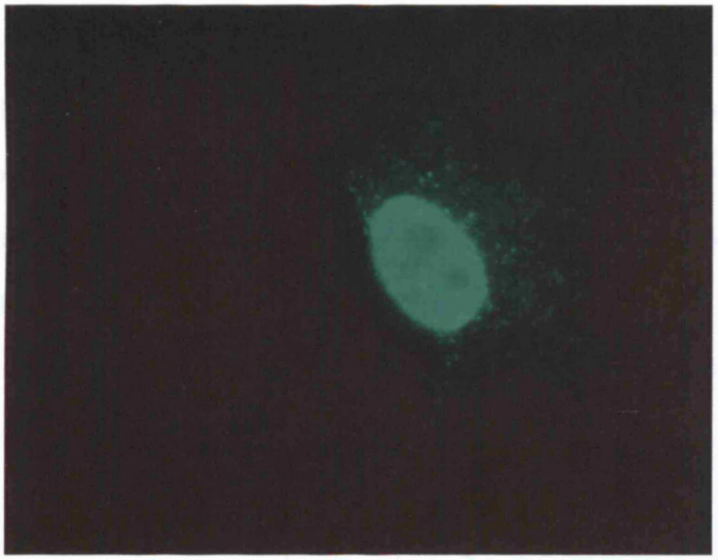


Figure 2.7: A549 cells infected with influenza virus strain A/Swine/1976/31 (X100) (A and B).

2.1.2.4.4 Plaque assay – titration of influenza virus

The plaque assay remains the gold standard for virus quantification. The plaque itself can be described as an area of dead cells or as area of infected cells in a cell monolayer. The production of plaques representing original titer under liquid medium is impossible. In case of influenza virus, new influenza virions detach from the host cells when neuraminidase cleaves sialic acid residues from the cell membrane. After this release, new influenza virus spreads over the cell monolayer with the liquid medium forming uneven secondary infected cells which do not represent the original titer. To overcome this problem different solid and semisolid overlay media are used. Amongst these, two the most favourable are agar and methylcellulose overlays. Recently, new Avicel-containing media were introduced, superior to both agar and methylcellulose (Matrosovich et al. 2006). Avicel is a off-white powder composed of insoluble microcrystalline cellulose to which water dispersible sodium carboxymethylcellulose is added. Different influenza virus subtypes (H1N1, H3N2, H5N1 and H7N7) have been shown to be able to form plaques under Avicel overlay. Plaque numbers were shown to be higher in the presence of Avicel than in the presence of agar. This might be due to inhibition of virus infectivity by heated agar. In addition plaques were found to be larger under Avicel when compared to agar and methylcellulose. The low viscosity of the Avicel also allowed reduction of the format of this assay to a 96 well plate (Matrosovich et al. 2006).

Avicel RC 581 was the kind gift from the manufacturer (Honeywill & Stein Ltd., UK). A stock suspension was prepared by dissolving 2.4 g of Avicel powder in 100 ml of distilled water on a standard magnetic stirrer for 1 hour. The Avicel stock solution was sterilized by autoclaving for 20 minutes at 121^{0}C and stored at room temperature. The overlays were prepared by mixing stock solutions of Avicel with equal volumes of medium.

BEAS-2B cells (4 x 10^4 cells/well) were plated into 96 well plates in F-12K with 10% FBS. After 24 hours, cells were infected with 50 μl/well of serial dilutions of influenza virus strain A/WSN/33 and A/PR/8/34 in F-12K. After 1 hour incubation, without removal of the inoculum, 100 μl/well of 1.2% Avicel RC 581 overlay media was added. After 24 hours of incubation the overlays were removed and the cells were fixed with 3% paraformaldehyde solution for 30 minutes at 4^0C and permeabilized with 0.5% Triton X-100 for 10 minutes at room temperature. Influenza virus infected BEAS-2B cells were immunostained by incubating for 1 hour at 37^0C with mouse monoclonal antibodies specific for the influenza A virus nucleoprotein (Chemicon, UK) (1:500 dilution in blocking buffer) followed by 1 hour incubation at 37^0C with peroxidase labelled anti-mouse antibodies (Chemicon, UK) (1:1000 dilution in blocking buffer). True Blue^{TM} (Kirkegaard & Perry Laboratories, UK), a peroxide substrate, was then added for 1 hour at 37^0C. Stained plates were washed with water to stop the reaction and dried. Plates were scanned on a flat bed scanner and the number of plaques was examined by using Corel PHOTO-PAINT X3 software (Figure 2.8).

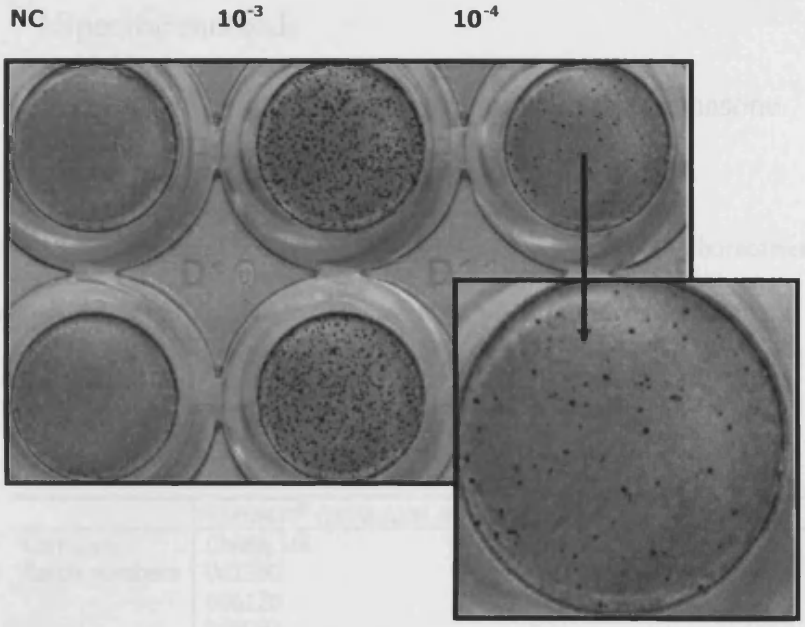


Figure 2.8: Plaque assay under Avicel overlay. BEAS-2B cells were infected with influenza virus strain A/PR/8/34; negative control (left column), 10-3 (middle column) and 10-4 dilution (right column). (NC – negative control)

2.1.2.4.5 Syncytia counting method – titration of measles virus

B95a cells ($1x10^5$ cells/well) were plated into 24 well plates (Nunc, Denmark) in DMEM with 5% FBS one day before infection. Measles virus stock was serially diluted from 10^{-1} to 10^{-5} in DMEM, and each dilution was plated across 4 wells (300 μl/well). After the adsorption period at 37^0C for 30 minutes, 700 μl of DMEM were added per each well. 72 hours later the cells were examined for the presence of multinucleated giant cells and syncytia were counted.

2.2 Specific methods

2.2.1 Preparation of pulmonary surfactant and dexamethasone

2.2.1.1 Preparation of pulmonary surfactant

In these studies Curosurf® (Chiesi, UK) and Survanta® (Abbott Laboratories Ltd., UK) were diluted in medium to obtain a specific concentration of phospholipid (from 31.25 to 500 μg/ml) (Table 2.3).

	Curosurf® (poractant alfa)	*Survanta® (beractant)*
Company	Chiesi, UK	Abbott Laboratories Ltd., UK
Batch numbers	061350 066120 069097	40-705-27 41-957-27
Origin	Porcine lung minces	Bovine lung minces
Composition	Polar lipids, mainly phospholipids, and hydrophobic proteins SP-B and SP-C	Phospholipids, neutral lipids, fatty acids, and hydrophobic proteins SP-B and SP-C supplemented with dipalmitoylphosphatidylcholine (DPPC), palmitic acid and tripalmitoylglycerol
Phospholipids concentration	80 mg/ml	25 mg/ml
Suspension solution	0.9% sodium chloride	0.9% sodium chloride
Colour	White to creamy white	White to light brown

Table 2.3: Comparison of Curosurf® and Survanta®.

2.2.1.2 Preparation of dexamethasone

The effects of pulmonary surfactant preparations were compared to those of dexamethasone. Dexamethasone is a potent synthetic type of steroid hormone which acts as an anti-inflammatory agent as well as immunosuppressant. Currently, dexamethasone is used to treat many inflammatory and autoimmune conditions associated with increased inflammatory activity (Nguyen et al. 2007; Scarborough et al. 2007).

Dexamethasone (Sigma, UK) was dissolved in 70% ethanol at stock concentration of 2 mg/ml and stored frozen at -20^{0}C, and diluted in medium when added to cell culture.

2.2.2 Preparation of stimulants

2.2.2.1 Preparation of polyinosinic-polycytidylic acid (poly(I-C))

Poly(I-C) is a synthetic analogue of viral dsRNA which is synthesised during replication of ssRNA viruses. Poly(I-C) was previously demonstrated to elicit innate immune response through the induction of high levels of proinflammatory cytokines and antiviral factors (Guillot et al. 2005h). Poly(I-C) (Sigma-Aldrich, UK) was dissolved in endotoxin-free water at stock concentrations of 1 mg/ml and 100 μg/ml and stored frozen at -20^{0}C.

2.2.2.2 Preparation of lipopolysaccharide (LPS)

LPS is a major component of the outer membrane of gram negative bacteria (Ziegler-Heitbrock 1995). This glycolipid contributes greatly to the structural integrity of bacteria and protects the membrane from damage. In addition, LPS acts as an endotoxin and induces strong innate immune responses *in vitro* and *in vivo*. LPS stimulates the cells to produce and release an array of inflammatory mediators, therefore it was used in this study as a positive control for the ability of cells to produce cytokines and chemokines.

LPS from Escherichia coli (serotype 0111:B4) (Sigma-Aldrich, UK) was dissolved in endotoxin-free water at stock concentrations of 1 mg/ml, 100 μg/ml and 10 μg/ml and stored at -20^{0}C.

2.2.3 Limulus amoebocyte lysate (LAL) assay for endotoxin

2.2.3.1 Limulus amoebocyte lysate assay

In order to exclude the possible effects of trace amounts of LPS on cytokine and chemokine secretion as well as on NF-κB and IFN-β promoter activation all virus samples as well as poly(I-C) preparations and plasmids were tested for the presence of endotoxin by LAL assay (BioWhittaker, UK).

The major component of the LAL assay is a protein derived from the blood of the horseshoe crab *Limulus polyphemus*. This proenzyme, part of the innate immune system, is activated upon endotoxin stimulation. As a result of this activation, the functional enzyme acts as the blood clotting agent (Bang 1956; Levin & Bang 1964a and b; Young et al. 1972). In the LAL assay L-nitroanilide is cleaved to produce the yellow compound p-nitroaniline. The p-nitroaniline release is measured photometrically at 405 nm (Figure 2.9).

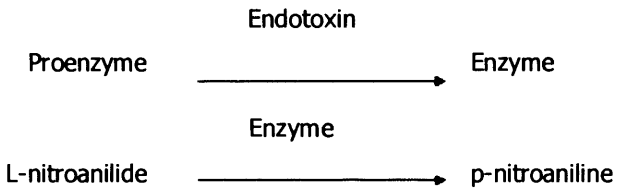


Figure 2.9: Schematic diagram of LAL assay principle (adapted from Bio Whittaker).

2.2.3.2 Preparation of limulus amoebocyte lysate assay

The assay was performed according to the manufacturer's instructions. Endotoxin standard was reconstituted in pyrogen-free water to a final concentration 50 endotoxin units (EU)/ml (1 EU corresponds to 100 pg of endotoxin). This solution was vortexed for 5 minutes and then serially diluted to give standard values of 0.005, 0.05 and 0.5 EU/ml. Each standard and tested sample (50 μl) was pipetted in duplicate into wells of a microtiter plate. The plate

was placed in the KQCL reader and set to warm up to 37^0C. The LAL reagent was reconstituted by adding 2.6 ml of pyrogen-free water. To each well 50 μl of reconstituted LAL reagent was added. Readings were taken for 100 minutes. Endotoxin concentrations in the test samples were determined from the standard curve.

2.2.4 CellTiter96® AQ_{ueous} one solution cell proliferation assay

2.2.4.1 CellTiter96® AQ_{ueous} one solution cell proliferation assay

The CellTiter 96® Aqueous Assay (Promega, UK) is a colorimetric method for the evaluation of cell death or toxicity. The CellTiter 96® Aqueous Assay is composed of the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt] (MTS) and an electron transfer reagent phenazine methosulfate (PMS). MTS (Owen's reagent) is reduced into a water-soluble formazan by enzymes found only in metabolically active cells (Cory et al. 1991). Formation of reducing agents such as nicotinamide adenine dinucleotide hydride (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) is required to accomplish the reduction of tetrazolium (Berridge & Tan 1993). Dehydrogenase enzymes which are found in the mitochondrial respiratory chain are thought to generate these pyridine nucleotide cofactors (Figure 2.10). The quantity of formazan product (measured by the amount of absorbance at 490 nm) is proportional to the number of viable cells in culture.

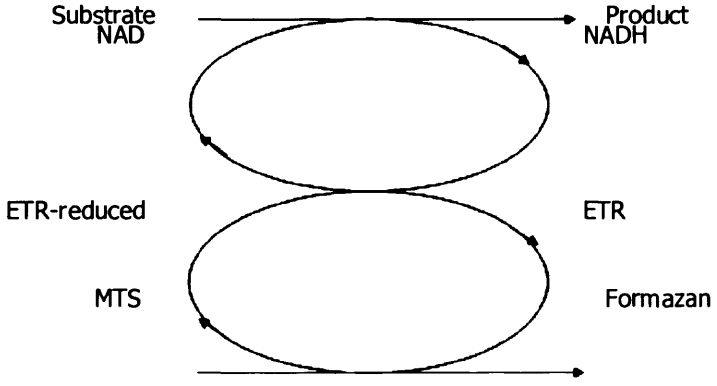


Figure 2.10: Schematic diagram of cellular metabolism by which MTS is converted to coloured formazan (adapted from Promega). (NADH - nicotinamide-adenine-dinucleotide hydride, NADPH - nicotinamide-adenine dinucleotide phosphate, ETR – electron transfer reagent, MTS - [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt])

2.2.4.2 Assessment of BEAS-2B and A549 cell viability following pulmonary surfactant treatment

BEAS-2B cells (2.5 x 10^3 cells/well) were plated into 96 well plates (Nunc, Denmark) in F-12K with 10% FBS. A549 cells (2.5 x 10^3 cells/well) were plated into 96 well plates (Nunc, Denmark) in DMEM with 5% FBS. After 24 hours cells were incubated with different concentrations of Curosurf® (batch # 066120 and 069097) or Survanta® (batch # 40-705-27 and 41-957-27) (500, 250, 125, 62.5 and 31.25 μg/ml) for 2 hours at 37^{0}C. Following incubation, Curosurf® or Survanta® solutions were removed, cells were washed 3 times with PBS and 100 μl of fresh F-12K/DMEM medium was added to each well. Each experimental condition was tested in quadruplicate. At different time points (24, 48 and 72 hours) equal volumes of CellTiter 96® AQ reagent were added (20 μl/well) and the plate was incubated for 1 hour at 37^{0}C. Absorbance was recorded at 492 nm (Titretec plate reader, Lab Systems, UK).

2.2.4.3 Assessment of BEAS-2B cell viability following Survanta® treatment and influenza virus infection

BEAS-2B cells (2.5 x 10^3 cells/well) were plated into 96 well plates (Nunc, Denmark) in F-12K with 10% FBS. After 24 hours cells were incubated with different concentrations of Survanta® (batch # 40-705-27) (500, 250, 125, 62.5, 31.25, 15.63 and 7.81 μg/ml) for 2 hours at 37^{0}C. Following incubation, Survanta® solutions were removed, cells were washed three times with PBS and were infected with influenza virus strain A/PR/8/34 and A/WSN/33 at an MOI 2. Each experimental condition was tested in quadruplicate. At different time points (24, 72 and 120 hours) equal volumes of CellTiter 96® AQ reagent were added (20 μl per well) and the plate was incubated for 1 hours at 37^{0}C. Absorbance was recorded at 492 nm (Titretec plate reader, Lab Systems, UK).

2.2.5 Enzyme linked immunosorbent assay (ELISA)

2.2.5.1 Enzyme linked immunosorbent assay (sandwich ELISA)

ELISA is a biochemical technique that measures the amount of antigen or antibody in a sample. In cytokine sandwich ELISA assays soluble cytokine or chemokine proteins are captured between two layers of antibodies; capture and biotinylated detection antibodies. The targeted cytokines are then quantified using a colorimetric reaction based on the activity of a streptavidin horseradish peroxidise (HRP) complex bound to the biotinylated detection antibody. Streptavidin is a tetrameric protein which shows high affinity to biotin. Each monomer of streptavidin binds one molecule of biotin therefore acting as signal amplification molecule. Horseradish peroxidase is an enzyme which catalyses the reaction with the substrate, for example tetramethylbenzidine (TMB). TMB is an analogue of benzidine which is oxidised during the enzymatic degradation of H_2O_2 by horseradish peroxidase. The oxidised product of this chromogen has a deep blue colour which is detected spectrophotomerically.

2.2.5.2 Determination of IL-8, RANTES and IL-6 production by ELISA

The level of cytokines produced by cells under various conditions was measured in a standard "sandwich" enzyme-linked immunosorbent assay (ELISA) using human paired antibodies (R&D Systems, UK) (Table 2.4). Monoclonal mouse anti-human antibody (capture antibody) was diluted in PBS to give an appropriate final concentration (Table 2.4). 100 μl of this unlabeled antibody was added to each well of maxisorb 96 well plates (Nunc, UK) and the plate was incubated overnight at room temperature to allow complete binding of antibody. The plate was then washed three times with wash buffer (PBS containing 0.05% Tween 20) and the remaining sites for protein binding on the plate were blocked by adding 300 μl of blocking buffer (PBS containing 1% BSA and 5% sucrose) to each well.

The plate was incubated for 1 hour at room temperature. During the incubation period, recombinant human cytokine standard was 2 fold serially diluted in diluent (de-ionised water containing 20 mM Tris, 150 mM NaCl, 0.1% BSA and 0.05% Tween 20, pH 7.3) to give an appropriate range of standard values (Table 2.4). The plate was washed three times with wash buffer and 100 μl of standard and samples were added to the plate in duplicate. In order to mix the standards and the samples the plate was gently tapped for 1 minute and then incubated for 2 hours at room temperature. The plate was then washed three times and 100 μl of biotinylated goat anti-human antibody (detection antibody) in diluent added to each well (Table 2.4). To allow the binding of this labelled antibody to antigen, completing the "sandwich", the plate was incubated for 2 hours at room temperature. Following three washes, 100 μl of 1:200 dilution of streptavidin horseradish peroxidase (HRP) conjugate in diluent was added. The plate was incubated for 20 minutes at room temperature and then washed again three times. The bound biotin-streptavidin complex was detected by adding 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories, UK). After incubation in darkness for 25 minutes at room temperature the reaction was stopped by adding 50 μl of 0.5 M H_2SO_4. A clear yellow colour was formed after addition of the acidic stop solution. This colorimetric endpoint was read spectrophotomerically at 450 nm (Titretec plate reader, Lab Systems, UK). The cytokine concentration was determined by comparing the signal of unknown samples against those of the standard curve.

		IL-8	*RANTES*	*IL-6*
Capture antibody	Clone	IL-8 DuoSet® ELISA kit DY208	MAB678	MAB206
	Final concentration	4 µg/ml	1 µg/ml	4 µg/ml
Standard	Clone	IL-8 DuoSet® ELISA kit DY208	278-RN	206-IL
	Final concentration	From 2000 to 31.25 pg/ml	From 1000 to 15.6 pg/ml	From 1200 to 18.75 pg/ml
Detection antibody	Clone	IL-8 DuoSet® ELISA kit DY208	BAF278	BAF206
	Final concentration	20 ng/ml	10 ng/ml	25 ng/ml

Table 2.4: Antibodies used to determine IL-8, RANTES and IL-6 concentration by ELISA.

2.2.5.3 Determination of IFN-beta production

The level of IFN-β was measured in a one step "sandwich" enzyme-linked immunosorbent assay (ELISA) using human IFN-β ELISA kit (Biosource, UK). Human IFN-β standard, after reconstitution in 1 ml of ice-cold endotoxin-free water, was prepared from 200 IU/ml to 3.125 IU/ml in doubling dilutions (200, 100, 50, 25, 12.5, 6.25 and 3.125 IU/ml) in ice-cold dilution buffer (phosphate buffer containing 0.1% BSA and 0.05% Tween 20) provided. The enzyme (HRP) labelled monoclonal mouse anti-human IFN-β antibody was prepared by dissolving lyophilizate with 6 ml of ice-cold dilution buffer. 96 well plate already coated with the affinity-purified polyclonal goat anti-human IFN-β antibody was primed by washing once with wash buffer (PBS containing 0.05% Tween 20) and 50 µl of enzyme-labelled antibody together with 100 µl of either standard or sample were added to each well. The plate was incubated for 2 hours at room temperature on a microplate shaker. After completion of the antigen-antibody reaction the plate was washed three times and 100 µl of colour developer solution added to each well. Colour developer was prepared by mixing 10 ml of sodium acetate-citrate buffer containing H_2O_2 with 0.5 ml of TMB solution. Covered plate was incubated for 30 minutes at room temperature on a microplate shaker. After incubation 100 µl of 0.5 mol/L H_2SO_4 were added and the absorbance was read

spectrophotomerically at 450 nm using Titretec plate reader (Lab Systems, UK). The IFN-β concentration was determined by comparing the signal of unknown samples against those of the standard curve.

2.2.5.4 Effect of influenza virus on IL-8, RANTES and IL-6 production in BEAS-2B cells

BEAS-2B cells (1×10^5 cells/well) were plated into 12 wells plates in F-12K medium with 10% FBS. After 24 hours monolayers were infected with decreasing multiplicity of infection (MOI) of influenza virus strains A/WSN/33 and A/PR/8/34 (1, 0.1, 0.01, 0.001 and 0.0001) for 12, 24 and 48 hours. Supernatants were assessed for IL-8, RANTES and IL-6 by ELISA as described in section 2.2.5.2.

2.2.5.5 Effect of pulmonary surfactant on IL-8, RANTES and IL-6 production induced by influenza virus in BEAS-2B cells

BEAS-2B cells were grown to confluence as described in section 2.2.5.4. After 24 hours cells were incubated with different concentrations of Curosurf® (batch # 066120) or Survanta® (batch # 41-957-27) (500, 250 and 125 μg/ml) for 2 hours at 37^0C. Following incubation, Curosurf® or Survanta® solutions were removed, cells were washed 3 times with PBS before infection with influenza virus, as described in section 2.2.5.4. Collected supernatants were assessed for cytokines as described in section 2.2.5.2.

2.2.5.6 Effect of dexamethasone on IL-8, RANTES and IFN-β production induced by poly(I-C), LPS, influenza virus and measles virus in BEAS-2B cells

BEAS-2B cells were grown to confluence as described in section 2.2.5.4. After 24 hours cells were incubated with 1 μg/ml of dexamethasone for 2 hours at 37^0C. Following

incubation, dexamethasone solution was removed, cells were washed 3 times with PBS before stimulation with poly(I-C) (50 μg/ml) and LPS (1 μg/ml) or infection with influenza virus strain A/WSN/33 (MOI 1), influenza virus strain A/PR/8/34 (MOI 1) and measles virus (MOI 0.01) for 24 hours. Supernatants were tested for IL-8, RANTES and IFN-β by ELISA as described in sections 2.2.5.2 and 2.2.5.3.

2.2.6 Plaque assay

2.2.6.1 Effect of pulmonary surfactant on viral infectivity in BEAS-2B cells

BEAS-2B cells (0.4 x 10^5 cells/well) were plated into 96 well plates in F-12K medium with 10% FBS. After 24 hours, cells were incubated with different concentrations of Curosurf® (batch # 066120) or Survanta® (batch # 41-957-27) (500, 250 and 125 μg/ml) for 2 hours at 37^0C. Following incubation, Curosurf® or Survanta® solutions were removed, cells were washed 3 times with PBS and were infected with 50 μl/well of serial dilutions of influenza virus strain A/WSN/33 and A/PR/8/34 in F-12K. Plaque assay was completed as described in section 2.1.2.4.4.

2.2.7 Liquid chromatography and mass spectrometry of phospholipids

2.2.7.1 Liquid chromatography and mass spectrometry

Chromatography is one of the most powerful methods for separating and analysing the composition of lipids, especially in combination with mass spectrometry which provides information about the chemical structure of lipid molecules (Maskrey et al. 2007). A chromatographic analysis involves passing a sample of lipids through a column that contains a matrix capable of retarding the flow of lipids. Lipids in the sample are separated because their affinities to the matrix differ. The stronger the affinity between the specific lipid and the matrix, the slower it passes through the column. After separation and identification, the concentration of each of the lipids is determined by a suitable detector.

After chromatography, mass spectrometry is applied to analyse lipid composition. Each lipid is exposed to ionization and then separated into a number of ions (peaks) based on differences in masses and charges, which are quantified using suitable detectors. This procedure is used to determine the total fatty acid profile and to calculate the type and concentration of fatty acids present in the original lipid sample.

Liquid chromatography and mass spectrometry can be used not only to determine the complete profile of the molecule present in a lipid but also to calculate the degree of lipid oxidation and the extent of heat or radiation damage or to determine the presence of antioxidants (Guardiola et al. 2007).

2.2.7.2 Liquid chromatography and mass spectrometry of phospholipids

Liquid chromatography and mass spectrometric analysis of Curosurf® (batch # 061350 and 069097) and Survanta® (batch # 41-957-27) was performed with the assistance of Dr Val O'Donnell, Department of Medical Biochemistry and Immunology, Cardiff University, Cardiff.

Briefly, pulmonary surfactant solutions were diluted 1:1000 in methanol and separated on a Spherisorb S5W 4.6 x 150-mm column (Waters Ltd., UK). Mass spectra were obtained on a Q-Trap instrument (Applied Biosystems 4000 Q-Trap, UK).

2.2.8 Dual luciferase reporter assay

2.2.8.1 Dual luciferase reporter assay

The Dual-Luciferase® Reporter Assay System (Promega, UK) allows for simultaneous measurement of two reporter enzyme activities (luciferases) within a single sample. One enzyme is derived from the light-emitting organ of the firefly (*Photinus pyralis*) another from sea pansy (*Renilla reniformis*). The reporter gene is under the control of the target promoter. The luciferase activity correlates with the target promoter activation. The *Renilla* reporter gene is under the control of the herpes simplex virus (HSV) thymidine kinase (TK) promoter. The *Renilla* luciferase activity provides an internal control to normalise against transfection efficiency. Firefly protein is a 62 kDa molecular weight oxidase. This enzyme requires ATP, molecular oxygen, Mg^{2+} and firefly luciferin as a substrate to generate light. *Renilla* luciferase is 36 kDa protein which catalyses coelenterazine oxidation by oxygen to produce light (Figure 2.11).

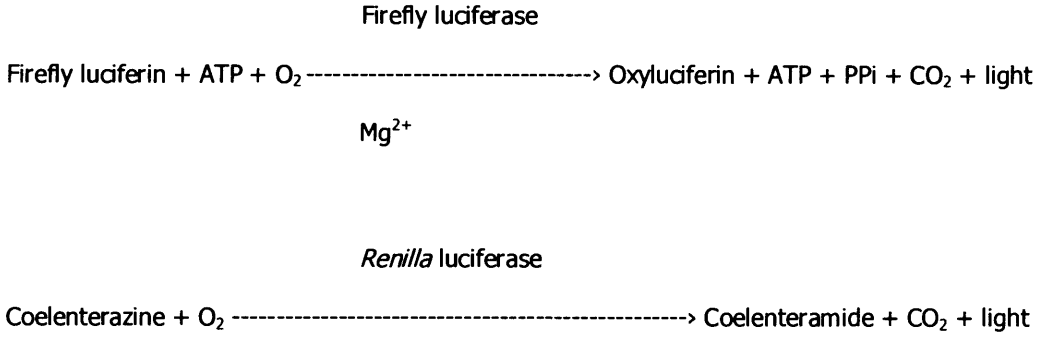


Figure 2.11: Bioluminescent reactions catalysed by firefly and *Renilla* luciferases (adapted from Promega).

2.2.8.2 Plasmids

NF-κB firefly reporter plasmid (ILR # 217)

The pNF-κB-Luc plasmid contains four tandem copies of the NF-κB consensus sequence located upstream of the TATA like promoter (P_{TAL}) region from the herpes simplex virus thymidine kinase (HSV-TK) promoter. The firefly reporter gene is located downstream from P_{TAL}. After endogenous NF-κB proteins bind to κ enhancer element (κB4), transcription is induced and the reporter gene is activated. The luciferase coding sequence is followed by the by the simian virus 40 (SV40) late polyadenylation signal to ensure proper processing of the luciferase transcript in eukaryotic cells. Located upstream of NF-κB is a synthetic transcription blocker (TB), which is composed of adjacent polyadenylation and transcription pause sites for reducing background transcription. Vector also contains f1 origin for ssDNA production, a pUC origin of replication and ampicillin resistance gene (amp^r) for propagation and selection in *E. coli*. This plasmid was referred to by us as NF-κB firefly reporter plasmid (internal laboratory reference number (ILR #) 217) (Baeuerle & Baltimore 1996; Peltz 1997; Baeuerle 1998) (Figure 2.12).

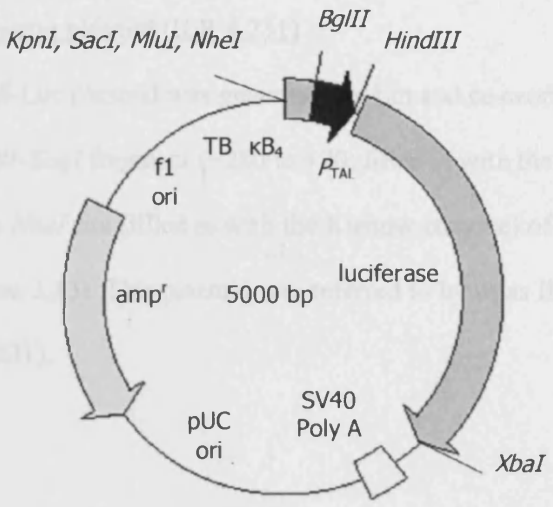


Figure 2.12: Schematic diagram of pNF-κB-Luc plasmid (adapted from Clontech).

IFN-β firefly reporter plasmid (ILR # 231)

The pGL3-IFN-β-Luc plasmid was generated by Lin and co-workers (Lin et al. 2000) by cloning the *EcoRI-TaqI* fragment (−280 to +20; filled in with the Klenow enzyme) from pUCβ26 into the *NheI* site (filled in with the Klenow enzyme) of the pGL3 basic vector (Promega) (Figure 2.13). This plasmid was referred to by us as IFN-β firefly reporter plasmid (ILR # 231).

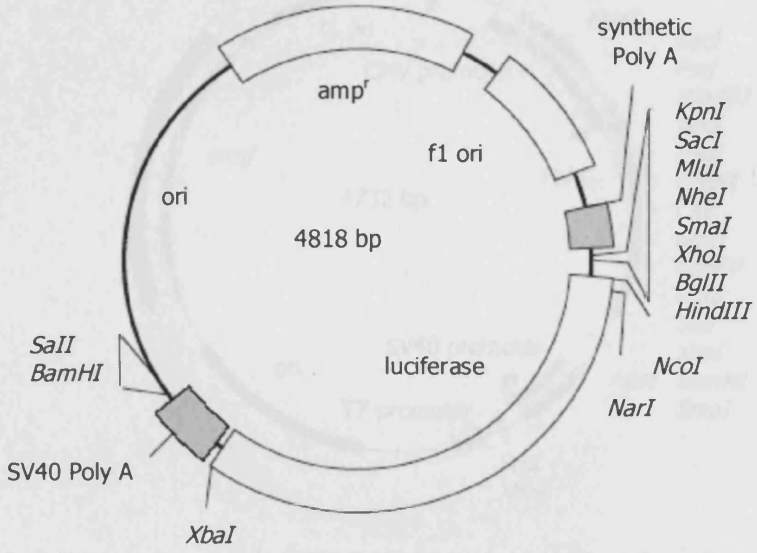


Figure 2.13: Schematic diagram of pGL3 vector (adapted from Promega).

TLR3 expression plasmid (ILR # 220)

The pFLAG-CMV1-TLR3 plasmid was generated by Schoenemeyer and co-workers (Schoenemeyer et al. 2005) by cloning PCR-amplified full length TLR3 cDNA into the pFLAG-CMV1 vector (Sigma) (Figure 2.14). This plasmid was referred to by us as TLR3 expression plasmid (ILR # 220).

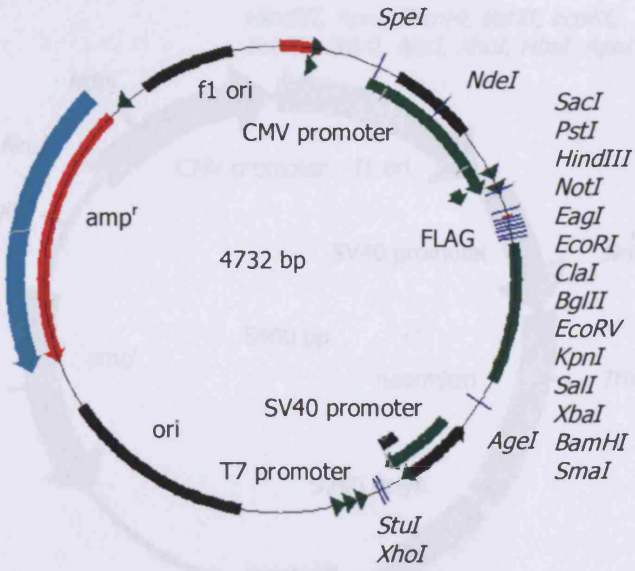


Figure 2.14: Schematic diagram of pFLAG-CMV1 vector (adapted from Sigma).

RIG-I expression plasmid (ILR # 227)

The pcDNA3-Neo-FLAG-RIG-I plasmid was generated by Venkataraman and co-workers (Venkataraman et al. 2007) by cloning PCR-amplified RIG-I cDNA into pcDNA3-Neo vector (Invitrogen Life Technologies) (Figure 2.15). This plasmid was referred to by us as RIG-I expression plasmid (ILR # 227).

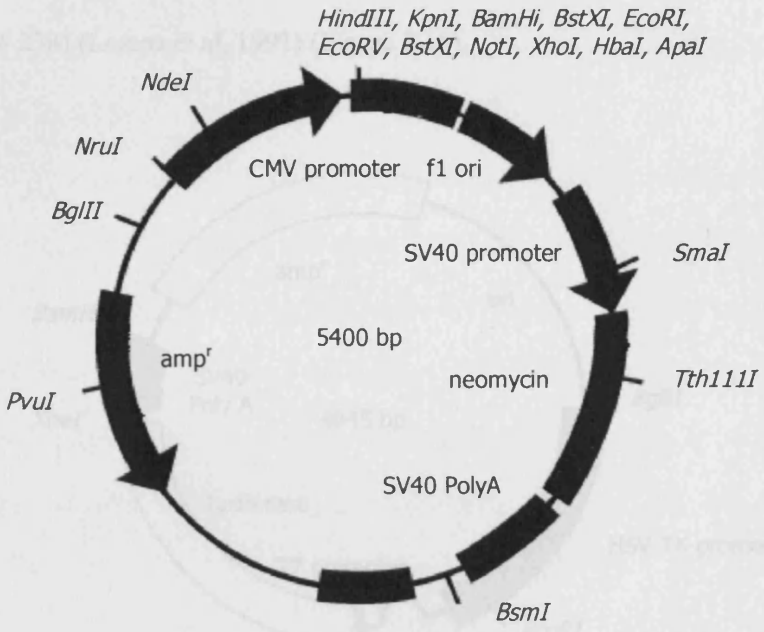


Figure 2.15: Schematic diagram of pcDNA3-Neo vector (adapted from Invitrogen Life Technologies).

Renilla reporter plasmid (ILR # 238)

The phRL-TK plasmid (Promega) contains the herpes simplex virus thymidine kinase (HSV-TK) promoter which provides low to moderate levels of *Renilla* luciferase expression. The *Renilla* reporter gene is located downstream HSV-TK promoter. The luciferase coding sequence is followed by the by the simian virus 40 (SV40) late polyadenylation signal. T7 promoter preceding the *Renilla* reporter gene can be used to synthesise RNA transcripts *in vitro* using T7 RNA polymerase. This plasmid was referred to by us as *Renilla* reporter plasmid (ILR # 238) (Lorenz et al. 1991) (Figure 2.16).

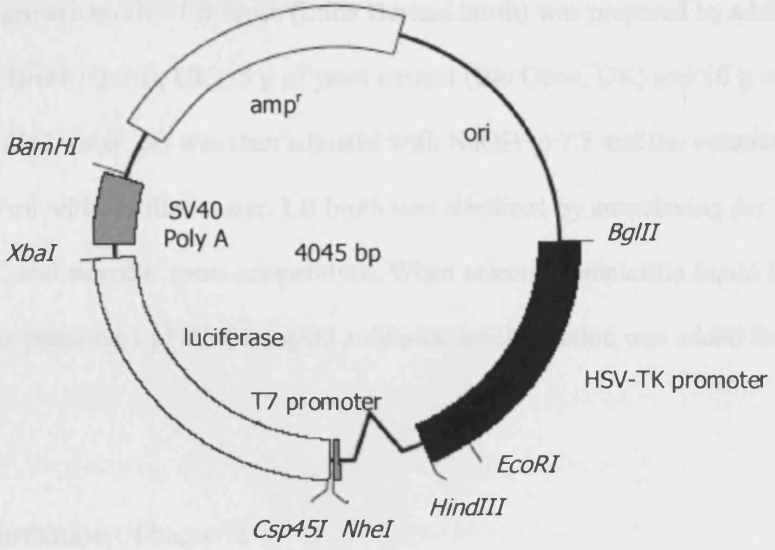


Figure 2.16: Schematic diagram of phRL-TK plasmid (adapted from Promega).

2.2.8.3 Preparation of agar plates and liquid bacterial growth medium

The 7.8 g of Columbia Agar Base (Oxoid, UK) was dissolved in 200 ml of distilled water. Agar solution was sterilized by autoclaving for 20 minutes at 121^{0}C. In order to prepare plates the agar solution was transferred from autoclave to water bath at 45^{0}C for at least 20 minutes and then 20 ml poured into each 8.25 cm diameter plastic disposable plate (Sterilin Ltd., UK) (20 ml creates a layer of agar 3.5 mm thick) and plates were allowed to cool down following overnight incubation at 37^{0}C. When selective ampicillin plates were prepared 1 µl of 100 mg/ml antibiotic stock solution was added for each 1 ml of media. Ampicillin was added at 42^{0}C in water bath.

Liquid bacterial growth broth – LB broth (Luria Bertani broth) was prepared by adding 10 g of Tryptone Soy Broth (Oxoid, UK), 5 g of yeast extract (Bio Gene, UK) and 10 g of NaCl to 800 ml of distilled water. pH was then adjusted with NaOH to 7.5 and the volume was adjusted to 1000 ml with distilled water. LB broth was sterilized by autoclaving for 20 minutes at 121^{0}C and stored at room temperature. When selective ampicillin liquid bacterial growth broth was prepared 1 µl of 100 mg/ml antibiotic stock solution was added for each 1 ml of media.

2.2.8.4 Transformation of bacteria

One bead of frozen competent cells Top 10 bacteria was placed onto agar plates and incubated overnight at 37^{0}C. The next day a single colony was selected for starter culture. The starter culture was grown in 10 ml of LB medium overnight in 37^{0}C shaker. The following day 1 ml of Top 10 bacteria was washed 3 times with endotoxin-free water by centrifugation for 1 minute at 500 x g and the pellet was resuspended with 40 µl of endotoxin-free water. 10 µl of plasmid DNA in TE buffer were added to bacteria. DNA suspended in bacteria was transferred gently to an electroporation cuvette and electroporated

with a Gene Pulser apparatus (Bio-Rad, UK) that was set at 2.5 kV, 25 μF, and 400 Ω. 400 µl of LB broth was added immediately to the cuvette and cells were incubated in an eppendorf tube for 30 minutes at 37^{0}C to recover. The electric pulse causes the formation of temporary aqueous pores in cell membrane by disruption of phospholipid bilayers. This damage results in increased electric potential across the membrane so that charged molecules like DNA move through the pores. As this movement takes place the cell membrane discharges and the pores quickly close. Transformed bacteria were then plated onto ampicillin selective agar plates (ampicillin corresponds with the resistance offered by plasmids used in these studies) and the plates were incubated overnight at 37^{0}C.
After incubation a single colony was selected and grown in 10 ml of selective ampicillin liquid bacterial broth overnight in 37^{0}C shaker. 100 µl of this culture were transferred in 100 ml of selective LB medium and grown again overnight in 37^{0}C shaker before plasmid purification.

2.2.8.5 Purification of plasmids

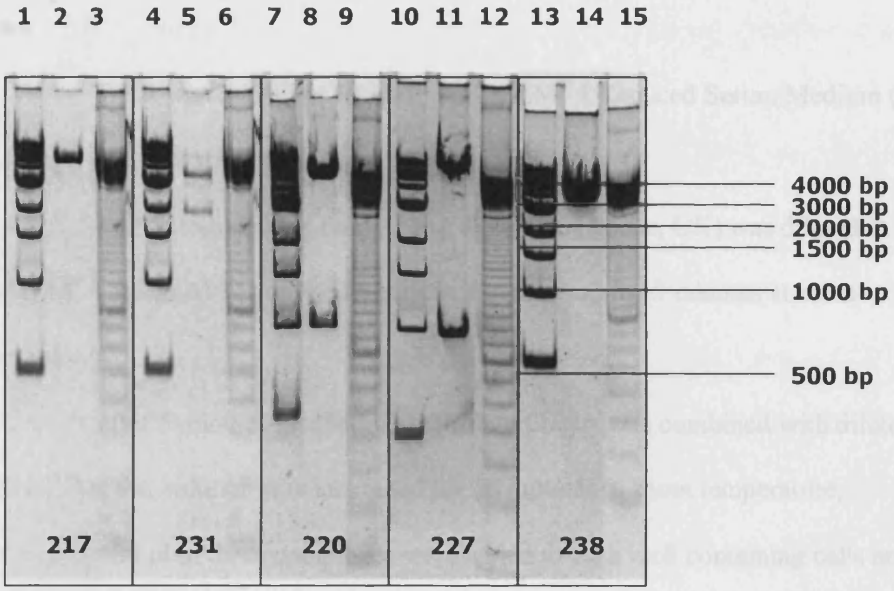
All plasmids were purified using the Pure Link^{TM} HiPure Plasmid DNA Purification Kit (Invitrogen, UK). The bacteria were harvested by centrifugation for 30 minutes at 3500 g and resuspended with 4 ml of resuspension buffer containing RNase prior to lysis with 4 ml of lysis buffer. The lysates were incubated for 5 minutes at room temperature. 4 ml of precipitation buffer were added and lysates were clarified at 18^{0}C for 10 minutes at 3500 x g. The cleared lysates were applied to pre-equilibrated anion exchange midi columns. DNA interacts with the positive charges on the surface of the resin. Under moderate salt condition, plasmid DNA binds to the resin while proteins, carbohydrates and other impurities are washed away with wash buffer. The washing was performed twice with 10 ml of wash buffer with high salt concentrations. The plasmid DNA was eluted under low salt condition

with 5 ml of elution buffer. The columns were allowed to drain by gravity flow. 3.5 ml of isopropanol were added to precipitate the plasmid DNA. The samples were centrifuged at 4^0C for 30 minutes at 15000 x g. The DNA pellet was washed in 3 ml 70% ethanol followed by centrifugation at 4^0C for 10 minutes at 15000 x g. The supernatant was decanted and the tube was allowed to dry horizontally at room temperature. The plasmid DNA was resuspended in 50 μl of TE buffer. The concentration of DNA was determined spectrophotometrically at 260 nm (Genequant, UK).

2.2.8.6 Restriction digest analysis and agarose gel electrophoresis

5 μl of plasmid DNA was digested in 50 μl reaction volume with 20 U of *XhoI* plus *BamII* and 10X Surecut buffer B at 370C for 2 hours and visualised via electrophoresis on 2% agarose gel (Figure 2.17). Digested DNA samples were mixed with 10 μl of loading buffer and loaded into the wells of a 2% agarose gel in vertical electrophoresis column (Hoeffer-Pharmacia SE280, UK) with 1X EPB as running buffer. A 1 kbp DNA ladder (Fermentas/NEB, UK) and a 100 bp ladder (Fermentas/NEB, UK) were also loaded to serve as size standards. The column was connected to a Consort E143 power supply and samples electrophoresed at 100 V for 60-90 minutes. Gels were stained with ethidium bromide (200 μg/ml) and DNA was visualised using BIORAD UV transiluminator.

A



B

Well(s)	*ILR # (Plasmid)*	*Size (bp)*
1, 4, 7, 10 and 13	1 kbp ladder	-
3, 6, 9, 12 and 15	100 bp ladder	-
2	217 (NF-κB firefly reporter)	5000
5	231 (IFN-β firefly reporter)	2000 + 5000
8	220 (TLR3 expression)	1000 + 5000
11	227 (RIG-I expression)	940 + 5200
14	238 (phRL-TK *Renilla* reporter)	4000

Figure 2.17: Agarose gel electrophoresis (panel A) and restriction digest analysis (panel B) of plasmids used to determine NF-κB and IFN-β promoter activation by Dual-Luciferase® Reporter Assay. (ILR # - internal laboratory reference number)

2.2.8.7 Transfection of HEK293

HEK293 cells (2 x 10^4 cells/well) were plated into 96 well plate in DMEM with 10% FBS. HEK293-TLR3 and HEK-RIG-I cells (2 x 10^4 cells/well) were plated into 96 well plate in DMEM with 10% FBS and 500 μg/ml of G418. After 24 hours cells were transfected with 0.3 μg of NF-κB or IFN-β firefly luciferase reporter plasmids, 0.3 μg of TLR3 or RIG-I expression vectors and 0.03 μg of *Renilla* luciferase reporter plasmids (at different

combinations). More precisely, for each transfection, sample complexes were prepared as follows:

- DNA was diluted in 50 μl of Opti-MEM® I Reduced Serum Medium (Gibco, UK),
- 1 μl of transfection reagent FuGENE™ 6 (Roche, UK) was diluted in 50 μl of Opti-MEM® I Reduced Serum Medium before incubation for 5 minutes at room temperature,
- after 5 minutes incubation, the diluted DNA was combined with diluted FuGENE™ 6, this solution was incubated for 20 minutes at room temperature,
- 100 μl of these complexes were added to each well containing cells and medium.

Cells were incubated for 24 hours at 37^0C prior to co-transfection with poly(I-C), as described above, or infection with influenza or measles virus. The reporter gene activity was assessed 6 (NF-κB) and 12 hours (IFN-β) later.

FuGENE™ 6 is a nonliposomal lipid-based transfection agent. After forming a complex with DNA/RNA FuGENE™ 6 interacts with the cell membrane. Unlike the liposomal transfection reagents, which envelopes DNA/RNA and then fuse with cell membrane, nonliposomal lipids form micelles of different sizes that are taken up by the cells by endocytosis and then released in the cytoplasm.

2.2.8.8 Transfection of BEAS-2B cells

Unlike HEK293 cells, BEAS-2B cells were transfected in suspension with 0.3 μg of NF-κB firefly luciferase reporter plasmids and 0.03 μg of *Renilla* luciferase reporter plasmids or IFN-β firefly luciferase reporter plasmids and 0.03 μg of *Renilla* luciferase reporter plasmids. First DNA/FuGENE™ 6 complexes were prepared in 96 well plate and then

BEAS-2B ($1x10^4$ cells/well) were plated in F-12K with 10% FBS. Cells were incubated for 24 hours at 37^0C prior to transfection with poly(I-C) or infection with respiratory viruses.

2.2.8.9 Measurement of luciferase activity

Briefly, transfection reactions were removed from the cells and 40 μl of 1X passive lysis buffer were dispensed into each culture well. Sufficient quantity of the 1X passive lysis buffer was prepared by adding 1 volume of 5X passive lysis buffer (Promega, UK) to 4 volumes of enotoxin-free water. The plate was placed on a rocking platform for 15 minutes at room temperature. 40 μl of cell lysate were dispensed to a luminometer plate (Nunc, Denmark). Two different methods and two different luminometers were applied to determine enzymes activity.

- 100 μl of luciferase assay reagent II were carefully transferred manually into a luminometer plate containing cell lysate (to all samples at the same time), the plate was placed in the luminometer (Microtiter plate luminometer, Dynex Technologies, UK) and the reading was initiated (1 second per each well). The firefly luciferase activity was recorded. Luciferase assay reagent II was prepared by resuspending the provided lyophilized luciferase assay substrate (Promega, UK) in 10 ml of the supplied luciferase assay buffer II (Promega, UK). Luciferase assay reagent II was aliquoted and stored frozen at -70^0C. Following luciferase activity measurement, the plate was removed from the luminometer and 100 μl of Stop & Glo reagent were added manually as described above. The plate was replaced in the luminometer and the reading was initiating (1 second per each well). This time the Renilla luciferase activity measurement was recorded. Sufficient quantity of the Stop & Glo reagent was prepared by adding 1 volume of 50X Stop & Glo substrate (Promega, UK) to 50 volumes of Stop & Glo buffer (Promega, UK) in a glass tube.

- The plate was placed in luminometer (FLUOstar Optima, BMG LABTECH, UK) and 100 μl of luciferase assay reagent II were injected automatically into each well shortly before the measurement. The reading was initiated (10 seconds per each well) and the firefly luciferase activity was recorded. Without removing the plate from the machine 100 μl of Stop & Glo reagent were injected automatically, again into each well shortly before the measurement. The reading was initiated (10 seconds per each well) and the *Renilla* luciferase activity was recorded.

2.2.8.10 Effect of poly(I-C) on NF-κB and IFN-β promoters activation in HEK293 cells

HEK293 cells transfected with NF-κB and TLR3, NF-κB and RIG-I, IFN-β and TLR3 or IFN-β and RIG-I were co-transfected with 1 μg/ml of poly(I-C). The reporter gene activity was assessed after 6 (NF-κB) or 12 hours (IFN-β).

2.2.8.11 Effect of poly(I-C), influenza virus and measles virus on NF- κB and IFN-β promoters activation in BEAS-2B cells

BEAS-2B cells transfected with NF-κB or IFN-β reporter plasmid were co-transfected with 1 μg/ml of poly(I-C), infected with influenza virus at an MOI 1 or infected with measles virus at the MOI 0.05. The reporter gene activity was assessed after 6 (NF-κB) or 12 hours (IFN-β).

2.2.8.12 Effect of poly(I-C), influenza virus and measles virus on NF- κB promoter activation in HEK293, HEK293-TLR3 and HEK293-RIG-I cells

HEK293, HEK293-TLR3 or HEK293-RIG cells transfected with NF-κB reporter plasmid were co-transfected with 50 μg/ml of poly(I-C), infected with influenza virus at an MOI 1 or

infected with measles virus at an MOI 0.05. The reporter gene activity was assessed after 6 hours.

2.2.8.13 Effect of pulmonary surfactant and dexamethasone on measles virus induced NF- κB promoter activation and poly(I-C), influenza virus and measles virus induced IFN-β promoter activation in BEAS-2B cells

BEAS-2B cells transfected with NF-κB reporter plasmid were incubated with 500 μg/ml of Curosurf® (batch # 069097), 500 μg/ml of Survanta® (batch # 41-957-27) or 1 μg/ml of dexamethasone for 2 hours. Following incubation, Curosurf®, Survanta® or dexamethasone solutions were removed and cell were washed once with F-12K medium before infection with measles virus at the MOI 0.05. The reporter gene activity was assessed after 6 hours.
BEAS-2B cells transfected with IFN-β reporter plasmid were incubated with 500 μg/ml of Curosurf® (batch # 069097), 500 μg/ml of Survanta® (batch # 41-957-27) or 1 μg/ml of dexamethasone for 2 hours. Following incubation, Curosurf®, Survanta® or dexamethasone solutions were removed and cell were washed once with F-12K medium before co-transfection with 1 μg/ml of poly(I-C), infection with influenza virus at the MOI 1 or infection with measles virus at the MOI 0.05. The reporter gene activity was assessed after 12 hours.

2.2.8.14 Effect of Curosurf® and dexamethasone on poly(I-C) and measles virus induced NF- κB promoter activation in HEK293-TLR3 and HEK293-RIG-I cells

HEK293, HEK293-TLR3 or HEK293-RIG-I cells transfected with NF-κB reporter plasmid were incubated with 500 μg/ml of Curosurf® (batch # 069097) or 1 μg/ml of dexamethasone for 2 hours. Following incubation, Curosurf® or dexamethasone solutions were removed and cell were washed once with DMEM medium before co-transfection with 1 μg/ml of poly(I-

C) or infection with measles virus at the MOI 0.05. The reporter gene activity was assessed after 6 hours.

2.2.9 IT equipment and software

Immunofluorescence was examined using the fluorescence microscope Olympus BX5I and AxioVision software version 4.4. Plates with haemagglutination tests, TCID50 and plaque assays were scanned on a flat bed scanner HP Scanjet 5470C, using HP Precisionscan Pro software version 3.1 and examined by using COREL® PHOTO – PAINT® X3 software version 3. Luciferase activity was measured using Microtiter Plate Luminometer DYNEX TECHNOLOGIES and Revelation MLX software version 4.25 or FLUOstar OPTIMA Luminometer BMG LABTECH and FLUOstar* OPTIMA software. Statistical analysis was performed using SPSS software version 12. Bibliography was created using Reference Manager software version 10.

3 Results

3.1 Generation and quantitation of viral stocks

Viral stocks of A/Swine/1976/31, A/WSN/33 and A/PR/8/34 influenza viruses were prepared in 7 day old embryonated chicken eggs (ECEs) of specific pathogen free origin. This method was previously reported to yield generally higher titers when compared to propagation in cell culture system (Clavijo et al. 2002). All strains of influenza virus used in this study grew to high titer as determined by haemagglutination test (Table 3.1). Although titers varied between preparations from different eggs, the titer of human influenza virus reached a value of 811920 HAU/ml in 9 out of 12 inoculated eggs (A/WSN/33: SPF-11, SPF-12, SPF-15 and A/PR/8/34: SPF-2, SPF-3, SPF-4, SPF-6, SPF-9, SPF-10). The titer of swine influenza virus was lower but still at a significant quantifiable level of 20480 HAU/ml, in all eggs from which allantoic fluids were obtained (A/Swine/1976/31: SPF-3, SPF-5, SPF-8, SPF-9).

For the first stock preparation of A/Swine/1976/31 amniotic and allantoic fluids were collected separately for analysis. The yield of amniotic fluid (1ml on average) was much lower than the yield of fluid obtained from allantoic cavity (7 ml on average) therefore A/WSN/33 and A/PR/8/34 inoculation infective amniotic fluid was harvested together with allantoic fluid for each ECE (4 ml on average for A/WSN/33 strain and 6 ml on average for A/PR/8/34 strain) (Table 3.1).

A/WSN/33 and A/PR/8/34 influenza viruses were also propagated in MDCK cells. Surprisingly, only the growth of A/WSN/33 was supported by these cells (Table 3.1). Both human strains were previously shown to be successfully propagated in MDCK cells (Someya et al. 1990;Turan et al. 1996), however the ability of influenza virus to replicate in ECEs and cell culture system depends on the origin of virus strain. These results indicate that A/PR/8/34 strain (obtained from ECACC) was ECE adapted whereas A/WSN/33 strain

(also obtain from ECACC) was both ECE and cell culture adapted. As expected, the titer of A/WSN/33 isolated in cell culture (5120 HAU/ml) was much lower than the titer of this strain grown in ECEs (81920 HAU/ml) (Table 3.1).

The infection of MDCK cells with influenza virus was performed in the presence of high purity TrypLE or TPCK-treated trypsin. The addition of trypsin to cell culture medium has been shown to stimulate the growth of influenza virus and to enable many influenza virus strains to form plaques with high efficiency (Tobita et al. 1975). This was achieved by enzymatic cleavage of the precursor HA molecule (HA0) into two subunits (HA1 and HA2) and by mediating the fusion of the viral envelope with the endosomal membrane. In our hands, the growth ability of A/WSN/33 strain in the presence of high purity TrypLE was identical to that in the presence of TPCK-treated trypsin (Table 3.1). Possible explanation for the observed result is that HA of ECE adapted A/WSN/33 strain was already activated by protease present in allantoic fluid of embryonated eggs (factor X-like enzyme), which has been previously shown for influenza virus (Gotoh et al. 1990), and not by addition of exogenous proteases.

Virus	*Host*	*Sample*	*Volume (ml)*	*HAU/50 µl*	*HAU/ml*
A/Swine/1976/31	ECE	SPF-1*	0.5	0	0
		SPF-2*	0.7	0	0
		SPF-3*	0.8	512	10240
		SPF-4*	1.5	64	1280
		SPF-8*	0.4	256	5120
		SPF-9*	2.6	512	10240
		SPF-3**	**5**	**1024**	**20480**
		SPF-5**	**5**	**1024**	**20480**
		SPF-8**	**9**	**1024**	**20480**
		SPF-9**	**8**	**1024**	**20480**
A/WSN/33		**SPF-11*****	**4**	**4096**	**81920**
		SPF-12***	**4**	**4096**	**81920**
		SPF-14***	3	16	320
		SPF-15***	**3**	**4096**	**81920**
		SPF-16***	5	8	160
		SPF-17***	4	2	40
A/PR/8/34		**SPF-2*****	**5**	**4096**	**81920**
		SPF-3***	**5**	**4096**	**81920**
		SPF-4***	**4**	**4096**	**81920**
		SPF-6***	**8**	**4096**	**81920**
		SPF-9***	**7**	**4096**	**81920**
		SPF-10***	**6**	**4096**	**81920**
A/WSN/33	MDCK	TrypLE	9	256	5120
		TPCK	9	256	5120
A/PR/8/34		TrypLE	9	0	0
		TPCK	9	0	0

ECE – embryonated chicken egg

* - amniotic fluid

** - allantoic fluid

*** - amniotic fluid + allantoic fluid

Table 3.1: Characterisation of influenza virus growth in embryonated chicken eggs and MDCK cells by haemagglutination test.

3.2 Characterisation of influenza virus infection in target cell lines by TCID50 and immunofluorescence

The biological properties associated with infectivity and pathogenicity of prepared influenza virus stocks were examined by immunofluorescence and TCID50, respectively (Table 3.2). At different stages of viral life cycle such as uptake by endocytosis (Sieczkarski & Whittaker 2003), transcription and replication in the cell nucleus (O'Neill et al. 1995) or assembly in the cytoplasm (O'Neill et al. 1998; Neumann et al. 2000) influenza virus proteins can be detected in different cellular compartments by immunofluorescence staining. The number of influenza positive cells correlates with active infection and entry of the virus. However, only after the release of new influenza virus the host cell dies and this cytotoxic effect which correlates with pathogenicity and virulence of the virus can be evaluated by TCID50. Therefore infection and pathogenicity of A/Swine/1976/31, A/WSN/33 and A/PR/8/34 viruses were assessed in kidney epithelial cells MDCK and two different lung epithelial cell lines BEAS-2B and A549. The MDCK cell line is the most efficient cell system for the plaque assay of influenza virus available at the present (Gaush & Smith 1968). Human bronchial and alveolar epithelial cells are the primary site for influenza virus infection in lungs. Therefore they were tested along with MDCK cells because they would constitrute the final airway model.

According to these results, A/WSN/33 grown in embryonated chicken eggs was characterised as the strain of highest pathogenicity ($1.3x10^7$ TCID50/ml) and infectivity ($2.5x10^7$ IC/ml) in MDCK cells. Similarly, virulence ($5.6x10^4$ TCID50) correlated with infectivity ($9.0x10^4$ IC/ml) of the same strain of influenza virus derived from cell culture in MDCK cells. The high pathogenicity of A/WSN/33 strain was previously reported in MDCK cells as well as in mice and was surprisingly associated with NA of this strain. The lack of conserved glycosylation sites of NA determines the virulence of influenza virus

A/WSN/33 strain (Li et al. 1993). On the other hand, A/Swine/1976/31 and A/PR/8/34 were found to exhibit lower pathogenicity ($3.2x10^3$ TCID50/ml and $3.2x10^4$ TCID50/ml, respectively) albeit high infectivity ($2.7x10^6$ IC/ml and $1.1x10^8$ IC/ml, respectively) in these cells. These results indicate that the life cycle of A/Swine/1976/31 and A/PR/8/34 strains was not completed in MDCK cells. The same pattern was observed for bronchial and alveolar epithelial cells. All influenza virus strains tested had lower pathogenicity than infectivity in these cells, suggesting that viral budding was also impaired in all lung cells tested. This might result in the lower yield of the virus at the site of inflammation and the suppression of cytokine production associated with the secondary infection of the influenza virus.

The infectivity of two human influenza viruses, measured by immunofluorescence, was higher than the infectivity found for swine influenza virus in all cell lines used therefore A/WSN/33 and A/PR/8/34 were chosen for further experiments. The observed effect could be due to receptor preference of different influenza virus strains. Human trachea contains sialic acids with α-(2,6) linkages which preferentially bind human strains of influenza virus. In addition, the viral titer, measured by immunofluorescence, for all strains used was higher in MDCK and BEAS-2B cells than in A549 cells therefore BEAS-2B cells were selected as a model for further studies. This result indicates that the main sites of entry for these strains of influenza virus in lungs may be bronchioles.

In the light of these results, the effect of pulmonary surfactant on influenza virus A/WSN/33 and A/PR/8/34 infected human bronchial epithelial cells BEAS-2B was the major focus of this work. For the purpose of this study all MOIs given were based on the "infectivity" titers determined by the immunofluorescence method.

To exclude any stimulatory effect associated with contamination by LPS, all viral stocks were shown to be free from bacterial endotoxins by LAL assay (data not shown).

Virus	*MDCK*		*BEAS-2B*		*A549*	
	TCID50/ml	IC/ml	TCID50/ml	IC/ml	TCID50/ml	IC/ml
A/Swine/1976/31*	$3.2x10^3$	$2.7x10^6$	0	$8.0x10^5$	$3.2x10^2$	$3.0x10^5$
A/WSN/33 SPF-11	$1.3x10^7$	$2.5x10^7$	$3.2x10^4$	$1.6x10^7$	$3.2x10^3$	$5.6x10^6$
A/PR/8/34 SPF-2	$3.2x10^4$	$1.1x10^8$	$3.2x10^4$	$4.0x10^8$	0	$5.0x10^6$
A/WSN/33 TrypLE	$5.6x10^4$	$9.0x10^4$	-	-	-	-
A/PR/8/34 TPCK	$3.2x10^2$	-	-	-	-	-

* - after Optiprep™ purification

Table 3.2: Characterisation of influenza virus in target cell lines by TCID50 and immunofluorescence.

3.3 Optiprep™ purification

The titer of A/Swine/1976/31 (20480 HAU/ml) determined by haemagglutination test was lower than the titer of A/WSN/33 and A/PR/8/34 (81920 HAU/ml) therefore this strain was further subjected to density gradient centrifugation to concentrate low titer virus from ECE fluids. As an alternative to sucrose, iodixanol was investigated as the density gradient medium. Iodixanol was successfully used previously for purification of various viruses, including respiratory syncytial virus (RSV) (Gias et al. 2007), human immunodeficiency virus type 1 (HIV-1) (Dettenhofer & Yu 1999), recombinant adeno associated virus (rAAV) (Hermens et al. 1999), Moloney murine leukemia virus (MoMLV)-derived retrovirus particles (Moller-Larsen & Christensen 1998; Segura et al. 2006) and hepatitis C virus (HCV) (Nielsen et al. 2006).

Allantoic fluid was underlayed with 10 ml of 15%, 8 ml of 25% and 7 ml of 40% iodixanol. After centrifugation, the gradient was collected by gravity flow into twenty 1 ml fractions. The titer of influenza virus in each fraction was determined by haemagglutination test (Table 3.3). A haemagglutination peak higher than the input titer was observed only in Optiprep™ gradient number 1, fraction 8 (40960 HAU/ml). The haemagglutination peaks equal to the input concentration were obtained in Optiprep™ gradient number 2, fractions 6 and 7 (20480 HAU/ml) (Figure3.1). The gradient fractions containing the most concentrated virus band were pooled and analysed by immunofluorescence and TCID50. Detection of influenza virus strain A/Swine/1976/31 after gradient centrifugation by immunofluorescence proved that purified particles were still infective. The apparent peaks seen in Optiprep™ gradients number 1 and 2 did not occur in other gradients therefore high concentrations obtained in two first gradients were not reproducible. In all ten gradients performed influenza virus was distributed through all density steps (15%, 25% and 40%) therefore concentration of influenza virus in a small volume of the iodixanol was also not achieved.

The presence of influenza virus in iodixanol medium at the lowest dilution (40%) showed that layer(s) of less diluted or undiluted Optiprep™ should also be included in this method in the future. In addition, an increased gradient centrifugation time might be more efficient than one hour gradients in the process of influenza virus purification. Interestingly, in Optiprep™ gradients number 4, 7, 8 and 9 two separated peaks were observed which might suggest the presence of two different morphological forms of this virus. Additional electron microscopy studies would be required to ascertain if this is the case.

Fraction number	*Optiprep™ gradient number*									
	1	2	3	4	5	6	7	8	9	10
	HAU/50 μl									
1	1	0	2	1	1	1	2	2	1	2
2	0	0	2	1	4	2	2	2	1	4
3	0	4	1	8	64	8	1	2	16	64
4	0	32	8	128	128	32	1	8	128	256
5	0	128	128	256	**256**	128	8	128	256	256
6	4	**1024**	256	**512**	**256**	128	**512**	**512**	**512**	256
7	256	**1024**	256	**512**	**256**	256	**512**	256	**512**	256
8	**2048**	512	256	256	**256**	256	256	256	256	256
9	512	512	256	256	**256**	**512**	256	**512**	**512**	**512**
10	256	512	256	**512**	**256**	**512**	256	**512**	**512**	**512**
11	256	256	256	**512**	**256**	**512**	256	**512**	**512**	256
12	128	256	**512**	256	**256**	**512**	256	**512**	**512**	256
13	128	128	256	128	128	**512**	**512**	256	256	256
14	128	64	128	128	128	256	256	256	256	256
15	128	64	128	128	128	256	256	256	128	256
16	128	64	128	128	128	256	256	256	128	128
17	128	64	128	128	128	256	256	256	128	128
18	128	64	128	128	128	128	256	256	128	128
19	128	128	128	128	64	128	256	128	128	128
20	64	64	128	64	64	128	256	128	128	64

Table 3.3: Characterisation of fractions from 10 different Optiprep™ gradients.

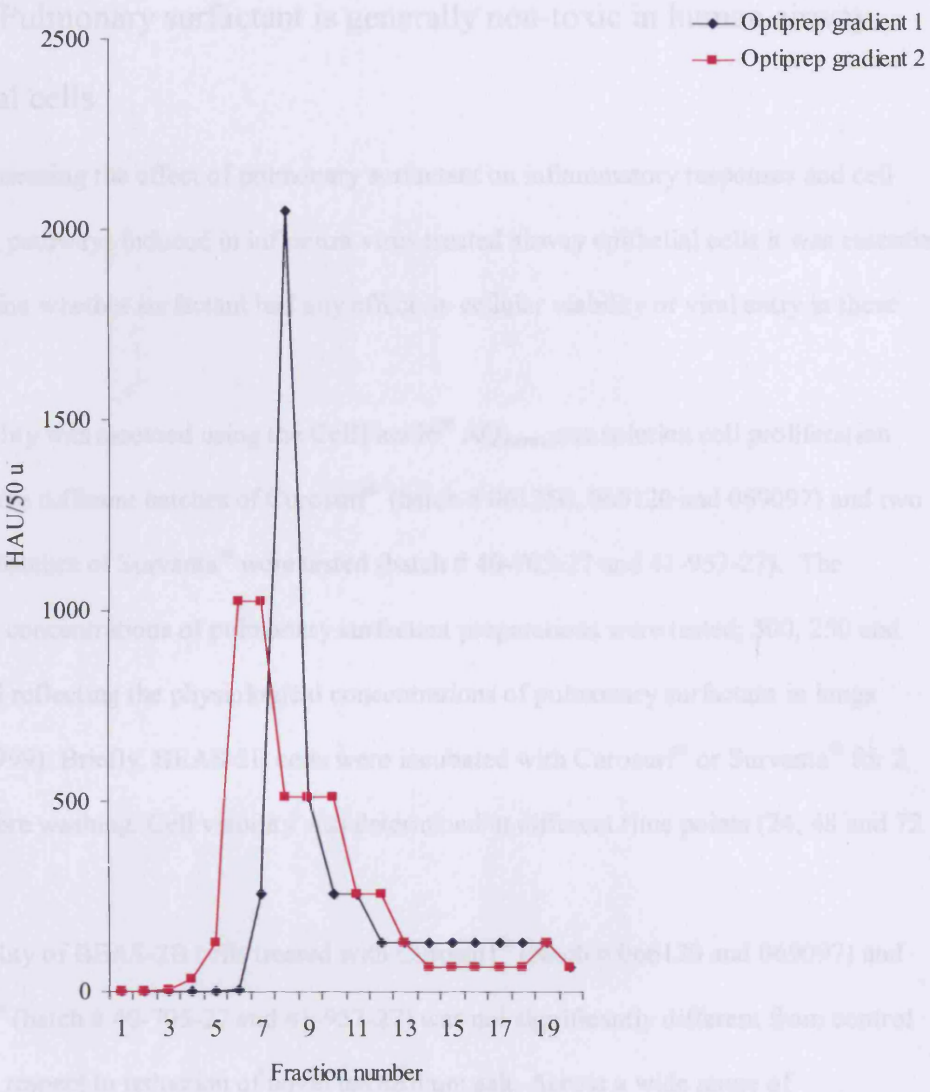


Figure 3.1: Characterisation of fractions from Optiprep™ gradient 1 and 2.

3.4 Pulmonary surfactant is generally non-toxic in human airway epithelial cells

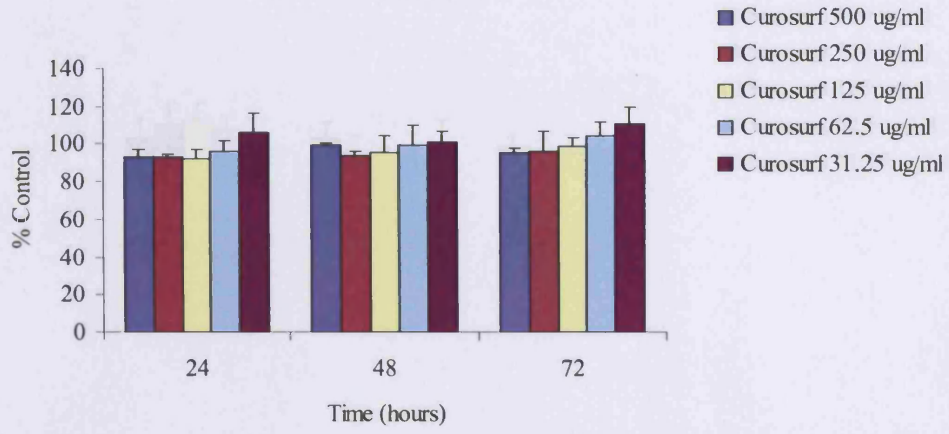
Prior to assessing the effect of pulmonary surfactant on inflammatory responses and cell signalling pathways induced in influenza virus treated airway epithelial cells it was essential to determine whether surfactant had any effect on cellular viability or viral entry in these cells.

Cell viability was assessed using the CellTiter96® AQ_{ueous} one solution cell proliferation assay. Three different batches of Curosurf® (batch # 061350, 066120 and 069097) and two different batches of Survanta® were tested (batch # 40-705-27 and 41-957-27). The following concentrations of pulmonary surfactant preparations were tested; 500, 250 and 125 μg/ml reflecting the physiological concentrations of pulmonary surfactant in lungs (Griese 1999). Briefly, BEAS-2B cells were incubated with Curosurf® or Survanta® for 2 hours before washing. Cell viability was determined at different time points (24, 48 and 72 hours).

The viability of BEAS-2B cells treated with Curosurf® (batch # 066120 and 069097) and Survanta® (batch # 40-705-27 and 41-957-27) was not significantly different from control cells with respect to reduction of novel tetrazolium salt. Across a wide range of concentrations of these pulmonary surfactant preparations were not toxic for BEAS-2B cells and viability of cells remained at greater than 90% under the various conditions assessed (Figure 3.2 and 3.3).

Surprisingly, Curosurf® batch # 061350 showed a strong cytotoxic effect on BEAS-2B cells, this was seen to be dose dependent. Viability of these cells incubated with 500 μg/ml of Curosurf® batch # 061350 for 2 hours was reduced to 47 and 43% after 48 and 72 hours, respectively. In addition, Curosurf® batch # 061350 also had a cytotoxic effect on A549 cells, however viability of these cells did not fall below 80% (Figure 3.4).

A



B

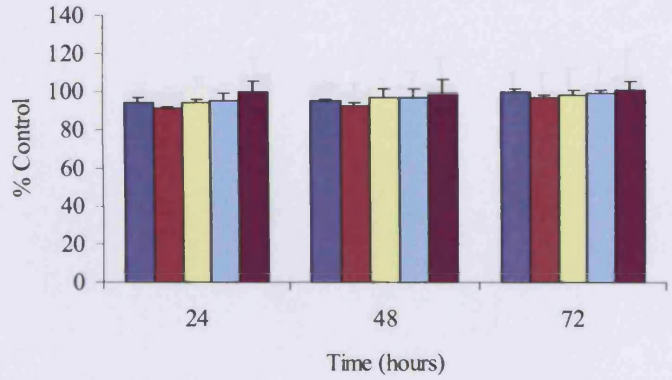
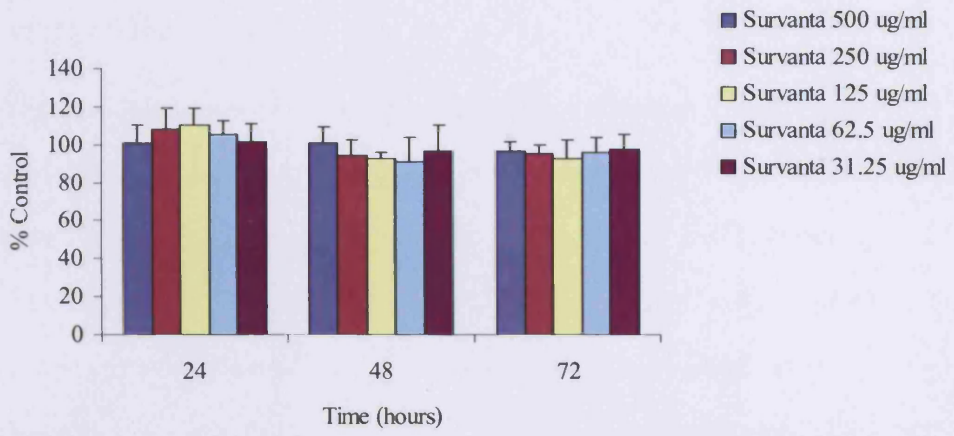


Figure 3.2: Effect of Curosurf® (batch # 066120 – panel A and batch # 069097 – panel B) on BEAS-2B cell viability. BEAS-2B cells were incubated with different concentrations of Curosurf® (500, 250, 125, 62.5 and 31.25 μg/ml) for 2 hours. Cell viability was determined at different time points (24, 48 and 72 hours). Each experimental condition was tested in quadruplicate. Results are expressed as mean ± 1 SD of 3 separate experiments. * $P < 0.05$ using unpaired t-test comparison.

A



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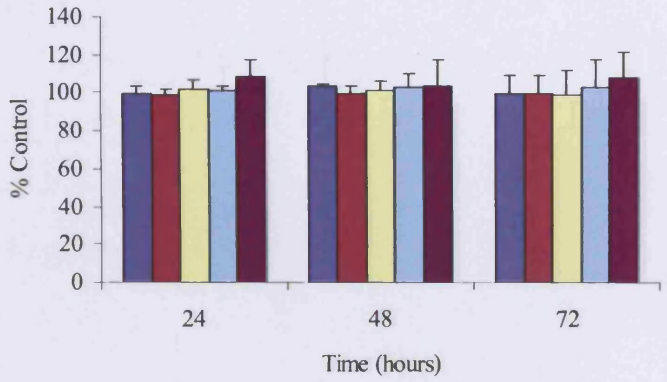
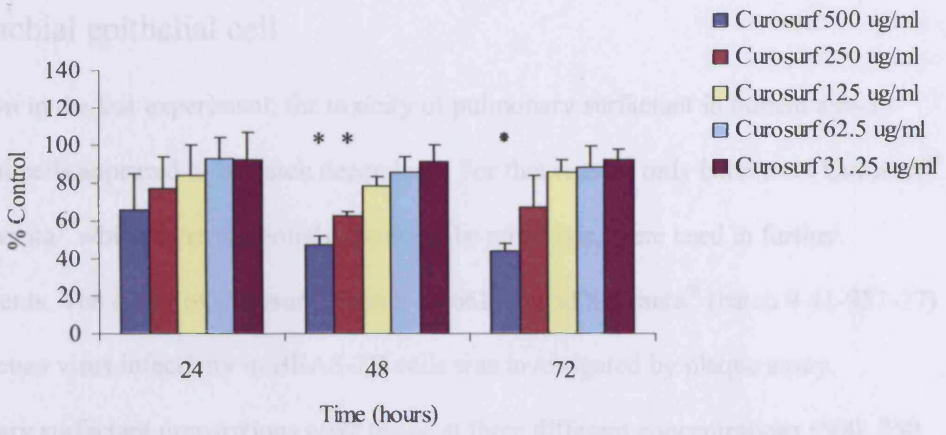


Figure 3.3: Effect of Survanta® (batch # 40-705-27 – panel A and batch # 41-957-27 – panel B) on BEAS-2B cell viability. BEAS-2B cells were incubated with different concentrations of Survanta® (500, 250, 125, 62.5 and 31.25 μg/ml) for 2 hours. Cell viability was determined at different time points (24, 48 and 72 hours). Each experimental condition was tested in quadruplicate. Results are expressed as mean ± 1 SD of 3 separate experiments. * $P < 0.05$ using unpaired t-test comparison.

A



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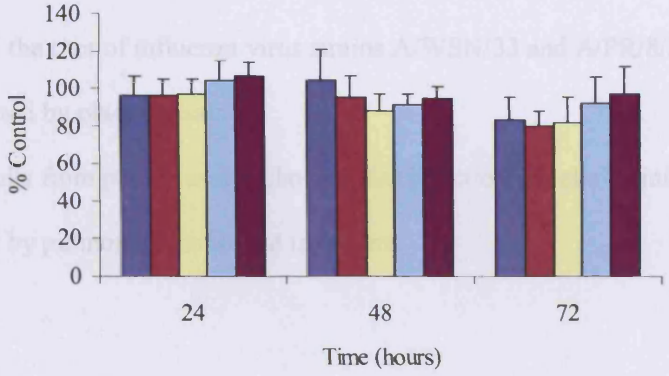


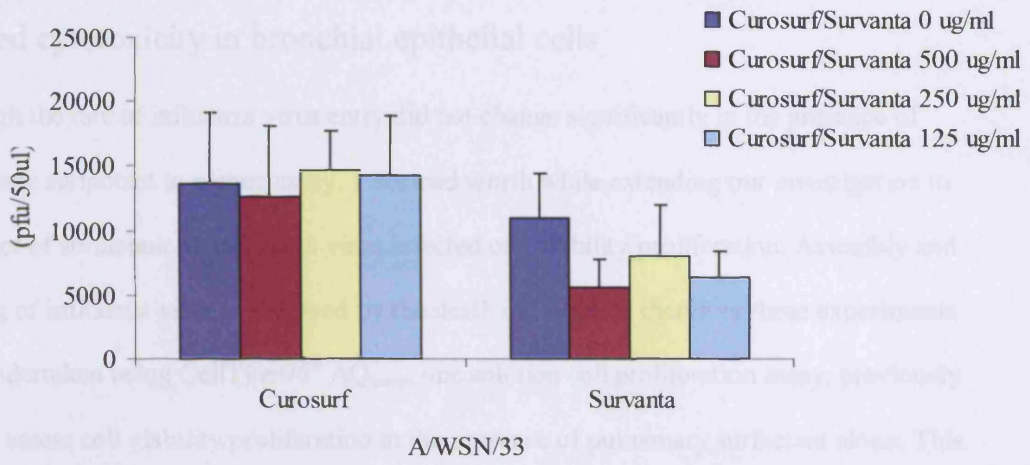
Figure 3.4: Effect of Curosurf® (batch # 061350) on BEAS-2B (panel A) and A549 cell (panel B) viability. BEAS-2B and A549 cells were incubated with different concentrations of Curosurf® (500, 250, 125, 62.5 and 31.25 μg/ml) for 2 hours. Cell viability was determined at different time points (24, 48 and 72 hours). Each experimental condition was tested in quadruplicate. Results are expressed as mean ± 1 SD of 3 separate experiments. * $P < 0.05$ using unpaired t-test comparison.

3.5 Pulmonary surfactant does not affect infectivity of influenza virus in bronchial epithelial cell

As shown in the last experiment, the toxicity of pulmonary surfactant in human airway epithelial cells appeared to be batch dependent. For that reason, only batches of Curosurf® and Survanta® which were previously shown to be non-toxic, were used in further experiments. The effect of Curosurf® (batch # 066120) and Survanta® (batch # 41-957-27) on influenza virus infectivity in BEAS-2B cells was investigated by plaque assay. Pulmonary surfactant preparations were tested at three different concentrations (500, 250 and 125 μg/ml). BEAS-2B cells were incubated with pulmonary surfactant for 2 hours and then, after washing, infected with influenza virus. The number of plaques was determined after 24 hours. Neither pre-incubation with Curosurf® nor pre-incubation with Survanta® changed the titer of influenza virus strains A/WSN/33 and A/PR/8/34 (Figure 3.5) as determined by plaque assay.

The results from plaque assays showed that infection of cells by influenza virus was not affected by pulmonary surfactant treatment.

A



B

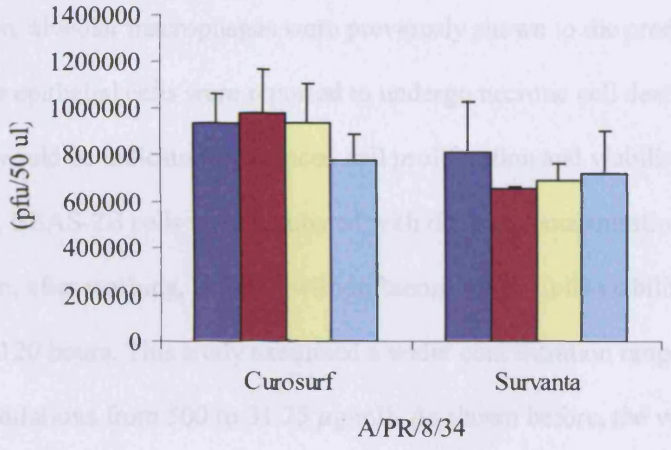


Figure 3.5: Effect of Curosurf® (batch # 066120) and Survanta® (batch # 41-957-27) on A/WSN/33 (panel A) and A/PR/8/34 (panel B) infectivity in BEAS-2B cells. BEAS-2B cells were incubated with different concentrations of Curosurf® or Survanta® (500, 250 and 125 μg/ml) for 2 hours, washed and then infected with influenza virus strain A/WSN/33 or A/PR/8/34. The number of plaques was determined after 24 hours. Results are expressed as mean ± 1 SD of 3 separate experiments.

3.6 Pre-treatment with Survanta® does not change influenza virus induced cytotoxicity in bronchial epithelial cells

Although the rate of influenza virus entry did not change significantly in the presence of pulmonary surfactant in plaque assay, it seemed worth while extending our investigation to the effect of surfactant on influenza virus infected cell viability/proliferation. Assembly and budding of influenza virus is followed by the death of host cell, therefore these experiments were undertaken using CellTiter96® AQ_{ueous} one solution cell proliferation assay, previously used to assess cell viability/proliferation in the presence of pulmonary surfactant alone. This colorimetric method was used to determine the effect of Survanta® (batch # 40-705-27) pre-treatment on influenza virus infected bronchial epithelial cells. As a result of influenza virus infection, alveolar macrophages were previously shown to die predominantly by apoptosis, whereas epithelial cells were reported to undergo necrotic cell death (Fesq et al. 1994), which would be indicated by reduced cell proliferation and viability.

Briefly, BEAS-2B cells were incubated with different concentration of Suvanta® for 2 hours and then, after washing, infected with influenza virus. Cells viability was measured after 24, 72 and 120 hours. This study examined a wider concentration range of Survanta® (five double dilutions from 500 to 31.25 μg/ml). As shown before, the viability of BEAS-2B cells treated with Survanta® batch # 40-705-27 was not significantly different from the viability of untreated cells with respect to their reductive capacity of tetrazolium compound. Different concentrations of this batch of pulmonary surfactant were not toxic for BEAS-2B cells and viability of these cells was greater than 90% at all time points tested (Figure 3.6 and 3.7).

In contrast, influenza virus strain A/WSN/33 and A/PR/8/34 showed a strong cytotoxic effect on BEAS-2B cells which was clearly time dependent. Although the titer determined by TCID50 was the same for both strains in BEAS-2B cells ($3.2x10^4$ TCID50/ml) (Table

3.2), this effect was more pronounced for strain A/WSN/33. This result suggests that CellTiter96® AQ_{ueous} one solution cell proliferation assay is a more sensitive method measuring the cytopathic effect of influenza virus than TCID50. Viability of BEAS-2B cells incubated with A/WSN/33 strain at the MOI of 2 was reduced to 80, 45 and 27% after 24, 72 and 120 hours, respectively (Figure 3.6). Viability of BEAS-2B cells incubated with A/PR/8/34 strain at the MOI 2 was reduced to 92, 66 and 65% after 24, 72 and 120 hours, respectively (Figure 3.7).

Pre-incubation with Survanta®, at all concentrations tested, did not change reduced proliferation rates induced by influenza virus induced death of BEAS-2B cells (Figure 3.6 and 3.7).

In summary, neither the rate of the virus infectivity (higher for A/PR/8/34 strain, as shown by plaque assay) nor the negative effect of virus on cell proliferation (higher for A/WSN/33, as shown by proliferation assay) were affected by pulmonary surfactant treatment therefore, in the next step, the main attention was directed to investigate the effect of pulmonary surfactant on the production of inflammatory mediators during influenza virus infection.

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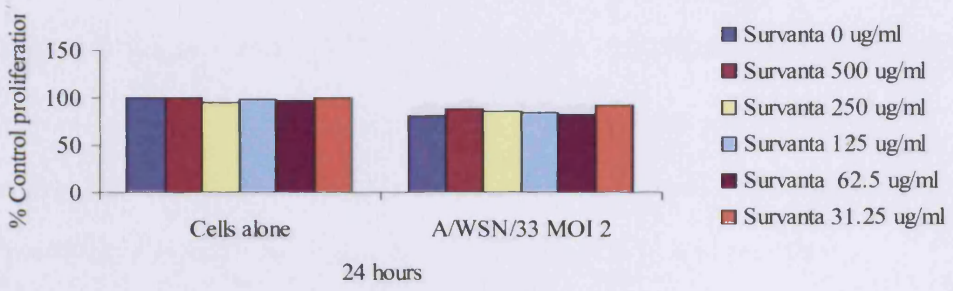

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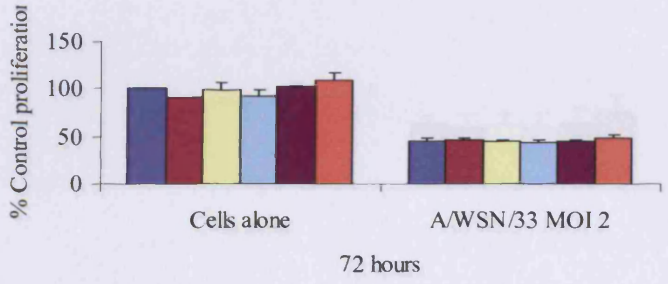

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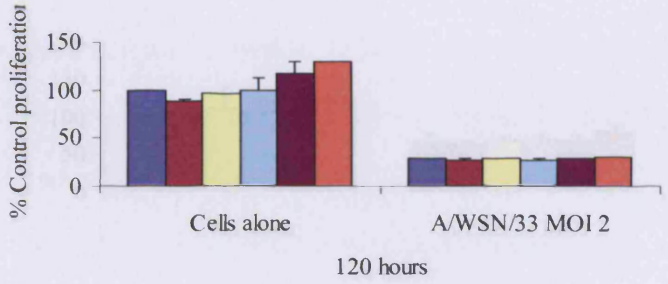

Figure 3.6: Effect of Survanta® (batch # 40-705-27) on BEAS-2B cell viability upon A/WSN/33 infection. BEAS-2B cells were incubated with different concentrations of Survanta® (500, 250125. 62.5 and 31.25 μg/ml) for 2 hours and then infected with influenza virus strain A/WSN/33 at MOI 2. Cell viability was determined after 24 (panel A), 72 (panel B) and 120 hours (panel C). Each experimental condition was tested in quadruplicate. Results are expressed as mean ± 1 SD of 2 separate experiments.

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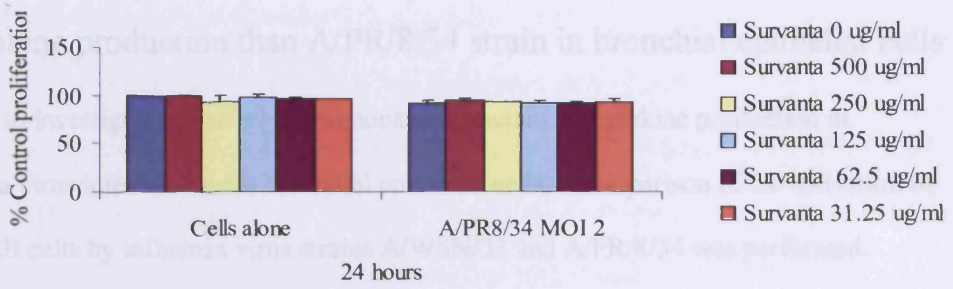

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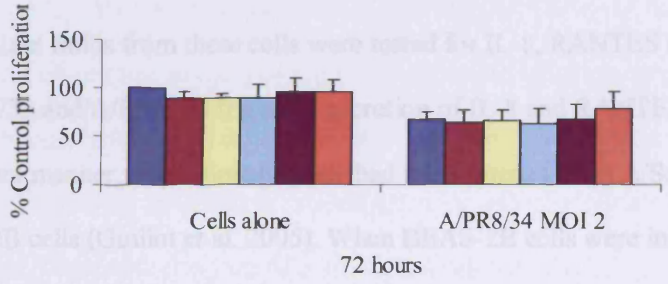

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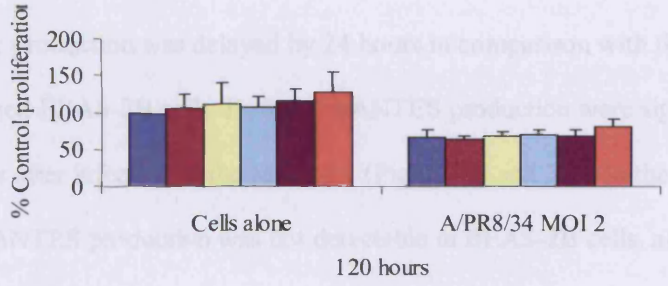

Figure 3.7: Effect of Survanta® (batch # 40-705-27) on BEAS-2B cell viability upon A/PR/8/34 infection. BEAS-2B cells were incubated with different concentrations of Survanta® (500, 250125. 62.5 and 31.25 μg/ml) for 2 hours and then infected with influenza virus strain A/PR/8/34 at MOI 2. Cell viability was determined after 24 (panel A), 72 (panel B) and 120 hours (panel C). Each experimental condition was tested in quadruplicate. Results are expressed as mean ± 1 SD of 2 separate experiments.

3.7 Influenza virus strain A/WSN/33 strain is a more potent inducer of cytokine production than A/PR/8/34 strain in bronchial epithelial cells

In order to investigate the effect of pulmonary surfactant on cytokine production in influenza virus infected human bronchial epithelial cells, a comparison of the activation of BEAS-2B cells by influenza virus strains A/WSN/33 and A/PR/8/34 was performed. Monolayers of BEAS-2B cells were challenged for various times (12, 24 and 48 hours) with increasing concentrations of influenza virus (MOI 0.0001, 0.001, 0.01, 0.1 and 1). Supernatant fluids from these cells were tested for IL-8, RANTES and IL-6 by ELISA. Both A/WSN/33 and A/PR/8/34 triggered secretion of IL-8 and RANTES in a MOI and time dependent manner, as previously described for influenza virus A/Scotland/20/74 (H3N2) in BEAS-2B cells (Guillot et al. 2005). When BEAS-2B cells were infected with A/WSN/33 strain IL-8 and RANTES secretion was detected after 24 hours with a MOI of 0.1, reaching the maximum 48 hours after infection at the MOI of 1. Upon infection with A/PR/8/34 cytokine production was delayed by 24 hours in comparison with that for A/WSN/33 challenged BEAS-2B cells. IL-8 and RANTES production were significantly induced only 48 hours after infection at the MOI of 1 (Figure 3.8 and 3.9). In the absence of influenza virus RANTES production was not detectable in BEAS-2B cells, although these cells demonstrated some background IL-8 release. BEAS-2B cells infected with influenza virus A/WSN/33 strain at the MOI 1 also produced higher level of IFN-β than BEAS-2B cells infected with influenza virus A/PR/8/34 strain at the same MOI, 24 hours post-infection (Figure 3.10). Surprisingly in contrast to influenza virus A/Scotland/20/74 strain, none of the influenza virus strains used in this study induced IL-6 production in BEAS-2B cells (data not shown). Influenza virus strain A/WSN/33 was a more potent inducer of IL-8, RANTES and IFN-β production than A/PR/8/34 strain in BEAS-2B cells which might be

associated with higher cytopathogenicity of this strain, as shown by us in CellTiter96® AQ_{ueous} one solution cell proliferation assay (section 3.6).

During influenza virus infection, dsRNA has been previously shown to accumulate and induce inflammatory and antiviral response within infected cells (Jacobs & Langland 1996; Majde 2000). For that reason it was important to determine whether influenza virus induced activation of bronchial epithelial cells resembles that stimulated by a synthetic dsRNA such as poly(I-C). In BEAS-2B cells, 50 μg/ml of poly(I-C) triggered higher levels of secretion of IL-8 and RANTES than influenza virus at the MOI of 1 after 24 hours (Figure 3.10). IL-8 and RANTES production peaked after 48 hours after influenza virus infection, suggesting that more time is required to generate dsRNA within the infected cells, during the replication of the virus, which is necessary for induction of these chemokines. These results are in agreement with previous data which indicates that poly(I-C) induced the secretion of IL-8 within 3 hours and accumulated up to 24 hours whereas the secretion of RANTES was induced within 6 hours and also accumulated in the culture medium up to 24 hours (Guillot et al. 2005). The same group showed that upon poly(I-C) stimulation IFN-β production reached the maximum level 6 hours post-stimulation but was not detected after 24 hours. Referring to these observations in our experiments, the production of IFN-β in poly(I-C) stimulated BEAS-2B cells was scarce in comparison to influenza virus treated cells after 24 hours (Figure 3.10).

Finally, influenza virus and poly(I-C) induced cytokine production was compared to that caused by measles virus Edmonston strain. Measles virus at the MOI as low as 0.01 triggered a stronger IL-8 production than influenza virus with MOI of 1 and 50 μg/ml of poly(I-C) after 24 hours in BEAS-2B cells (Figure 3.10). These results are in agreement with data by Sato and co-workers, who showed that in contrast to influenza virus, replication of measles virus Edmonston strain was not required for induction of IL-8 protein,

in alveolar epithelial cells A549 (Sato et al. 2005). Induced synthesis of IL-8 is probably caused by interaction of measles virus with cell surface TLRs and/or incorporation of viral particles. This also affects RANTES production since measles virus at the MOI 0.01 triggered lower RANTES production than 50 μg/ml of poly(I-C) after 24 hours in BEAS-2B cells (Figure 3.10). Measles virus did not induce IFN-β in BEAS-2B, 24 hours post infection (p.i.).

Two receptors have been proposed to be involved in inflammatory and anti-viral response in influenza infected BEAS-2B cells (Le Goffic et al. 2007). TLR3 has been shown to mediate NF-κB dependent gene expression whereas RIG-I has been shown to mediate both pro-inflammatory and anti-viral signalling pathways.

In summary, the best ELISA MOI for IL-8 and RANTES induction by the two influenza virus strains used was an MOI of 1. The best time was 24 hours p.i. for A/WSN/33 and 48 hours p.i. for A/PR/8/34.

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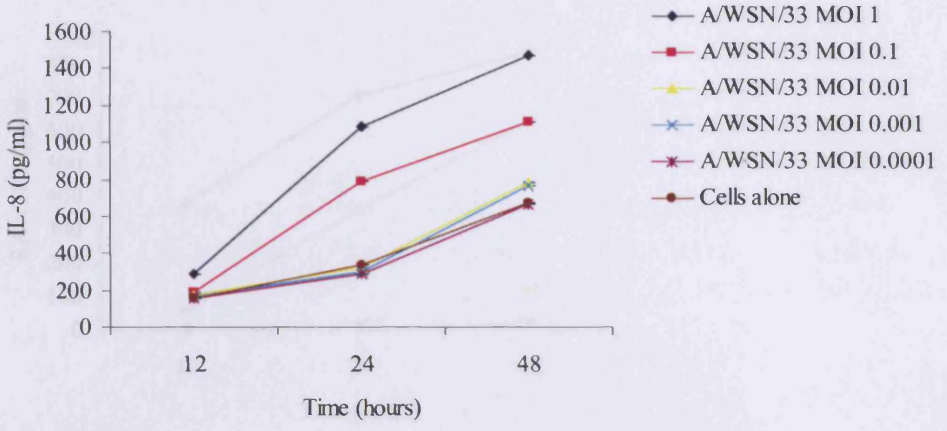

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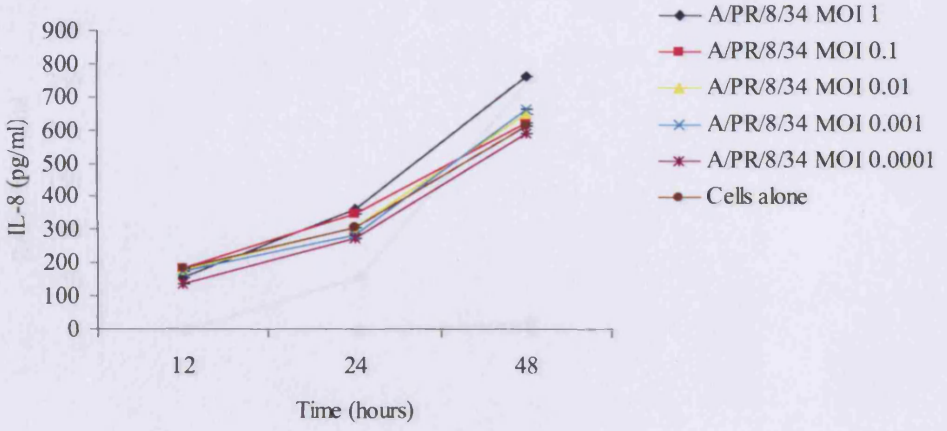

Figure 3.8: Effect of A/WSN/33 (panel A) and A/PR/8/34 (panel B) on BEAS-2B cell IL-8 production. BEAS-2B cells were infected with influenza virus strain A/WSN/33 or A/PR/8/34 at different MOIs (1, 0.1, 0.01, 0.001 and 0.0001). Cell IL-8 production was determined after 12, 24 and 48 hours. Each experimental condition was tested in duplicate. Results are expressed as mean ± 1 SD of representative of 3 separately performed experiments with similar results.

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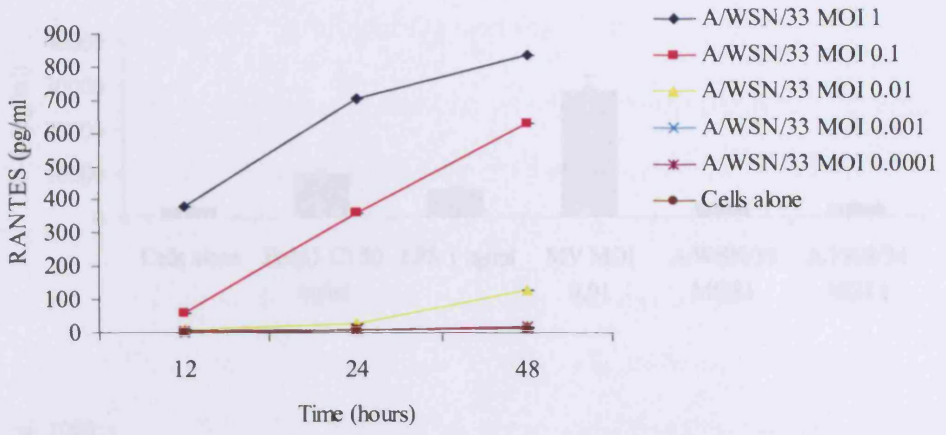

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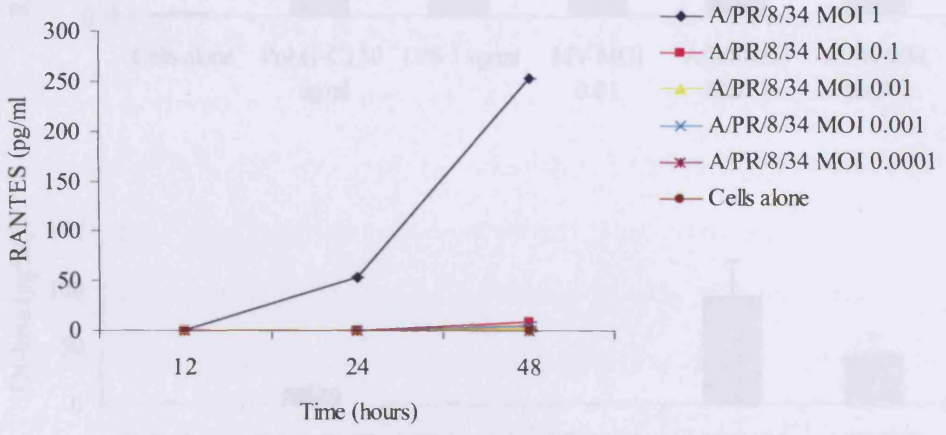

Figure 3.9: Effect of A/WSN/33 (panel A) and A/PR/8/34 (panel B) on BEAS-2B cell RANTES production. BEAS-2B cells were infected with influenza virus strain A/WSN/33 or A/PR/8/34 at different MOIs (1, 0.1, 0.01, 0.001 and 0.0001). Cell RANTES production was determined after 12, 24 and 48 hours. Each experimental condition was tested in duplicate. Results are expressed as mean ± 1 SD of representative of 3 separately performed experiments with similar results.

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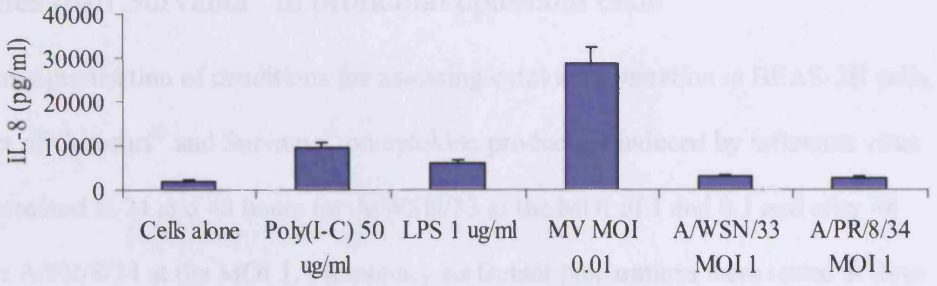

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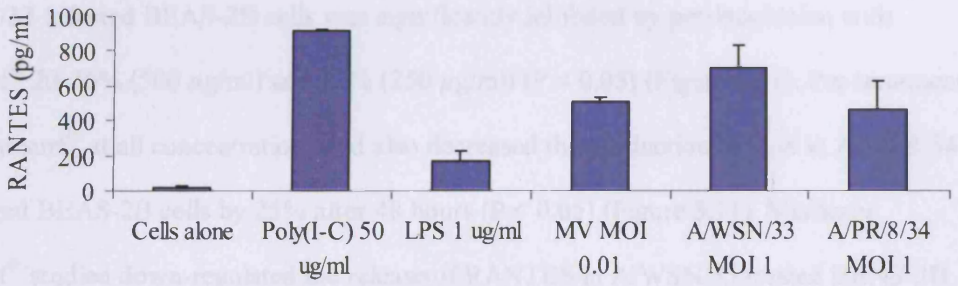

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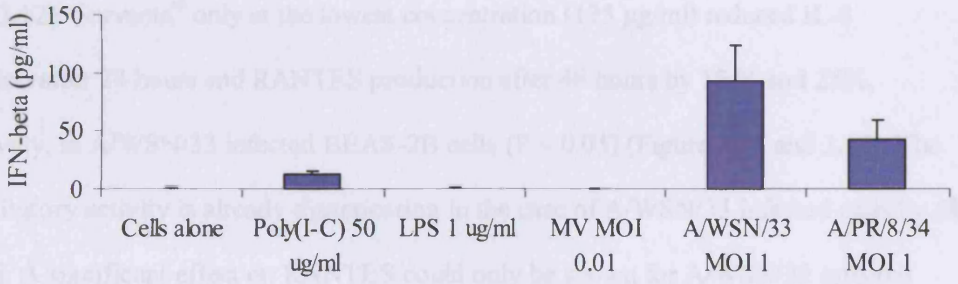

Figure 3.10: Effect of poly(I-C), LPS, measles virus and influenza virus on BEAS-2B cell IL-8, RANTES and IFN-β production. BEAS-2B cells were stimulated with poly(I-C) (50 μg/ml) or LPS (1 μg/ml) or infected with measles virus (MOI 0.01) or influenza virus (MOI 1). Cytokine production was determined after 24 hours. Results are expressed as mean ± 1 SD of 3 separate experiments.

3.8 Curosurf® is a more potent inhibitor of key inflammatory cytokines than Survanta® in bronchial epithelial cells

Following optimisation of conditions for assessing cytokine generation in BEAS-2B cells, the effect of Curosurf® and Survanta® on cytokine production induced by influenza virus was determined at 24 and 48 hours for A/WSN/33 at the MOI of 1 and 0.1 and after 48 hours for A/PR/8/34 at the MOI 1. Pulmonary surfactant preparations were tested at three different concentrations (500, 250 and 125 μg/ml). After 24 hours the release of IL-8 in A/WSN/33 infected BEAS-2B cells was significantly inhibited by pre-incubation with Curosurf® 20-30% (500 μg/ml) and 12% (250 μg/ml) ($P < 0.05$) (Figure 3.11). Pre-treatment with Curosurf® at all concentration used also decreased the production of IL-8 in A/PR/8/34 challenged BEAS-2B cells by 25% after 48 hours ($P < 0.05$) (Figure 3.11). Similarly Curosurf® studied down-regulated the release of RANTES in A/WSN/33 treated BEAS-2B cells after 24 hours by 35% (500 μg/ml), 12% (250 μg/ml) and 22% (125 μg/ml) ($P < 0.05$) (Figure 3.12). Survanta® only at the lowest concentration (125 μg/ml) reduced IL-8 production after 24 hours and RANTES production after 48 hours by 15 % and 25%, respectively, in A/WSN/33 infected BEAS-2B cells ($P < 0.05$) (Figure 3.13 and 3.14). The IL-8 inhibitory activity is already disappearing in the case of A/WSN/33 infected cells by 48 hours p.i. A significant effect on RANTES could only be shown for A/WSN/33 infected BEAS-2B cells.

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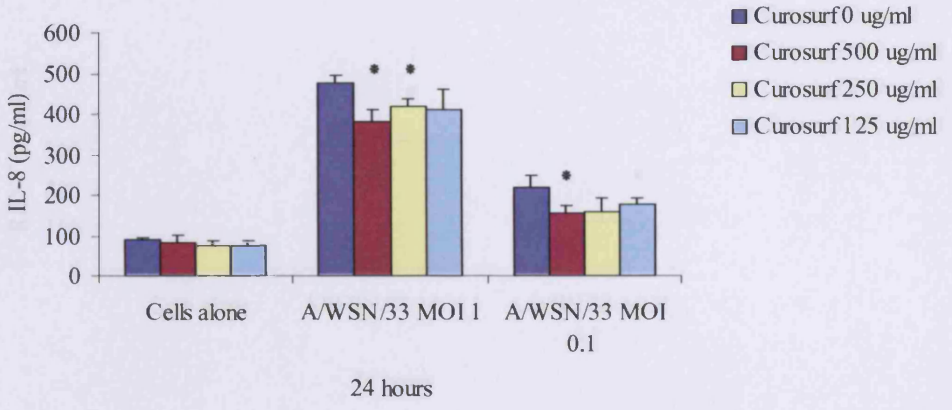

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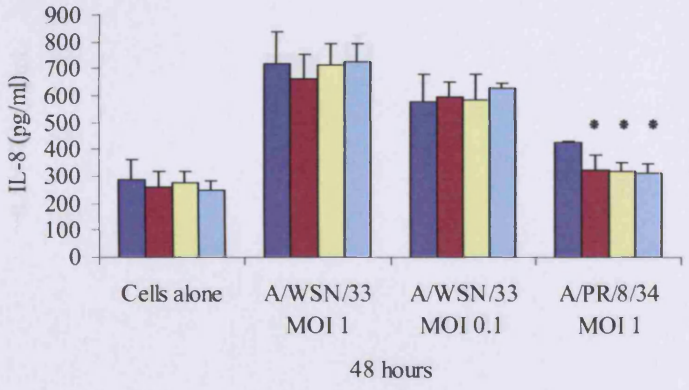

Figure 3.11: Effect of Curosurf® (batch # 066120) on BEAS-2B cell IL-8 production upon A/WSN/33 and A/PR/8/34 infection. BEAS-2B cells were incubated with different concentrations of Curosurf® (500, 250 and 125 μg/ml) for 2 hours, washed and then infected with influenza virus strain A/WSN/33 or A/PR/8/34. IL-8 production was determined after 24 (panel A) and 48 hours (panel B). Each experimental condition was tested in duplicate. Results are expressed as mean ± 1 SD of 3 separate experiments. * $P < 0.05$ using unpaired t-test comparison.

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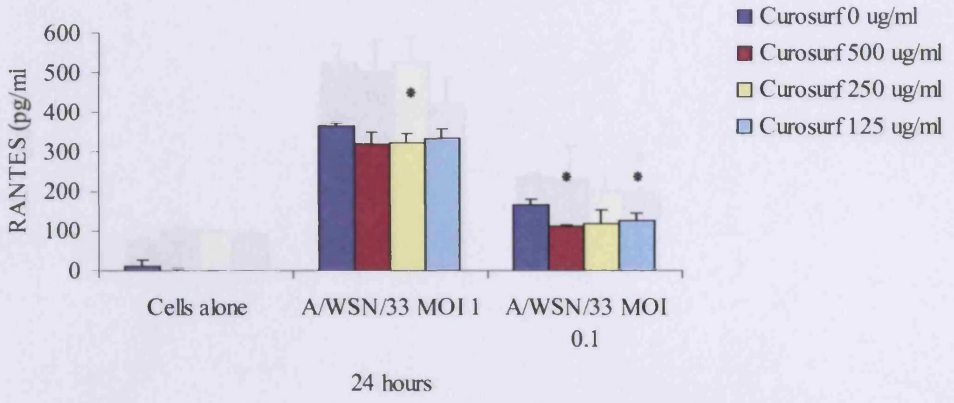

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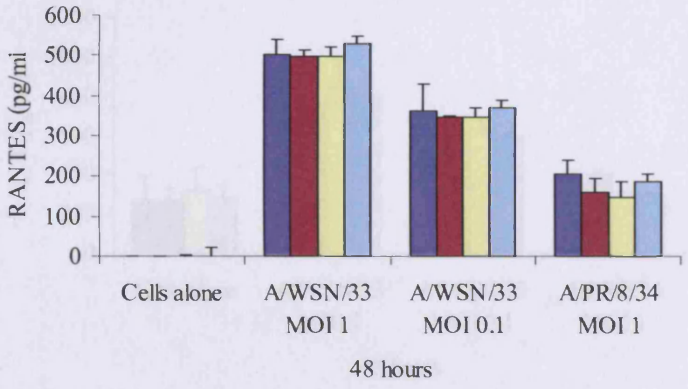

Figure 3.12: Effect of Curosurf® (batch # 066120) on BEAS-2B cell RANTES production upon A/WSN/33 and A/PR/8/34 infection. BEAS-2B cells were incubated with different concentrations of Curosurf® (500, 250 and 125 μg/ml) for 2 hours, washed and then infected with influenza virus strain A/WSN/33 or A/PR/8/34. RANTES production was determined after 24 hours. Each experimental condition was tested in duplicate. Results are expressed as mean ± 1 SD of 3 separate experiments. * $P < 0.05$ using unpaired t-test comparison.

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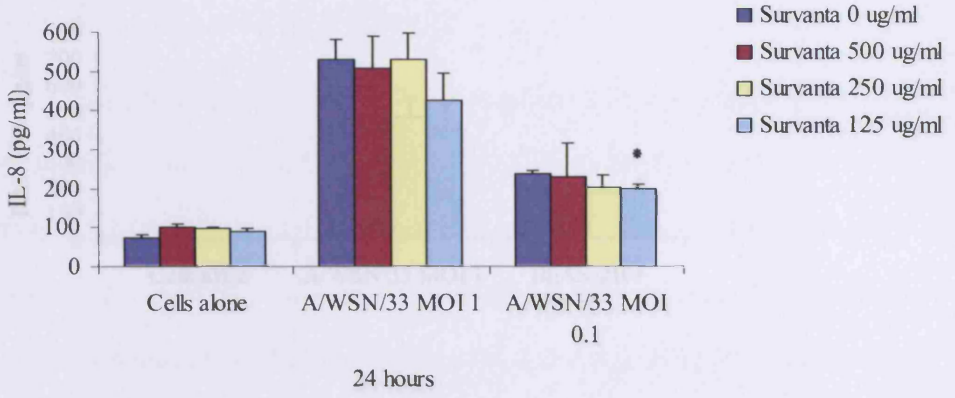

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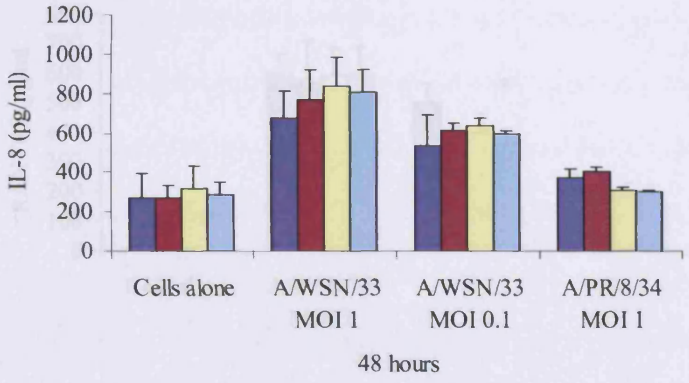

Figure 3.13: Effect of Survanta® (batch # 41-957-27) on BEAS-2B cell IL-8 production upon A/WSN/33 and A/PR/8/34 infection. BEAS-2B cells were incubated with different concentrations of Survanta® (500, 250 and 125 μg/ml) for 2 hours, washed and then infected with influenza virus strain A/WSN/33 or A/PR/8/34. IL-8 production was determined after 24 (panel A and 48 hours (panel B). Each experimental condition was tested in duplicate. Results are expressed as mean ± 1 SD of 3 separate experiments. * $P < 0.05$ using unpaired t-test comparison.

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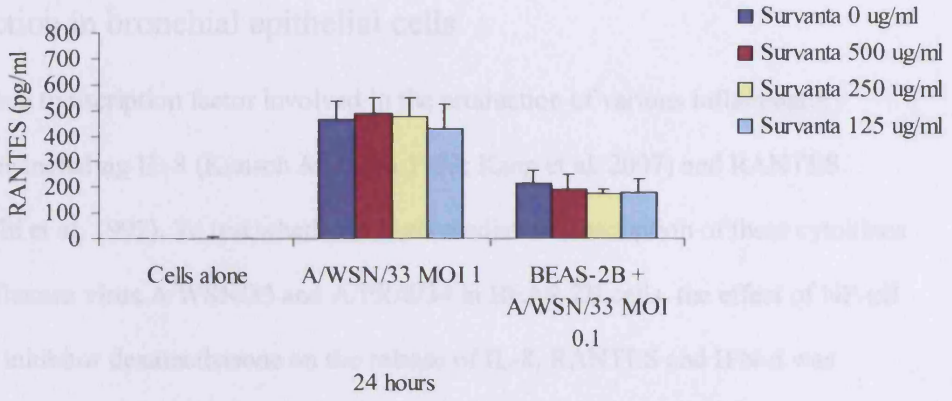

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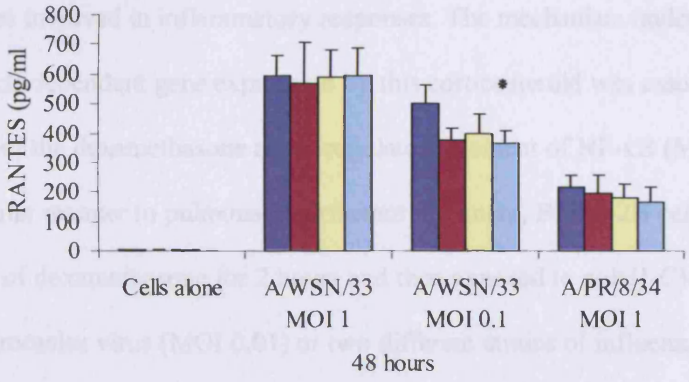

Figure 3.14: Effect of Survanta® (batch # 41-957-27) on BEAS-2B cell RANTES production upon A/WSN/33 and A/PR/8/34 infection. BEAS-2B cells were incubated with different concentrations of Survanta® (batch # 41-957-27) (500, 250 and 125 μg/ml) for 2 hours, washed and then infected with influenza virus strain A/WSN/33 or A/PR/8/34. RANTES production was determined after 24 (panel A) and 48 hours (panel B). Each experimental condition was tested in duplicate. Results are expressed as mean ± 1 SD of 3 separate experiments. * $P < 0.05$ using unpaired t-test comparison.

3.9 Dexamethasone inhibits IL-8 but not RANTES or IFN-β production in bronchial epithelial cells

NF-κB is a transcription factor involved in the production of various inflammatory cytokines including IL-8 (Kunsch & Rosen 1993; Kang et al. 2007) and RANTES (Moriuchi et al. 1997). To test whether NF-κB mediates transcription of these cytokines upon influenza virus A/WSN/33 and A/PR/8/34 in BEAS-2B cells, the effect of NF-κB specific inhibitor dexamethasone on the release of IL-8, RANTES and IFN-β was investigated in these cells. Dexamethasone is a corticosteroid which is widely used as an anti-inflammatory agent. It is able to down-regulate the cellular release of different cytokines involved in inflammatory responses. The mechanism underlying the suppression of NF-κB dependent gene expression by this corticosteroid was associated with the direct binding of the dexamethasone to cis-regulatory element of NF-κB (Mukaida et al. 1994). In a similar manner to pulmonary surfactant treatment, BEAS-2B cells were incubated with 1 μg/ml of dexamethasone for 2 hours and then exposed to poly(I-C) (50 μg/ml), LPS (1 μg/ml), measles virus (MOI 0.01) or two different strains of influenza virus (MOI 1) (A/WSN/33 and A/PR/8/34). The concentration of dexamethasone which showed the strongest inhibitory effect on LPS induced IL-8 production in BEAS-2B cells without showing any toxic effect in these cells was used in this experiment (data not shown). Only IL-8 production, triggered by all stimulants, was significantly inhibited by pre-incubation with 1 μg/ml of dexamethasone (by 37, 30, 26, 33 and 49%, respectively) ($P < 0.05$) (Figure 3.15). In all cases, the inhibition was not complete, suggesting participation of other transcription factors in IL-8 production. No effect of dexamethasone pre-treatment on RANTES and IFN-β release was observed (Figure 3.15), suggesting that different transcription factors are involved in expression of these cytokines expression. Interestingly,

the range of inhibition found in the presence of dexamethasone was similar to that found in the presence of Curosurf®.

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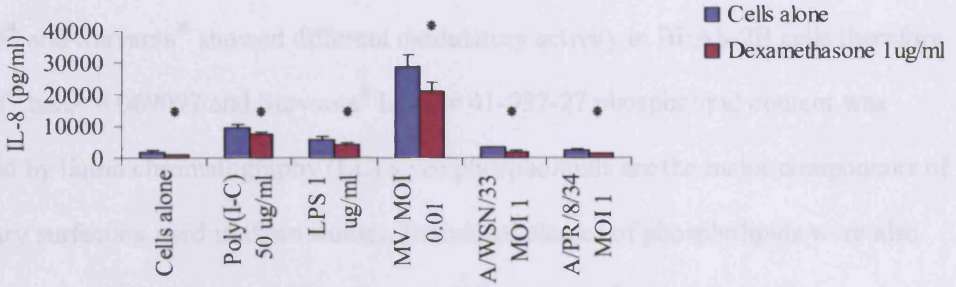

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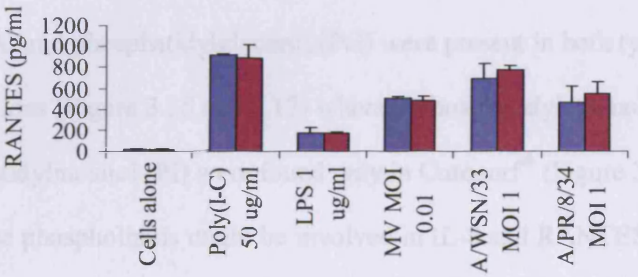

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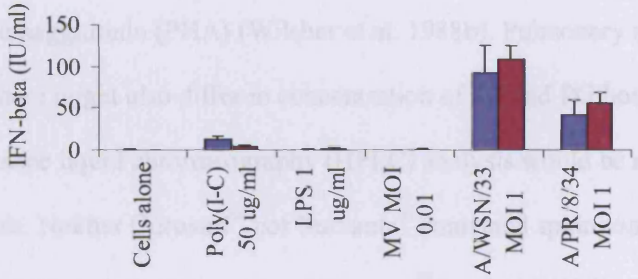


Figure 3.15: Effect of dexamethasone on BEAS-2B cell IL-8, RANTES and IFN-β production. BEAS-2B cells were incubated with 1 μg/ml of dexamethasone for 2 hours and then stimulated with poly(I-C) (50 μg/ml) or LPS (1 μg/ml) or infected with measles virus (MOI 0.01) or influenza virus (MOI 1). Cytokine production was determined after 24 hours. Results are expressed as mean ± SD of 3 separate experiments. * $P < 0.05$ using unpaired t-test comparison.

3.10 Curosurf® and Survanta® differ in phospholipid content

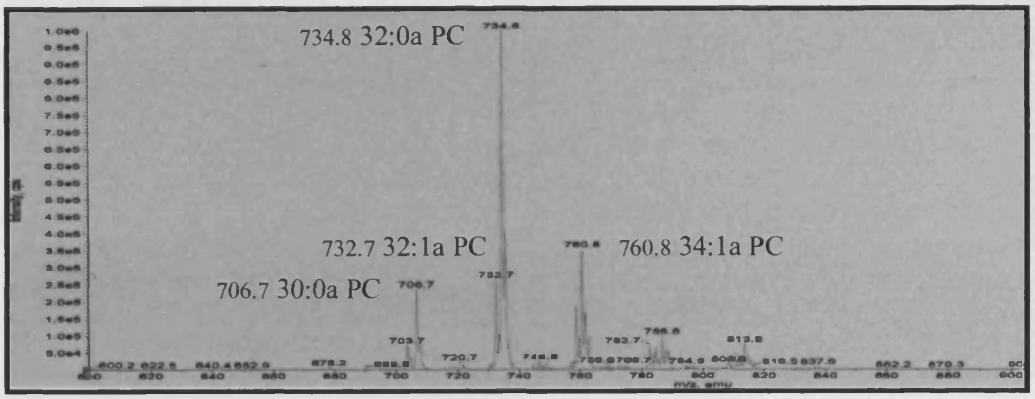
Curosurf® and Survanta® showed different modulatory activity in BEAS-2B cells therefore Curosurf® batch # 069097 and Survanta® batch # 41-957-27 phospholipid content was compared by liquid chromatography (LC) since phospholipids are the major components of pulmonary surfactant used in these studies. Individual classes of phospholipids were also assessed by mass spectrometry (MS) and some differences in fatty acids profiles were detected. Analysis of LC-MS profiles indicates that phosphatidylcholine (PC), phosphatidic acid (PA) and phosphatidylglycerol (PG) were present in both types pulmonary surfactant preparations (Figure 3.16 and 3.17) whereas phosphatidylethanolamine (PE) phosphatidylinositol (PI) were found only in Curosurf® (Figure 3.18 and 3.19), suggesting that these phospholipids might be involved in IL-8 and RANTES inhibition in influenza virus infected BEAS-2B cells. Previously, PC, PG and PI were shown to be the key phospholipids of pulmonary surfactant suppressing lymphocytes response to phytohaemagglutinin (PHA) (Wilsher et al. 1988b). Pulmonary surfactant preparations studied here might also differ in concentration of PC and PG however additional high performance liquid chromatography (HPLC) analysis would be necessary to conclude if this is the case. Neither Curosurf® nor Survanta® contained sphingomyelin (SM) (Figure 3.16). In addition phospholipids profile of Curosurf® batch # 061350 which showed strong cytotoxic effect on BEAS-2B cells was compared to non-toxic Curosurf® batch # 069097, however no differences between these preparations were observed (Figure 3.16, 3.17, 3.18 and 3.19).

The observed variations in phoslipid composition between Curosurf® and Survanta® might be caused by different source of pulmonary surfactant (porcine versus bovine) or different preparation methods. Production of Curosurf® includes removing of neutral lipids by gel-

liquid chromatography whereas Survanta® is supplemented with DPPC, triglicerides and palmitic acid.

Identification of components responsible for observed differences would require significant chemical investigation and assessment of individual components biological activities which is beyond scope of this study.

A



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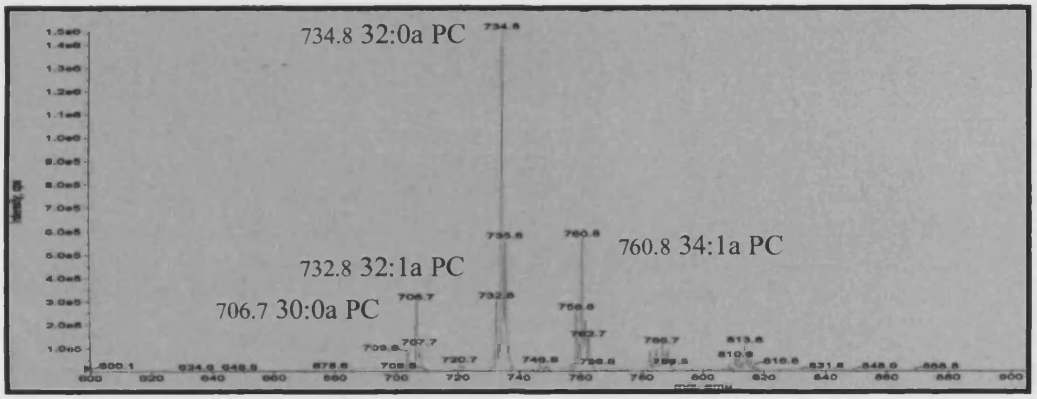

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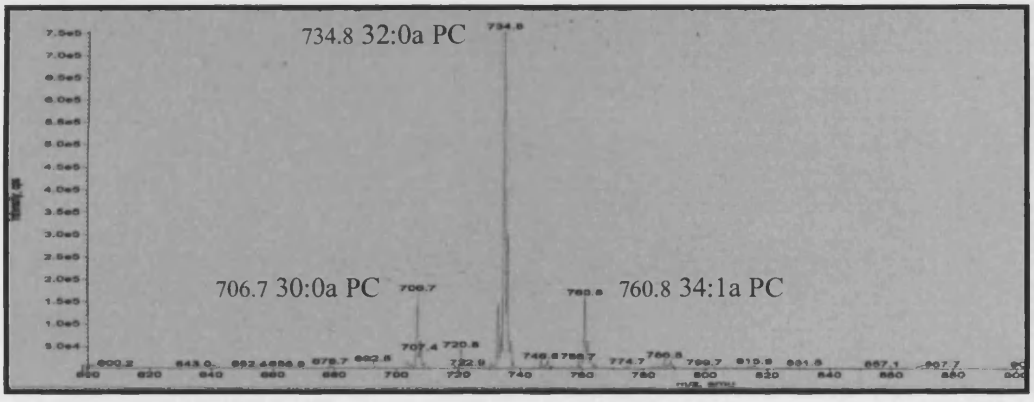
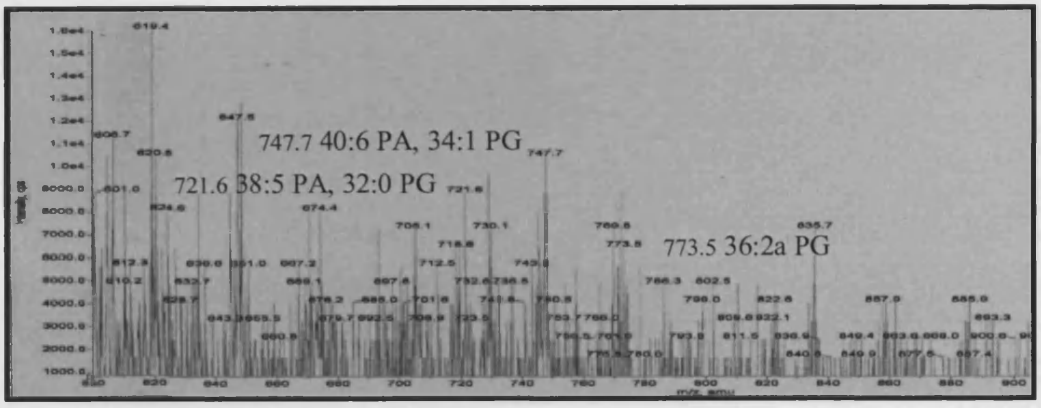


Figure 3.16: Mass spectrometry of Curosurf® (batch # 061350) (panel A), Curosurf® (batch # 069097) (panel B) and Survanta® (batch # 41-957-27) (panel C) – phosphatidylcholine (PC) / sphingomyelin (SM).

A



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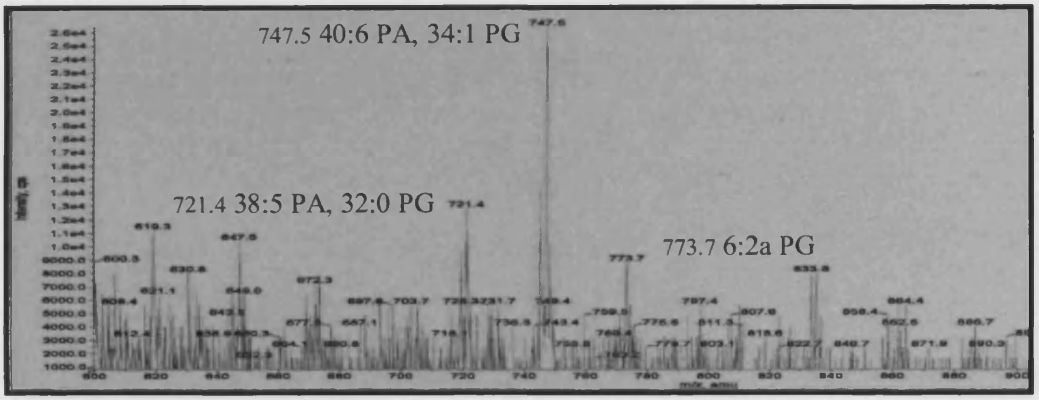

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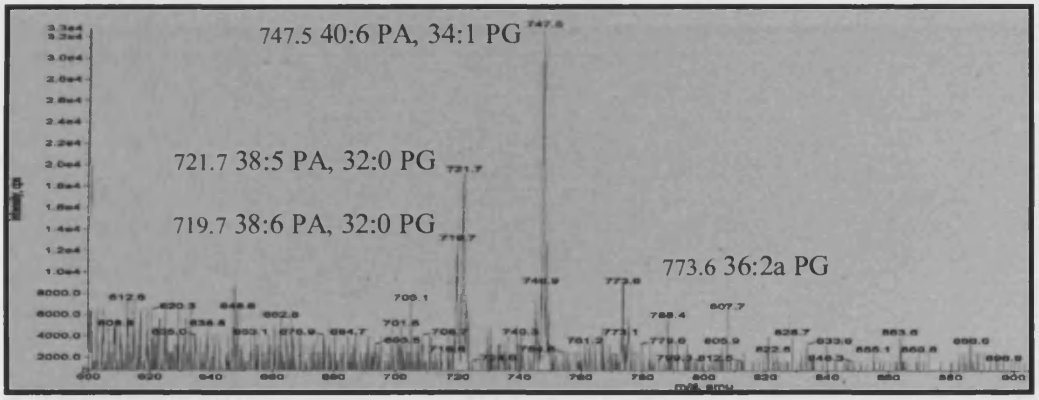
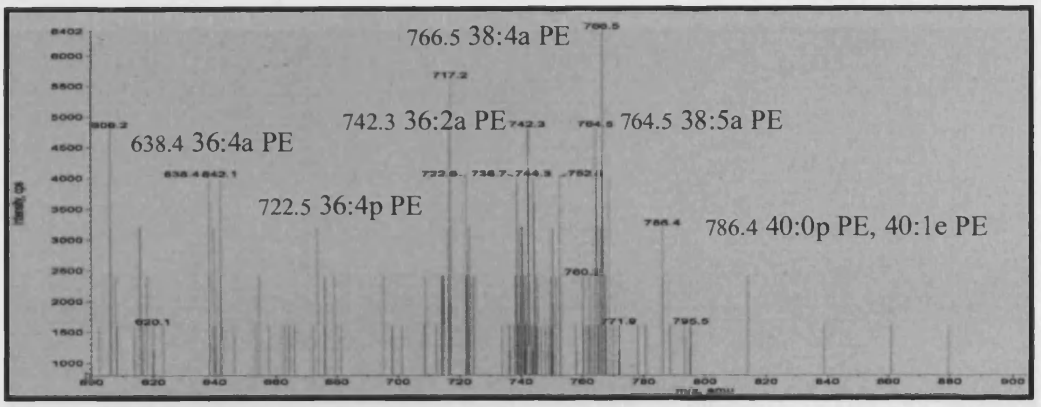


Figure 3.17: Mass spectrometry of Curosurf® (batch # 061350) (panel A), Curosurf® (batch # 069097) (panel B) and Survanta® (batch # 41-957-27) (panel C) – phosphatidic acid (PA) / phosphatidylglycerol (PG).

A



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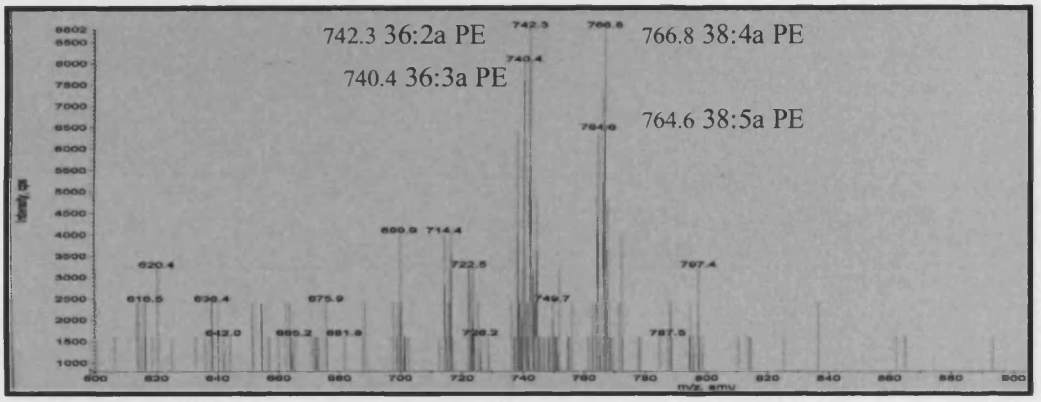

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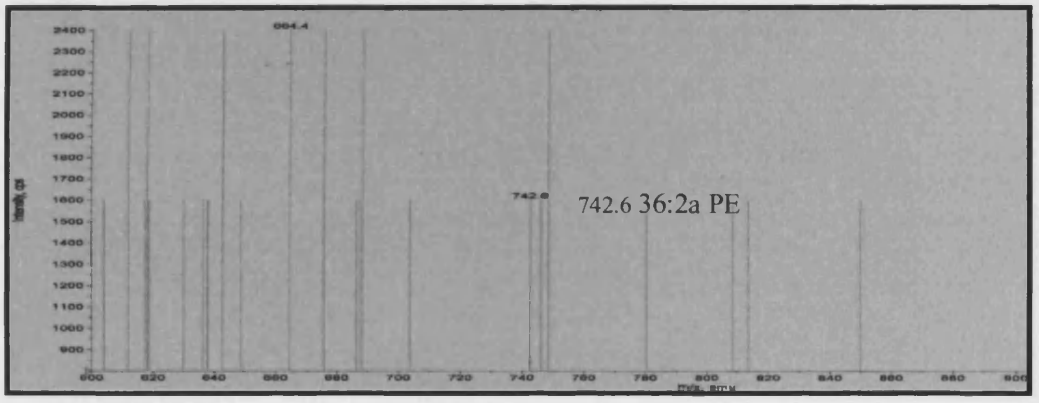
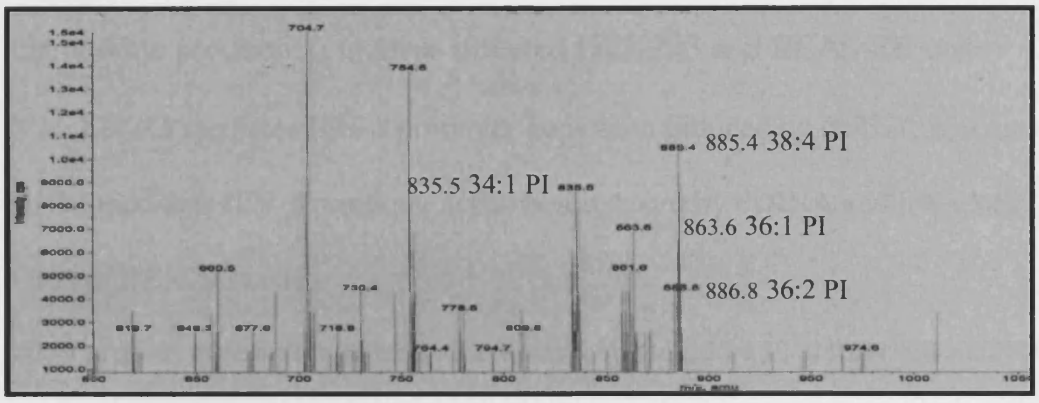


Figure 3.18: Mass spectrometry of Curosurf® (batch # 061350) (panel A), Curosurf® (batch # 069097) (panel B) and Survanta® (batch # 41-957-27) (panel C) – phosphatidylethanolamine (PE).

A



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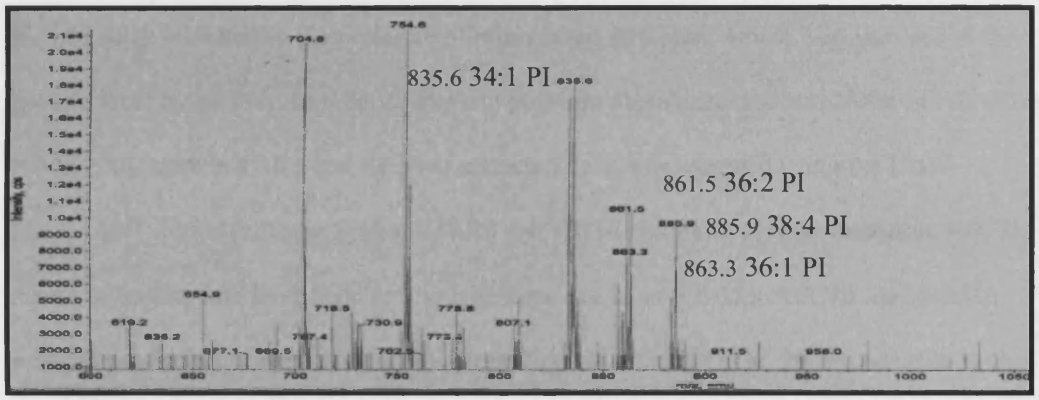

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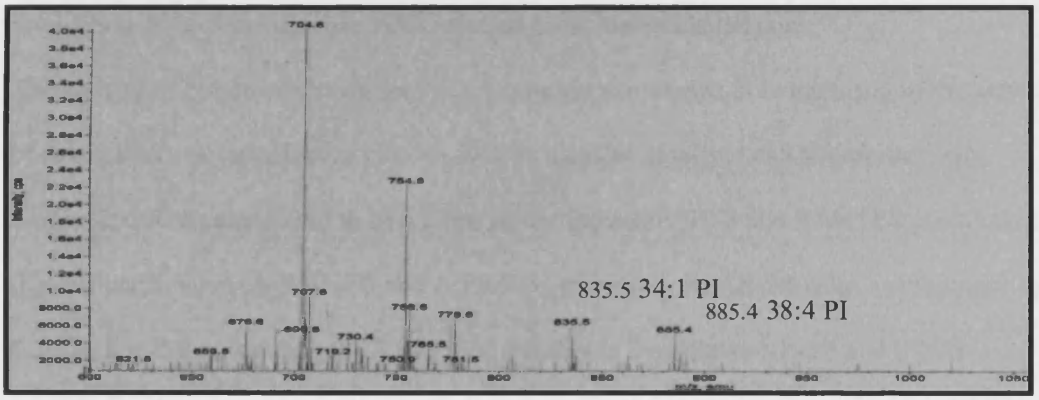

Figure 3.19: Mass spectrometry of Curosurf® (batch # 061350) (panel A), Curosurf® (batch # 069097) (panel B) and Survanta® (batch # 41-957-27) (panel C) – phosphatidylinositol (PI).

3.11 Characterisation of signalling pathways involved in cytokine and chemokine production in virus infected HEK293 and BEAS-2B cells

3.11.1 TLR3 mediates IFN-β promoter activation induced by dsRNA whereas RIG-I mediates IFN-β promoter activation induced by dsRNA and influenza virus in HEK293 cells

In our previous experiments, influenza virus was shown to induce IL-8, RANTES and IFN-β production in bronchial epithelial cells. The production of IL-8 and RANTES was significantly inhibited in the presence of pulmonary surfactant which was assessed at the protein level by ELISA. In order to explore possible mechanism the activation of NF-κB and IFN-β promoters in TLR3 and RIG-I transfected cells was assessed using the Dual-Luciferase® Reporter Assay System. TLR3 and RIG-I receptors play an important role in innate immunity and have been previously shown to be involved in ssRNA and dsRNA induced pro-inflammatory and anti-viral responses (Le Goffic et al. 2007), whereas in this study, activation of NF-κB transcription factor was found to be directly involved in the production of IL-8 in influenza virus infected bronchial epithelial cells.

The activity of pulmonary surfactant in this assay was assessed in comparison to the activity of dexamethasone in influenza virus as well as measles virus and dsRNA treated cells. Synthetic dsRNA was found to be a more potent inducer of IL-8 and RANTES production than influenza virus (A/WSN/33 and A/PR/8/34 strains) in BEAS-2B cells, as measured by ELISA. For that reason poly(I-C) was used initially to investigate NF-κB and IFN-β promoter activation, both TLR3 and RIG-I mediated, in transiently transfected HEK293 cells. Briefly, HEK293 cells were transfected with 300 ng of expression vectors for TLR3 or RIG-I, 300 ng of NF-κB or IFN-β luciferase reporter gene and 30 ng of Renilla luciferase internal control. After 24 hours cells were co-transfected with poly(I-C) (1 μg/ml). In

comparison to Genejuice and Metafectene, FuGENE 6 was shown to be the most efficient transfection agent (data not shown) therefore this reagent was selected for all transfection experiments, including synthetic dsRNA administration. A comparison study shown that IFN-β promoter activation was stronger in HEK293 cells co-transfected with poly(I-C) than in HEK293 cells stimulated with poly(I-C) (4 folds vs.1 fold of induction via TLR3 and 10 folds vs. 4 folds of induction via RIG-I) (data not shown).

Luciferase activity was measured 6 (NF-κB) and 12 (IFN-β) hours later. Firefly luciferase values were divided by *Renilla* luciferase values to normalise for transfection efficiency. For this series of experiments the results were presented as firefly luciferase values divided by *Renilla* luciferase values presented as fold induction over mock.

Surprisingly, in this luciferase reporter assay, neither TLR3 nor RIG-I expression mediated NF-κB promoter activity in HEK293 cells after poly(I-C) co-transfection. However, both TLR3 and RIG-I expression mediated IFN-β promoter activation in these cells upon transfection with poly(I-C) (Figure 3.20). Induction of IFN-β by dsRNA was both TLR3- and RIG-I-mediated however in the presence of RIG-I, IFN-β promoter activation was stronger (14 folds vs. 12 folds of induction) which was in agreement with earlier studies by Opitz and co-workers (Opitz et al. 2007). The same pattern of activation was observed using influenza virus A/WSN/33 strain as a stimulant. Although it was shown that this strain induced significant amounts of IL-8, NF-κB promoter was not activated in this assay. Only transient expression of RIG-I but not TLR3 mediated IFN-β promoter activation upon A/WSN/33 infection (2 folds of induction) in HEK293 cells (data not shown). Detectable luciferase activity from IFN-β reporter plasmid in the presence of A/WS/33 suggests that HEK293 cells are permissive for influenza virus infection. No effect was observed with influenza virus A/PR/8/34 strain (data not shown). Lack of the signal from NF-κB luciferase reporter gene might be associated with the method of transfection. In transient transfection,

certain plasmids might be lost from the cells during cell mitosis. It was decided to use stably transfected cell lines as an alternative option. In addition, functionality of this reporter might have been impaired therefore additional studies with different cell lines were also included in the next stage of this work.

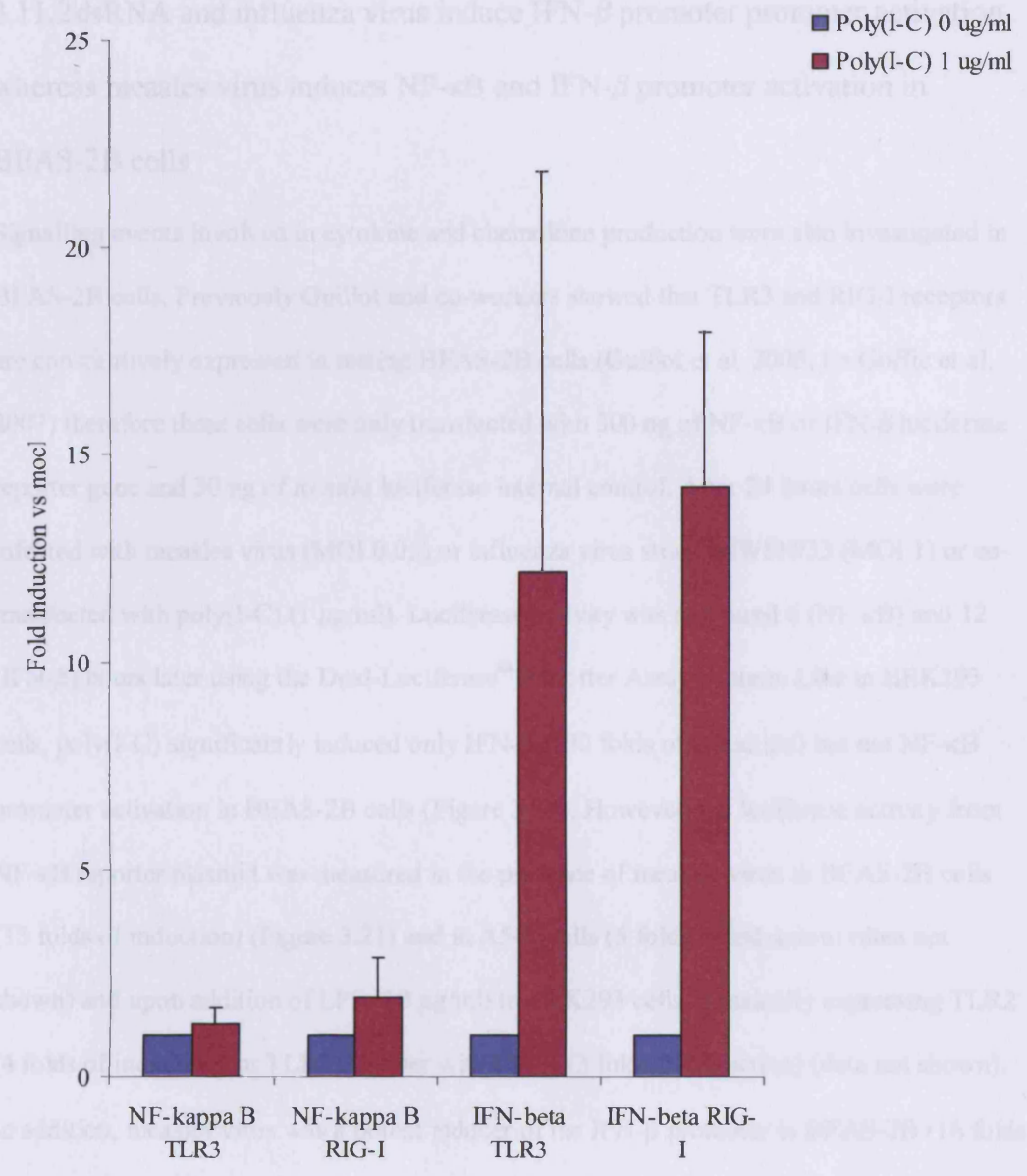

Figure 3.20: Effect of poly(I-C) on HEK293 cells NF-κB promoter and IFN-β promoter activation. HEK293 cells were transfected with 1 μg/ml of poly(I-C). NF-κB promoter and IFN-β promoter activation were measured after 6 and 12 hours, respectively. Results are expressed as mean ± 1 SD of 2 separate experiments.

3.11.2 dsRNA and influenza virus induce IFN-β promoter promoter activation whereas measles virus induces NF-κB and IFN-β promoter activation in BEAS-2B cells

Signalling events involved in cytokine and chemokine production were also investigated in BEAS-2B cells. Previously Guillot and co-workers showed that TLR3 and RIG-I receptors are constitutively expressed in resting BEAS-2B cells (Guillot et al. 2005; Le Goffic et al. 2007) therefore these cells were only transfected with 300 ng of NF-κB or IFN-β luciferase reporter gene and 30 ng of *Renilla* luciferase internal control. After 24 hours cells were infected with measles virus (MOI 0.05) or influenza virus strain A/WSN/33 (MOI 1) or co-transfected with poly(I-C) (1 μg/ml). Luciferase activity was measured 6 (NF-κB) and 12 (IFN-β) hours later using the Dual-Luciferase® Reporter Assay System. Like in HEK293 cells, poly(I-C) significantly induced only IFN-β (190 folds of induction) but not NF-κB promoter activation in BEAS-2B cells (Figure 3.21). However, the luciferase activity from NF-κB reporter plasmid was measured in the presence of measles virus in BEAS-2B cells (13 folds of induction) (Figure 3.21) and in A549 cells (5 folds of induction) (data not shown) and upon addition of LPS (10 μg/ml) to HEK293 cells, transiently expressing TLR2 (4 folds of induction) or TLR2 together with CD14 (3 folds of induction) (data not shown). In addition, measles virus was a potent inducer of the IFN-β promoter in BEAS-2B (16 folds of induction) (Figure 3.21). Again like in HEK293 cells, influenza virus A/WSN/33 strain induced IFN-β (10 folds of induction) but not NF-κB promoter activation in BEAS-2B cells (Figure 3.21).

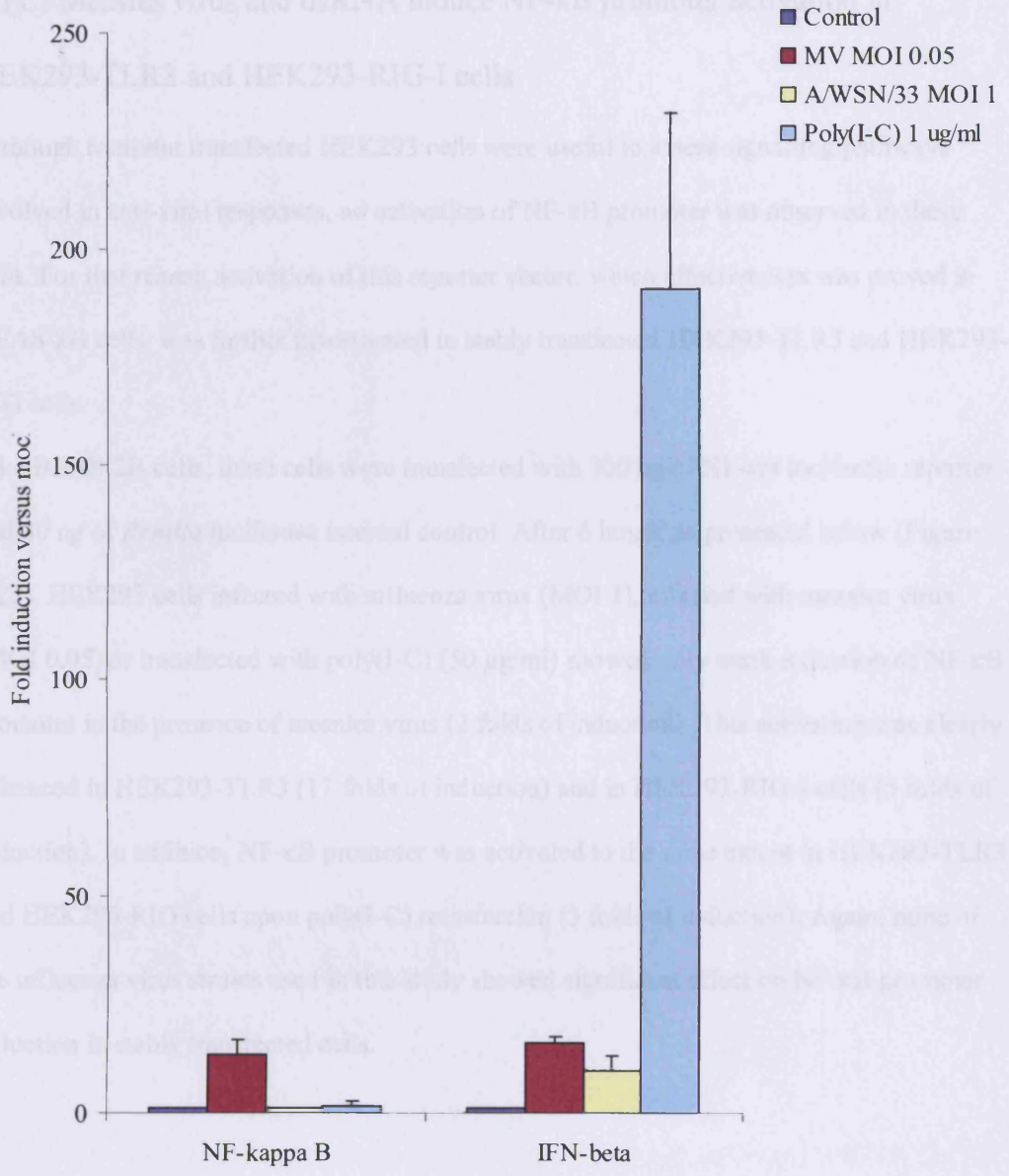

Figure 3.21: Effect of poly(I-C) on BEAS-2B cells NF-κB promoter and IFN-β promoter activation. BEAS-2B cells were infected with measles virus at the MOI 0.05 or influenza virus at the MOI 1 or transfected with 1 μg/ml of poly(I-C). NF-κB promoter and IFN-β promoter activation were measured after 6 and 12 hours, respectively. Results are expressed as mean ± 1 SD of of 2 separate experiments.

3.11.3 Measles virus and dsRNA induce NF-κB promoter activation in HEK293-TLR3 and HEK293-RIG-I cells

Although transient transfected HEK293 cells were useful to assess signalling pathways involved in anti-viral responses, no activation of NF-κB promoter was observed in these cells. For that reason activation of this reporter vector, which effectiveness was proved in BEAS-2B cells, was further investigated in stably transfected HEK293-TLR3 and HEK293-RIG cells.

Like BEAS-2B cells, these cells were transfected with 300 ng of NF-κB luciferase reporter and 30 ng of *Renilla* luciferase internal control. After 6 hours, as presented below (Figure 3.22), HEK293 cells infected with influenza virus (MOI 1), infected with measles virus (MOI 0.05) or transfected with poly(I-C) (50 μg/ml) showed only weak induction of NF-κB promoter in the presence of measles virus (2 folds of induction). This activation was clearly enhanced in HEK293-TLR3 (17 folds of induction) and in HEK293-RIG-I cells (5 folds of induction). In addition, NF-κB promoter was activated to the same extent in HEK293-TLR3 and HEK293-RIG cells upon poly(I-C) transfection (3 folds of induction). Again, none of the influenza virus strains used in this study showed significant effect on NF-κB promoter induction in stably transfected cells.

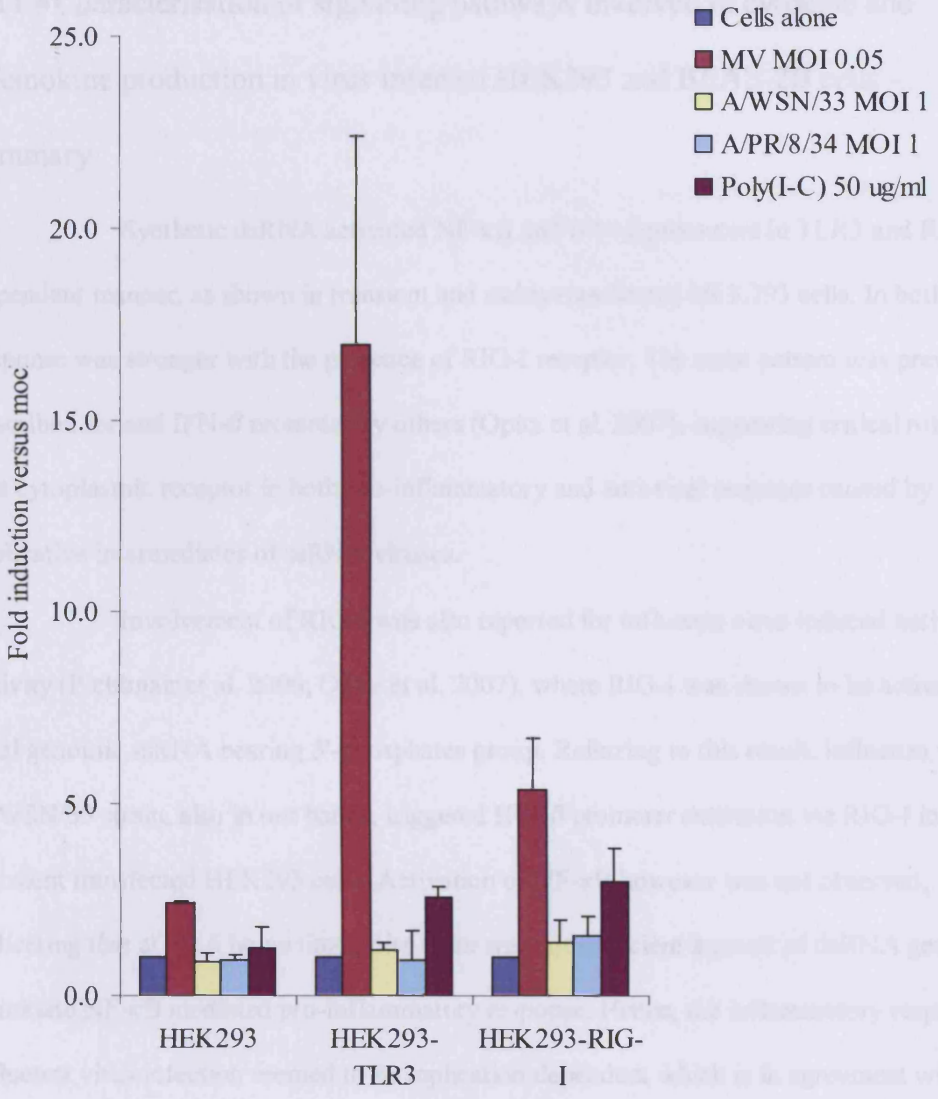

Figure 3.22: Effect of measles virus, influenza virus and poly(I-C) on HEK293, HEK-293-TLR3 and HEK293-RIG-I cells NF-κB promoter activation. Cells were infected with measles virus at the MOI 0.05 or influenza virus at the MOI 1 or transfected with 1 μg/ml of poly(I-C). NF-κB promoter promoter activation was measured after 6 hours. Results are expressed as mean ± 1 SD of 2 separate experiments.

3.11.4 Characterisation of signalling pathways involved in cytokine and chemokine production in virus infected HEK293 and BEAS-2B cells - summary

- Synthetic dsRNA activated NF-κB and IFN-β promoters in TLR3 and RIG-I dependent manner, as shown in transient and stably transfected HEK293 cells. In both cases response was stronger with the presence of RIG-I receptor. The same pattern was previously described for and IFN-β promoter by others (Opitz et al. 2007), suggesting critical role of this cytoplasmic receptor in both pro-inflammatory and anti-viral response caused by replicative intermediates of ssRNA viruses.
- Involvement of RIG-I was also reported for influenza virus induced antiviral activity (Pichlmair et al. 2006; Opitz et al. 2007), where RIG-I was shown to be activated by viral genomic ssRNA bearing 5'-phosphates group. Referring to this result, influenza virus A/WSN/33 strain, also in our hands, triggered IFN-β promoter activation via RIG-I in transient transfected HEK293 cells. Activation of NF-κB however was not observed, indicating that at the 6 hours time point there was not sufficient amount of dsRNA generated to initiate NF-κB mediated pro-inflammatory response. Hence, the inflammatory response to influenza virus infection seemed to be replication dependent which is in agreement with previous work (Guillot et al. 2005).
- After 6 hours NF-κB promoter activation was clearly detected in measles virus infected TLR3 and RIG-I stably transfected HEK293 cells, suggesting that production of inflammatory mediators in response to measles virus infection occurred in the absence of multiplication of this pathogen which was also shown at the protein level in this work. In addition, NF- κB promoter activation was induced in non-transfected HEK293 cells, suggesting that additional signalling pathways were involved in measles virus induced pro-inflammatory mediato production. Interaction of measles virus with cell surface receptors

pathogen detection was suggested in pro-inflammatory cytokine production by Sato and co-workers (Sato et al. 2005). The role of TLR3 and RIG-I receptors in measles virus induced IFN-β production should be investigated further.

- A similar pattern of activation was achieved in BEAS-2B cells where NF- κB promoter activation was observed in the presence of measles virus, whereas IFN-β promoter activation was observed in the presence of poly(I-C), influenza virus and measles virus.

3.12 The effect of pulmonary surfactant on signalling pathways involved in cytokine and chemokine production in virus infected HEK293 and BEAS-2B cells

3.12.1 Survanta®, Curosurf® and dexamethasone inhibit NF-κB promoter activation induced by measles virus whereas Survanta® and Curosurf® inhibit IFN-β promoter activation induced by dsRNA and influenza virus in BEAS-2B cells

Referring to the previous experiment the effect of pulmonary surfactant preparations (Survanta® batch # 41-957-27 and Curosurf® batch # 069097) at the concentration of 500 μg/ml and dexamethasone at the concentration 1 μg/ml on NF-κB and IFN-β promoter activation was investigated in bronchial epithelial cells.

After 6 hours, Survanta® and Curosurf® and dexamethasone down-regulated NF-κB promoter activation in measles virus infected BEAS-2B cells (by 27, 48 and 60%, respectively) (Figure 3.25).

After 12 hours, also IFN-β promoter activation in poly(I-C) (Figure 3.23) and influenza virus (Figure 3.24) treated cells was clearly inhibited by pre-incubation with 500 μg/ml of Survanta® (by 63 and 67%, respectively) as well as by pre-incubation with 500 μg/ml of Curosurf® (by 49 and 72%, respectively). In contrast, no effect of pulmonary surfactant preparations was observed on IFN-β promoter activation in measles virus (Figure 3.26) challenged cells. NF-κB specific inhibitor dexamethasone did not affect IFN-β induction in all conditions tested here.

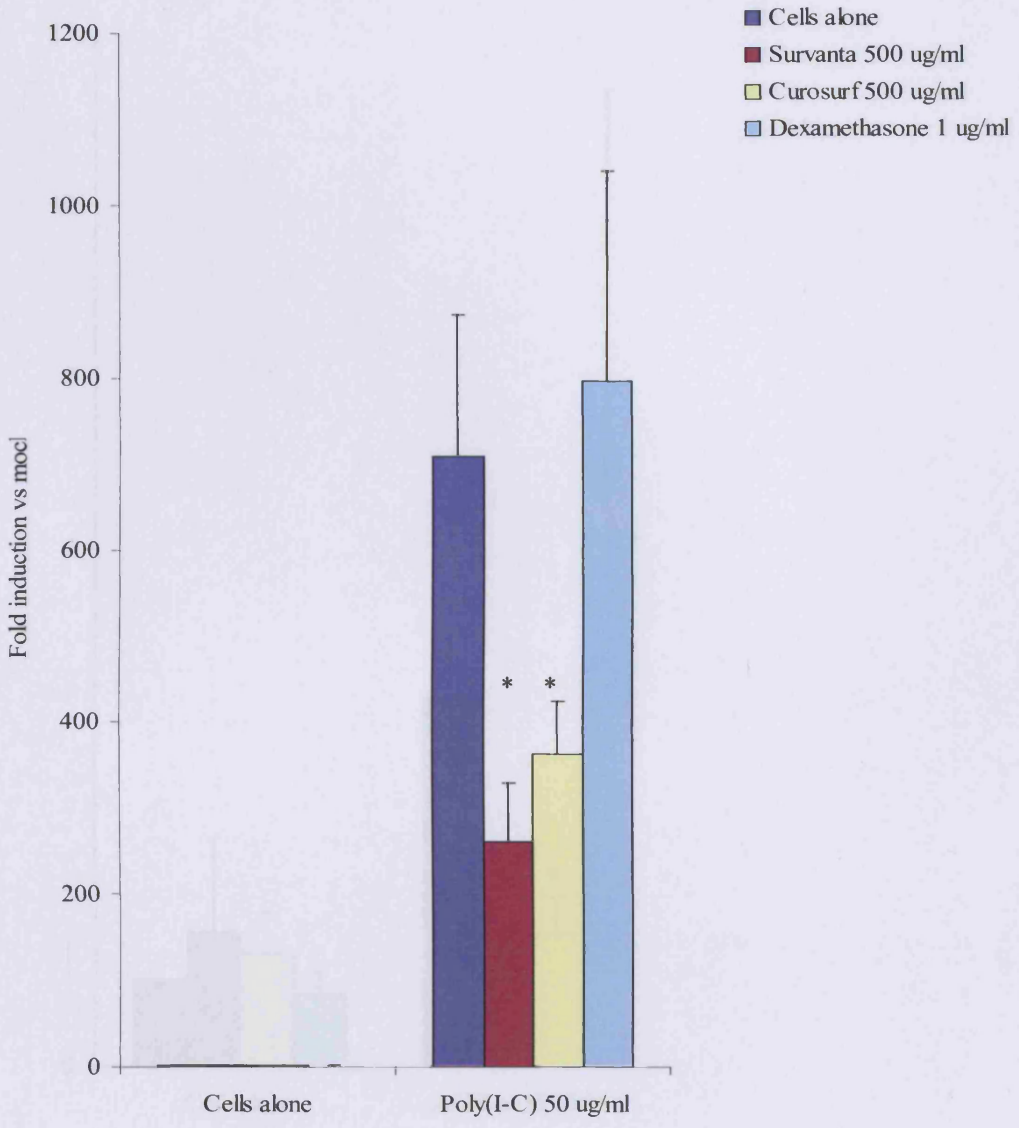

Figure 3.23: Effect of pulmonary surfactant and dexamethasone on poly (I-C) induced BEAS-2B cells IFN-β promoter activation. BEAS-2B cells were incubated with 500 μg/ml of Survanta®, 500 μg/ml of Curosurf® or 1 μg/ml of dexamethasone for 2 hours and then transfected with 50 μg/ml of poly(I-C). IFN-β promoter activation was measured after 12 hours. Results are expressed as mean ± 1 SD of 3 separate experiments. * $P < 0.05$ using unpaired t-test comparison to cells without pulmonary surfactant/dexamethasone treatment.

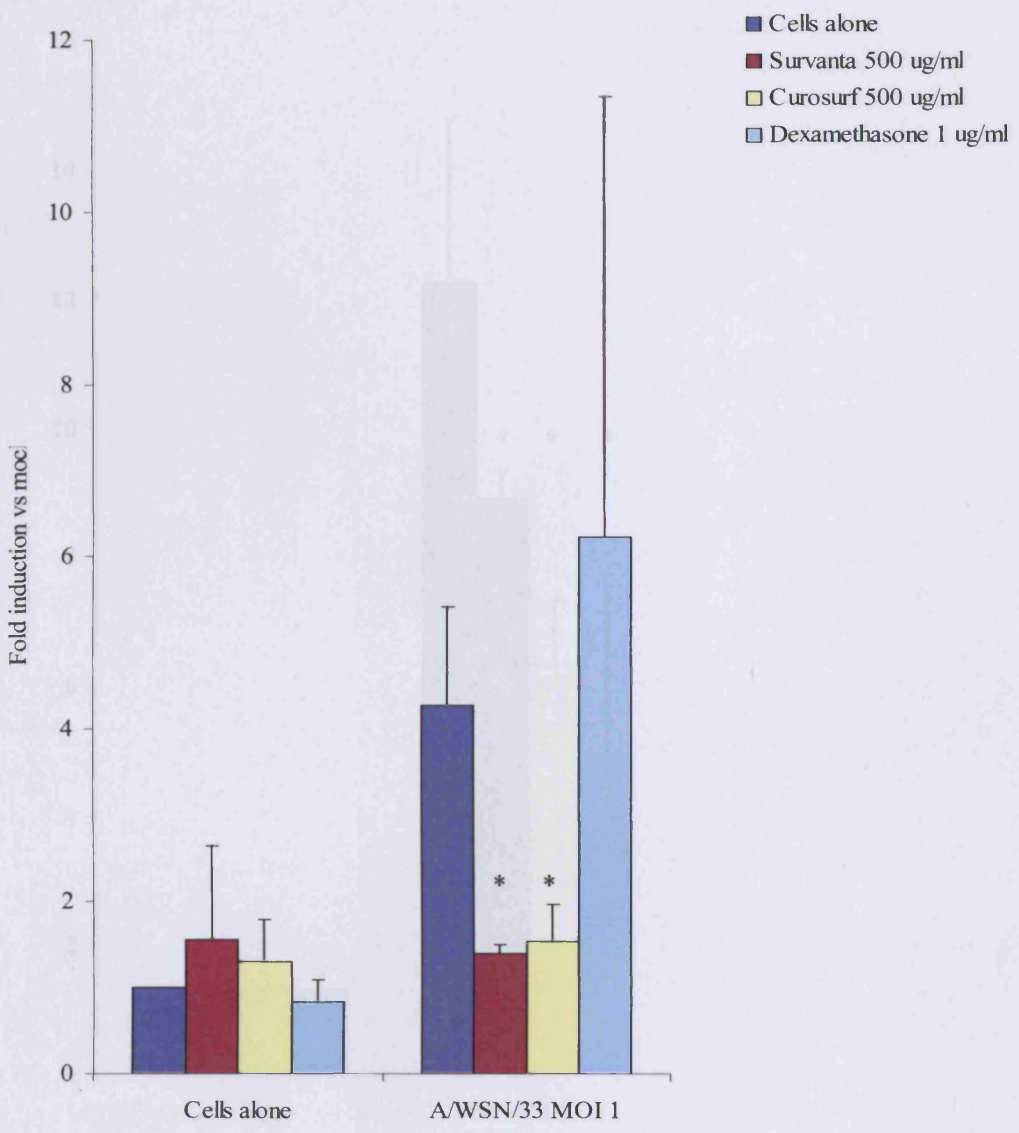

Figure 3.24: Effect of pulmonary surfactant and dexamethasone on A/WSN/33 induced BEAS-2B cells IFN-β promoter activation. BEAS-2B cells were incubated with 500 μg/ml of Survanta®, 500 μg/ml of Curosurf® or 1 μg/ml of dexamethasone for 2 hours and then infected with influenza virus A/WSN/33 strain at the MOI 1. IFN-β promoter activation was measured after 12 hours. Results are expressed as mean ± 1 SD of 3 separate experiments. * $P < 0.05$ using unpaired t-test comparison to cells without pulmonary surfactant/dexamethasone treatment.

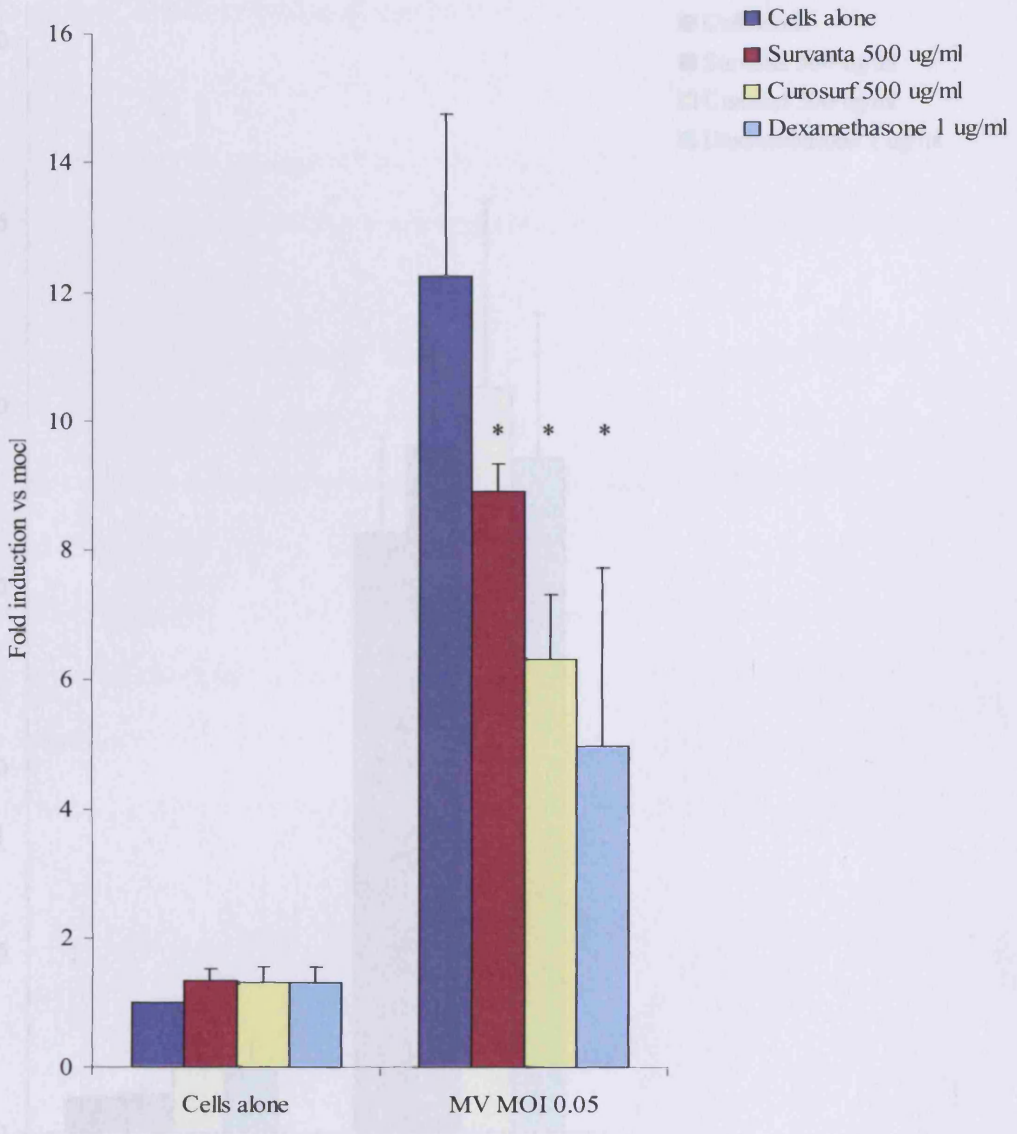

Figure 3.25: Effect of pulmonary surfactant and dexamethasone on measles virus induced BEAS-2B cells NF-κB promoter activation. BEAS-2B cells were incubated with 500 μg/ml of Survanta®, 500 μg/ml of Curosurf® or 1 μg/ml of dexamethasone for 2 hours and then infected with measles virus at the MOI 0.05. NF-κB promoter activation was measured after 6 hours. Results are expressed as mean ± 1 SD of 3 separate experiments. * $P < 0.05$ using unpaired t-test comparison to cells without pulmonary surfactant/dexamethasone treatment.

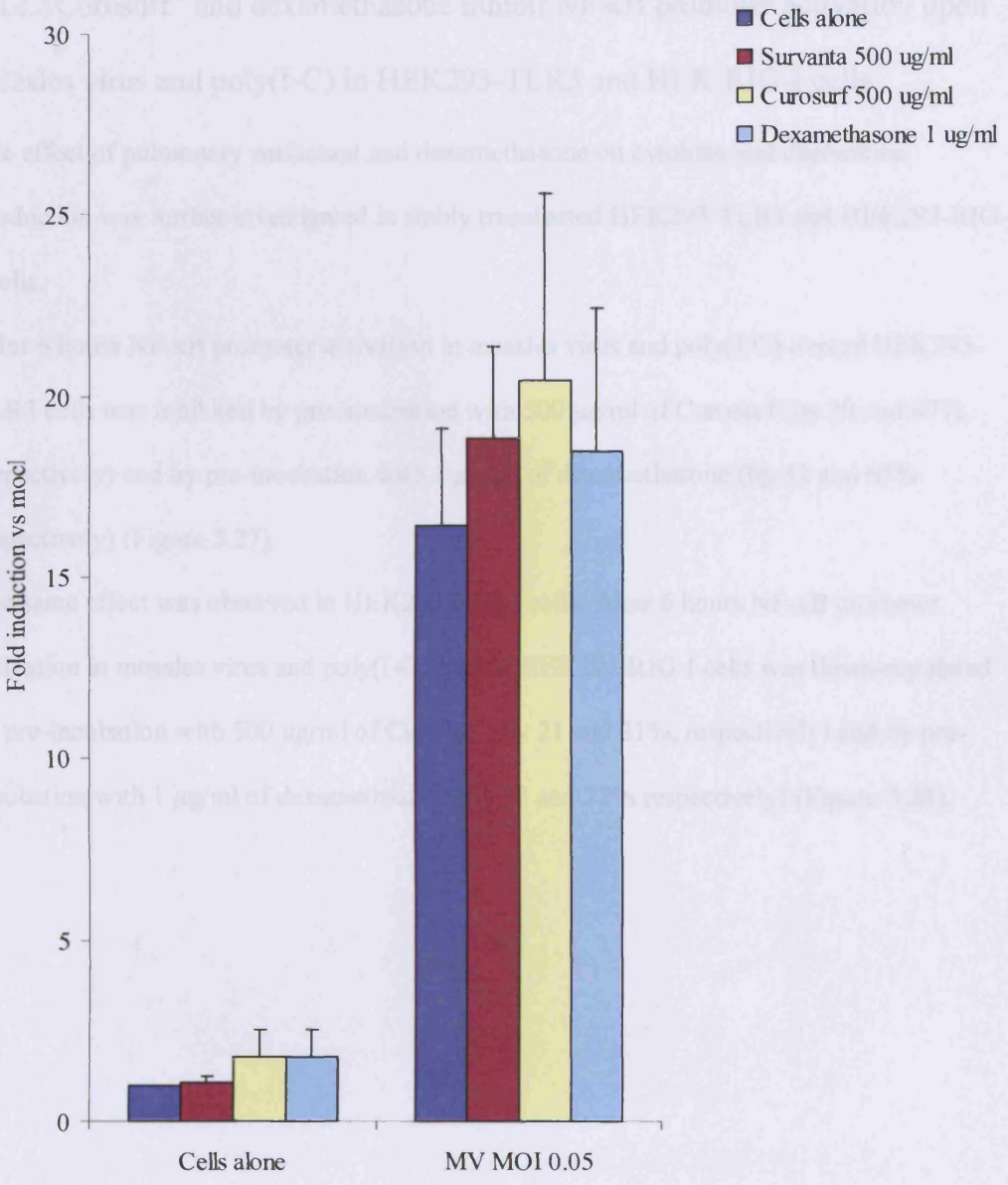

Figure 3.26: Effect of pulmonary surfactant and dexamethasone on measles virus induced BEAS-2B cells IFN-β promoter activation. BEAS-2B cells were incubated with 500 μg/ml of Survanta®, 500 μg/ml of Curosurf® or 1 μg/ml of dexamethasone for 2 hours and then infected with measles virus at the MOI 0.05. IFN-β promoter activation was measured after 12 hours. Results are expressed as mean ± 1 SD of 3 separate experiments.

3.12.2 Curosurf® and dexamethasone inhibit NF-κB promoter activation upon measles virus and poly(I-C) in HEK293-TLR3 and HEK-RIG-I cells

The effect of pulmonary surfactant and dexamethasone on cytokine and chemokine production was further investigated in stably transfected HEK293-TLR3 and HEK293-RIG-I cells.

After 6 hours NF-κB promoter activation in measles virus and poly(I-C) treated HEK293-TLR3 cells was inhibited by pre-incubation with 500 μg/ml of Curosurf (by 29 and 47%, respectively) and by pre-incubation with 1 μg/ml of dexamethasone (by 52 and 65% respectively) (Figure 3.27).

The same effect was observed in HEK293-RIG-I cells. After 6 hours NF-κB promoter activation in measles virus and poly(I-C) treated HEK293-RIG-I cells was down-regulated by pre-incubation with 500 μg/ml of Curosurf (by 21 and 31%, respectively) and by pre-incubation with 1 μg/ml of dexamethasone (by 32 and 72% respectively) (Figure 3.28).

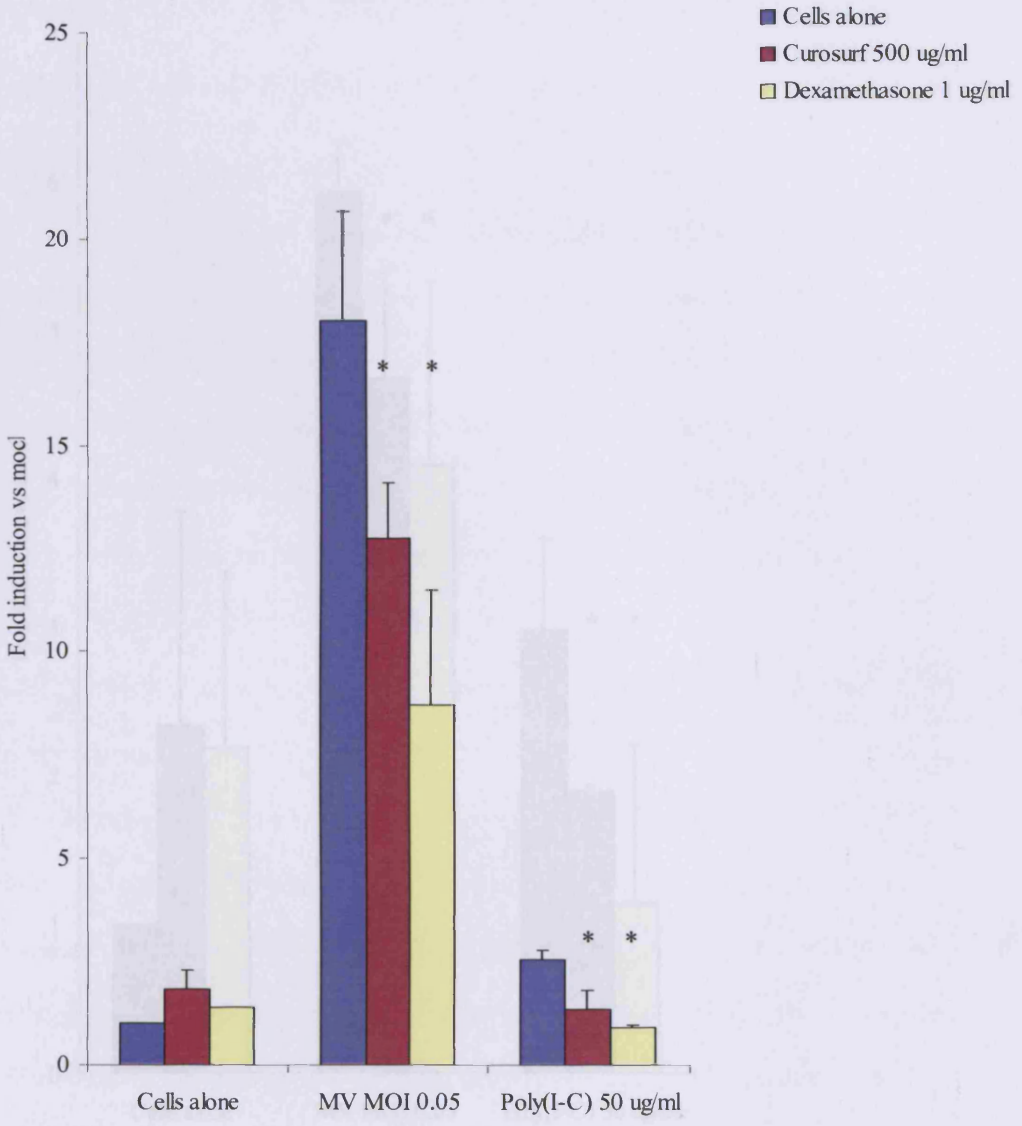

Figure 3.27: Effect of Curosurf® and dexamethasone on HEK293-TLR3 cells NF-κB promoter activation. Cells were incubated with 500 μg/ml of Curosurf® or 1 μg/ml of dexamethasone for 2 hours and then infected with measles virus at the MOI 0.05 or transfected with 50 μg/ml of poly(I-C). NF-κB promoter activation was measured after 6 hours. Results are expressed as mean ± 1 SD of 3 separate experiments. * $P < 0.05$ using unpaired t-test comparison to cells without pulmonary surfactant/dexamethasone treatment.

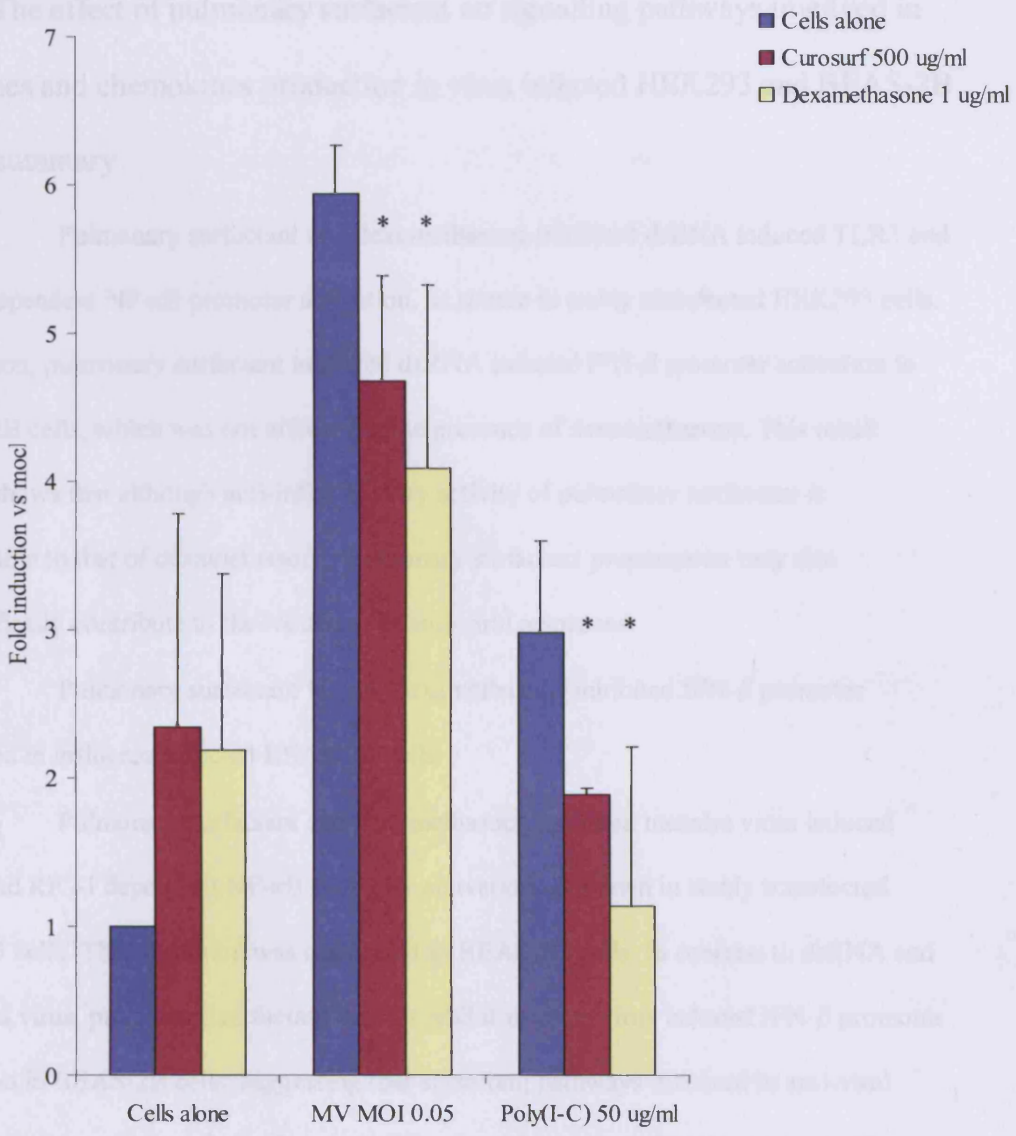

Figure 3.28: Effect of Curosurf® and dexamethasone on HEK293-RIG-I cells NF-κB promoter activation. Cells were incubated with 500 μg/ml of Curosurf® or 1 μg/ml of dexamethasone for 2 hours and then infected with measles virus at the MOI 0.05 or transfected with 50 μg/ml of poly(I-C). NF-κB promoter activation was measured after 6 hours. Results are expressed as mean ± 1 SD of 3 separate experiments. * $P < 0.05$ using unpaired t-test comparison to cells without pulmonary surfactant/dexamethasone treatment.

3.12.3 The effect of pulmonary surfactant on signalling pathways involved in cytokines and chemokines production in virus infected HEK293 and BEAS-2B cells - summary

- Pulmonary surfactant and dexamethasone inhibited dsRNA induced TLR3 and RIG-I dependent NF-κB promoter activation, as shown in stably transfected HEK293 cells. In addition, pulmonary surfactant inhibited dsRNA induced IFN-β promoter activation in BEAS-2B cells, which was not affected in the presence of dexamethasone. This result clearly shows that although anti-inflammatory activity of pulmonary surfactant is comparable to that of dexamethasone, pulmonary surfactant preparations may also unspecifically contribute to the reduction of anti-viral responses.
- Pulmonary surfactant but not dexamethasone inhibited IFN-β promoter activation in influenza infected BEAS-2B cells.
- Pulmonary surfactant and dexamethasone inhibited measles virus induced TLR3 and RIG-I dependent NF-κB promoter activation, as shown in stably transfected HEK293 cells. This inhibition was confirmed in BEAS-2B cells. In contrast to dsRNA and influenza virus, pulmonary surfactant did not inhibit measles virus induced IFN-β promoter activation in BEAS-2B cells, suggesting that signalling pathways involved in anti-viral response in the presence of measles virus differ from that activated in the presence of influenza virus or replicative intermediates of ssRNA viruses.

4 Discussion

Influenza virus has a worldwide distribution and causes annual epidemics of variable intensity. In the 20th century three pandemics of influenza virus have occurred, characterised by a large portion of influenza related deaths (>99% in 1918, 36% in 1957 and 48% in 1968) (Simonsen et al. 1998), caused by H1N1, H2N2 and H3N2 subtypes, respectively. The possibility of re-emergence of a highly pathogenic influenza virus was brought to public attention in 1997, by the sudden appearance of lethal avian influenza virus H5N1 in Asia (Hsieh et al. 2006), able to transmit directly from birds to humans.

Influenza virus infection results in a range of clinical syndromes which can manifest locally in the upper (common cold or pharyngitis) and lower (croup, bronchiolitis or pneumonia) respiratory tract or less often systemically (Richman et al. 2002). Clinical outcomes caused by influenza virus infection include chronic respiratory diseases (asthma, chronic obstructive pulmonary disease or acute respiratory distress syndrome), are often associated with excessive host inflammatory response (Van Reeth 2000). The initial release of inflammatory mediators by influenza virus infected airway epithelial cells and alveolar macrophages results in the recruitment of neutrophils, lymphocytes and macrophages from the peripheral blood to the infected lung tissue which is associated with massive production of cytokines (IFN-α/β, TNF-α, IL-1β and IL-6) and chemokines (IL-8, RANTES, MCP-1 and MIP-1) at the site of infection (Julkunen et al. 2000).

Currently, the principal method to control influenza virus infection is vaccination, however, vaccine induced immunity decreases over time and annual re-immunisation is necessary even if the vaccine antigens remain unchanged (Centers for Disease Control and Prevention). The target group for immunisation is limited and includes persons at increased risk of influenza related complications (persons aged 50 years and older, patients with

chronic cardiovascular and pulmonary disorders or residents of nursing homes) and persons who are in close contact with high risk individuals, including health care workers (Richman et al. 2002). Uncomplicated influenza does not require specific anti-viral therapy, but such therapy needs to be considered when complications of influenza occur, including bacterial infections (Schwarzmann et al. 1971), viral pneumonia (Kaiser & Hayden 1999), cardiac and other systems abnormalities (Hayase & Tobita 1997).

A few specific anti-viral drugs are used to treat infection caused by influenza virus, including replication inhibitors (amantadine and rimantadine) (Wintermeyer & Nahata 1995) and neuraminidase inhibitors (oseltamivir and zanamivir) (Monto et al. 1999). The appearance of drug resistant influenza virus strains has significantly limited the effectiveness of these drugs (Deyde et al. 2007). Another inhibitor of influenza virus replication is a nucleoside analog ribavirin (Oxford 1975), however intravenous administration of ribavirin may cause dose dependent hemolytic anemia whereas aerosolised administration may cause broncho-spasm (Ison & Hayden 2001). Clearly, alternative anti-viral drugs are needed. Recently the anti-malarial drug, chloroquine, was reported to significantly inhibit pH dependent influenza virus replication *in vitro* (Ooi et al. 2006). Moreover, chloroquine was shown to exhibit an immunomodulatory activity *in vitro*, suppressing the production of TNF-α (Jeong & Jue 1997), IL-1β (Bondeson & Sundler 1998), IL-6 (Karres et al. 1998) and IFN-γ (van den Borne et al. 1997) in monocytes and macrophages stimulated with various bacterial components. The anti-inflammatory action of chloroquine could potentially reduce influenza virus associated inflammation and thus reduce pathogenic effects. Similar to chloroquine, animal derived pulmonary surfactant preparations are clinically approved products which have been shown to have immunomodulatory activity *in vitro* (Walti et al. 1997), indicating potential beneficial

effects in the treatment of influenza virus infection. Previous clinical use of chloroquine and pulmonary surfactant preparations may shorten the duration required for the various phases of clinical trials.

Pulmonary surfactant is a phospholipid rich material that plays an essential role in pulmonary homeostasis. The composition of pulmonary surfactant may be altered, specifically with regard to quantity and composition, in a range of pulmonary diseases, including infant respiratory distress syndrome (IRDS) (Shelley et al. 1979) and in some which may develop secondary to or be exacerbated by influenza virus infection, such as acute respiratory distress syndrome (ARDS) (Nakos et al. 1998), pneumonia (Griese 1999), chronic obstructive pulmonary diseases (COPD) (Griese 1999) and asthma (Heeley et al. 2000). Administration of pulmonary surfactant preparations, also known as exogenous replacement therapy, was found to be an effective treatment in IRDS (Berggren et al. 1984) and has been assessed in clinical trials in ARDS with limited success, mainly because of the complexity of this disorder. Although surfactant replacement therapy was shown to improve oxygenation (Spragg et al. 2004) and lung compliance (Perez-Benavides et al. 1995), and to reduce the need for ventilatory support (Perez-Benavides et al. 1995) in patients with ARDS, it was unclear whether administration of pulmonary surfactant reduced ARDS associated mortality. The possible explanation for that is that deaths in ARDS may be due to multiple organ disfunction syndrome (MODS) and systemic pathology rather than respiratory failure. Previous *in vitro* studies have also indicated the modulatory action of commercially available pulmonary surfactant and its lipid components in inflammatory responses. Surfactant phospholipids were shown to suppress proliferation of lymphocytes (Wilsher et al. 1988b) and cytotoxicity of natural killer cells (Wilsher et al. 1988c), and to attenuate the production of various inflammatory mediators, including cytokines (TNF-α)

(Morris et al. 2000), lipid mediators (PAF and PGE_2) (Tonks et al. 2003) and reactive oxygen intermediates (ROIs) (Tonks 2001; Tonks et al. 2005) in human monocytes stimulated with LPS from gram negative bacteria. Similarly, animal derived pulmonary surfactant preparation was also reported to inhibit the production cytokines (TNF-α), arachidonic acid metabolites (PGE_2, TxB_2 and LTC_4) and superoxide anions in human monocytes stimulated with various bacterial components (Walti et al. 1997).

These observations led us to investigate the effect of pulmonary surfactant on the release of inflammatory mediators in response to influenza virus infection in human airway epithelial cells, and the mechanism by which pulmonary surfactant may affect this release. Furthermore, the effect of pulmonary surfactant on cellular viability and entry of influenza virus into human airway epithelial cells was assessed.

The data generated in the current study have shown that:

(i) influenza virus strain A/WSN/33 is a more potent inducer of cytokine production than strain A/PR/8/34 in bronchial epithelial cells,

(ii) pulmonary surfactant preparations reduce inflammatory cytokine production in bronchial epithelial cells, and that this anti-inflammatory activity is comparable to that of dexamethasone,

(iii) reduction of cytokine production is not a consequence of altered cell viability since pulmonary surfactant is non-toxic in human airway epithelial cells,

(iv) reduction of cytokine production is not a consequence of altered infectivity of influenza virus since pulmonary surfactant does not affect infectivity of influenza virus in bronchial epithelial cells,

(v) pulmonary surfactant inhibits NF-κB and IFN-β promoter activation in a variety of cell types whereas dexamethasone inhibits only NF-κB promoter activation.

Influenza virus strain A/WSN/33 is a more potent inducer of cytokine production than A/PR/8/34 strain in bronchial epithelial cells

Various experimental *in vitro* approaches and different animal models have been used to determine cytokine transcription and secretion profile during influenza virus infection (Julkunen et al. 2000).

Human airway epithelial cells were previously reported to produce significant amounts of mononuclear cell attracting CC chemokines (RANTES and MCP-1) and polymorphonuclear leukocytes chemoattractant CXC (IL-8) in response to influenza virus infection *in vitro* (Matsukura et al. 1996; Adachi et al. 1997) whereas the production of IFNα/β, IL-1β, IL-6 and TNF-α was shown to be limited in these cells (Ronni et al. 1997). Utilising a human bronchial epithelial cell line, Guillot and co-workers found these cells to be a good *in vitro* lung model which produced IL-8, RANTES, IL-6 and IFN-β following influenza virus strain A/Scotland/20/74 (H3N2) infection (Guillot et al. 2005). Therefore, we infected BEAS-2B with influenza virus strain A/WSN/33 and A/PR/8/34 and the cytokine profile was assessed. In our model influenza virus infected BEAS-2B cells also produced range of inflammatory mediators, including IL-8, RANTES as well as IFN-β and the production of these cytokines was found to be concentration and time dependent. Interestingly, in the present study influenza virus strain A/WSN/33 was a more potent inducer of IL-8, RANTES and IFN-β production than A/PR/8/34 strain which may be due to higher pathogenicity of this strain, as shown by CellTiter96® AQ_{ueous} one solution proliferation assay in BEAS-2B cells and by TCID50 in MDCK and A549 cells.

The pathogenicity of A/WSN/33 has also been reported previously in mice (Sugiura & Ueda 1980) and in MDCK cells (Schulman & Palese 1977) and was found to be related to the lack of a conserved glycosylation site at the position 130 of virus neuraminidase (Li et al. 1993).

The role of A/WSN/33 neuraminidase in the removal of sialic acid from haemagglutinin and facilitating the cleavage of haemagglutinin by endogenous proteases was suggested by Schulman and Palese (Schulman & Palese 1977). The glycosylation of neuraminidase at the position 130 could possibly inhibit the removal of sialic acid form haemagglutinin by neuraminidase of A/WSN/33. As reported by Guillot and others (Guillot et al. 2005), influenza virus induced cytokine release was delayed in comparison to the dsRNA stimulated response, suggesting that the observed production of inflammatory mediators was replication dependent. Our experiments with synthetic dsRNA also indicate that replication of influenza virus may be necessary for the observed cytokine biosynthesis, however experiments with UV treated virus or inhibitors of influenza virus replication (amantadine or rimantadine) would be required to ascertain if this is the case.

The present study provides evidence that influenza virus and dsRNA induced IL-8 production is partially mediated by NF-κB in BEAS-2B cells whereas different transcription factors are probably involved in RANTES and IFN-β. In our experiments, IL-8 but not RANTES and IFN-β production was inhibited by dexamethasone, specific inhibitor of NF-κB dependent gene expression (Mukaida et al. 1994). This is supported by findings of Bernasconi and others (Bernasconi 2005) who reported that activation of NF-κB is essential for A/WSN/33 mediated IL-8 production in alveolar epithelial cells A549. RANTES (Lin et al. 1999; Genin et al. 2000) and IFN-β (Sato et al. 1998; Sato et al. 2000) were previously shown to be regulated mostly by IRF3 and IRF7, although additional transcription factors may also play a role, including NF-κB. Nucleic acids of influenza virus are sensed by Toll-like receptors (TLRs) and RIG-like receptors (RLRs), well known pathogen recognition receptors (PRRs). Among them, TLR3 (Alexopoulou et al. 2001), TLR7 (Diebold et al. 2004) and RIG-I (Pichlmair et al. 2006) have been shown to be involved in the recognition

of ssRNA whereas TLR3 (Marshall-Clarke et al. 2007) and RIG-I (Yoneyama et al. 2004) were also reported to recognise dsRNA. Different receptors have been proposed to be involved in IL-8 and RANTES/IFN-β production in influenza virus infected BEAS-2B cells which may ultimately result in the activation of different transcription factors. Le Goffic and co-workers showed that the secretion of IL-8 was severely impaired in influenza virus infected BEAS-2B cells expressing an altered form of TLR3 whereas the secretion of RANTES and IFN-β was only partially inhibited (Le Goffic et al. 2007). This data indicate a critical role of TLR3 in the expression of IL-8 but not RANTES or IFN-β in bronchial epithelial cells infected by influenza virus. Influenza virus activates various signalling pathways, including dynamic protein phosphorylations (Wang et al. 2001) and ubiquitinations (Deng et al. 2000), which control the induction of pro-inflammatory and antiviral responses. The kinases involved in phosphorylation incude extracellular signal regulated kinase 1/2 (ERK1/2) (Pleschka et al. 2001), p38 mitogen activating protein (MAP) kinase (p38) (Kujime et al. 2000), phosphatidylinositol 3 kinase (PI3K) (Ehrhardt et al. 2006), dsRNA dependent protein kinase (PKR) (Balachandran et al. 2000) c-jun-NH2-terminal kinase (JNK) (Kujime et al. 2000) and others. As reported by Guillot and co-workers, influenza virus and dsRNA use a more complex signalling mechanism to induce IL-8 production than to induce RANTES in BEAS-2B cells (Guillot et al. 2005), which may also result in the activation of different transcription factors. They found that in influenza infected BEAS-2B cells, IL-8 production required ERK1/2, p38 and PI3K activation whereas RANTES secretion required p38 and PI3K signal transducing molecules activation. Similarly in dsRNA stimulated cells, IL-8 production was mediated by all of these kinases whereas RANTES release was PI3K dependent (Guillot et al. 2005).

The results obtained in the present study indicate that BEAS-2B cells provide a relevant *in vitro* model to study influenza virus infection. Although *in vitro* data allows us to make assumptions about the *in vivo* situation, the *in vitro* cytokine responses are greatly influenced by the cell type used. This limitation results from the lack of complex interactions between different cell types and cellular mediators, therefore our *in vitro* study should be validated in an animal model in the future.

Previous studies indicated the involvement of TLR3 and RIG-I receptor in pro-inflammatory and anti-viral immune responses to influenza virus and dsRNA. The TLR3 and its adaptor molecule TRIF were shown to be essential for dsRNA and influenza virus induced NF-κB activation, and dsRNA induced IRF/IRSE activation in BEAS-2B cells (Guillot et al. 2005; Le Goffic et al. 2007). IRFs are regulators of the IFN-α/β gene promoters (Nguyen et al. 1997) whereas ISRE can regulate various IFN stimulated genes (Williams 1991). TLR3 was reported not to be involved in IRF3 (Le Goffic et al. 2007) and IFN-β (Siren et al. 2006) activation in influenza virus infected BEAS-2B and HEK293 cells, respectively. Cytoplasmic RIG-I receptor and its adaptor molecule CARDIF were shown to mediate NF-κB and IRF3 activation in BEAS-2B cells infected with influenza virus. A critical role of RIG-I receptor in anti-viral responses to influenza virus was reported by Pichlmair and co-workers who showed that influenza virus genomic ssRNA bearing 5'-phosphates group is a ligand for RIG-I receptor (Pichlmair et al. 2006). Moreover, RIG-I was shown to mediate signals triggered by dsRNA, resulting in activation of NF-κB and IRF3 (Yoneyama et al. 2004).

In agreement with previous work the present study showed that TLR3 and RIG-I are essential for dsRNA induced NF-κB and IFN-β promoter activation, and that RIG-I but not TLR3 triggers IFN-β promoter activation in the presence of influenza virus strain A/WSN/33, as assessed in HEK293 cells by Dual Luciferase® Reporter Assay. Surprisingly,

in our experiments, neither TLR3 nor RIG-I expressing cells infected with influenza virus showed NF-κB promoter activation, suggesting the involvement of different receptors, possibly TLR7. Our results indicate a critical role of TLR3 and RIG-I in pro-inflammatory and anti-viral response to dsRNA and the importance of RIG-I receptor in anti-viral response to influenza virus strain A/WSN/33. This will be discussed in greater detail below.

Pulmonary surfactant preparations reduce cytokine production in bronchial epithelial cells

Clinically approved animal derived pulmonary surfactant undergoes extraction and purification procedures which remove hydrophilic proteins SP-A and SP-D and preserve the presence of phospholipids and hydrophobic proteins SP-B and SP-C (Blanco & Perez-Gil 2007). Phospholipids, especially DPPC, are the surface tension reducing components at the air liquid interface in the alveolus (Klaus et al. 1961) whereas surfactant hydrophobic proteins promote rapid adsorption of phospholipids at the air liquid interface (Curstedt et al. 1987), which is important for IRDS treatment. Commercially available SP-A and SP-D depleted pulmonary surfactant preparations (Speer et al. 1991) and surfactant associated lipids (Wilsher et al. 1988b) have been shown to have immunomodulatory activity *in vitro*. These studies reported diverse findings depending on the stimulus and the cell type considered. The vast majority of work to date focused on modulatory function of pulmonary surfactant in human monocytes and macrophages stimulated with bacterial components (Speer et al. 1991).

The present study provides evidence that pulmonary surfactant modulates the biosynthesis of IL-8 and RANTES in human bronchial epithelial cells infected with influenza virus. In the presence of physiological concentrations of Curosurf® (500 μg/ml), IL-8 and RANTES responses of BEAS-2B cells to infection with influenza virus strain A/WSN/33 were reduced by 30 and 35%, respectively. This is of relevance to the pathogenesis of influenza

virus infection since *in vivo* IL-8 is a potent chemoattractant for neutrophils (Mukaida 2003) and the production of IL-8 during influenza virus infection *in vivo* was shown to correlate with systemic (muscle aches, fatigue, headache and fever) and lower respiratory symptoms (cough, breathing difficulty, hoarseness and chest discomfort) (Hayden et al. 1998), indicating that pulmonary surfactant could be a good treatment option for severe influenza infection in lower respiratory tract. RANTES recruits monocytes, T lymphocytes, basophils and eosinophils (Schall 1991) however the role of RANTES in the pathology of influenza virus infection is unknown.

Our present study demonstrates the greater ability of porcine Curosurf®, compared with bovine Survanta®, to reduce production of pro-inflammatory cytokines. The wide spectrum of anti-inflammatory activity of Curosurf® has been reported previously. Speers and co-workers reported that in the presence of Curosurf® phagocytosis of *Staphylococcus aureus* was impaired and that this porcine preparation effectively suppressed the production of TNF-α in LPS stimulated human monocytes. Decreased TNF-α production was later associated with significantly reduced TNF-α mRNA expression by Curosurf® (Baur et al. 1998). Walti and co-workers showed that in addition to TNF-α production, Curosurf® attenuated the production of reactive oxygen intermediates (ROIs) and lipid mediators, including PGE_2, TxB_2 and LTC_4 induced by various bacterial components in human peripheral blood monocytes (Walti et al. 1997). Natural porcine surfactant was also shown to modulate the expression of heat shock proteins and the production of superoxide anions caused by tobacco smoke (Pinot et al. 1999).

Our liquid chromatography/mass spectrometry (LC/MS) phospholipid analysis revealed variations between these two types of commercially available pulmonary surfactant

preparation, indicating that the differences in potency may be due, at least in part, to differences in phospholipid composition. This is supported by findings of Wilsher co-workers who reported that the immunosuppressive properties of pulmonary surfactant are influenced by variations of the phospholipid composition profile (Wilsher et al. 1988). They showed that phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylinosytol (PI) were the most potent inhibitors of the lymphoproliferative response to phytohaemagglutinin (PHA). Differences in the chemical composition of these surfactants could be due to differences in source and the method used to extract and purify the surfactant material. Curosurf® undergoes neutral lipid extraction by gel-liquid chromatography whereas production of Survanta® includes supplementation with DPPC, triglycerides and palmitic acid (Suresh & Soll 2002). Applied methods of extraction and purification may also result in incomplete removal of pulmonary surfactant associated proteins SP-A and SP-D. White and co-workers showed that when neutrophils were incubated with SP-D before influenza virus was added, the oxidative response to the virus was reduced (White et al. 2005). One argument that residual SP-A and SP-D are not the reason for the observed effect of Curosurf® is that SP-A and SP-D depleted pulmonary surfactant should not affect entry of influenza virus in bronchial epithelial cells. This was shown by us in an influenza plaque assays (discussed later). Both SP-A and SP-D were shown to reduce influenza virus infectivity *in vitro* (Benne et al. 1995) and in *vivo* (Li et al. 2002). It is possible that material extracted from porcine lung tissue is better tolerated by human cells than surfactant complexes obtained from bovine lung minces. Compatibility of swine and human tissues is used in xenotransplantation to prevent rejection problems (Trucco et al. 2007).

Interestingly, our *in vitro* study is also consistent with previous *in vivo* studies, which demonstrate that porcine Curosurf® has higher clinical effectiveness than bovine Survanta® in the treatment of IRDS (Speer et al. 1995; Baroutis et al. 2003; Ramanathan et al. 2004). Premature infants treated with Curosurf® have shorter ventilation, oxygen administration and hospitalisation, when compared to those treated with Survanta®. The identification of components responsible for observed differences between Curosurf® and Survanta® *in vitro* was beyond the scope of this study, but is planned for the future. This study would require significant chemical investigation and assessment of individual components biological activities. The role of pure phospholipids of the major type found in pulmonary surfactant preparations, such as phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylinositol (PI), and the role of pure surfactant associated hydrophobic proteins SP-B and SP-C in the reduction of cytokine production in influenza virus infected bronchial epithelial cells should be assessed in the future. Our analysis of LC/MS profiles showed that PC, PA and PG were present in both types of pulmonary surfactant preparation whereas PE and PI were found only in Curosurf®, suggesting that these phospholipids might be involved in IL-8 and RANTES inhibition in influenza virus infected BEAS-2B cells.

Reduction of influenza virus induced cytokine production is not a consequence of altered cell viability

The results obtained in the present study show that SP-A and SP-D depleted pulmonary surfactant preparations affect influenza virus induced cytokine production at the protein level in bronchial epithelial cells BEAS-2B. The observed reduction could be possibly due to: (i) reduced viability of BEAS-2B cells treated with pulmonary surfactant, (ii) reduced entry of influenza virus into BEAS-2B cells treated with pulmonary surfactant or (iii) due to

the effect of pulmonary surfactant on cellular responses of BEAS-2B cells induced by influenza virus.

The effect of pulmonary surfactant on the viability of human airway epithelial cells was assessed by CellTiter96® AQ_{ueous} one solution proliferation assay. Although the kit is called proliferation assay, what it actually measures is the cellular reductive capacity of a tetrazolium salt (MTS) to a coloured formazan product. Bioreduction of MTS has been shown to occur extramitochondrially, in the presence of two reducing agents NADH and NADPH (Berridge & Tan 1993), and to be dependent on the mitochondrial dehydrogenase enzymes found only in metabolically active cells (Cory et al. 1991), therefore CellTiter96® AQ_{ueous} one solution proliferation assay can be use as an indirect method to evaluate cell viability if cell densities are standardised at the outset.

Curosurf® and Survanta® are the two major pulmonary surfactant preparations recommended in the UK (British National Formulary (BNF) BNF54-2007) which have been proven to be effective in the treatment of infant respiratory distress syndrome (IRDS) (Speer et al. 1995; Ramanathan et al. 2004). Although these animal products are subjected to chemical quality controls, aiming to standardise each batch by phosphatidylcholine (PC) content, important in reducing surface tension (Klaus et al. 1961), it is unclear which biological quality controls are performed before approval of final product release.

Our study indicates that pulmonary surfactant is predominantly non-toxic in human airway epithelial cells. Four out of five batches of pulmonary surfactant analysed, including batches which affected influenza virus induced chemokine production in BEAS-2B cells, were not cytotoxic and the viability of cells in the presence of surfactants was not significantly different from the viability of control cells with respect to reduction of MTS. These results indicate that reduction of influenza virus induced cytokine production observed was not a

consequence of altered cell viability by pulmonary surfactant treatment. Only one batch of pulmonary surfactant (Curosurf® batch # 061350) showed a strong cytotoxic effect in bronchial epithelial cells and this was seen to be time and dose dependent, suggesting that toxicity of pulmonary surfactant here may be batch related. The role of Curosurf® in pulmonary defence has been previously studied *in vitro* in human monocytes and macrophages (Speer et al. 1991; Hidi et al. 1997; Walti et al. 1997), however toxicity of this pulmonary surfactant preparation has not been reported. Also *in vivo* studies regarding Curosurf® treatment in various respiratory conditions, including impaired mucus clearance in a dog model (De Sanctis et al. 1994), bacterial pneumonia in a rabbit model (Herting et al. 1997) and acute respiratory distress syndrome (ARDS) in a rat model (van Helden et al. 1998) have not revealed any evidence of toxicity of this preparation of porcine origin. Administration of exogenous pulmonary surfactant could potentially cause lung injury, leading to subsequent leakage of pulmonary surfactant associated proteins SP-B and SP-C into the vascular space and formation of immune complexes between surfactant proteins and antibodies. The immunogenicity of Curosurf® has been tested in rabbit model (Precioso et al. 2006) where Curosurf® has been shown not to trigger antibody dependent immune response against its components, indicating that Curosurf® treatment is safe and does not produce lung injury. On the other hand, endotoxin (Whitelaw 1996) and platelet activating factor (PAF) (Moya et al. 1993) contaminations were identified in other natural surfactants, which could possibly act as stimulants, leading to reduced cell viability. There is also concern regarding the hypothetical risk of prions transmission from bovine sources (Ryou 2007).

The cytotoxicity of Curosurf® observed by us may have been caused by inappropriate storage and/or transport condition, resulting in possible oxidation of pulmonary surfactant associated lipids. Oxidised lipids, especially oxidised phospholipids have been shown to

possess pro-inflammatory potential (Bochkov 2007), which could ultimately cause cell death. The complexity of pulmonary surfactant of animal origin makes it difficult to identify possible harmful component in it. The possible batch to batch variability of animal derived pulmonary surfactant preparations underlies the importance of development of new, clinically more active artificial surfactants. Comparison studies between available natural and synthetic surfactants have shown greater potency of animal products in the prevention and treatment of IRDS (Soll & Blanco 2001). These studies reported that animal derived pulmonary surfactant therapy as opposed to synthetic surfactant therapy was associated with shorter ventilation, fewer pneumothoraces and higher survival rate in premature infants, probably due to the presence of hydrophobic proteins SP-B and SP-C. More efficient synthetic pulmonary surfactant therapy would offer better quality control and greater chemical purity than treatments based on animal products.

Reduction of influenza virus induced chemokine production is not a consequence of altered infectivity of influenza virus

Pulmonary surfactant associated proteins SP-A and SP-D, which are depleted from animal surfactant preparations, were previously demonstrated to control various viral infections, including influenza virus (Benne et al. 1995), CMV (Weyer et al. 2000), HIV-1 (Meschi et al. 2005), RSV (Levine et al. 1999; Levine et al. 2004) and adenovirus (Harrod et al. 1999) infections. These proteins were shown to increase viral phagocytic clearance (Benne et al. 1997) and to modulate innate responses during viral infections (Hartshorn et al. 1994) *in vitro* and *in vivo*. In contrast, little is known about the function of pulmonary surfactant lipids in viral infections *in vitro*. Balakireva and co-workers (Balakireva et al. 2003) reported that entry of adenovirus in alveolar epithelial cells to be increased in the presence of DPPC, the major component of natural and synthetic pulmonary surfactants. In their

experimental model cells were incubated with adenovirus in the presence of DPPC. The phospholipid binding activity of adenovirus, associated with increased entry in alveolar epithelial cells, may result from the absence of a lipid envelope in adenovirus particles (Balakireva et al. 2003), suggesting that entry of influenza virus would not be affected in the presence of pulmonary surfactant preparations. Further experiments were carried out to verify this assumption.

In our model bronchial epithelial cells were incubated with pulmonary surfactant preparations prior to influenza virus infection and then the effect of Curosurf® and Survanta® on influenza virus entry was assessed. Results from plaque assay and CellTiter96® AQ_{ueous} one solution proliferation assay show that infection of bronchial epithelial cells by influenza virus was not altered by Curosurf® and Survanta® pre-treatment *in vitro*, which suggests that reduction of influenza virus induced cytokine production was not a consequence of altered infectivity of influenza virus and that pulmonary surfactant possibly affected cellular responses of BEAS-2B cells induced by influenza virus. Our *in vitro* findings suggest that exogenous pulmonary surfactant administration will probably not facilitate entry of influenza virus into respiratory epithelial cells in distal parts of lungs *in vivo*. However uptake of other, especially non-enveloped viruses (like adenoviruses), respiratory pathogens could be increased by pulmonary surfactant. Adenoviruses cause rarely severe respiratory infections.

Pulmonary surfactant can inhibit NF-κB and IFN-β promoter activation

The present study showed the importance of TLR3 and RIG-I in pro-inflammatory and anti-viral responses to dsRNA, and the involvement of RIG-I receptor in anti-viral response to influenza virus A/WSN/33 in bronchial epithelial cells. These results suggest that after

binding of influenza virus to cell surface sialic acid (Rogers et al. 1983), endosomal internalisation (Sieczkarski & Whittaker 2003) and subsequent release of vRNPs into cytoplasm, the ssRNA of influenza virus is sensed by cytoplasmic RIG-I receptor. The RIG-I receptor activates mitochondrial membrane associated CARDIF adaptor molecule (Lin et al. 2006), mediating IFN-β related anti-viral response. After replication and transcription in the nucleus (Braam et al. 1983), and translation and assembly in the cytoplasm (Ali et al. 2000), the mature virus detach from the cell, using the host cytoplasmic membrane as an envelope. Possibly due to excessive membrane damage lung epithelial cells undergo necrotic cell death (Seo et al. 2001), releasing intracellular materials, including the dsRNA intermediates produced during virus replication, into the surrounding environment. Internalised dsRNA could be recognised in endosomal compartments by TLR3, which recruits adaptor molecule TRIF (Oshiumi et al. 2003), and by cytoplamic RIG-I receptor, leading to subsequent activation of NF-κB mediated pro-inflammatory and IFN-β dependent anti-viral response.

Using different stimulants, including synthetic dsRNA, influenza virus and measles virus as a control, the mechanism by which pulmonary surfactant affect cellular responses leading to the production of inflammatory mediators was assessed by us in BEAS-2B and HEK293 cells, using Dual-Lusiferase® Reporter Assay System. Like influenza virus, measles virus is ssRNA respiratory virus, which was shown in our *in vitro* model to be a potent inducer of chemokine production in human bronchial epithelial cells. As shown in HEK293 cells, porcine Curosurf® inhibited both TLR3 and RIG-I dependent dsRNA and measles virus induced NF-κB promoter activation. These findings indicate that in the presence of pulmonary surfactant presumably endosomal membrane associated TLR3 signalling pathways and mitochondrial membrane associated RIG-I signalling pathways may be

impaired. This is supported by findings of Tonks and co-workers who have reported that incorporation of DPPC into MM6 cells significantly decreased membrane fluidity (Tonks et al. 2001). In addition, several membrane associated enzymes have been shown to be affected by DPPC and pulmonary surfactant treatment. Curosurf® was shown to inhibit intracellular accumulation of cyclic adenosine monophosphate (cAMP) in monocytes stimulated with membrane dependent (cholera toxin and forskolin) but not membrane independent agents (Pinot et al. 2000). DPPC suppressed LPS stimulated production of platelet activating factor (PAF) in MM6 cells by inhibiting two membrane associated enzymes (coenzyme A (CoA) independent transacylase and CoA:lyso PAF acetyltransferase) involved in PAF synthesis (Tonks et al. 2003). Using the same *in vitro* model, DPPC was shown to modulate release of ROIs by inhibiting the activity of protein kinase C (PKC), membrane related enzyme involved in ROIs production (Tonks et al. 2005). Also human pulmonary surfactant, isolated from broncho-alveolar lavage fluid, was reported to affect bactericidal functions of monocytes by altering the activity of PKC (Geertsma et al. 1994). Pulmonary surfactant possibly may impair the activity of membrane associated enzymes or receptors by disruption of specific membrane rafts. Raft domains, particularly rich in glycosphingolipid and cholesterol, have been shown to be present in cell membrane (Harder et al. 1998). They have been implicated in important cellular processes such as signalling transduction, by acting as platforms able to concentrate various signalling molecules and receptors (Kurzchalia & Parton 1999). Decreasing membrane fluidity was shown to retard the formation of such clusters and inhibit cellular activation (Peck 1994).

In order to completely elucidate whether mechanisms by which pulmonary surfactant influences TLR3 and RIG-I signalling pathways are caused by endosomal and mitochondrial membrane disruption, additional experiments would be required. This could be achieved by investigating the effect of pulmonary surfactant on NF-κB and IFN-β promoter activation, in

our *in vitro* model, in the presence of vector expressing either truncated TRIF protein (adaptor molecule of TLR3) containing the TIR region only, able to bind to TIR domain of TLR3 and to prevent its down-stream signalling or truncated TRAF3/6 proteins containing TRAF domain only, able to bind to TRAF interaction motif (TIM) of CARDIF (adaptor molecule of RIG-I) and to prevent its down-stream signalling.

The present study also showed that pulmonary surfactant inhibits NF-κB promoter activation in measles virus infected BEAS-2B cells and IFN-β promoter activation in dsRNA transfected and influenza virus infected BEAS-2B cells. The inhibition of NF-κB activation by bovine Survanta® has been previously shown in LPS stimulated alveolar macrophages and monocytic cell line (Antal et al. 1996) which was evident in the suppression of TNF-α, IL-1 and IL-6 production in these cells (Thomassen et al. 1992). The role of pulmonary surfactant in the modulation of anti-viral response is unknown. Non-specific inhibition of both NF-κB and IFN-β promoter activation indicates that pulmonary surfactant may attenuate both pro-inflammatory and anti-viral responses *in vitro* and *in vivo*. Interestingly, the activation of IFN-β promoter in measles virus infected BEAS-2B cells was not affected by pulmonary surfactant treatment, indicating that other than TLR3/RIG-I mediated signalling pathways may be involved in anti-viral response to measles viruses in bronchial epithelial cells. This is adding a possible new pathway compared to what was observed by others. According to these results, expression of RIG-I/MDA5 but not TLR3 was important for IFN-β promoter activation in measles virus (Berghall et al. 2006) and influenza virus (Siren et al. 2006) infected endothelial and epithelial cells.

The observed inhibitory effect of Curosurf® on NF-κB promoter activation in dsRNA transfected and measles virus infected BEAS-2B and HEK293 cells was comparable to that

of dexamethasone (synthetic member of corticosteroid hormones), suggesting similar anti-inflammatory potential *in vitro* and *in vivo*. Corticosteroids are potent inhibitors for various inflammatory agents *in vitro* (Mukaida et al. 1994) and exogenous corticosteroids therapy, like pulmonary surfactant treatment, was shown to improve oxygenation (Steinberg et al. 2006), lung injury (Meduri et al. 2007) and to reduce the requirement for ventilatory support (Steinberg 2006) in patients with ARDS. It was unclear whether administration of corticosteroids increased ARDS associated survival rate, however, long term exogenous corticosteroids therapy has been shown to be associated with severe side effects, including immunosuppression, and metabolic and hormonal imbalance (Deshmukh 2007). Pulmonary surfactant treatment could potentially offer safer anti-inflammatory action only at the local level of the lung.

Relevance of the study

To assess the effect of pulmonary surfactant on influenza virus infected human airway epithelial cells, the cells were treated with Curosurf® or Survanta® prior to influenza virus infection. In this setup what was actually determined was the role of pulmonary surfactant in healthy lung prior to influenza induced inflammatory damage. It would be interesting to see, whether in cells infected with influenza virus prior to pulmonary surfactant treatment, cellular responses induced by influenza virus would be affected in the same manner.
As mentioned above, data obtained from the *in vitro* experiments are greatly influenced by the cell type used. Comparison studies between different types of lung cell lines (including monocytic cell lines), as well as between cell lines and cells isolated from primary tissue would help to make assumptions about the *in vivo* situation. Similarly, studies with laboratory adapted strains of influenza virus should be extended to wild type strains of influenza virus. To overcome limitations which result from the lack of complex interactions

between different cell types and cellular mediators, our *in vitro* studies should be validated in a more complex *in vitro* lung model an animal model in the future.

Weaknesses of the work

1) In order to examine whether synthetic dsRNA (poly(I-C)) induced activation of pulmonary epithelial cells BEAS-2B shares any characteristics with that stimulated by influenza virus or measles virus and whether production of inflammatory mediators in response to a viral infections can occur in the presence or absence of multiplication of the pathogen, a complete comparison of effects from these inducers in the human bronchial epithelial cell line BEAS-2B should be performed. This could be achieved by doing a time course and concentration curve for cytokine and chemokine production in the presence of these stimulants.

2) In order to ascertain that dexamethasone inhibits virus induced IL-8 production in BEAS-2B cells by preventing NF-κB activation (activation of this transcription factor manifest by nuclear translocation), the level of NF-κB should be determined in nuclear and cytoplasmic extracts before and after infection, without and in the presence of dexamethasone. This could be achieved by Western blot analysis.

3) In order to characterise signalling pathways involved in cytokine and chemokine production in virus infected HEK293 and BEAS-2B cells and the effect of pulmonary surfactant on signalling pathways involved in cytokine and chemokine production in virus infected HEK293 and BEAS-2B cells,

- expression and localisation of TLR3 and RIG-I receptors should be analysed in unstimulated and stimulated cells before and after transfection (RT-PCR could be used to

test the presence of TLR3 and RIG-I mRNA in these cells whereas protein expression level of TLR3 and RIG-I receptors could be examined by flow cytometry),

- the optimal time for assessing reporter activation in cells of interest should be ascertained (this could be achieved by doing a time course and concentration curve for the luciferase assay data set).

Summary

Present *in vitro* studies demonstrate that commercially available SP-A and SP-D depleted pulmonary surfactant preparations can reduce IL-8 and RANTES production in influenza virus infected human bronchial epithelial cell line BEAS-2B. Porcine Curosurf® had more pronounced inhibitory effect on cytokine production than bovine Survanta®. Liquid chromatography and mass spectrometry revealed that these two pulmonary surfactant preparations differed in phospholipid content therefore this may be at least in part responsible for differences seen. Pulmonary surfactant was not cytotoxic in BEAS-2B cells and had no effect on influenza virus infectivity, suggesting that in the presence of pulmonary surfactant cellular responses of BEAS-2B cells induced by influenza virus were altered. This was supported by Dual-Luciferase® Reporter Assay data which show that pulmonary surfactant suppressed TLR3 and RIG-I mediated NF-κB promoter activation in HEK293 cells, and NF-κB and IFN-β promoter activation in BEAS-2B cells. Hence, exogenous pulmonary surfactant therapy may be of clinical benefit by reducing NF-κB dependent pro-inflammatory responses during influenza virus infection; however, a weakening of IFN-β dependent anti-viral responses can not be excluded.

The present *in vitro* studies on the function of clinically approved pulmonary surfactant preparations during influenza virus infection in human airway epithelial cells may help the

development of new therapies aimed at achieving cytokine balance at the site of inflammation, thus reducing the severity of influenza disease.

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Project for MSc degree: Analysis of monocytes subpopulation – investigation of the $CD16^-$ and $CD16^+$ monocytes ability to bind to endothelial cells, obtained from lungs, skin and lymph nodes; led by supervisor Professor JULIUSZ PRYJMA (Jagiellonian University).

Conferences and presentation

- 2008; American Society for Virology 27th Annual Scientific Meeting, Ithaca, NY USA; **a poster.**
- 2007; Third European Congress of Virology, Nurnberg, Germany; **a poster.**
- 2006; American Society for Virology 25th Annual Scientific Meeting, Madison, Wisconsin, USA; **a poster.**
- 2005; 20th Annual Postgraduate Research Day, Cardiff, United Kingdom; **a poster.**

ABSTRACT

(2008 American Society for Virology 27th Annual Scientific Meeting, Ithaca, NY, USA)

This *in vitro* study of commercially available SpA/D depleted surfactant preparations shows that i. surfactant preparations approved for clinical use are generally not cytotoxic in human airway epithelial cells, ii. have no effect on influenza virus infectivity, and iii. downregulate pro-inflammatory cytokine secretion comparable to a glucocorticoid (dexamethasone) control. Human lung type II alveolar epithelial cells A549 and BEAS-2B human bronchial epithelial cells were infected with endotoxin-free influenza A virus H1N1 strains A/Swine/1976/31, A/WSN/33, and A/PR/8/34. Pro-inflammatory cytokines IL-8 and RANTES, as well as IFN-β were quantified by ELISA. Measles virus strain Edmonston, poly I:C and *Escherichia coli* 0111:B4 LPS were used as cytokine stimulation controls. In the presence of physiological concentrations of commercially available SpA/D depleted surfactant preparations (up to 500 μg/ml), IL-8 and RANTES responses of human airway epithelial cells to infection with influenza virus were reduced up to 30% ($p < 0.05$) between 12 and 48 hours following infection in comparison to uninfected controls and comparable to treatment with 1 μg/ml dexamethasone ($p < 0.05$). The reduction of pro-inflammatory cytokine responses were also observed when LPS was added as an additional stimulant ($p < 0.05$). However, in contrast to dexamethasone, commercially available SpA/D depleted surfactant preparations inhibited both NF-κB and IFN-β promoter activity in luciferase reporter assays. Surfactants from different manufacturers and even different batches from the same manufacturer showed considerable biological variability. This data may explain in part the widely different clinical outcomes when selected commercially available SpA/D depleted surfactant preparations were used as a non-systemic alternative for anti-inflammatory treatment of virally induced ARDS.