REGULATION OF THE EXPRESSION OF SECRETORY COMPONENT BY HUMAN AIRWAY EPITHELIAL CELLS *IN VITRO*

GEMMA SLINN

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ASTON UNIVERSITY

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SUMMMARY

Secretory component (SC) is a member of the immunoglobulin superfamily and is secreted uniquely by epithelial cells. Its primary role is the transcytosis of secretory immunoglobulin A (SIgA) from the basolateral to the apical side of epithelial cells. Membrane bound SC (polymeric immunoglobulin receptor; pIgR) forms a complex with dimeric IgA at the basolateral side of the cell, the complex is transcytosed and SIgA is released following proteolytic cleavage at the luminal side of the cell. When in complex with IgA, SC confers resistance and stability to the antibody. In addition pIgR can transcytose by itself and it is proteolytically cleaved and released at the apical side of the cell as free SC.

The role of free SC in mucosal protection remains elusive with both anti-inflammatory and anti-infective properties proposed. Previous studies investigating the role of SC in mucosal immunity have shown that SC is upregulated in airway epithelia by the inflammatory cytokines interferon gamma (IFN•) and interleukin-4 (IL-4). Both IFN• and IL-4 are found in inflamed airways which suggest that SC could play an important anti-inflammatory role. Bacterial stimuli such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA) have been shown to cause inflammation *in vitro* and *in vivo* and therefore the work presented in this thesis aimed to establish whether SC production by human airway epithelial cells *in vitro* was up regulated following stimulation with LPS or LTA.

An immortalized adherent epithelial cell line (CALU-3) that secretes SC constitutively was used as an *in vitro* model to investigate whether SC expression was up regulated following stimulation with either LPS from *E. coli, B. cepacia, P. aeruginosa*, or LTA from *S. aureus.* Analytical techniques were developed to enable the analysis and quantification of SC released from CALU-3 cells. It was found that SC expression was upregulated following stimulation with LPS or LTA.

Furthermore it was observed constitutively released SC was detected only after 48 hours, and did not accumulate in culture supernatants. Foetal calf serum and/ or protease inhibitor cocktail was reintroduced into the cell culture model and it was found that following this, SC was detected at the earlier time point of 24 hours and the concentration of SC detected in unstimulated culture supernatants increased as time increased. Additionally medium extracted from CALU-3 cell cultures was shown to degrade SC isolated from colostrum. Previous studies have shown that SC is degraded in the chronically inflamed lungs of CF patients and that this degraded SC no longer binds interleukin-8 (IL-8). The findings presented in this thesis could suggest that as SC is readily degraded by serine proteases *in vitro*, the degradation of SC observed in inflamed airways *in vivo* could be a result of proteolytic degradation of SC and potentially this could impact SCs immunological function.

Key words: Secretory component, epithelial cells, lipopolysaccharide, lipoteichoic acid

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For Elliot

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ABBREVIATIONS

APS	Ammonium persulphate
AU	Arbitrary units
BSA	Bovine serum albumin
CF	Cystic fibrosis
cm^2	Centimetre squared
dIgA	Dimeric immunoglobulin A
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ELISpot	Enzyme linked immuno spot assay
EU	Endotoxin units
FCS	Foetal calf serum
H_2O_2	Hydrogen peroxide
HAT	Human airway trypsin-like protease
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL-1	Interleukin 1
IL-1•	Interleukin 1 beta
IL-4	Interleukin 4
IL-8	Interleukin 8
INF-•	Interferon gamma
ITS	Insulin/ transferrin/ sodium selenite supplement
kDa	kilo Daltons
KDO	2-keto-3-deoxyoctonate
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
M	Molar
MEM	Minimum essential medium
mg	Milligrams
MH	Mueller Hinton
µg	Micrograms
ml	Millilitres
mM	Millimolar
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NaCl	Sodium chloride
NF•B	Nuclear factor kappa B
ng	Nanograms
nm	Nanometres
nM	Nanomolar
°C	Degrees Celsius
OD	Optical density
OPD	*o*-phenylenediamine dihydrochloride
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
pg	Picograms
PIC	Protease inhibitor cocktail
pIgR	Polymeric immunoglobulin receptor
R_F	Relative front
SC	Secretory component
SDS PAGE	Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis
SIgA	Secretory immunoglobulin A
TEMED	N,N,N',N'- tetramethylenediamine
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNF-•	Tumour necrosis factor alpha
V	Volts
v/v	Volume for volume
w/v	Weight for volume

1 Introduction

1.1 Secretory component

The N-linked glycoprotein secretory component (SC) is a member of the immunoglobulin superfamily and is secreted uniquely by epithelial cells (Kühn and Kraehenbuhl, 1979). The molecular weight of free SC was determined by (Kobayashi, 1971) and (van Munster *et al.*, 1971) to be approximately 74 ± 6 kDa. SC in mucosal secretions can be found as either free SC or SC in association with dimeric IgA (dIgA) as secretory IgA (SIgA). SIgA is composed of two monomeric IgA units, a linking 'J chain' and SC. The IgA units and the J chain are synthesised by plasma cells, SC is derived from the polymeric immunoglobululin receptor (pIgR).

1.2 The Role of SC in Humoral Defence

The primary role of SC is the binding and transcytosis of SIgA from the basolateral to the mucosal side of the epithelial cell (Mostov, 1994). Briefly, dimeric IgA is secreted by plasma cells in the lamina propria; it then forms a complex with pIgR at the basolateral side of the cell. The complex is transcytosed and SIgA is released when the complex is proteolytically cleaved at the luminal side of the cell, in addition pIgR that has not bound to IgA can also transcytose and is found in mucosal secretions as free SC (figure 1.1).

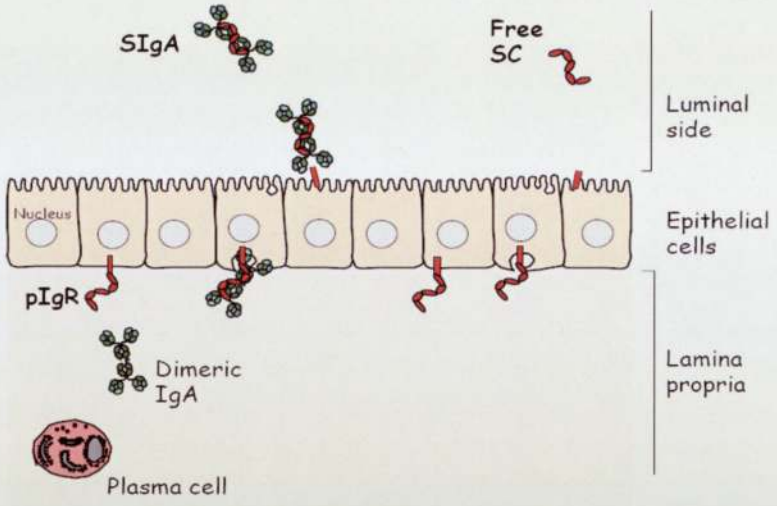

Figure 1.1 Schematic adapted from (Perrier *et al.*, 2006) illustrating the transcytosis of SIgA across the epithelium. Dimeric IgA secreted by plasma cells in the lamina propria binds to membrane bound pIgR at the basolateral side of the cell. The complex is transcytosed and SIgA is released following proteolytic cleavage. Transcytosis of unoccupied pIgR can also occur and this leads to the release of free SC into mucosal secretions.

SIgA plays a central role in the airways' humoral defence system as it acts a barrier, effectively protecting mucosal cells from pathogenic colonisation and invasion by binding to antigens, thus preventing their attachment known as 'immune exclusion' (Brandtzaeg, 1996 and Corthésy and Kraehenbuhl, 1999). When in complex with SIgA, SC confers both stability (by anchoring IgA through its seven carbohydrate sidechains to the luminal side of the cell) and proteolytic resistance (protection from degradation by proteolytic enzymes released by pathogens) to the complex (Brown *et al.*, 1970 and, Lindh, 1975).

1.3 The Role of Free SC in Mucosal Defence

The role of free SC in mucosal protection however, remains elusive with both anti-inflammatory and anti-infective properties proposed. It has been shown that SC is upregulated in airway epithelial cells *in vitro* by inflammatory cytokines including interferon gamma (IFN•: Loman, *et al.*, 1997; Godding *et al.*, 1998; Loman *et al.*, 1999 and Ackermann *et al.*, 1999) and interleukin-4 (IL-4; Loman *et al.*, 1999 and Ackermann *et al.*, 1999).

1.4 Anti-inflammatory Properties of SC

Nihei *et al.*, (1994) proposed that SC played an anti-inflammatory role in the pathogenesis of inflammatory skin diseases by showing that SC inhibits INF• induced inflammation in human keratinocytes *in vitro*. Marshall *et al.*, (2001) provided further evidence that SC displays anti-inflammatory properties by showing that SC binds to and reduces the neutrophil chemoattractant properties of IL-8, thus potentially reducing neutrophilic inflammation in human airways.

1.5 Anti-infective Properties of SC

Research by Giugliano *et al.*, (1995) and by de Araujo and Giugliano, (2001) demonstrated that free SC extracted from human milk inhibited the adhesion of enterotoxic *Escherichia coli* to intestinal epithelial cells *in vitro*. In addition it was demonstrated by Dallas and Rolfe, (1998) and Perrier *et al.*, (2006) that SC interacts with toxin A from *Clostridium difficile* and prevents its cytopathic effects upon epithelial cells; however when toxin A was preincubated with deglycosylated SC, SC appeared to exhibit no such protective properties on epithelial cells. A study in 2002 by Phalipon *et al.* demonstrated that dimeric IgA is more efficient when in complex with SC in protecting mice from bacterial infection. It was observed that the glycans displayed by SC enabled anchorage of SIgA to the mucous linings of the epithelial surface, thus increasing localisation and clearance of bacteria.

1.6 Infection and Inflammation in the Airways

1.6.1 Gram Negative Infection

Lipopolysaccharide (LPS) is a heat stable constituent of the gram negative bacteria's architecture and is thought to be the centre point of gram negative infection. Rietschel and Brade (1992) proposed that LPS molecules consist of a lipid A core attached to a polysaccharide moiety linked via a 2-keto-3-deoxyoctonate (KDO) to the variable oligosaccharide region or the 'O antigen'. It was demonstrated that whilst the lipid A core is responsible for the biological effects of LPS and activated the alternative complement pathway, the O antigen is responsible for the heterogeneity of the response between LPS from varying bacteria strains and activated the classical complement pathway (Rietschel *et al.*, 1994).

Lipid A binds at epithelial surfaces and elicits a cytokine cascade which is responsible for the host inflammatory response to the pathogen (Rietschel *et al.*, 1994). The O antigen of LPS is non-toxic but it is still thought to play a role in gram-negative bacteria's virulence (Rietschel *et al.*, 1994).

1.6.1.1 *Burkholderia cepacia*

B.cepacia as first described by Burkholderia in 1949 is a Gram-negative aerobic, motile rod that causes opportunistic infection in the lungs of cystic fibrosis (CF) patients and is resistant to many antibiotics (Zughaier *et al.*, 1999). It is estimated that 10 % of CF patients lungs are colonised by *B. cepacia* and *B. cepacia* is often present in co-colonisation with *P. aeruginosa*

another important pathogen in CF (Sajjan *et al.*, 1992). Co-colonisation with both *B. cepacia* and *P.aeruginosa* is associated with a decreased life expectancy (Hart and Winstanley, 2002)

1.6.1.2 *Psuedomonas aeruginosa*

P. aeruginosa is major pathogen in CF lungs, it is a Gram-negative, oxidase positive rod that is motile by means of polar flagella and is ubiquitous in moist environments (Hart and Winstanley, 2002). Burns *et al.*, (2001) found that 97.5 % of children with CF in three centres in the US were infected with *P. aeruginosa* by the age of three years.

1.6.2 The Role of LPS from *B. cepacia* and *P. aeruginosa* in Inflammation

LPS plays an important role in inflammation. In 1999 Zughaier *et al.*, showed that *B. cepacia* LPS stimulates TNF-• production from a human monocyte cell line to the same extent as LPS extracted from *E. coli*, but the activity of *B. cepacia* LPS was four to eightfold greater than LPS extracted from *P. aeruginosa*. Also it has been shown that the LPS from the strain used in this study (the ET12 lineage) upregulates the production of nitric oxide synthase and pro inflammatory cytokine mRNA in human leukocytes *in vitro* (Hutchison *et al.*, 1998)

1.6.3 Gram Positive Infection

The pathogenicity of gram positive bacteria depends on the production of virulence factors for example surface proteins such as 'clumping factor' that is found on most strains of *S. aureus* and aids adherence to the host (Ginsberg 2002). In addition pathogen-associated molecular patterns (PAMPs) for example LTA play an important role in pathogenesis as they enable the bacterium to bind to host cells causing them to synthesize and secrete inflammatory cytokines and chemokines. Once adhesion and invasion has taken place many gram positive bacteria secrete toxins for example • – toxin secreted by *S. aureus*. These toxins are harmful to many types of mammalian cells and are associated with the many pathological symptoms observed in gram positive infection (Ginsberg 2002).

1.6.3.1 *Staphylococcus aureus*

S. aureus is catalase positive, gram positive cocci that are arranged in clusters. It is a facultative anaerobe and displays species specific teichoic acid and a slime layer. *S. aureus* forms part of the normal flora on skin and mucosal surfaces. *S. aureus* most commonly causes infections around foreign bodies for example shunts and catheters, however it frequently colonises children with CF and is a prominent cause of pneumonia in CF patients (Ginsberg 2002).

1.6.3.2 The Role of LTA from *S.aureus* in Inflammation

Lipoteichoic acid (LTA) is a common surface antigen that distinguishes bacterial serotypes and promotes attachment to other bacteria and to specific receptors on mammalian cell surfaces. Teichoic acids are important factors in virulence (Ginsberg 2002) and are believed to 'shed' into the host. Although weaker than the response to endotoxin, the response is thought to be a similar mechanism to the host's response to endotoxin. *In vitro* models have suggested that LTA owes some of its virulence to its capacity to stimulate pro inflammatory cytokines such as TNF-• and IL-1 (Ginsburg, 2002).

1.7 Aims of the Project

Previously published work by Loman, *et al.*, (1997) and (1999), Godding *et al.*, (1998), Ackermann *et al.*, (1999) have found that SC is upregulated by INF• and IL-4 in *in vitro* models of human airway epithelia. In addition it was found that SC may play a potentially important anti-inflammatory role *in vivo* Nihei *et al.*, (1994) and Marshall *et al.*, (2001). It has not yet been established whether LPS and LTA, both of which are important components in bacterial derived inflammation up regulate SC production in human airways. This study therefore aimed to examine whether LPS and LTA are responsible for an up regulation of SC.

1. The initial aim of the project was to verify SC secretion constitutively from CALU-3 cells and to establish whether SC expression from CALU-3 cells is up regulated by LPS and LTA.

2. The next aim was to develop analytical techniques to analyse and quantify SC release from CALU-3 cells. Western Blotting was used to analyse the SC released from CALU-3 cells. In addition a capture ELISA was developed to quantify the amount SC released from CALU-3 cells to test the hypothesis that SC is up regulated following stimulation with LPS or LTA.

3. The final aim was to establish whether conditioned media (media that had previously been incubated with CALU-3 cells) contained proteases that were causing cleavage of the SC released by CALU-3 cells prior to its analysis.

2 Materials and Methods

2.1 Materials

2.1.1 Standard Human SC

Human Standard Milk Reference from Nordic Immunology (Autogen Bioclear, UK) was used as standard SC. The standard was prepared from colostrum from a person that lacks all immunoglobulins. The manufacturers provided the standard at a concentration of SC of 820 µg/ml. The lyophilized powder was mixed with sterile distilled water and allowed to equilibrate at ambient temperature for 10 minutes, before being aliquoted and stored at -20 ºC.

2.1.2 Antibodies

Mouse monoclonal anti-human SC; clone GA-1 (ascites fluid) was obtained from Sigma Aldrich (Poole, UK). The antibody was immunospecific for SIgA and free SC as determined by ELISA. Sigma confirmed that the IgG concentration was 6.7 mg/ml.

Polyclonal rabbit antiserum to human SC was obtained from Nordic Immunology (Autogen Bioclear, UK). The antibody is specific for both SC bound to IgA and free SC. The antibody was delivered as delipidated, heat inactivated, stable lyophilized antiserum. Lyophilized antiserum was reconstituted by adding 1 ml sterile distilled water, it was then aliquoted into 2 and 10 •l aliquots and stored at -20 °C. Nordic Immunology confirmed that the IgG concentration was 5 mg/ml.

Horseradish peroxidase-linked anti-mouse IgG from sheep and horseradish peroxidase-linked anti-rabbit IgG from donkey were obtained from Amersham Biosciences (Buckinghamshire, UK) and stored at 4 °C.

Mouse IgG1 kappa from murine myeloma, clone •MOPC-21 ascites, obtained from Sigma Aldrich (Poole, UK) was used as a negative control. This antibody recognises the kappa light chain of IgG1 and was derived from a mouse plasma cytoma. The antibody was delivered as ascites fluid lyophilized with no preservatives added. To reconstitute, 1 ml of sterile distilled water and sodium azide were added to a final concentration of 15 mM. Sigma confirmed that the myeloma protein content was 17 mg/ml.

2.1.3 Tissue Culture

CALU-3 cells were purchased from the American Type Culture Collection (ATCC).

Minimum essential medium (MEM), foetal calf serum (FCS), penicillin/streptomycin, $_{L}$-glutamine and trypsin:EDTA were all from PAA (Buckinghamshire, UK).

Sodium pyruvate, non essential amino acids, insulin-transferrin-sodium selenite (ITS) liquid media supplement and protease inhibitor cocktail were all from Sigma Aldrich (Poole, UK).

2.1.4 Control Proteins

Bovine serum albumin (BSA) Fraction V, 96 % pure (A2153-50G), BSA Fraction V 99 % pure (A3059-50G), gelatin from cold water fish were all from Sigma Aldrich (Poole, UK).

Powered skimmed milk was reconstituted in sterile PBS and then syringe filtered using a 25 mm Millex filter unit (0.45 micron).

2.1.5 Lipoteichoic Acid

LTA from *S. aureus* B4 was purchased from Sigma Aldrich (Poole, UK).

QCL-1000® Endpoint Chromogenic LAL Assay (50-647U) was purchased from Lonza (Basel, Switzerland).

2.1.6 Lipopolysaccharide

LPS from *E. coli* (strain 0111:B4) was purchased from Sigma Aldrich (Poole, UK).

B. cepacia (strain J2315) and *P. aeruginosa* (strain O5D) were obtained from the Microbiology Research Group culture collection, Aston University. Both strains were isolated from sputum samples from CF patients. The strain of *B. cepacia* used in this project is from the ET12 lineage (strain J2315) and this is known to be the most highly transmissible strain of *B .cepacia*, in addition to containing LPS that plays a "considerable role" in CF lung inflammation and is significantly more toxic than LPS from the *P. aeruginosa* strain used here Zughaier *et al.*, (1999).

2.1.7 Reagents and Buffers

Trypsin from bovine pancreas, sodium chloride (NaCl), Polyoxyethylene (20) sorbitan monolaurate solution (Tween® 20), 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), hydrochloric acid (HCl), carbonate-bicarbonate buffer capsules, SIGMA*FAST*™ *o*-phenylenediamine dihydrochloride (OPD) ELISA colour substrate tablets, Kodak processing chemicals for autoradiography, (GBX developer and replenisher and GBX fixer and replenisher), Kodak XLS X-ray film, butan-1-ol, glycine, glycerol, N,N,N',N'-tetramethylenediamine (TEMED), ammonium persulphate (APS), sodium dodecyl sulphate (SDS), 30 % acrylamide:bisacrylamide solution and bromophenol blue were all from Sigma (Poole, UK).

Mueller Hinton Broth and 1 x Dulbecco's Phosphate Buffered Saline (PBS) tablets were from Oxoid (Cambridge, UK).

ProSieve® colour protein markers 9 – 173 kDa were from Lonza (Basel, Switzerland).

Schleicher and Schuell Protran® nitrocellulose blotting membrane (0.45 μm pore size) and Schleicher and Schuell blotting paper were from Geneflow (Staffordshire, UK).

SuperSignal® West Pico Chemiluminescent Substrate was from Pierce (Chester, UK).

2.1.8 Equipment

Maxisorp 96 well microtitre plates were from Nunc™ (Rochester, NY).

Costar® Mylar® plate sealing tape was from Sigma Aldrich (Poole, UK).

Anthos reader 2001 (microtitre plate reader), was from Anthos Labtec Instruments (Eugendorf, Austria).

Epson LX-850 printer (with plate reader).

Vertical gel electrophoresis units, gel multicaster and powerpack were all from Geneflow (Staffordshire, UK).

Transblot® SD semidry electrophoretic transfer cell was from Bio-Rad (Hertfordshire, UK).

Scanner and protein quantification software were both from Syngene (Cambridge, UK).

2.2 Methods

2.2.1 Cell Culture

CALU-3 cells are an immortalized adherent epithelial cell line derived from an adenocarcinoma of the lung. Cells were grown in 75 cm^2 tissue culture flasks and maintained in 12 ml of minimum essential medium (MEM) supplemented with 10 % foetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM $_L$-glutamine, 1 % sodium pyruvate and 1 % non essential amino acids (complete medium). Cells were incubated in a 37 °C, 5 % CO_2/ 95 % air mixture incubator until the cells reached 80 % confluence at which time the cells were passaged. Cells were passaged by removing the medium and washing the cells in approximately 15 ml of PBS, then incubating the cells with 2.5 ml of 720 µg/ml trypsin: EDTA for 20 minutes at 37 °C. When cells had detached 9.5 ml of complete growth medium was added to each flask, this was transferred to a 50 ml test tube and centrifuged at 500 *g* for 5 minutes at room temperature. The supernatants were discarded, then the cell pellet was resuspended in 1 ml of fresh medium the cells were then counted using a haemocytometer. The cells were sub cultured at a ratio of 1:3 and plated in fresh 75 cm^2 flasks. For challenge experiments, cells were plated at a density of 1 x 10^5 cells per well in 24 well plates and were maintained in 1 ml per well of complete growth medium for 11 days until 100 % confluence was reached.

2.2.2 Preparation of Purified LPS From *Psuedomonas aeruginosa* and *Burkholderia cepacia*

LPS was extracted from whole cells that had been cultured overnight in Mueller Hinton (MH) Broth, using the hot phenol extraction method described by Westphal and Jann (1965). Strains of *P. aeruginosa* (O5D) and *B. cepacia* (J2315) were harvested by centrifugation at 10,000 *g* for 10 minutes. The pellet was resuspended in approximately 100 ml distilled water, sonicated for three minutes on ice and incubated with 125 μg/ml of DNase for one hour at 37 °C. An equal volume of 80 % (w/v) phenol was added and the mixture was heated to 80 °C with stirring. The two phases were separated by centrifugation at 10, 000 *g* for 10 minutes, the upper aqueous phase was removed and retained. The lower phenol layer was re-extracted with an equal volume of water, following centrifugation the upper aqueous phase was removed and recovered, this was repeated four times in total. All recovered upper aqueous phases were combined and dialysed overnight against three changes of water to remove the phenol. 10 mM magnesium sulphate was then added to the dialysate to aid aggregation of the LPS micelles. LPS was centrifuged at 50, 000 *g* using a Beckman J8 ultracentrifuge with a Ti 80 rotor at for three hours in total. The subsequent LPS pellet was then freeze-dried and stored at -20 °C.

2.2.3 QCL-1000® Endpoint Chromogenic LAL Assay

The QCL-1000® Endpoint Chromogenic LAL Assay was used a quantitative test for gram negative bacterial endotoxin. Test kit provided endotoxin standards (concentrations ranging from 0.1 to 1 EU/ml), 10 μg/ml of LTA from *S. aureus* or 10 μg/ml LPS from *E. coli*, *B. cepacia* or *P. aeruginosa* was mixed with the LAL that was supplied in the test kit and

incubated for 10 minutes at 37 °C. A 2 mM chromogenic substrate solution was then mixed with the LAL and LTA or LPS mixture and incubated for 6 minutes at 37 °C. The reaction was stopped by adding 25 % v/v glacial acetic acid in water and the absorbance of the end product was determined spectrophotometrically at 410 nm. Endotoxin concentrations (EU/ml) of the samples were interpolated from the endotoxin standard curve which may be found in Appendix 3.

2.2.4 Statistical Analysis

Data were statistically analysed using the Student's two-tailed *t* test in Microsoft Excel and statistical significance was assigned at $p \bullet 0.05$.

3 Detection and Analysis of SC Using Western Blotting

3.1 Introduction

The purposes of the Western blotting experiments were to develop a method that showed both the size and integrity of SC and to establish whether Western blotting was as sensitive a method of detecting SC as an ELISA designed to detect SC.

The standard reference for SC is prepared from human colostrum, details can be found in section 2.2.1. Previous work by van Munster *et al.*, (1970) and Kobayashi, (1970) determined the molecular weight of free SC isolated from human milk to be 74 ± 6 kDa.

Western blotting is a method adopted to detect and quantify the presence of antigen in a sample, in addition it can be used to determine the size and quantity of protein antigens which react with a specific antibody. Proteins are firstly separated according to their molecular weight using SDS-PAGE as first described by Laemmli, (1970). Once separated, proteins in the polyacrylamide gel can be stained using a protein stain such as Coomassie blue to ascertain the purity of the preparation or they can be electrophoretically transferred to a nitrocellulose membrane using either a submerged or the semi dry transfer method described by Towbin *et al.*, (1979). Following transfer, the nitrocellulose membrane is incubated with a primary antibody raised against the antigen of interest. This 'primary' antibody will bind to the antigen that has been immobilised onto the membrane, then an enzyme conjugated secondary antibody is added; for example antibody conjugated to horseradish peroxidase (HRP). Following incubation with the secondary antibody, the membrane is incubated with a chemiluminescent substrate solution containing luminol and hydrogen peroxide (H_2O_2),

luminol is oxidized in the presence of H_2O_2 and creates an excited state product called 3-aminophthalate. The product decays to a lower energy state by releasing photons of light.

3.2 Methods

3.2.1 Analysis of SC Using SDS-PAGE

SC was analysed on a 10 % resolving gel using SDS-PAGE according to the method described by Laemmli, (1970) using a discontinuous buffer system. The SC was first solubilised in non reducing 2 X Laemmli sample buffer and 10 µl of the sample was loaded per lane. A voltage of 90 V was applied across the gel until the protein stack reached the resolving gel at which point the voltage was increased to 120 V. Voltage was applied until the bromophenol blue leading edge reached the bottom of the gel.

3.2.2 Electrophoretic Transfer of SC to Nitrocellulose

SC was transferred from the polyacrylamide gel to 0.45 µm nitrocellulose membrane using the semi dry transfer method described by Towbin *et al.*, (1979) in transfer buffer (150 mM Tris, 20 mM glycine, SDS and 20 % methanol) for 80 minutes at a constant current of 250 mA. Following transfer membranes were blocked in blocking buffer (PBS/ 2 % Tween® v/v) overnight at 4 °C.

3.2.3 Western Blotting Using Monoclonal Anti-SC

The membrane was removed from the blocking buffer and washed three times for ten minutes each with agitation in wash buffer (PBS/ 0.05 % Tween® v/v). Following washing the membrane was incubated with the primary antibody (670 µg/ml monoclonal anti-SC in PBS/ 2 % Tween® v/v) for 90 minutes at ambient temperature. The washing step was repeated following incubation with primary antibody, and then the membrane was incubated with

secondary antibody HRP conjugate at 0.19 μg/ml in PBS/ 2 % Tween® v/v for one hour at ambient temperature. After an hour the membrane was washed five times for ten minutes each and developed. The membrane was incubated with chemiluminescent substrate for five minutes and then placed in a clear sealable bag, excess moisture was removed and the membrane was exposed to X-ray film overnight. Densitometric Analysis of Western blot images was preformed using the Syngene scanner and Gene Genius protein quantification software.

3.2.4 Western Blotting Using Polyclonal Anti-SC

The protocol in section 3.2.3 was repeated with the exception that the blot was incubated with 1.25 μg/ml polyclonal anti-SC as the primary antibody and 0.19 μg/ml secondary antibody HRP conjugate.

3.2.5 Densitometric Analysis of Western blots

Semi-quantitative analysis of the protein was achieved using densitometric measurement, the X-ray films were scanned and the intensity of the protein band was measured. Following scanning a densitogram was produced and the area under each peak was used to determine the intensity of the protein band (Bromage *et al.*, 2007). Semi quantitative estimates of the concentration of protein in an unknown sample can be determined by comparison with a curve of the band density against the concentration of the known standard. SC standard curves for both monoclonal and polyclonal anti-SC may be found in sections 3.3.2 and 3.3.3. For reference an R_F graph of molecular weight markers is provided in appendix 1.

3.3 Results

3.3.1 Determination of the Purity of the SC Preparation using Coomassie Blue Staining

In order to determine the purity of the commercial preparation of SC, the preparation was electrophoretically separated on a 10 % SDS PAGE gel and then stained with Coomassie Blue. The results of the staining are shown in figure 3.1.

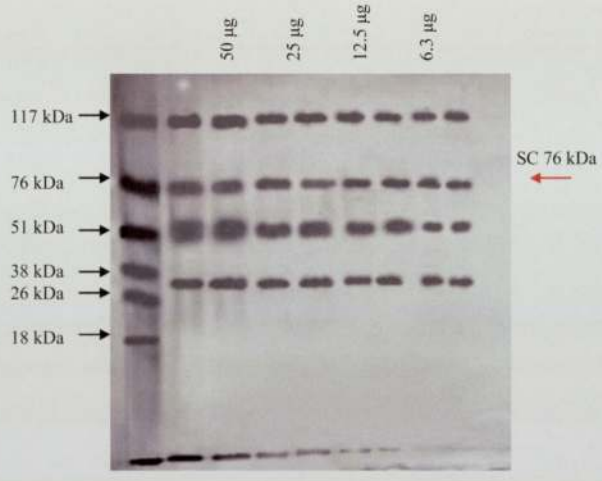

Figure 3.1 Scanned image of a 10 % SDS PAGE gel following staining with Coomassie Blue. The amounts of SC loaded onto the gel are shown above the lanes. Positions of molecular weight markers are shown to the left of the image. The expected position of SC is indicated by the arrow to the right of the image. The scanned image is representative of four replicate gels.

Figure 3.1 shows the electrophoretic separation of Nordic standard SC preparation on an SDS PAGE gel. In this image it was shown that a protein was stained at 76 kDa, which could suggest that the protein was SC. However lactoferrin which is also present in this preparation is approximately 80 kDa. Therefore it is not possible to give a conclusive answer about the identity of the 76 kDa protein from Coomassie blue staining. The staining did however show that the preparation was relatively impure and therefore incubation of a Western blot with an anti-SC antibody was required to confirm that SC was in the preparation.

3.3.2 Western Blotting Using Monoclonal Anti-SC

Monoclonal anti-SC was used to detect SC immobilised on a nitrocellulose membrane, the results of which are illustrated in figure 3.2.

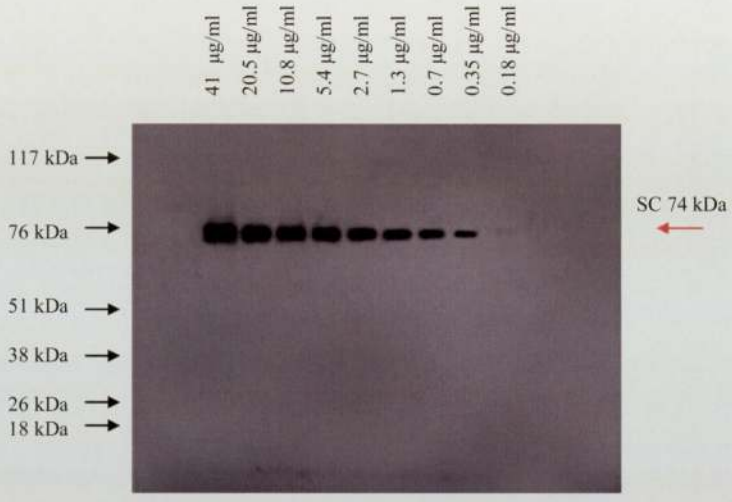

Figure 3.2 Scanned image of a Western blot following incubation with monoclonal antibody to SC and development with HRP-conjugated secondary antibodies and chemiluminescence. The concentration of SC loaded per lane is shown above the lanes, the volume of SC loaded per lane was 10 µl. Positions of molecular markers are shown to the left of the image. The molecular weight of SC is indicated by an arrow to the right of the image. The scanned image is representative of six independent gels.

Data presented in figure 3.2 shows that SC at a molecular weight of 74 kDa was detected using monoclonal anti-SC. There was intra assay variation between the six experiments and overall it was found that the molecular weight of SC was 74 ± 5 kDa. SC was detected between concentrations of 0.35 µg/ml and 41 µg/ml.

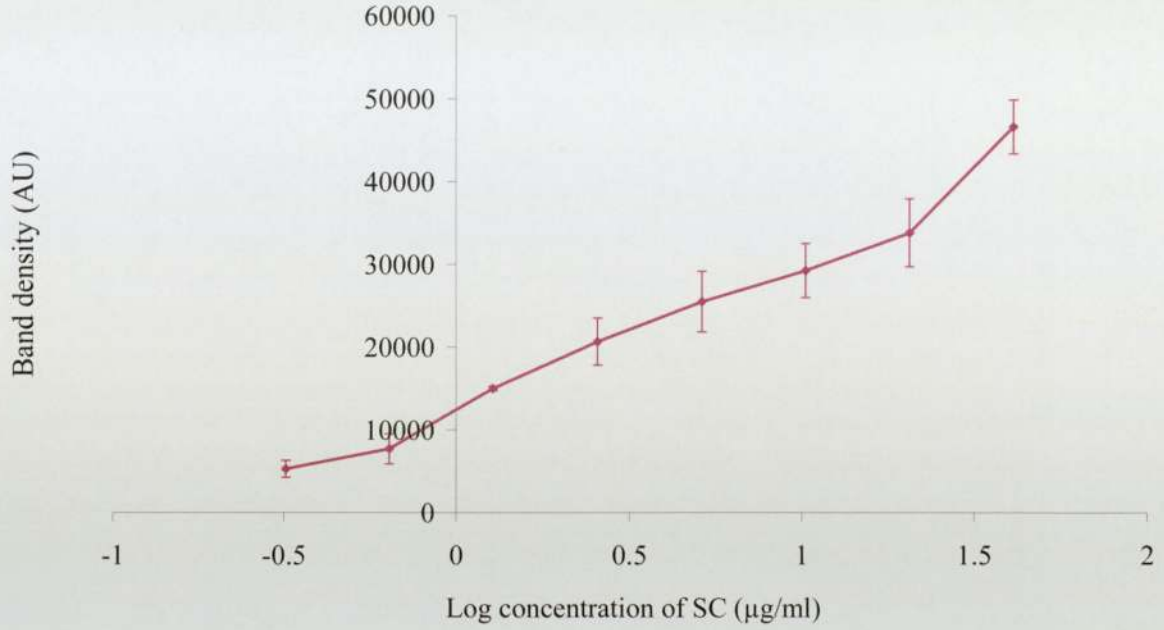

Figure 3.3 Densitometric analysis of a Western blot that had been incubated with a monoclonal antibody to SC and developed with HRP-conjugated secondary antibodies and chemiluminescence. Data are expressed as mean ± standard error (SE) of six independent experiments.

The data presented in figure 3.3 shows the densitometric analysis of Western blots repeated on six occasions. The band density increased as the amount of SC loaded per lane increased. There was a linear increase in band density between SC concentrations of 0.35 and 41 µg/ml. These data can be used as a measurement of relative expression of SC.

3.3.3 Western Blotting Using Polyclonal Antibodies to SC

Polyclonal antibodies to SC were used to detect SC immobilised on a nitrocellulose membrane, the results of which are illustrated in figure 3.4.

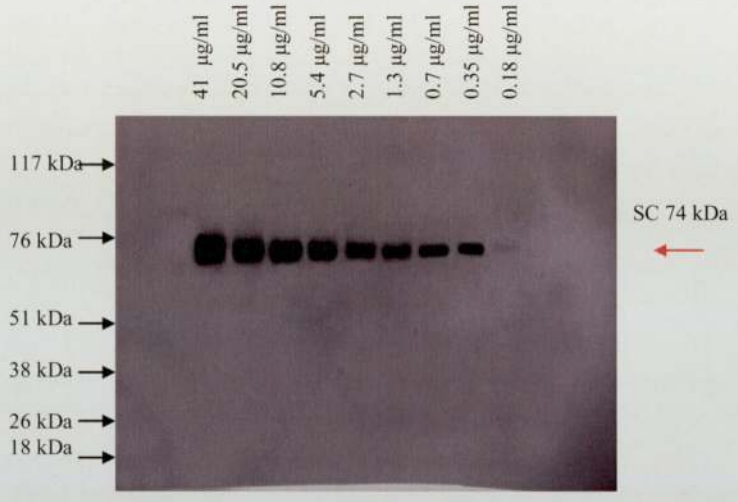

Figure 3.4 Scanned image of a Western blot following incubation with polyclonal antibody to SC and development with HRP-conjugated secondary antibodies and chemiluminescence. The concentration of SC loaded per lane is shown above the lanes, the volume of SC loaded per lane was 10 µl . Positions of molecular markers are shown to the left of the image. The molecular weight of SC is indicated by an arrow to the right of the image. The scanned image is representative of six independent gels.

Data presented in figure 3.4 shows that SC at a molecular weight of 74 kDa was detected using polyclonal anti-SC. There was intra assay variation between the six experiments and overall it was found that the molecular weight of SC was 74 ± 5 kDa. SC was detected between the concentrations of 0.35 µg/ml and 41 µg/ml.

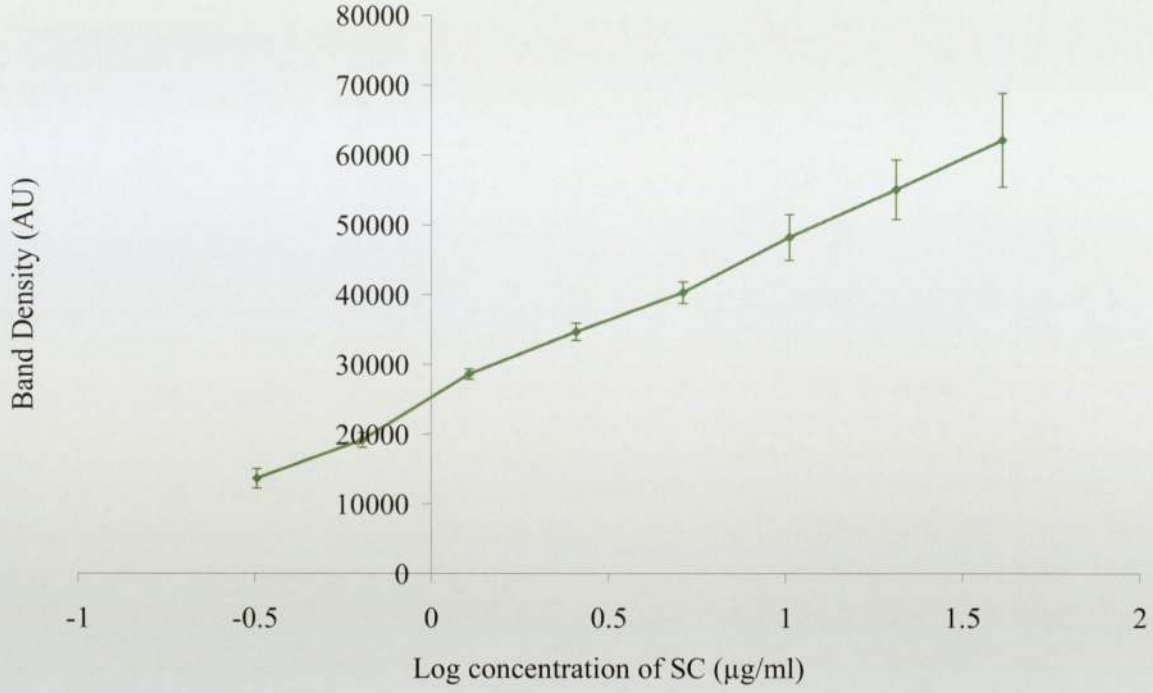

Figure 3.5 Densitometric analysis of a Western blot that had been incubated with a polyclonal antibody to SC and developed with HRP-conjugated secondary antibodies and chemiluminescence. Data are expressed as mean ± SE of six independent experiments.

The data presented in figure 3.5 shows the densitometric analysis of Western blots repeated on six occasions. The band density increased as the amount of SC loaded per lane increased. There was a linear increase in band density between the concentrations of 1.3 and 41 µg/ml. These data can be used as a measurement of relative expression of SC.

3.4 Discussion

The Coomassie blue stained image of colostral SC (figure 3.1) indicated that the preparation was not pure. It was not possible to conclusively identify SC in this preparation and as such it was necessary to use antibodies to SC to detect it.

SC was detected using monoclonal and polyclonal anti-SC at a molecular weight of 74 ± 5 kDa (figures 3.2 and 3.4). These findings agree with previous work by Kobayashi, (1971) and van Munster *et al.*, (1971) that state that the molecular weight of free SC isolated from human colostrum is 74 kDa ± 6 kDa. There was some intra assay variability observed and this was evident in the differences in molecular weight of SC between each blot.

The data presented here also show that Western blotting using either monoclonal anti-SC or polyclonal anti-SC detects SC to a limit no lower than 0.35 μg/ml. It is possible that protein transfer from the polyacrylamide gel onto the membrane varies between each Western blot. For example incomplete transfer of the protein would result in less protein being immobilised on the membrane, this would affect the amount of antigen accessible to the antibody and would thus be reflected in the densitometric measurement. Increasing the sensitivity of the Western blot could potentially result in detecting lower limits of SC. However increasing sensitivity in Western blotting can result in a decrease of specificity shown by high background from non specific binding.

Western blotting cannot be used accurately to determine concentrations of SC unless all standards are run on the same gel and unless the pixel intensities of all of the protein bands

are not saturated. Nonetheless, Western blotting is useful for obtaining information as to the size of the protein.

4 Development of an ELISA for SC

4.1 Introduction

The purpose of these experiments was to design a capture enzyme linked immunosorbent assay (ELISA) to detect SC. Ultimately the ELISA would be used to detect and quantify SC secreted by CALU-3 cells. There are two types of ELISA commonly used, direct ELISA and the capture or sandwich ELISA.

In the direct ELISA, a microtitre plate is coated with antigen that has been diluted in 0.5 M carbonate-bicarbonate buffer (coating buffer). The following day the plate is washed and incubated with either a monoclonal or polyclonal antibody that has been raised against the antigen. Following this, the plate is incubated with an enzyme labelled secondary antibody, for example a horseradish peroxidase (HRP) conjugate. Following incubation with the secondary antibody a colour substrate is added. If an HRP conjugate has been used as the secondary antibody, the colour substrate of choice is *o*-phenylenediamine dihydrochloride (OPD). Peroxidase catalyses the oxidation of OPD, resulting in a colour change from clear to yellow. The optical density (OD) of the enzyme reaction end product can be measured at an appropriate wavelength. In the capture ELISA, the capture antibody (so called because it 'captures' or 'pulls down' the antigen) is coated onto the plate first after which the antigen is added to the assay. The capture antibody may be either a monoclonal or polyclonal antibody that has been raised against the antigen of interest. Following antigen incubation the detection antibody is then used to detect the amount of antigen that has been bound to the capture antibody, the detection antibody can also be monoclonal or polyclonal. A secondary antibody enzyme conjugate is added after the detection antibody followed by a colour

substrate and the optical density of the enzyme reaction end product in each well is measured at an appropriate wavelength.

Quantification of samples measured by the ELISA can be achieved with comparison to known standards of the antigen. This can be done as a standard curve by where increasing dilutions of the known standard are assayed, in addition to the samples. The optical densities of samples can be compared to the optical densities of the known standard; the result is that the antigen concentration per sample can be determined.

4.2 Methods

4.2.1 Direct ELISA Using Monoclonal Anti-SC

In order to ascertain the optimal working conditions and the specificity of monoclonal and polyclonal anti-SC the following investigation was conducted. A 96 well microtitre plate was coated overnight at 4 °C with 100 µl per well of SC at 10 µg/ml diluted in coating buffer. The plate was sealed with Mylar® tape to prevent evaporation of the antigen. Following overnight coating, the plate was washed three times using wash buffer (PBS with 0.05% (v/v) Tween®). The plate was incubated with 100 •l per well of mouse monoclonal anti-secretory component (monoclonal anti-SC) for one hour at ambient temperature. The monoclonal antibody was tested at concentrations of 0.067, 0.67, 6.7, 67 and 670 µg/ml. Antibody working dilutions were done in wash buffer. Following incubation the wells were washed three times with wash buffer and then tapped vigorously to remove any remaining liquid or bubbles. The plate was incubated with 100 •l per well of 0.75 µg/ml of secondary antibody HRP conjugate diluted in wash buffer for 30 minutes at 37 °C. The plate was washed three times with wash buffer and tapped vigorously. 100 •l of SIGMA*FAST*™ OPD ELISA colour substrate was added to each well and plates were incubated for 15 minutes at ambient temperature in the dark. After 15 minutes a colour change from clear to yellow was observed and 100 •l of 1 M HCl was added to each well to stop the reaction. The optical density of the plate was read at 492 nm using the Anthos reader 2001.

4.2.2 Direct ELISA Using Polyclonal Anti-SC

Using the protocol described in section 4.2.1 a microtitre plate was coated with SC overnight and then washed and incubated with 100 •l per well of rabbit polyclonal anti-secretory component (polyclonal anti-SC), for one hour at ambient temperature. Dilutions were done in wash buffer and the concentrations of the antibody used were 0.05, 0.5, 5, 50 and 500 μg/ml. After an hour, the plate was incubated with a secondary antibody HRP conjugate, developed with colour substrate and the optical density of the end product was read as described in section 4.2.1.

4.2.3 Direct ELISA Using Irrelevant Control Antibody, MOPC21

A microtitre plate was coated with SC overnight, and then washed using the protocol described in section 4.2.1. 100 •l per well of MOPC21, diluted in wash buffer was added for one hour at ambient temperature at concentrations ranging from 0.0170 to 1700 μg/ml. Following incubation with MOPC21, the plate was incubated with a secondary antibody HRP conjugate, developed with colour substrate and the optical density of the end product was read as described in section 4.2.1.

4.2.4 Determining the Specificity of Monoclonal and Polyclonal Anti-SC

In order to determine the specificity of both monoclonal and polyclonal anti-SC, a microtitre plate was coated with 100 µl per well of either 96% pure BSA, 99% pure BSA, gelatine, SC or skimmed milk at a concentration of 10 µg/ml, the schematic of the plate can be found in figure 4.1.

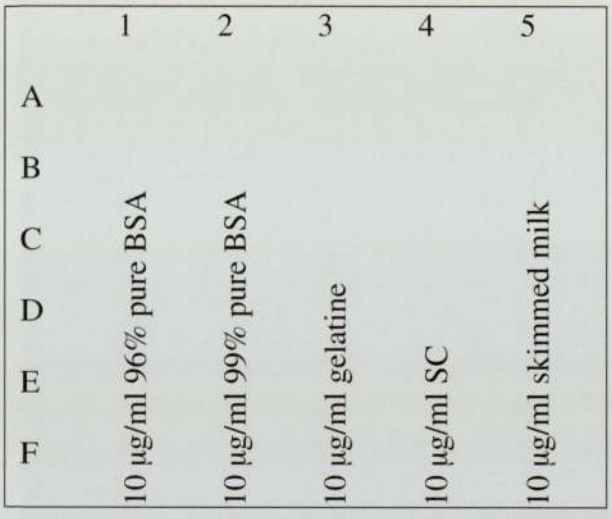

Figure 4.1 Schematic illustrating the layout of proteins coated on a 96 well plate.

All proteins were diluted in coating buffer. Following overnight incubation the wells were washed and the plate was incubated with monoclonal anti-SC using the protocol given in section 4.2.1. After an hour the plate was incubated with a secondary HRP conjugate, developed with colour substrate and the optical density of the end product was read at 492 nm. This protocol was repeated using polyclonal anti-SC and an appropriate secondary HRP conjugate.

4.2.5 Checkerboard ELISAs

The aim of the checkerboard capture ELISA is to elucidate the optimum working concentrations of the capture and detection antibodies. The checkerboard ELISA is achieved by coating the 96 well microtitre plate horizontally with various concentrations of capture antibody, then incubating the plate vertically with different concentrations of the detection antibody.

A microtitre plate was coated overnight at 4 °C with 100 µl of either monoclonal anti-SC (concentrations ranging from 0.067 to 670 µg/ml) or polyclonal anti-SC (concentrations ranging from 0.05 to 500 µg/ml), diluted in coating buffer. After coating, the plate was washed and then incubated with 100 µl per well of either 10 µg/ml SC or 10 µg/ml BSA, (diluted in wash buffer) for one hour at ambient temperature. Following incubation with the antigen, the plate was incubated for 30 minutes at room temperature with polyclonal anti-SC (concentrations ranging from 0.05 to 500 µg/ml) diluted in wash buffer, if the capture antibody was monoclonal anti-SC. If the capture antibody used was polyclonal anti-SC then the plate was incubated with monoclonal anti-SC (concentrations ranging from 0.067 to 670 µg/ml). After 30 minutes the plate was washed and then incubated with the appropriate secondary antibody HRP conjugate, developed with colour substrate and the OD of the end product was read as described in section 4.2.1.

4.2.6 ELISA Using Monoclonal Anti-SC as Capture and Polyclonal Anti-SC as Detection

A microtitre plate was coated overnight at 4 °C with 100 µl per well of 670 µg/ml of monoclonal anti-SC, diluted in coating buffer. Following washes the next morning the plate was incubated for one hour at ambient temperature with 100 µl per well of SC, at concentrations ranging from 0.17 to 10 µg/ml, diluted in wash buffer. After an hour the plate was washed and then incubated with 100 µl per well of 5 µg/ml polyclonal anti-SC, diluted in wash buffer, for 30 minutes at room temperature. Following incubation with the detection antibody, a secondary antibody HRP conjugate was added and the plate was developed using the protocol described in 4.2.1.

4.2.7 Investigating the Efficacy of Various Blocking Agents on Non Specific Binding

Addition of a blocking step into the ELISA can be beneficial where there is a high background signal due to non specific binding. Vogt *et al.*, (1987) showed that the use of a blocking step could prove effective at decreasing non specific binding. A series of potential blocking agents were investigated to establish which blocking agents successfully decreased non specific binding in the ELISA presented here.

A microtitre plate was coated with SC, and then washed using the protocol described in section 4.2.6. Each plate was then split into four sections, ensuring that three repeats could be done per blocking agent. A schematic illustrating the layout of the microtitre plate is shown in figure 4.2.

	1	2	3	4	5	6	7	8	9	10	11	12

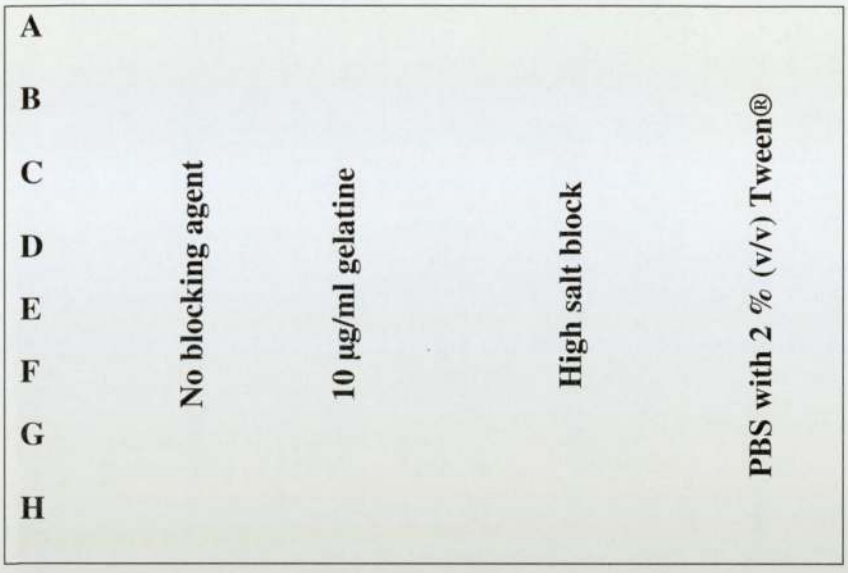

Figure 4.2 Schematic illustrating the layout of blocking agents used in a monoclonal capture, polyclonal detection ELISA.

Each section would test a different blocking agent; the first section would not be blocked at all, the next section was blocked with 315 •l per well of 10 µg/ml gelatine solution. Another section was blocked with 315 •l per well of a high salt block (50 mM Tris-HCl, with 100 mM sodium chloride (NaCl) and 2 % Tween®). The final section was blocked using 315 •l per well of PBS with 2 % (v/v) Tween®. Plates were blocked at 37 °C for one hour. 100 •l per well of increasing concentrations (ranging from 0.17 to 10 µg/ml) of either SC or BSA diluted in wash buffer were added to the plate following blocking, for one hour at room temperature. The plate was incubated with polyclonal anti-SC as described in section 2.2.7, and then incubated with secondary antibody HRP conjugate and developed using the protocol described in section 4.2.1.

4.2.8 Investigating the Cross Reactivity and Specificity of Polyclonal Anti-SC

In order to demonstrate the specificity and lack of cross reactivity of polyclonal anti-SC, the following investigation was conducted. Three samples containing polyclonal anti-SC were prepared, the first contained 0.25 µg of polyclonal anti-SC and 200 µl of wash buffer, the second contained 0.25 µg of polyclonal anti-SC with 8 µg of SC in a total volume of 200 µl of wash buffer and the third contained 0.25 µg of polyclonal anti-SC with 8 µg of BSA made up to a final volume of 200 µl in wash buffer. The samples were incubated with gentle rocking for two hours at ambient temperature.

As described in section 4.2.6, a microtitre plate was coated overnight with monoclonal anti-SC and then incubated with either SC or BSA, at concentrations ranging from 0.17 to 10 µg/ml. Following incubation with the proteins, the plate was washed and then blocked for one hour at 37 °C using 315 •l per well of PBS/2% Tween® (blocking buffer). After two hours the antibodies that had been prepared earlier were centrifuged for 2 minutes at 500 *g*. 195 •l of the antibody samples were made up to a final volume of 5 ml in wash buffer in order to make the final concentration of polyclonal anti-SC 10 µg/ml. Following blocking, plates were washed and then incubated with either 100µl per well of polyclonal anti-SC that had been incubated with wash buffer only, polyclonal anti-SC that had been incubated with SC, or polyclonal anti-SC that had been incubated with BSA. The plate was incubated with the detection antibody for 30 minutes at ambient temperature. After 30 minutes the plate was washed and then incubated with a secondary HRP conjugate, developed with colour substrate and the optical density of the end product was read as described in section 4.2.1.

4.3 Results

4.3.1 Direct ELISAs

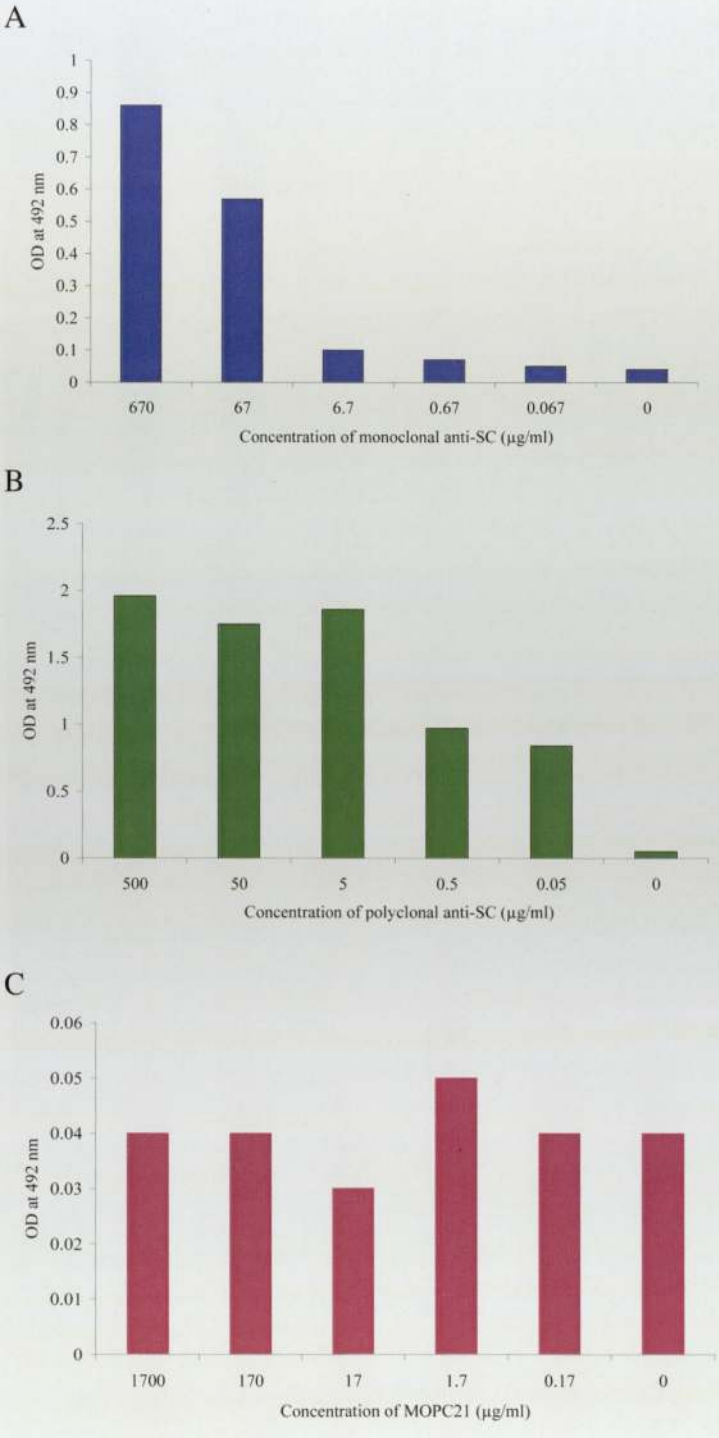

Figure 4.3 The mean OD measurement in relation to the concentration of antibody used on a direct ELISA. A = direct ELISA using monoclonal anti-SC, B = direct ELISA using polyclonal anti-SC, C = direct ELISA using MOPC21 (n=2).

The results presented in figure 4.3A show that the highest OD measurement was recorded when 670 μg/ml of monoclonal anti-SC was used to detect SC. As the concentration of monoclonal anti-SC decreased the OD measurements decreased.

Figure 4.3B presents the findings of a direct ELISA using polyclonal anti-SC and it was observed here that the OD did not decrease as the concentration of polyclonal anti-SC decreased.

Data recorded from the direct ELISA using MOPC21 (figure 4.3C) showed no change in the OD with the changing concentration of antibody.

From these findings it was concluded that the ELISA using monoclonal anti-SC to capture and polyclonal anti-SC to detect would prove most effective at detecting and quantifying SC expressed by epithelial cells.

4.3.2 Determining the Specificity of Monoclonal and Polyclonal Anti-SC

Table 4.1 The mean OD read at 492 nm in relation to the concentration of monoclonal anti-SC (n=2).

Concentration of protein (µg/ml)	Mean OD at 492nm (n=2)				
	96% pure BSA	99% pure BSA	Gelatine	SC	Skimmed Milk
10	0.09	0.05	0.05	0.89	0.54
5	0.07	0.05	0.04	0.60	0.56
2.5	0.07	0.04	0.04	0.12	0.47
1.25	0.05	0.05	0.04	0.06	0.36
0	0.02	0.02	0.02	0.02	0.02

The data presented in table 4.1 shows that the amount of SC detected decreased as the concentration of SC immobilised on the plate decreased. SC was not detected in the wells that were coated with 99% pure BSA and gelatine. There was evidence of antibody binding in wells that were coated with 96% pure BSA, however the OD measurements did not decrease as the concentration of BSA decreased. There was also evidence antibody binding in wells that were coated with skimmed milk however OD measurements did not decrease as the concentration of protein decreased.

Table 4.2 The mean OD measured at 492 nm in relation to the concentration of polyclonal anti-SC (n=2).

Concentration of protein (µg/ml)	Mean OD at 492nm (n=2)				
	96% pure BSA	99% pure BSA	Gelatine	SC	Skimmed Milk
10	0.25	0.05	0.47	1.54	1.29
5	0.31	0.04	0.51	1.02	1.38
2.5	0.29	0.04	0.36	0.97	1.0
1.25	0.29	0.04	0.60	0.85	0.98
0	0.02	0.02	0.02	0.02	0.02

The data presented in table 4.2 shows that the amount of SC detected decreased as the concentration of SC immobilised on the plate decreased. SC was not detected in the wells

that were coated with 99% pure BSA. There was evidence of antibody binding in wells that were coated with gelatine, 96% pure BSA and skimmed milk however the OD measurements did not decrease as the concentration of protein decreased.

4.3.3 Checkerboard ELISA

In order to establish the optimum working concentrations of both monoclonal and polyclonal anti-SC and to elucidate which antibody would be most effective as the capture and which antibody would be most effective as the detection antibody, checkerboard ELISAs were conducted, the data for which are presented in tables 4.3 and 4.4.

Table 4.3 The mean OD measured at 492 nm in relation to the concentration of antibodies used in monoclonal anti-SC capture with polyclonal anti-SC detection ELISA (n=2).

Concentration of polyclonal anti-SC (µg/ml)	Concentration of monoclonal anti-SC (µg/ml)			
	670	**67**	**6.7**	**0.67**
500	1.98	1.39	1.24	0.97
50	1.97	1.25	0.98	0.70
5	0.92	0.74	0.63	0.40
0.5	0.62	0.41	0.38	0.32

The data presented in table 4.3 from the checkerboard ELISA that used monoclonal anti-SC to capture and polyclonal anti-SC to detect, indicated that the optimum working concentration of monoclonal anti-SC was 670 µg/ml as the amount of SC detected was highest when 670 µg/ml monoclonal anti-SC was used as a capture antibody and either 50 or 500 µg/ml polyclonal anti-SC was used as the detection antibody (table 4.3). It was concluded that the optimum working concentration of polyclonal anti-SC was 50 µg/ml as there was very little difference between the amounts of SC detected when using either 50 or 500 µg/ml of polyclonal anti-SC (table 4.3).

Table 4.4 The mean OD measured at 492 nm in relation to the concentration of antibodies used in a polyclonal anti-SC capture with monoclonal anti-SC detection ELISA (n=2).

Concentration of monoclonal anti-SC (µg/ml)	Concentration of polyclonal anti-SC (µg/ml)			
	500	**50**	**5**	**0.5**
670	0.79	0.70	0.64	0.54
67	0.69	0.61	0.55	0.49
6.7	0.58	0.48	0.37	0.28
0.67	0.49	0.32	0.22	0.19

The data presented in table 4.4 from the checkerboard ELISA that used polyclonal anti-SC to capture and monoclonal anti-SC to detect showed that overall lower amounts of SC were detected compared to when the ELISA was done as a monoclonal anti-SC capture and polyclonal anti-SC detection (tables 4.3 and 4.4). Indicating that the most sensitive ELISA to detect SC was one using monoclonal anti-SC to capture and polyclonal anti-SC to detect.

4.3.4 ELISA Using Monoclonal Anti-SC as Capture and Polyclonal Anti-SC as Detection

The checkerboard ELISA data in section 4.3.3 suggested that the most sensitive ELISA to detect SC would be a monoclonal capture with polyclonal detection ELISA. In addition the data showed that in that system the optimum working concentrations of monoclonal anti-SC was 670 μg/ml and the optimum working concentration was of polyclonal anti-SC was 50 μg/ml (table 4.3). Therefore it was decided to construct a standard SC curve that would allow the quantification of SC in unknown samples. The results are presented in figure 4.3.

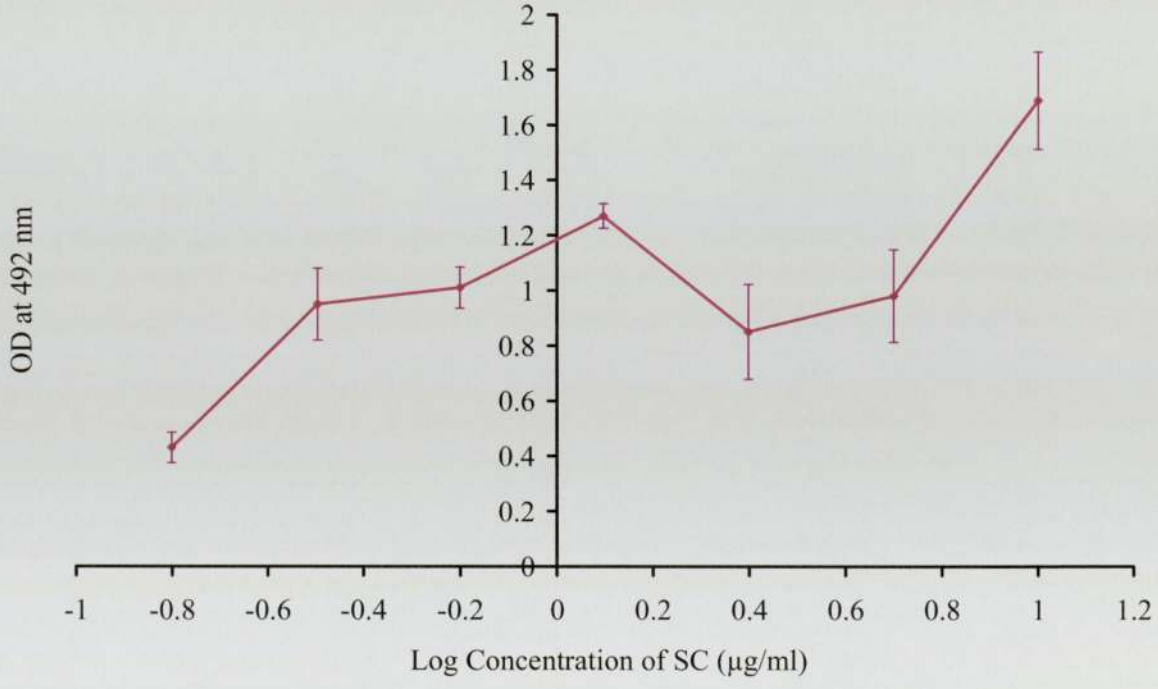

Figure 4.4 The mean OD measurements in relation to the concentration of SC as measured by a an ELISA using monoclonal anti-SC to capture and polyclonal anti-SC to detect. Data are expressed as mean ± SD (n = 2).

The data obtained from the ELISA using monoclonal anti-SC as a capture antibody and polyclonal anti-SC as a detection antibody showed SC was detected between the concentration of 0.17 and 10 μg/ml. It was however noted that whilst the amount of SC detected initially decreased as the concentration of SC coated on the plate decreased, this

observation was not consistent (figure 4.4). There was evidence of non specific binding in the wells that contained 0.31, 0.62 and 1.25 μg/ml SC (figure 4.4). From these findings it was decided to investigate how to reduce non specific binding.

4.3.5 Investigating the Efficacy of Various Blocking Agents on Non Specific Binding

The results of the investigation into the efficacy of various blocking agents are shown in figure 4.5.

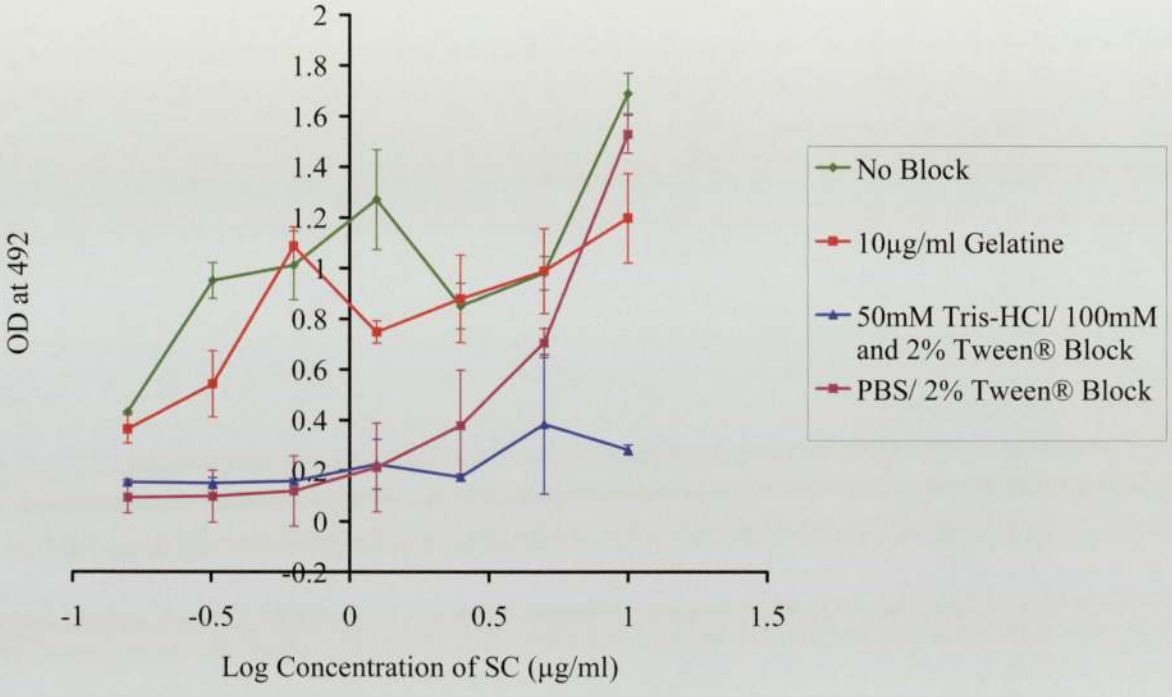

Figure 4.5 The mean OD measurements at 492 nm in relation to the concentration of SC used in an ELISA using monoclonal anti-SC to capture and polyclonal anti-SC to detect. The microtitre plate was blocked for an hour using either a 10 µg/ml gelatine, a 50 mM Tris-HCl/ 100 mM NaCl and 2% Tween® block (high salt) or PBS with 2% Tween® following coating with SC. Data are expressed as mean ± SD (n = 2).

The data presented in figure 4.5 show that when no block was used there was evidence of non specific binding in the wells that contained 0.31, 0.62 and 1.25 µg/ml SC. When the 10 µg/ml gelatine block was used there was evidence of non specific binding in wells that contained 0.62 µg/ml SC. Non specific binding was not observed in the ELISA when the high salt block was used however when this block was used SC did not appear to be detected (figure 4.5).

The PBS/ 2 % Tween® block did appear to reduce non specific binding in comparison to the findings presented in figure 4.4, when this block was used the amount of SC detected decreased as the concentration of SC decreased (figure 4.5). These findings suggested that the most effective block to use was PBS/ 2 % Tween®.

4.3.6 Investigating the Cross Reactivity and Specificity of Polyclonal Anti-SC

The results of the investigation into the specificity of polyclonal anti-SC are shown in figure 4.6.

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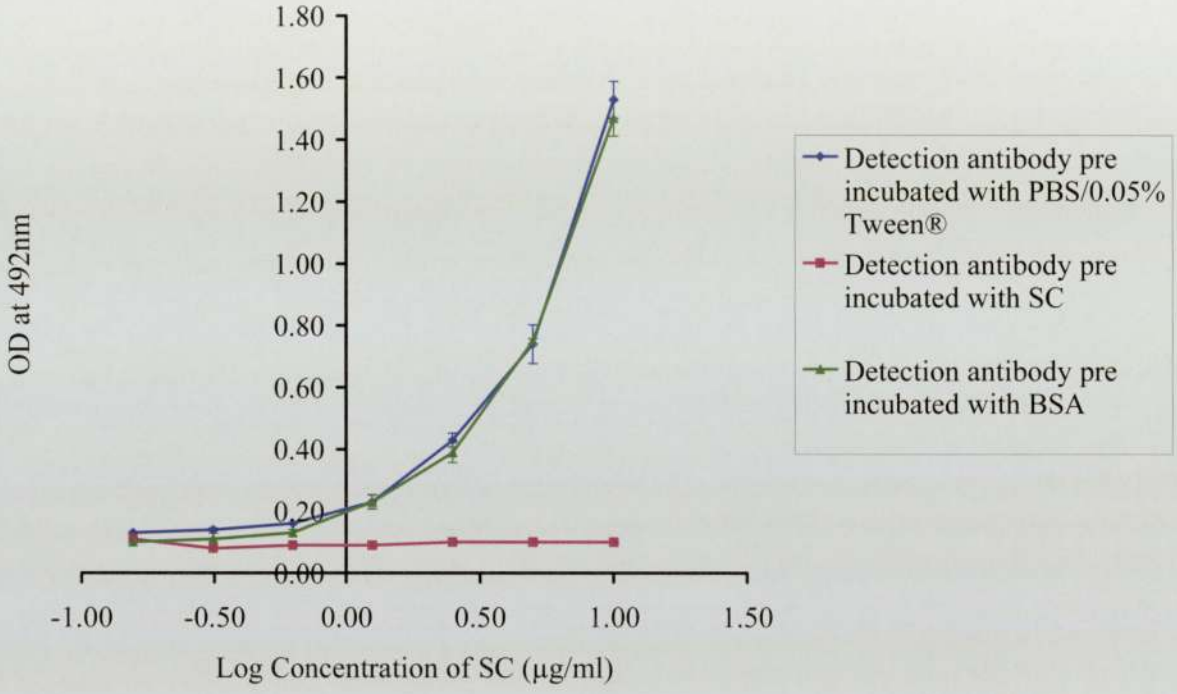

Figure 4.6 The mean OD measurements at 492 nm in relation to the concentration of SC used in an ELISA using monoclonal anti-SC to capture and polyclonal anti-SC to detect. The polyclonal antibody was first incubated with either wash buffer, SC or BSA before use as a detection antibody. Data are expressed as mean ± SD (n = 2).

The data presented in figure 4.6 shows that polyclonal anti-SC bound specifically to SC and exhibited no cross reactivity. When polyclonal anti-SC was incubated with either wash buffer or BSA prior to its use as a detection antibody, the amount of SC detected decreased as the concentration of SC immobilised on the plate decreased (figure 4.6). When polyclonal anti-SC was pre incubated with SC, the SC that had been coated on the plate was not detected and there was no evidence of non specific binding (figure 4.6).

4.4 Discussion

The data presented in figure 4.3A, indicated that monoclonal anti-SC was specifically binding to SC, as the concentration of SC immobilised on the plate decreased, the amount of SC detected decreased. The data also indicated that the optimum working concentration of monoclonal anti-SC was 670 μg/ml. The data shown in figure 4.3B indicated that polyclonal anti-SC was binding to SC however it was observed that the amount of SC detected did not decrease as the concentration of SC immobilised on the plate decreased. These findings suggested that there was some non specific binding exhibited by polyclonal anti-SC. The observation from the direct ELISA using an irrelevant antibody (MOPC21) were that compared to when using either monoclonal or polyclonal anti-SC, SC was not detected (figure 4.3C). MOPC21 specifically binds to the kappa light chain of IgG1; therefore its paratopes will only recognise the epitopes of the kappa light chain. These data indicated that only antibodies raised against SC could detect SC in an ELISA.

The data in table 4.1 demonstrates the specificity of the monoclonal anti-SC to human SC as the amount of SC detected decreased as the concentration of SC immobilised on the plate decreased. These findings indicate that binding is specific and that the antibody will only bind to its recognised antigen. In addition it was apparent that no antigen was detected in the wells that contained 99% pure BSA, this was also the finding for wells that were coated with gelatine (table 4.1). There was some evidence of antigen – antibody binding in the wells that were coated with 96% pure BSA and skimmed milk however as the OD measurement did not decrease as the concentration of protein decreased, this suggested that the binding was non specific and not due to the presence of SC in the preparations (table 4.1).

The data presented in table 4.2 indicate that although polyclonal anti-SC appears to specifically bind to SC, there is some evidence of non specific binding as the amount of SC detected did not decrease consistently as the concentration of SC immobilised on the plate decreased. There was evidence of non specific binding in the wells that were coated with skimmed milk, gelatin and 96% pure BSA (table 4.2).

The data presented in table 4.3 shows that the optimum working concentration of monoclonal anti-SC was 670 µg/ml and the optimum working concentration of polyclonal anti-SC was 50 µg/ml. It was evident that the amount of SC detected overall was lower when the ELISA using polyclonal anti-SC to capture and monoclonal anti-SC to detect was used compared to when the monoclonal capture, polyclonal detection ELISA was used. These findings indicated that the most sensitive ELISA to detect SC was one using monoclonal anti-SC to capture and polyclonal anti-SC to detect (tables 4.3 and 4.4).

The data obtained from the ELISA that used monoclonal anti-SC to capture and polyclonal anti-SC to detect, indicated that whilst SC was being detected, there was also some non specific binding as the amount of SC detected did not decrease as the concentration of SC immobilised on the plate decreased (figure 4.4). Based on these findings a blocking step was added to the assay and it was found that using PBS with 2% Tween® as a block after the addition of antigen reduced non specific binding and this was evident as the amount of SC detected decreased as the concentration of SC immobilised on the plate decreased (figure 4.5). There was still evidence of non specific binding when the gelatine block was used. The amount of SC detected when the high salt block was used was lower than the amount of SC detected when other blocking agents were used (figure 4.5). These findings suggested that the high concentration of salt interfered with the antigens epitopes or that the antigen was washed away by the block.

The specificity of polyclonal anti-SC and its lack of cross reactivity with other proteins is evident in the data found in figure 4.6. These data indicated that when polyclonal anti-SC had been pre incubated with SC, it was no longer able to bind to the SC that had been coated on the ELISA plate (figure 4.6). In addition, it was observed that when polyclonal anti-SC had been pre incubated with BSA, the antibody was still able to bind to the SC that had been coated on the plate. Also as expected the amount of SC detected decreased as the concentration of SC immobilised on the plate decreased. This was also the finding for polyclonal anti-SC that had been pre incubated with wash buffer only (figure 4.6). These findings demonstrate that when polyclonal anti-SC was pre incubated with SC, the antibodies paratopes were occupied through specific interactions with SC epitopes. When the antibody was pre incubated with BSA, it did not bind to BSA as it did not recognise the epitopes displayed by BSA. Therefore the antibodies paratopes remained unoccupied until it was incubated with the SC that was immobilised on the plate.

Taken together all of the data indicated that the best ELISA to detect SC was an ELISA using monoclonal anti-SC to capture and polyclonal anti-SC to detect, with a PBS 2% Tween® block as it has been demonstrated that this particular capture ELISA can specifically detect SC with minimal non specific binding allowing more accurate quantification of unknown samples.

5 The Effect of Lipopolysaccharide and Lipoteichoic Acid on SC Release

5.1 Introduction

The work presented in this chapter aims to establish whether SC production by human airway epithelial cells *in vitro* is up regulated following stimulation with infectious stimuli, LPS or LTA. LPS is released from gram negative bacteria during cell lysis and it is thought that LTA is 'shed' from gram positive bacteria into the host during infection. Both LPS and LTA have been shown to cause inflammation *in vitro* and *in vivo* through the stimulation of inflammatory cytokines. Previous studies investigating the role of SC in mucosal immunity have shown that SC is up regulated in airway epithelia by a variety of inflammatory cytokines including IFN• (Loman *et al.*, 1997; Godding *et al.*, 1998 and Loman *et al.*, 1999) and IL-4 (Loman *et al.*, 1999). Both IFN• and IL-4 are found in inflamed airways which suggests that SC could play an important anti-inflammatory role.

The CALU-3 cell line was used as an *in vitro* model to investigate whether SC expression is up regulated following stimulation with either LPS from *E. coli, B. cepacia, P. aeruginosa*, or LTA from *S. aureus.* CALU-3 cells are an immortalized adherent epithelial cell line derived from an adenocarcinoma of the lung that secrete SC constitutively (Loman, *et al.*, 1997; Godding *et al.*, 1998 and Loman *et al.*, 1999), which makes them a particularly good model for investigating SC expression.

Commercially available strains of LPS (*E. coli* 0111) and LTA (*S. aureus*) were used in addition to LPS that had been extracted from clinically isolated pathogens (*B. cepacia* and *P. aeruginosa*).

5.2 Methods

5.2.1 Challenge of CALU-3 Cell Line with LPS and LTA

CALU-3 were set up in 24 well plates at a cell density of 1 X 10^5 cells per well. Once cells had reached 100 % confluence, complete growth medium was removed and replaced with 500 µl per well of 'quiescent medium' (basal MEM supplemented with ITS (10 µg/ml insulin, 5.5 µg/ml transferrin and 0.5 pg/ml sodium selenite) supplement, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM $_{L}$-glutamine, 1 % sodium pyruvate and 1 % non essential amino acids). Cells were incubated in quiescent medium overnight at 37 ºC prior to stimulation. CALU-3 cells were stimulated with either commercially available LPS from *E. coli* strain 0111:B4, LPS from a clinical isolate of *B. cepacia* (J2315), LPS from a clinical isolate of *P. aeruginosa* (O5D) or a commercially available LTA from *S. aureus*. CALU-3 cells that were stimulated with either *E. coli* LPS or *S. aureus* LTA were incubated with the stimuli for 3, 6, 24 and 48 hours. CALU-3 cells that were stimulated with either LPS from *B. cepacia* or LPS from *P. aeruginosa,* were incubated for 3, 6, 24, 48, 72 and 96 hours. The concentrations of LPS and LTA used were 10, 1 and 0.1 µg/ml. Control wells were set up in the absence of bacterial stimuli. Following stimulation, the medium was removed and centrifuged at 850 *g* for 10 minutes at 4 ºC. Cleared supernatants were transferred, without disturbing the pellet of cell debris, these were stored at -20 ºC and analysed by the monoclonal capture, polyclonal detection ELISA with PBS/ 2 % Tween® block described in chapter 4 and SDS PAGE and Western blotting using monoclonal anti-SC described in chapter 3. The cells were washed once with 1 ml of ice cold PBS per well, then lysed with 500 µl per well of cell lysis buffer (PBS with 1 % (v/v) Triton x-100 and protease inhibitor cocktail). The cells were incubated

in cell lysis buffer for 20 minutes at 4 °C, cell lysis was confirmed by viewing wells with the light microscope. Once cells had lysed they were harvested and stored at -20 °C.

5.2.2 Densitometric Analysis of Western blots

Semi-quantitative analysis of SC released from CALU-3 cells and detected using Western blotting was achieved using densitometric measurement. X-ray films of Western blots were scanned and the intensity of the protein band was measured. Following scanning a densitogram was produced and the area under each peak was used to determine the intensity of the protein band. The number of pixels in each protein band were converted to a percentage so that relative expression of SC could be determined.

5.2.3 Quantification of SC Released by CALU-3 Using ELISA Analysis

SC concentrations in the supernatants were interpolated from an ELISA standard curve, an example of which may be found in Appendix 2.

5.3 Results

5.3.1 The Effect of *E. coli* 0111:B4 LPS on Release of SC

CALU-3 cells were stimulated for three, six, 24 and 48 hours with LPS from *E. coli* and the resulting supernatants were collected and analysed using ELISA and Western blotting.

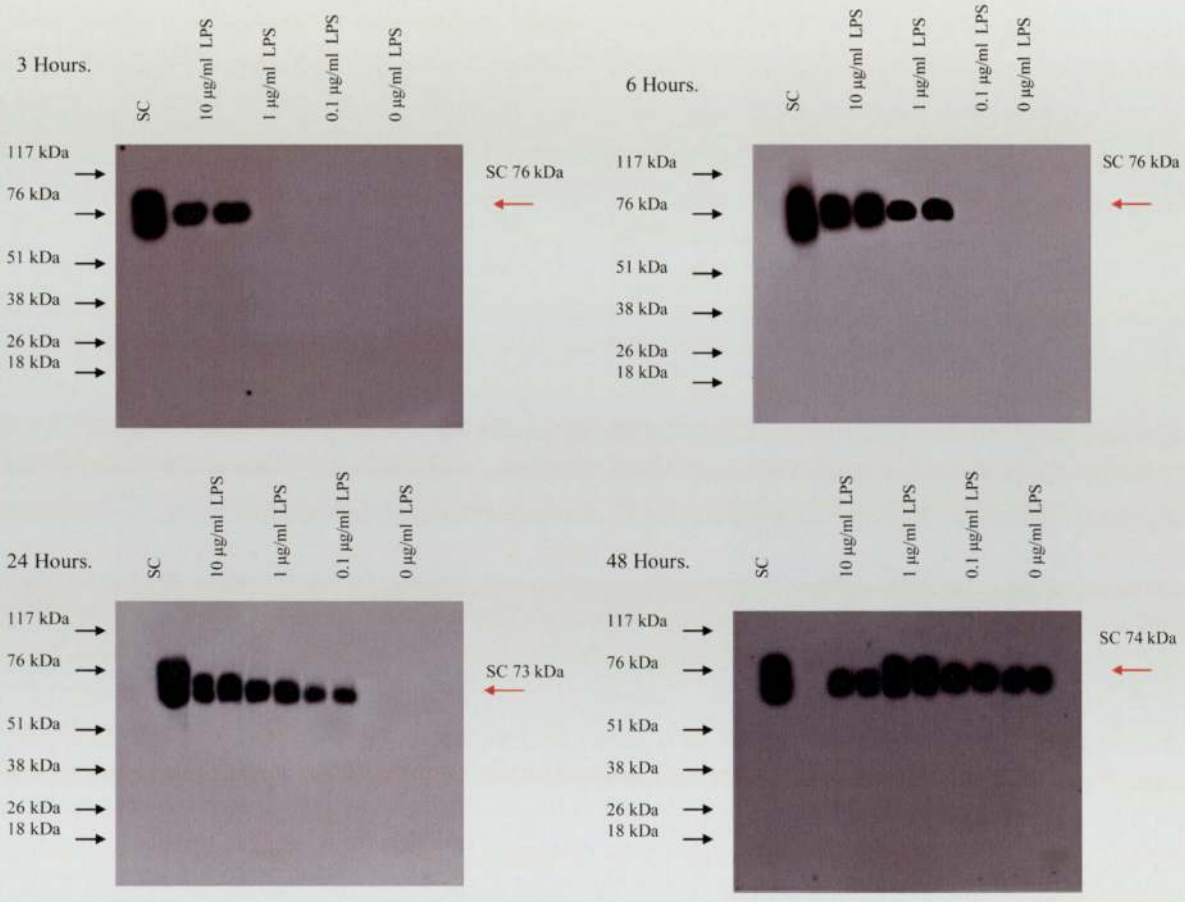

Figure 5.1 Scanned images of Western blots following incubation with monoclonal antibody to SC and development with HRP-conjugated secondary antibodies and enhanced chemiluminescence. Positions of molecular weight markers are shown to the left of the image. The molecular weight is of SC indicated by an arrow to the right of the images. The concentration of *E. coli* LPS used to stimulate CALU-3 cells is shown above each image. 5 µg/ml of the standard preparation of SC was loaded in the lane marked SC. Time of stimulation is shown above each image and duplicate wells of each harvested supernatant were loaded (10 µl per well). Representative images of 4 experiments are shown.

The images shown in figure 5.1 show that SC was detected in the culture supernatants of cells that were stimulated with 10 µg/ml LPS for three hours. The molecular weight of SC detected

was 76 ± 3 kDa. Following three hours incubation, Western blot analysis showed that SC release was not detected in the supernatants of cells that were either not stimulated with LPS or that were stimulated with 0.1 or 1 μg/ml (figure 5.2). These observations are the same as the observations made when using ELISA analysis (figure 5.3).

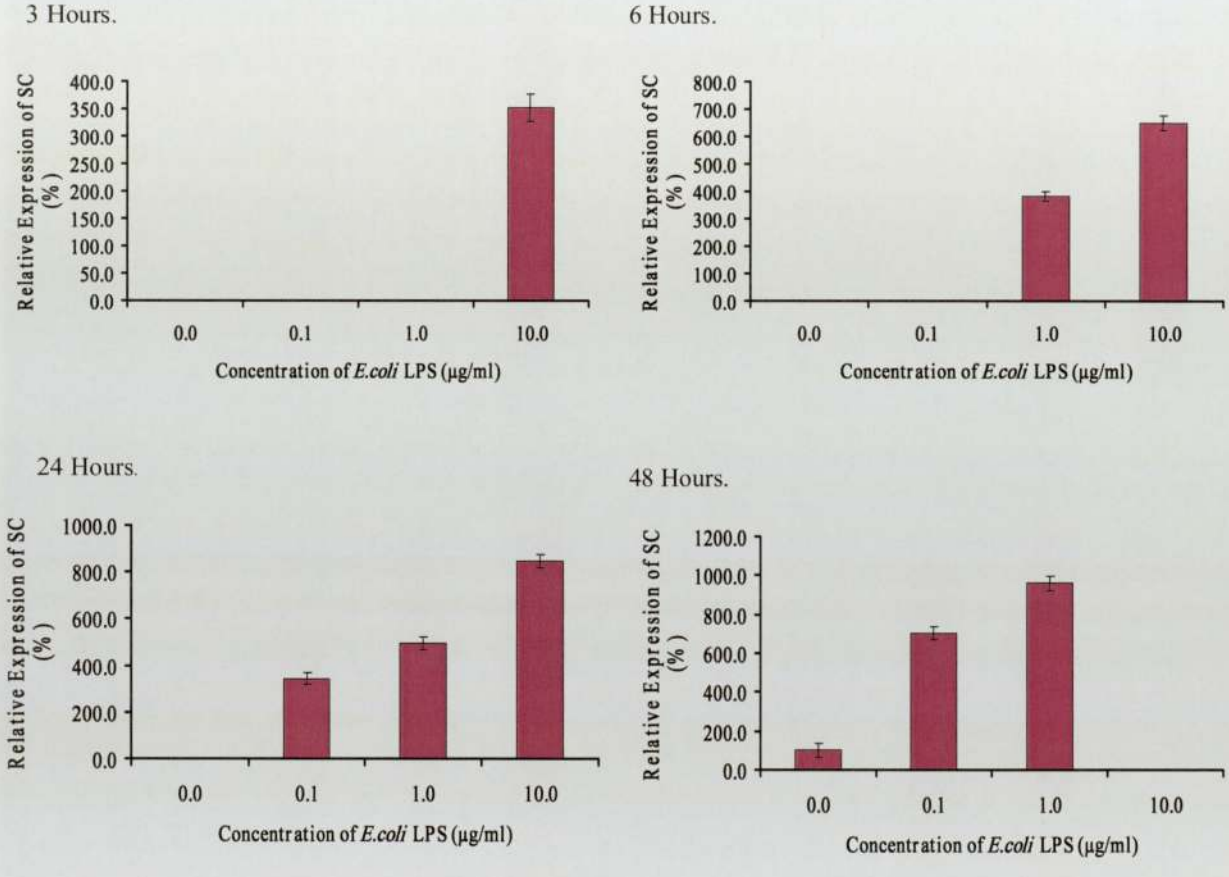

Figure 5.2 The effect of the concentration of *E. coli* LPS and time on the relative expression of SC detected in CALU-3 cell supernatants as analysed by Western blotting. The number of pixels within each protein band was converted to a percentage. Constitutive release is expressed as 100%. Where constitutive release is not detected, constitutive release at 48 hours was defined as 100% and stimulated release of SC is expressed relative to this. Data are expressed Data are expressed as mean ± standard error (SE) of four independent experiments with three replicates. * = p • 0.05, ** = p • 0.01 vs. SC release from unstimulated CALU-3 cells.

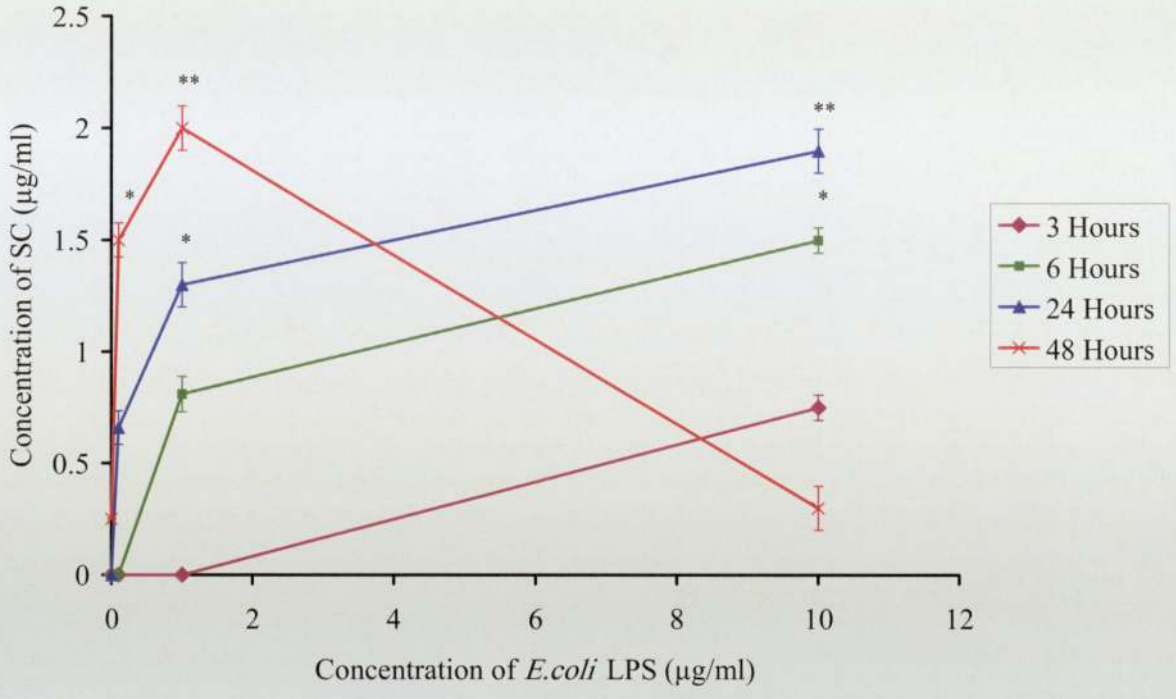

Figure 5.3 The effect of the concentration of *E. coli* LPS and time on the concentration of SC detected in CALU-3 cell supernatants as analysed by ELISA. Data are expressed as mean ± SE of four independent experiments with three replicates. * = $p \bullet 0.05$, ** = $p \bullet 0.01$ vs. SC release from unstimulated CALU-3 cells.

Following stimulation of cells with 10 µg/ml LPS for three hours the mean relative expression of SC detected using Western blotting was 352 ± 25.2 % (figure 5.3). Under the same conditions the mean concentration of SC detected by ELISA for this time point was 0.83 ± 0.01 µg/ml (figure 5.3).

Following stimulation with 1 and 10 µg/ml LPS for 6 hours, SC at a molecular weight of 76 ± 5 kDa was detected in culture supernatants (figure 5.1). Densitometric analysis of Western blots showed that no detectable amounts of SC were released from cells that were either not incubated with LPS or cells that were stimulated with 0.1 µg/ml of LPS (figure 5.2), these were also the findings of ELISA analysis (figure 5.3). A mean relative expression of 382 ± 19.8 % of SC was detected by Western blotting in the supernatants of cells that were stimulated with 1 µg/ml of LPS for 6 hours (figure 5.3). The mean concentration of SC released under the same conditions as analysed by ELISA was 0.85 ± 0.06 µg/ml. When

cells were stimulated with 10 μg/ml of LPS for 6 hours the mean relative expression of SC detected by Western blotting was 652 ± 24.9 % (figure 5.2). Under the same conditions the concentration of SC measured by ELISA was 1.53 ± 0.02 μg/ml and this concentration of SC was significantly higher than the concentration of SC detected when cells were not stimulated, (p • 0.05; figure 5.3).

SC at molecular weight of 76 ± 4 kDa was detected in the culture supernatants of cells that were stimulated with 0.1, 1 and 10 μg/ml LPS for 24 hours (figure 5.1). Following 24 hour incubation, it was shown through both Western blot and ELISA analysis that cells that were not stimulated with LPS did not release a detectable amount of SC (figures 5.2 and 5.3). Relative expression of SC of 343 ± 26.8 % was detected by Western blotting in the supernatants of cells that were stimulated with 0.1 μg/ml LPS for 24 hours (figure 5.2), The concentration of SC detected when supernatants from cells under the same conditions were analysed using ELISA was 0.80 ± 0.02 μg/ml (figure 5.3). The mean relative expression of SC detected by Western blotting when cells were stimulated with 1 μg/ml of LPS for 24 hours was 495 ± 25.4 % (figure 5.2). The concentration of SC detected in the supernatants of cells that were incubated under the same conditions, but analysed using an ELISA was 1.13 ± 0.05 μg/ml and this concentration was significantly higher than the concentration of SC detected when cells were not exposed to bacterial stimuli, (p • 0.05). The mean relative expression of SC detected by Western blotting in the supernatants of cells stimulated with 10 μg/ml of LPS for 24 hours was 847 ± 39.1 %. The concentration of SC detected by ELISA under the same conditions was 1.94 ± 0.05 μg/ml and this concentration was significantly higher compared to constitutive release of SC (p • 0.01).

Following incubation for 48 hours, SC at a molecular weight of 74 ± 3 kDa was detected in the supernatants of cells that were stimulated with 0.1, 1 and 10 μg/ml LPS (figure 5.1).

Constitutive release of SC was also detected in cells that were incubated for 48 hours (figure 5.1). ELISA analysis detected SC at a concentration of 0.26 ± 0.01 μg/ml in the supernatants of cells that had not been exposed to bacterial stimuli (figure 5.3). On average the relative expression of SC, detected using Western blotting in the supernatants of cells that were stimulated with 0.1 μg/ml LPS for 48 hours was 704 ± 34 %. The concentration of SC detected under the same conditions and analysed using an ELISA was 1.63 ± 0.05 μg/ml and this concentration of SC detected was significantly higher than the amount of SC detected in the supernatants of cells that were not stimulated, (p • 0.05;figure 5.3). The relative expression of SC detected by Western blotting in the supernatants of cells stimulated with 1 μg/ml of LPS was 960 ± 37.5 % (figure 5.2). It was noted that the concentration of SC detected using the ELISA was 2.14 ± 0.09 μg/ml figure 5.3. This concentration was significantly higher than the production of SC by cells that were not stimulated with LPS, (figure 5.3; p • 0.01).

During the assay it was observed when cells were stimulated with 10 μg/ml LPS for 48 hours, cell detachment and necrosis occurred. It was apparent that approximately 30 % of the cells were viable at this time point.

It was determined using the LAL assay described in section 2.2.3 that the endotoxin concentration in the 10 μg/ml preparation of *E. coli* LPS was 0.9 ± 0.037 EU/ml.

5.3.2 The Effect of *S. aureus* B4 LTA on Release of SC

CALU-3 cells were stimulated for 3, 6, 24 and 48 hours with LTA from *S.aureus* and the resulting supernatants were collected and analysed using ELISA and Western blotting, the results are presented below.

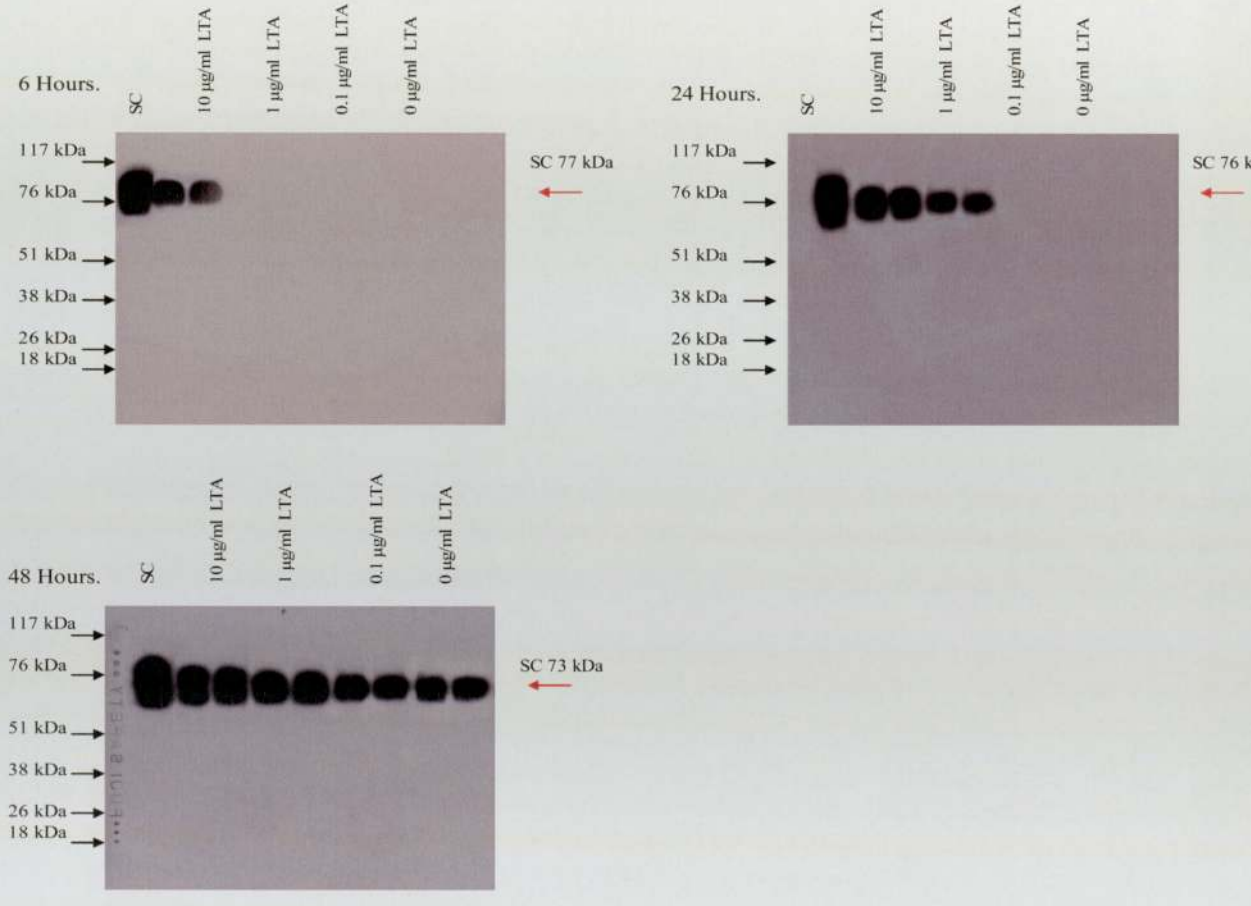

Figure 5.4 Scanned images of Western blots following incubation with monoclonal antibody to SC and development with HRP-conjugated secondary antibodies and enhanced chemiluminescence. Positions of molecular weight markers are shown to the left of the image. The molecular weight is of SC indicated by an arrow to the right of the images. The concentration of *S. aureus* LTA used to stimulate CALU-3 cells is shown above each image. 5 µg/ml of the standard preparation of SC was loaded in the lane marked SC. Time of stimulation is shown above each image and duplicate wells of each harvested supernatant were loaded (10 µl per well). Representative images of 4 experiments are shown.

SC was not detected in the supernatants of cells that were stimulated with 0.1, 1 and 10 µg/ml LTA for three hours and constitutive release of SC was not detected. As a result of these

observations, the three hours Western blot image is not shown as only standard SC was visible on the blot.

The images shown in figure 5.4 show that SC was detected in the culture supernatants of cells that were stimulated with 10 μg/ml LTA for six hours. The molecular weight of SC detected was 77 ± 5 kDa. Western blot and ELISA analysis showed that SC release was not detected in the supernatants of cells that were maintained in quiescent medium only or from cells that were stimulated with 0.1 or 1 μg/ml LTA (figures 5.5 and 5.6).

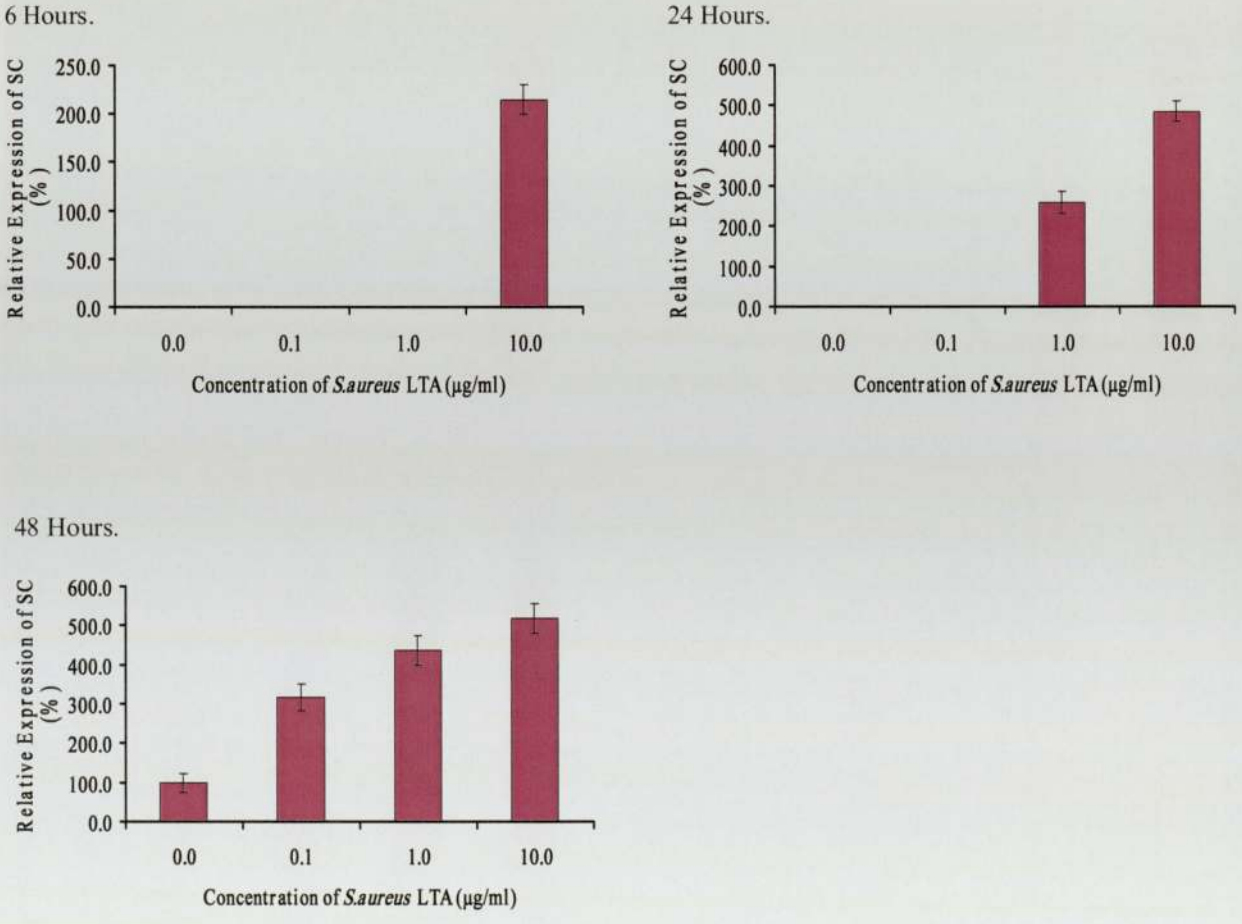

Figure 5.5 The effect of the concentration of *S. aureus* LTA and time on the relative expression of SC detected in CALU-3 cell supernatants as analysed by Western blotting. The number of pixels within each protein band was converted to a percentage. Constitutive release is expressed as 100%. Where constitutive release is not detected, constitutive release at 48 hours was defined as 100% and stimulated release of SC is expressed relative to this. Data are expressed Data are expressed as mean ± SE of four independent experiments with three replicates. * = $p \bullet 0.05$ vs. SC release from unstimulated CALU-3 cells.

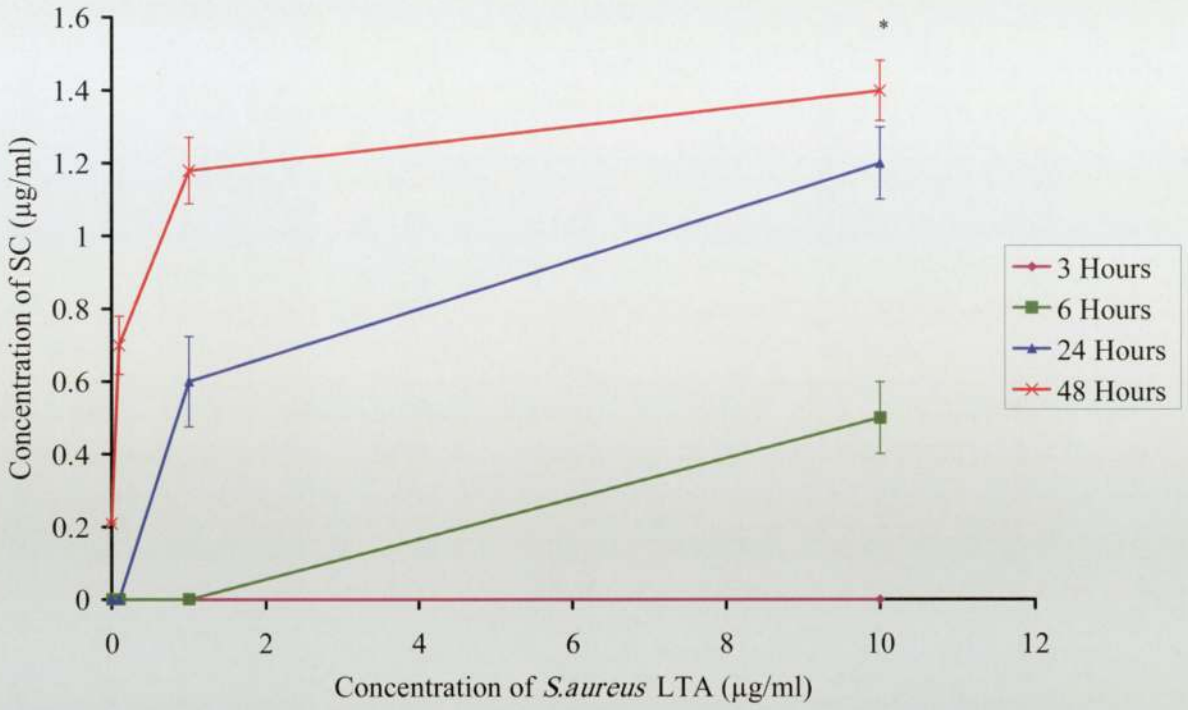

Figure 5.6 The effect of the concentration of *S. aureus* LTA and time on the concentration of SC detected in CALU-3 cell supernatants as analysed by ELISA. Data are expressed as mean ± SE of four independent experiments with three replicates. * = p • 0.05 vs. SC release from unstimulated CALU-3 cells.

The relative expression of SC detected by Western blotting from cells that were stimulated with 10 µg/ml LTA for six hours was 215 ± 15.3 (figure 5.5). The concentration of SC release detected under the same conditions using an ELISA (0.60 ± 0.08 µg/ml; figure 5.6).

Following stimulation with 1 and 10 µg/ml LTA for 24 hours, SC at a molecular weight of 76 ± 3 kDa was detected in culture supernatants (figure 5.4). Western blot and ELISA analysis showed that SC was not detected in the supernatants of cells that were either not stimulated with LTA, or that were stimulated with 0.1 µg/ml LTA for 24 hours (figures 5.5 and 5.6). Following incubation for 24 hours with 1 µg/ml LTA the mean relative expression of SC detected by Western blotting was 259 ± 27.2 % (figure 5.5). Under the same conditions the mean concentration of SC detected by ELISA was 0.70 ± 0.10 µg/ml (figure 5.6). The mean relative expression of SC detected by Western blotting in the supernatants of cells that that

were stimulated with 10 μg/ml LTA for 24 hours was 485 ± 26 % (figure 5.5). It was observed that the mean concentration of SC detected by ELISA in the supernatants of cells that were stimulated for 24 hours with 10 μg/ml LTA was 1.30 ± 0.09 μg/ml (figure 5.6).

SC at a molecular weight of 73 ± 5 kDa was detected in the culture supernatants of cells that were stimulated with 0.1, 1 and 10 μg/ml LTA for 48 hours (figure 5.4). In addition constitutive release of SC was detected in cells that were incubated for 48 hours; the molecular weight was 73 ± 5 kDa (figure 5.4). Analysis by ELISA found that the concentration of constitutively released SC was 0.28 ± 0.009 μg/ml. The relative expression of SC detected by Western blotting in the culture supernatants of cells that were stimulated with 0.1 μg/ml LTA for 48 hours was 316 ± 24.5 % (figure 5.5). The concentration of SC detected when the supernatants of cells under the same conditions and analysed using ELISA was 0.78 ± 0.08 μg/ml (figure 5.6). The relative expression of SC detected by Western blotting in the supernatants of cells that were stimulated with 1 μg/ml LPS for 48 hours was 437 ± 34.2 % (figure 5.5). 1.26 ± 0.12 μg/ml of SC was detected when cells under the same conditions were analysed using an ELISA (figure 5.6). Following stimulation with 10 μg/ml LTA for 48 hours the relative expression of SC in culture supernatants detected by Western blotting was 518 ± 38.5 % (figure 5.5). When supernatants from cells incubated under the same conditions were analysed using an ELISA the concentration of SC detected was 1.41 ± 0.09 μg/ml (figure 5.6). This concentration of SC was significantly higher than the concentration of SC released from CALU-3 cells constitutively ($p \bullet 0.05$).

It was determined using the LAL assay described in section 2.2.3 that the endotoxin concentration in the 10 μg/ml preparation of *S. aureus* LTA was 0.1 ± 0.051 EU/ml.

5.3.3 The Effect of *B. cepacia* J2315 LPS on Release of SC

CALU-3 cells were stimulated for 3, 6, 24, 48, 72 and 96 hours with LPS from *B.cepacia* and the resulting supernatants were collected and analysed using an ELISA and Western blotting, the results are presented below.

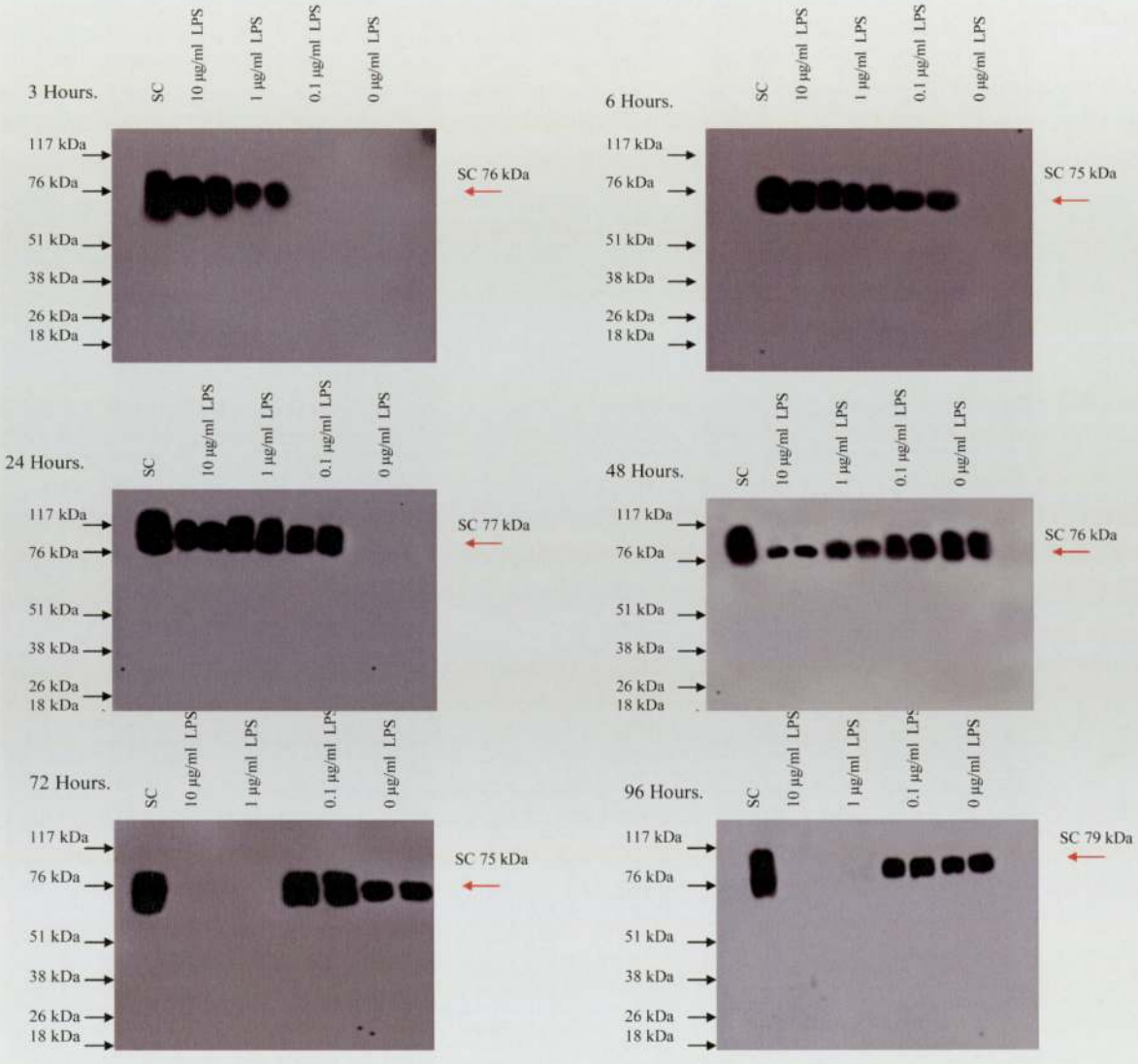

Figure 5.7 Scanned images of Western blots following incubation with monoclonal antibody to SC and development with HRP-conjugated secondary antibodies and enhanced chemiluminescence. The molecular weight of SC is indicated by an arrow to the right of the images. The concentration of *B. cepacia* LPS used to stimulate CALU-3 cells is shown above each image. 5 µg/ml of the standard preparation of SC was loaded in the lane marked SC. Time of stimulation is shown above each image and duplicate wells of each harvested supernatant were loaded. Representative images of 4 experiments are shown.

The images shown in figure 5.7 show that SC at a molecular weight of 76 ± 4 kDa was detected in the culture supernatants of cells that were stimulated with 10 μg/ml LPS for three hours. Western blot and ELISA analysis showed that when the cells were stimulated with 0.1 μg/ml LPS for three hours, SC was not detected in culture supernatants (figures 5.8 and 5.9).

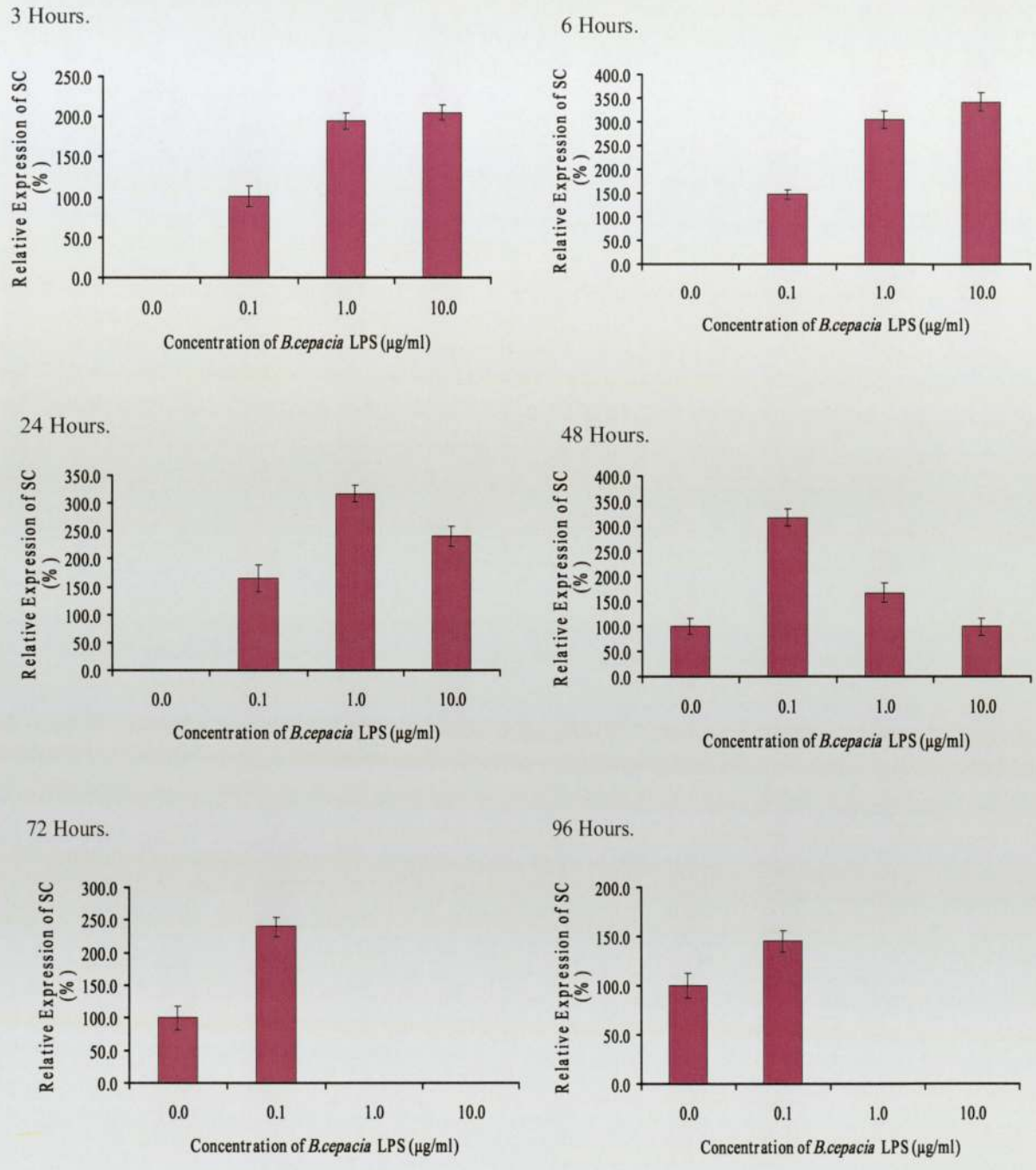

Figure 5.8 The effect of the concentration of *B. cepacia* LPS and time on the relative expression of SC detected in CALU-3 cell supernatants as analysed by Western blotting. The number of pixels within each protein band was converted to a percentage. Constitutive releases is expressed as 100%. Where constitutive release is not detected, constitutive release at 48 hours was defined as 100% and stimulated release of SC is expressed relative to this. Data are expressed Data are expressed as mean ± SE of three independent experiments with three replicates. ** = $p \bullet 0.01$, *** = $p \bullet 0.001$ vs. SC release from unstimulated CALU-3 cells.

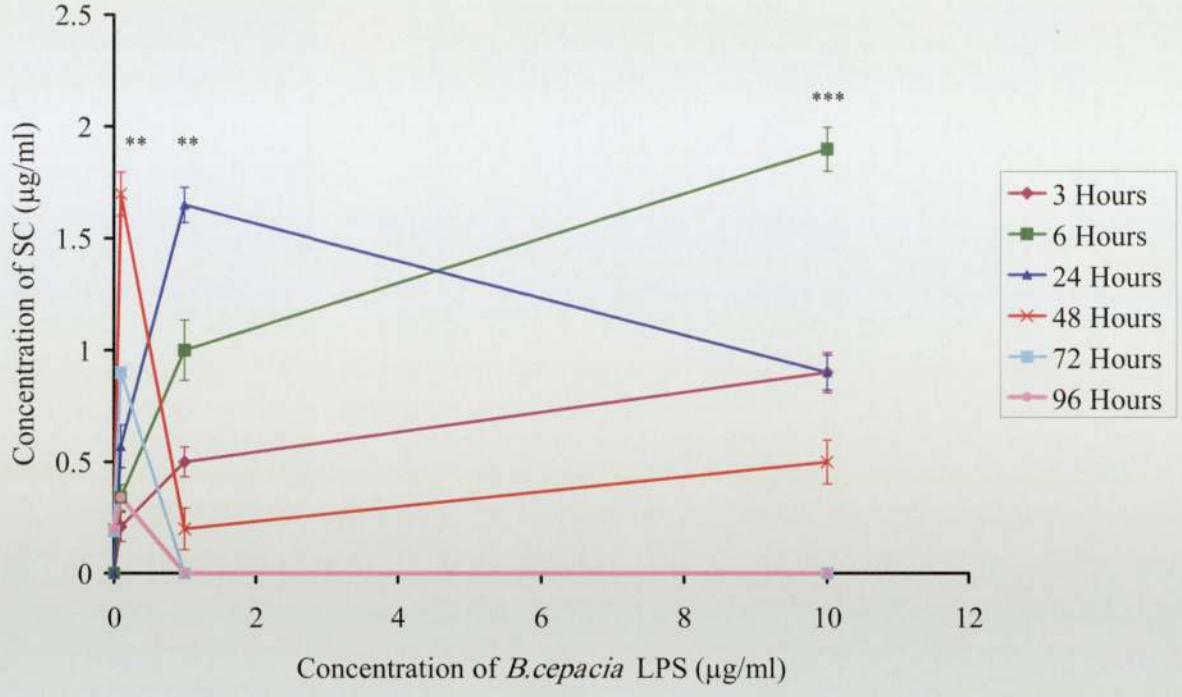

Figure 5.9 The effect of the concentration of *B. cepacia* LPS and time on the concentration of SC detected in CALU-3 cell supernatants as analysed by ELISA. Data are expressed as mean ± SE of three independent experiments with three replicates. ** = $p \bullet 0.01$, *** = $p \bullet 0.001$ vs. SC release from unstimulated CALU-3 cells.

When cells were stimulated with 1 µg/ml LPS for three hours a mean SC concentration of 0.66 ± 0.05 µg/ml was detected using ELISA analysis (figure 5.9). The relative expression of SC detected in the supernatants of cells that were incubated under the same conditions, but analysed using Western blotting was 342 ± 12.3 % (figure 5.8). The relative expression of SC when detected by Western blotting in the supernatants of cells that were stimulated with 10 µg/ml LPS for three hours was 317 ± 9.9 % (figure 5.8). ELISA analysis detected 0.09 ± 0.06 µg/ml of SC in culture supernatants of cells that were incubated under the same conditions (figure 5.9).

Following stimulation with 0.1, 1 and 10 µg/ml LPS for six hours, SC at a molecular weight of 75 ± 5 kDa was detected in culture supernatants (figure 5.7). Figure 5.8 shows that when cells were stimulated with 0.1 µg/ml LPS for six hours, the relative expression of SC detected by Western blotting was 317 ± 8.7 %, the concentration of SC detected in the same culture

supernatants by ELISA analysis was 0.36 ± 0.05 μg/ml (figure 5.9). The relative expression of SC in the supernatants of cells that were stimulated with 1 μg/ml LPS for six hours detected by Western blotting was 304 ± 10.5 % (figure 5.8). In the same culture supernatants, SC at a concentration of 1.29 ± 0.06 μg/ml was detected using an ELISA (figure 5.9). The relative expression of SC detected by Western blotting in the culture supernatants of cells stimulated with 10 μg/ml LPS for six hours was 240 ± 18.7 % (figure 5.8). The concentration of SC detected in the supernatants from cells that were incubated under the same condition and analysed using an ELISA was 2.10 ± 0.09 μg/ml (figure 5.9). Following stimulation with 10 μg/ml LPS for six hours, the concentration of SC detected was significantly higher than the concentration of SC detected in the supernatants of cells that were not stimulated ($p \bullet 0.001$).

SC at a molecular weight of 77 ± 3 kDa was detected in the culture supernatants of cells that were stimulated with 0.1, 1 and 10 μg/ml LPS for 24 hours (figure 5.7). The relative expression of SC detected in the supernatants of cells that were stimulated with 0.1 μg/ml LPS for 24 hours and analysed using Western blotting was 240 ± 21.2 % (figure 5.8). When the same culture supernatants were analysed using an ELISA the concentration of SC detected was 0.45 ± 0.07 μg/ml (figure 5.9). The relative expression of SC detected by Western blotting in the supernatants of cells that were stimulated with 1 μg/ml LPS for 24 hours was 205 ± 23.4 % (figure 5.8). In the same culture supernatants, SC was detected at a concentration of 1.67 ± 0.08 μg/ml using an ELISA (figure 5.9). In addition, the concentration of SC detected at this time point was significantly higher than the concentration of SC detected in the supernatants of cells that were not exposed to bacterial stimuli ($p \bullet 0.01$). The mean relative expression of SC detected by Western blotting in the culture supernatants of cells that were stimulated with 10 μg/ml for 24 hours was 195 ± 15.2 % (figure 5.8). The concentration of SC detected by an ELISA in the supernatants of cells that were incubated under the same conditions was 0.09 ± 0.06 μg/ml (figure 5.9).

76 ± 4 kDa SC was detected in the culture supernatants of cells that were stimulated with 0.1, 1 and 10 μg/ml LPS for 48 hours (figure 5.7) Additionally constitutive release of SC was detected, the molecular weight was also 76 ± 4 kDa (figure 5.7). The data presented in figure 5.8 shows that constitutive release of SC was detected by Western blotting at 48 hours and that the mean relative expression was 167 ± 18.2 % (figure 5.8). The concentration of SC detected in the same supernatants when analysed using an ELISA was 0.21 ± 0.03 μg/ml (figure 5.9). The mean relative expression of SC detected by Western blotting in culture supernatants following stimulation with 0.1 μg/ml LPS for 48 hours was 165 ± 16.5 % (figure 5.8). When the same supernatants were analysed using an ELISA, the concentration of SC detected was 1.72 ± 0.06 μg/ml (figure 5.9). This concentration of SC was significantly higher than the concentration of SC detected when cells were not stimulated ($p \bullet 0.01$). When cells were stimulated with 1 μg/ml LPS for 48 hours the mean relative expression of SC detected using Western blot analysis was 145 ± 17.1 % (figure 5.8). The mean concentration of SC detected in the same supernatants using an ELISA was 0.50 ± 0.04 μg/ml (figure 5.9). The mean relative expression of SC detected by Western blotting in the culture supernatants of cells stimulated with 10 μg/ml LPS for 48 hours was 147 ± 19 % (figure 5.8). 0.20 ± 0.05 μg/ml of SC was detected in the same culture supernatants, when analysed using an ELISA (figure 5.9).

SC at molecular weight of 75 ± 2 kDa was detected in the culture supernatants of cells that were stimulated with 0.1 μg/ml LPS for 72 hours (figure 5.7). Constitutively released SC was also detected at 75 ± 2 kDa (figure 5.7). Western blot and ELISA analysis showed that SC was not detected in the supernatants of cells that were stimulated with 1 or 10 μg/ml LPS for 72 hours (figures 5.8 and 5.9). At 72 hours the mean relative expression of SC detected by Western blotting in the supernatants of cells that were stimulated with 0.1 μg/ml LPS was 101

± 16.6 % (figure 5.8). The concentration of SC detected in the same supernatants using an ELISA was 1.0 ± 0.08 μg/ml (figure 5.9). The mean concentration of SC detected by ELISA in the supernatants of cells that were not exposed to bacterial stimuli was 0.21 ± 0.02 μg/ml (figure 5.9).

Following 96 hours incubation, 76 ± 4 kDa SC was detected in the supernatants of cells that were stimulated with 0.1 μg/ml LPS and in the supernatants of cells that were not exposed to bacterial stimuli (figure 5.7). SC was not detected using either Western blotting or ELISA analysis in the supernatants of cells that were stimulated with either 1 or 10 μg/ml LPS for 96 hours (figures 5.8 and 5.9). The mean relative expression of SC detected by Western blotting in the supernatant of cells stimulated with 0.1 μg/ml LPS was 99 ± 18.2 % (figure 5.8). The concentration of SC detected in the same supernatants by ELISA was 0.43 ± 0.08 μg/ml (figure 5.9). Constitutively released SC was detected at a concentration of 0.20 ± 0.05 μg/ml, when analysed using an ELISA (figure 5.9).

It was observed that during this assay that cell detachment and necrosis occurred in some of the cultures. It was found that when cells were stimulated with 10 μg/ml LPS for 24 hours, approximately 70 % of the cells were viable. In cultures that were incubated with 1 μg/ml LPS for 48 hours approximately 50 % cells were viable. Cell detachment was also observed in cultures that were incubated with 10 μg/ml LPS for 48 hours, it was noted that approximately 20 % of the cells were viable. There appeared to be no viable cells in cultures that were incubated with 1 or 10 μg/ml LPS for periods of time points exceeding 72 hours. Approximately 50% of the cells were viable in cultures that were incubated with 0.1 μg/ml LPS. Following 96 hours stimulation with LPS, approximately 30 % of cells were still viable.

It was determined using the LAL assay described in section 2.2.3 that the endotoxin concentration in the 10 μg/ml preparation of *B. cepacia* LPS was 0.85 ± 0.064 EU/ml.

5.3.4 The Effect of *P. aeruginosa* 05D LPS on Release of SC

CALU-3 cells were stimulated for 3, 6, 24, 48, 72 and 96 hour time points with LPS from *P.aeruginosa* and the resulting supernatants were collected and analysed using an ELISA and Western blotting, the results are presented below.

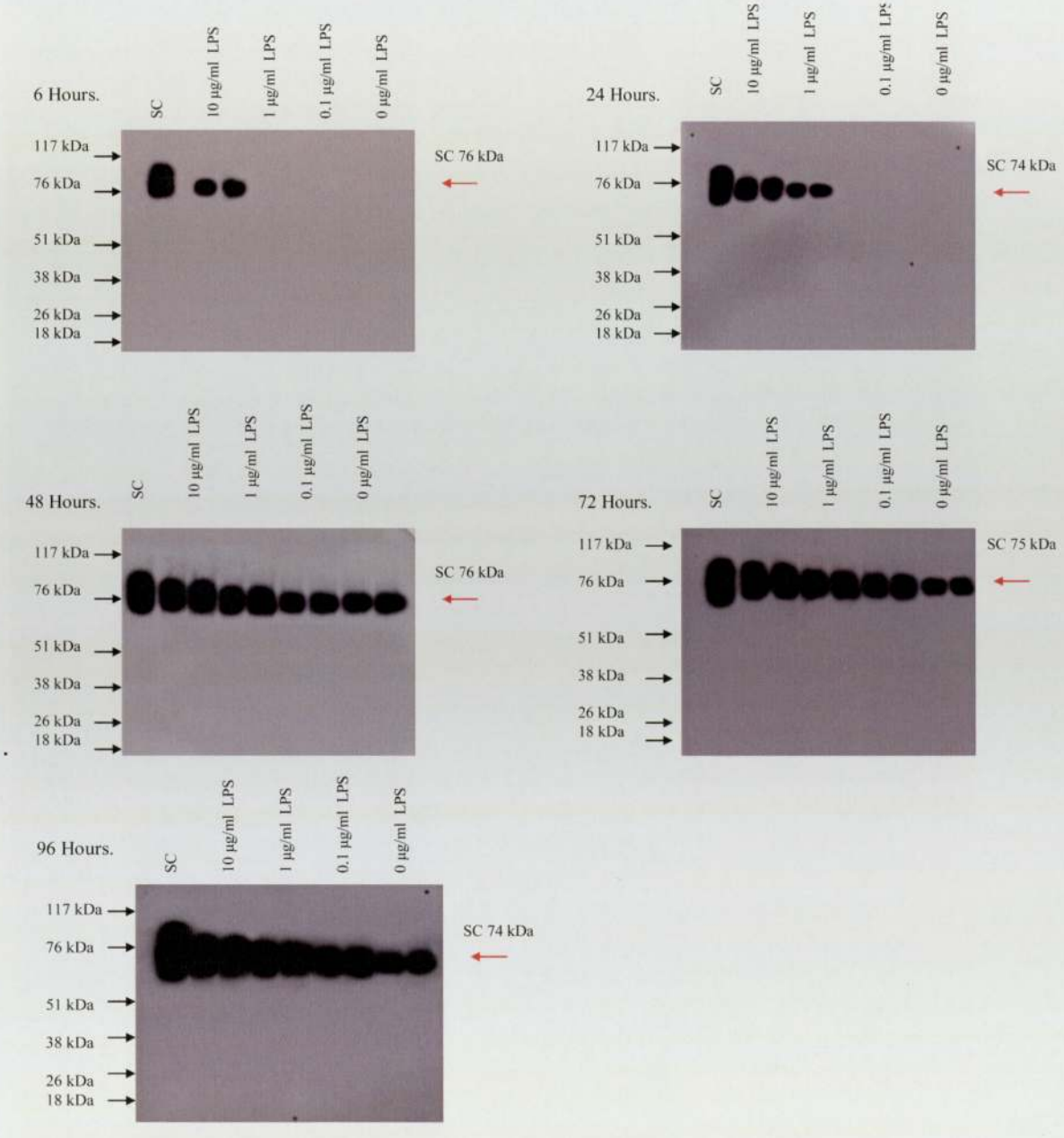

Figure 5.10 Scanned images of Western blots following incubation with monoclonal antibody to SC and development with HRP-conjugated secondary antibodies and enhanced chemiluminescence. The molecular weight of SC is indicated by an arrow to the right of the images. The concentration of *P. aeruginosa* LPS used to stimulate CALU-3 cells is shown above each image. 5 µg/ml of the standard preparation of SC was loaded in the lane marked SC. Time stimulation is shown above each image and duplicate wells of each harvested supernatant were loaded. Representative images of 4 experiments are shown.

SC was not detected in the supernatants of cells that were stimulated with 0.1, 1 and 10 μg/ml LPS for three hours and constitutive release of SC was not detected. As a result of these observations, the three hours Western blot image is not shown as only standard SC was visible on the blot.

Figure 5.10 shows that following stimulation with 10 μg/ml LPS for six hours, SC at a molecular weight of 76 ± 3 kDa was detected. Western blot and ELISA analysis showed that SC was not detected in the culture supernatants of cells that were stimulated with either 0.1 or 1 μg/ml LPS (figure 5.11 and 5.12).

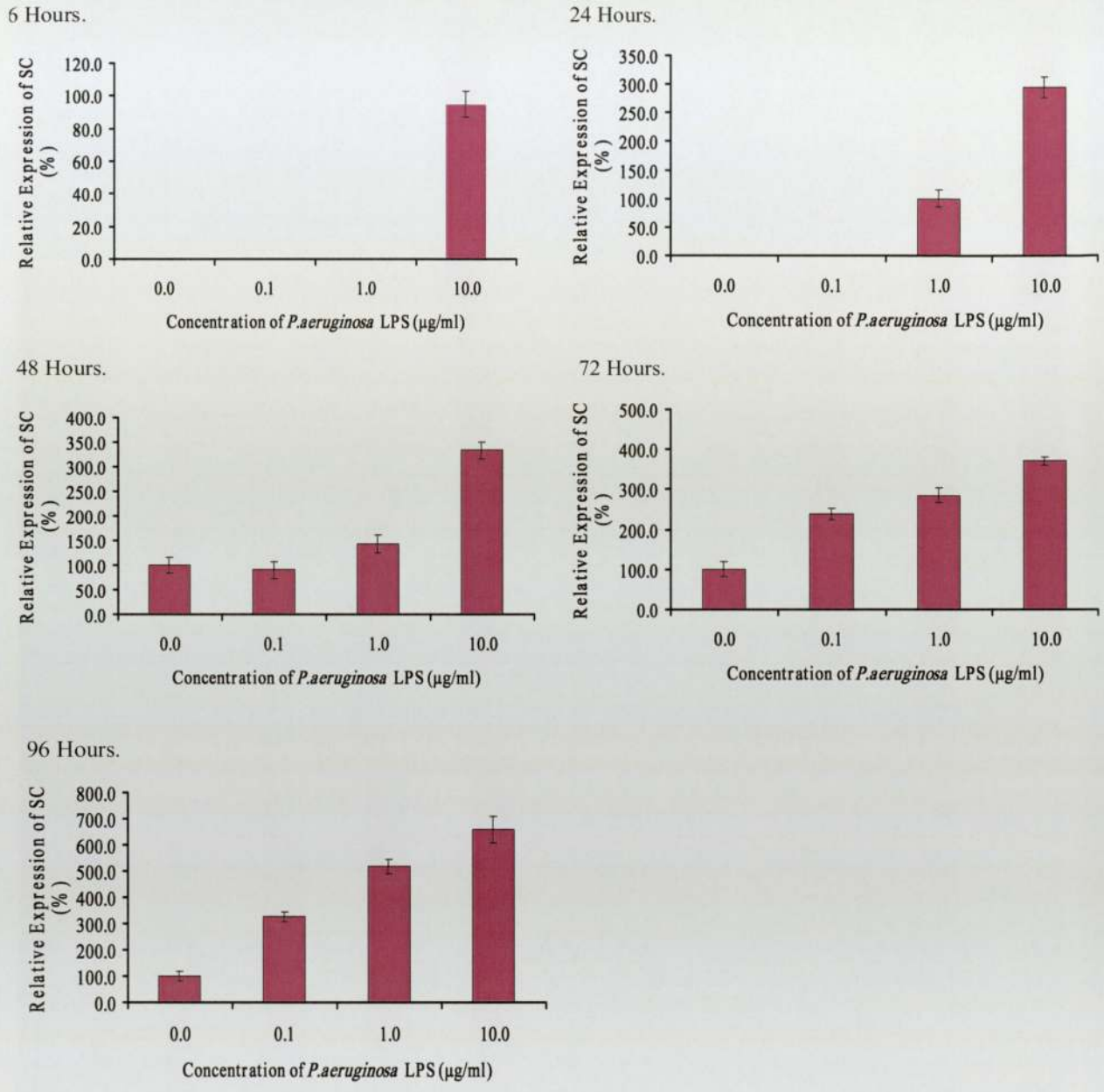

Figure 5.11 The effect of different concentrations of *P. aeruginosa* LPS and time on the relative expression of SC detected in CALU-3 culture supernatants as measured by Western blotting. The number of pixels within each protein band was converted to a percentage. Constitutive release is expressed as 100%. Where constitutive release is not detected, constitutive release at 48 hours was defined as 100% constitutive release of SC is expressed as 100 % and stimulated release of SC is expressed relative to this. Data are expressed as mean ± SE of three independent experiments with three replicates. * = p • 0.05, ** = p • 0.01 vs. SC release from unstimulated CALU-3 cells.

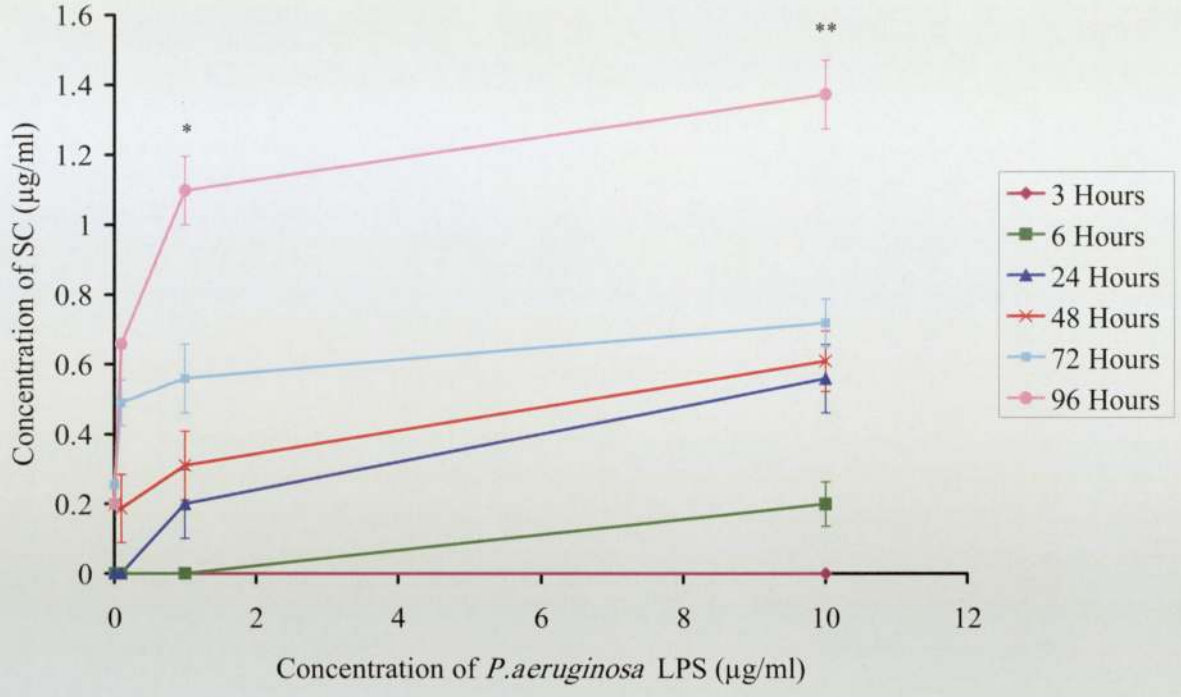

Figure 5.12 The effect of the concentration of *P. aeruginosa* LPS and time on the concentration of SC detected in CALU-3 cell supernatants as analysed by ELISA. Data are expressed as mean ± SE of three independent experiments with three replicates. * = $p \bullet 0.05$, ** = $p \bullet 0.01$ vs. SC release from unstimulated CALU-3 cells.

The mean relative expression of SC in the supernatants of cells that were incubated with 10 µg/ml LPS for six hours was 95 ± 15.2 % when analysed using Western blotting (figure 5.11). The mean concentration of SC detected in the same supernatants using ELISA analysis was 0.21 ± 0.07 µg/ml (figure 5.12).

The data presented in figure 5.10 shows that SC at a molecular weight of 74 ± 5 kDa was detected in the supernatants of cells that were stimulated with 1 and 10 µg/ml. Western blot and ELISA analysis did not reveal SC in the culture supernatants of cells that were stimulated with 0.1 µg/ml LPS for 24 hours (figures 5.11 and 5.12). The mean relative expression of SC detected by Western blotting when cells were stimulated with 1 µg/ml LPS for 24 hours was 100 ± 17.9 %. When the same supernatants were analysed using an ELISA the concentration of SC detected 0.21 ± 0.04 µg/ml. Following stimulation with 10 µg/ml LPS for 24 hours, the

relative expression was found to be 295 ± 16.6 % when analysed by Western blotting (figure 5.11). The concentration of SC detected by an ELISA in the same culture supernatants was 0.61 ± 0.06 μg/ml (figure 5.12).

Constitutive release of SC was detected by Western blotting at 48 hours in culture supernatants; the molecular weight of SC was 76 ± 2 kDa (figure 5.10). 76 ± 2 kDa SC was also detected in the supernatants of cells that were stimulated with 0.1, 1 and 10 μg/ml LPS (figure 5.10). The mean relative expression of SC detected in the culture supernatants of cells incubated with 0.1 μg/ml LPS for 48 hours was 90 ± 14.7 %. The concentration of SC detected in the same culture supernatants when analysed with an ELISA was 0.19 ± 0.07 μg/ml (figure 5.12). The concentration of SC detected by ELISA in the same supernatants was 0.21 ± 0.08 μg/ml (figure 5.12). When cells were stimulated with 1 μg/ml LPS for 48 hours the mean relative expression of SC was 143 ± 19.8 % when analysed by Western blotting (figure 5.11). When the same supernatants were analysed using an ELISA the concentration of SC detected was 0.31 ± 0.07 μg/ml. The mean relative expression of SC was found to be 333 ± 16.6 % in the supernatants of cells that were stimulated with 10 μg/ml LPS for 48 hours and analysed using Western blotting (figure 5.11). The concentration of SC detected by ELISA in the same supernatants was 0.71 ± 0.05 μg/ml (figure 5.12). Constitutive release of SC was detected by ELISA at a concentration of 0.21 ± 0.03 (figure 5.12).

Western blot analysis showed that 75 ± 3 kDa SC was detected in the culture supernatants of cells that were stimulated with 0.1, 1 and 10 μg/ml LPS and in the supernatants of cells that were not exposed to bacterial stimuli (figure 5.10). The mean relative expression of SC in the supernatants of cell that were stimulated with 0.1 μg/ml LPS for 72 hours was 238 ± 14.7 % (figure 5.11). In the same culture supernatants 0.49 ± 0.06 μg/ml of SC was detected using an

ELISA (figure 5.12). A mean relative expression of 285 ± 19.1 % of SC was detected by Western blotting in the culture supernatants of cells that were stimulated with 1 μg/ml LPS for 72 hours (figure 5.11). The concentration of SC detected by ELISA in the same culture supernatants was 0.61 ± 0.05 μg/ml (figure 5.12). SC was detected by Western blotting in culture supernatants from cells that had been stimulated with 10 μg/ml LPS for 72 hours at a mean relative expression of 371 ± 11 % (figure 5.11). The mean concentration of SC detected by ELISA in the same supernatants was 0.79 ± 0.08 μg/ml (figure 5.12). Constitutive release of SC was detected by ELISA at a mean concentration of 0.21 ± 0.06 μg/ml (figure 5.12).

Western blot analysis showed that 74 ± 3 kDa SC was detected in the culture supernatants of cells that were stimulated with 0.1, 1 and 10 μg/ml LPS and in the supernatants of cells that were not exposed to bacterial stimuli for 96 hours (figure 5.10). Constitutive release of SC was detected by ELISA at a mean concentration of 0.21 ± 0.05 μg/ml (figure 5.12). The mean relative expression of SC in the culture supernatants of cells stimulated with 0.1 μg/ml LPS for 96 hours was 371 ± 11.2 % (figure 5.11). A concentration of 0.73 ± 0.11 μg/ml of SC was detected by ELISA in the same culture supernatants (figure 5.12). Following stimulation with 1 μg/ml LPS for 96 hours the mean relative expression of SC was 328 ± 17.6 % when culture supernatants were analysed using Western blotting (figure 5.11). The mean concentration of SC was 1.10 ± 0.07 μg/ml when detected in the same culture supernatants by an ELISA (figure 5.12). The concentration of SC released under these conditions was significantly higher than the concentration of SC released from cells that were not stimulated ($p \bullet 0.05$). When cells were stimulated with 10 μg/ml LPS for 96 hours the mean relative expression of SC detected by Western blotting was 519 ± 27.5 % (figure 5.11). The concentration of SC detected in the same culture supernatants by ELISA was 1.40 ± 0.07 μg/ml (figure 5.12). The concentration of SC released following stimulation with 10 μg/ml LPS for 96 hours was significantly higher than the concentration of SC released from cells constitutively ($p \bullet 0.01$).

It was determined using the LAL assay described in section 2.2.3 that the endotoxin concentration in the 10 μg/ml preparation of *P. aeruginosa* LPS was 0.82 ± 0.031 EU/ml.

5.4 Discussion

The CALU-3 cell line expresses SC constitutively Loman *et al.*, (1997) and (1999) and Godding *et al.*, (1998), they therefore provided an excellent model for investigating SC expression *in vitro*. Data from a study published by Godding *et al.*, (1998) showed that constitutive release of SC from CALU- 3 cultures was detected after 24 hours, in this instance 12.4 ng/ml of SC was detected. The data presented in this report show that SC released from unstimulated cells is detected only after 48 hours, but at higher concentrations. A mean concentration of 0.22 ± 0.027 μg/ml of constitutively released SC was detected. Loman *et al.*, (1999) found that SC released constitutively from CALU-3 cells was detected in supernatants at 48 hours but at a lower concentration of approximately 120 ng/ml. However in a study undertaken previously in 1997 by Loman *et al.*, it was found that SC was detected at 24 hours at a concentration of 110 ng/ml. Differences in findings between the data presented here and previous studies could possibly be explained by the difference in growth conditions of CALU-3 cells. Similar to the cell culture model presented in this thesis, CALU-3 cells were plated at 10^5 cells per well, however they were grown on collagen coated Transwells inserts. Fielder *et al.*, (1991) found that the production of SC by human tracheal explant (HTE) cells was significantly higher in cultures that were grown either on nitrocellulose Transwells or collagen coated Transwells, compared to SC production by cells that were grown on plastic. These findings could therefore explain why, in this thesis it is shown that SC was detected only after 48 hours. In addition it was noted that, in the studies published by Loman *et al.*, (1997) and (1999) and Godding *et al.*, (1998) free SC was detected in supernatants harvested from the apical side of the cells, whereas in this study supernatants were harvested from submerged cell cultures. Harvesting supernatants at the apical side of the cell could possibly result in SC release from unstimulated cells being detected at an earlier time point, as free SC

is transcytosed from the basolateral to the apical side. It is also possible that as there is less medium at the apical surface of air liquid interface cultures, the SC in the medium would have been more concentrated.

It is shown in here that when LPS or LTA were used to stimulate CALU-3 cells there was an up regulation of SC expression. LPS signals through Toll-like receptor 4 (TLR4) Polterak *et al.,* (1998) and LTA is thought to signal through Toll-like receptor 2 (TLR2) Ginsburg, (2002), both of which are expressed by airway epithelial cells. When TLR4 is activated by LPS it stimulates a nuclear factor kappa B (NF•B) mediated cytokine cascade of inflammatory cytokines Ginsburg, (2002). Although less is known about the *in vivo* activities of LTA, *in vitro* LTA has been shown to stimulate inflammatory cytokines Ginsburg, (2002). It has been shown that SC is up regulated in airway epithelial cells *in vitro* by inflammatory cytokines IFN• Loman, *et al.*, (1997), Godding *et al.*, (1998), Loman *et al.*, (1999), Ackermann *et al.*, (1999) and IL-4 Loman *et al.*, (1999) and Ackermann *et al.*, (1999). It is known that there is an over expression of these inflammatory mediators in the lungs of CF Berger, (2002) and asthmatic Holgate *et al.*, (1999) patients, however it is not known whether LPS and/or LTA up regulate IL-4 and IFN•. Characteristically the lungs of asthmatic patients are inflamed and the lungs of CF subjects are both chronically inflamed and colonised with bacteria, particularly *P. aeruginosa* and *B. cepacia* Zughaier *et al.*, (1999) which could indicate that IL-4 and IFN• are up regulated *in vivo* during infection and inflammation. It is an important finding, therefore that IL-4 and IFN• up regulate expression of SC *in vitro*, as it is known that LPS and LTA cause inflammation *in vivo* and have been shown in this thesis to up regulate SC *in vitro.* It is possible that the increased expression of SC observed following stimulation of CALU-3 cells with LPS or LTA is a result of up regulation of IL-4 and IFN•. It is also important to note the observations made by Nihei *et al.*, (1995), who showed that SC was an inhibitor of IFN• function in keratinocytes. These findings could suggest the reason

SC expression was up regulated in the cell culture system presented in this thesis following stimulation with LPS or LTA. It is therefore plausible to suggest that SC expression is up regulated to counteract inflammation. In order to elucidate whether LPS or LTA up regulate expression of IL-4 and IFN• in CALU-3 cells, culture supernatants from cells that had been stimulated with LPS or LTA could be analysed for IL-4 and IFN• using an ELISA, an enzyme linked immuno spot (ELISpot) assay or a polymerase chain reaction (PCR) assay. If it was shown that LPS or LTA up regulate the expression of IL-4 and IFN•, a link between the up regulation of SC by bacterial products and the up regulation of SC by inflammatory cytokines could be drawn. An important observation was made by Pilette *et al.*, (2003) in that they showed that NF•B up regulated SC expression in airway epithelial cells. It has been shown here that LPS and LTA up regulate SC expression and it is known that LPS and LTA activate NF•B. Therefore it could be hypothesised that the reason SC was up regulated in the cell culture model presented in this thesis was because of the activation of NF•B by LPS and LTA. In order to confirm these findings it would be necessary to inhibit the actions of NF•B in cell cultures to see whether the upregulation of SC expression is independent of NF•B activation.

Another interesting observation made during this work was that when LPS from *E. coli* or *B. cepacia* was used to stimulate cells SC expression was rapidly up regulated, but as the concentration of LPS used to stimulate cells increased and the period of incubation increased the expression of SC decreased. When LPS from *P. aeruginosa* or LTA from *S. aureus* was used to stimulate cells an up regulation in SC expression was observed, but only when cells were stimulated for longer time periods with higher concentrations of bacterial stimuli. In addition it was observed that unlike when cells were stimulated with higher concentrations of LPS from *E. coli* or *B. cepacia* for longer incubation periods, expression of SC was not down regulated. It was observed during the work presented in this thesis that stimulating cells with

10 μg/ml *E. coli* LPS for over 24 hours, 1 μg/ml *B. cepacia* LPS for over 24 hours or 10 μg/ml *B. cepacia* LPS for over 6 hours resulted in cell detachment and necrosis. These observations explain why there was a decrease in the concentration of SC detected in the CALU-3 cell model used in the thesis. A cell viability assay for example the lactate dehydrogenase (LDH) assay could be used to investigate the integrity of the CALU-3 cell membrane to ascertain whether or not stimulation with concentrations of LPS has resulted in cell death.

It was found that approximately 0.1 ± 0.051 EU/ml of endotoxin was detected in the 10 μg/ml preparation of LTA, compared to 0.9 ± 0.037 EU/ml measured in the 10μg/ml *E. coli* LPS preparation, 0.85 ± 0.064 EU/ml measured in the 10 μg/ml preparation of *B. cepacia* LPS and 0.82 ± 0.032 EU/ml detected in the 10 μg/ml *P. aeruginosa* LPS preparation. Therefore it could be hypothesised that the delayed inflammatory reaction observed in cells stimulated with LTA, was due to the contamination of endotoxin. As the endotoxin in the LTA preparation was more dilute than the LPS used in this assay, it could explain why SC expression was not up regulated until after cells had been incubated with 10 μg/ml for six hours.

It is also important to consider the concentration of SC *in vivo,* Marshall *et al.*, (2004) found that there is a higher concentration of SC in the sputa of asthma and CF patients (24.5 μg/ml and 40.32 μg/ml respectively) compared to the concentration of SC in the sputa of normal subjects (7.84 μg/ml). It is interesting that the concentration of SC in the sputa of patients with inflamed and infected lungs is higher than the concentration of SC measured in normal sputa because this indicates that there is an upregulation of SC *in vivo* in response to inflammation.

Data presented here shows that SC expression is up regulated by airway epithelial cells in response to stimulation with LPS or LTA. However the observation that SC does not accumulate between 48 and 96 hours and the observation that unstimulated release of SC was detected only after 48 hours now requires further investigation

6 Fragmentation of SC by Serine Proteases

6.1 Introduction

Loman *et al.*, (1997) and (1999) and Godding *et al.*, (1998) all found that constitutive release of SC by CALU-3 cells increased as the period of incubation increased. However the data presented in chapter 5 showed that constitutively released SC did not accumulate in culture supernatants between 48 and 96 hours. In addition it was found that constitutive release of SC was detected only after 48 hours, these findings required further investigation.

A possible explanation for this finding could be that as the cells were maintained in quiescent medium, there was no cell proliferation. It could be hypothesised that an increase in the number of cells would result in an increase in the concentration of SC detected as there would be more cells synthesising and secreting SC. Therefore it was decided to introduce growth factor containing FCS into quiescent medium and investigate the effect of this on cell proliferation and subsequent SC secretion.

Another explanation could be that a proportion of the SC released was cleaved by proteases expressed by CALU-3 cells. It is possible that this fragmented SC was not detected using Western blot analysis with a monoclonal antibody to SC or ELISA analysis. It has been shown that serine proteases, specifically trypsin, degrade SC by cleaving it after lysine and before arginine in the amino acid sequence, Cunningham-Rundles *et al.*, (1973), Beale and Hopley., (1984) and Frutiger *et al.*, (1986). It has been shown that trypsin is expressed in lung tissue Koshikawa *et al.*, (1998) and specifically, human airway trypsin-like protease (HAT) is expressed by CALU-3 cells. In addition it has been shown that other membrane anchored serine proteases are expressed by human airway epithelial cells Tong *et al.*, (2004).

It was therefore hypothesised that the addition of protease inhibitor cocktail (PIC) into cell culture medium would maintain SC in an intact form which could then be detected using Western blot and ELISA analysis.

6.2 Materials and Methods

6.2.1 The Effect of PIC and FCS on CALU-3 Proliferation

Following overnight incubation in quiescent medium, cells were incubated with either protease inhibitor cocktail (PIC) in quiescent medium, PIC and 10 % FCS in medium, 10 % FCS in medium or quiescent medium alone. The cells were incubated for 24, 48, 72 and 96 hour time points. Following each time point the supernatants were harvested and the cells were washed once with 1 ml of ice cold PBS then incubated with 50 µl per well of trypsin:EDTA for 10 minutes at 37 °C. When the cells had detached, 450 µl of complete growth medium was added to each well, this medium was then collected and centrifuged at 500 *g* for 5 minutes at room temperature. Following centrifugation the supernatants were discarded and the cell pellet was resuspended in 1 ml of fresh complete medium. Samples were diluted 1:1 in trypan blue and cells were counted using a haemocytometer.

6.2.2 The Effect of Protease Inhibitor Cocktail on SC Production

Using the protocol described in section 6.2.1, cells were incubated with either PIC, PIC and 10 % FCS, 10 % FCS or quiescent medium alone for 24, 48, 72 and 96 hours. After each time point 24 well plates were removed from the incubator and put onto ice to minimise further protein degradation then supernatants were removed and spun at 850 *g* for 10 minutes at 4 °C. The cells were washed once with 1 ml of ice cold PBS per well, then lysed with 500 µl per well of cell lysis buffer (PBS containing PIC and 1 % (v/v) Triton X-100). The cells were incubated in cell lysis buffer for 20 minutes; cell lysis was confirmed by viewing wells with the light microscope. Once cells had lysed they were harvested and stored at -20 °C

ready for analysis. Cleared supernatants were analysed immediately using the monoclonal capture, polyclonal detection ELISA with PBS/ 2 % Tween® block described in chapter 4.

6.2.3 The Effect of Conditioned Medium on the Integrity of SC

The medium was removed from a 100 % confluent 75 cm^2 tissue culture flask and the cells were washed with PBS and then incubated in quiescent medium for 48 hours. After 48 hours the flask was put on ice and the medium was harvested and stored at 4 °C. Prior to Western blot analysis 10 μg of SC was incubated for 24 hours at 37 °C with either conditioned medium, 50 nM trypsin, conditioned medium and 100 mg/ml PIC, 50 nM trypsin with 100 mg/ml of PIC or 100 mg/ml of PIC. All samples were made up to a total volume of 26 μl in conditioned media. The reaction was stopped by the addition of an equal volume of 2 X Laemmli sample buffer. Samples were immediately analysed using the SDS-PAGE protocol and polyclonal anti-SC Western blotting protocols described in chapter 3.

6.3 Results

6.3.1 The Effect of FCS and PIC on CALU-3 Proliferation and SC Production

The data presented below show the effect that adding PIC and/or 10 % FCS to CALU-3 cell cultures has on the number of cells counted.

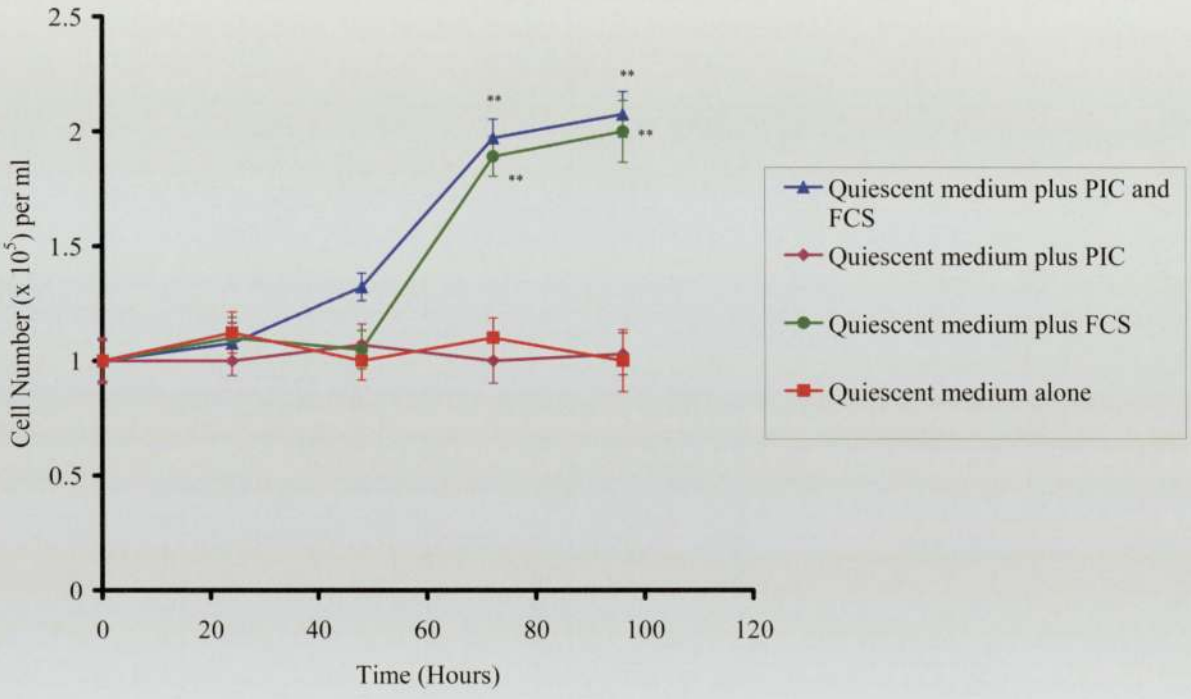

Figure 6.1 The number of CALU-3 cells counted per well following maintenance in quiescent medium or incubation with PIC and/or 10% FCS. Data are expressed as mean ± SD of three independent experiments with three replicates. ** = $p \bullet 0.01$, vs. growth of CALU-3 cells maintained in quiescent medium only.

The data presented in figure 6.1 shows that cell number did not increase as time increased in cultures that were maintained in quiescent medium. The number of cells per ml at 24 hours was 1 x 10^5 ± 0.064. At 48 hours the number of cells per ml was 1 x 10^5 ± 0.089. 1 x 10^5 ± 0.097 cells/ml were counted at 72 hours and 1 x 10^5 ± 0.091 cells/ml were counted at 96 hours. In addition Figure 6.1 shows that cell proliferation was not observed in cultures that were incubated with PIC but not FCS. At 24 hours 1 x 10^5 ± 0.072 cells/ml were counted.

Following 48 hours incubation 1 x 10^5 ± 0.1 cells/ml were counted. 1 x 10^5 ± 0.098 cells/ml were counted following 72 hours incubation and at 96 hours 1 x 10^5 ± 0.092 cells/ml were counted.

Cell proliferation was evident in cultures that were incubated with medium containing FCS with PIC or medium with FCS alone. The number of cells was significantly higher (p • 0.01) at 72 and 96 hours compared to cultures that were not incubated with FCS. At 24 hours the number of cells in cultures incubated with FCS was 1 x 10^5 ± 0.090 cells/ml, following 48 hour incubation with FCS the number of cells remained at 1 x 10^5 ± 0.084 cells/ml (figure 6.1). After incubation FCS for 72 hours the number of cells counted was 1.75 x 10^5 ± 0.087 cells/ml, the number of cells continued to increase up to 2 x 10^5 ± 0.134 cells/ml after incubation with FCS for 96 hours (figure 6.1). This trend was also evident in the presence of FCS and PIC, following 24 hours incubation the number of cells per ml counted was 1 x 10^5 ± 0.089, but after 48 hours incubation the number of cells per ml increased to 1.25 x 10^5 ± 0.060. Following incubation with FCS and PIC for 72 hours 2 x 10^5 ± 0.082 cells/ml were counted and this number increased again following 96 hour incubation to 2.2 x 10^5 ± 0.098 cells/ml.

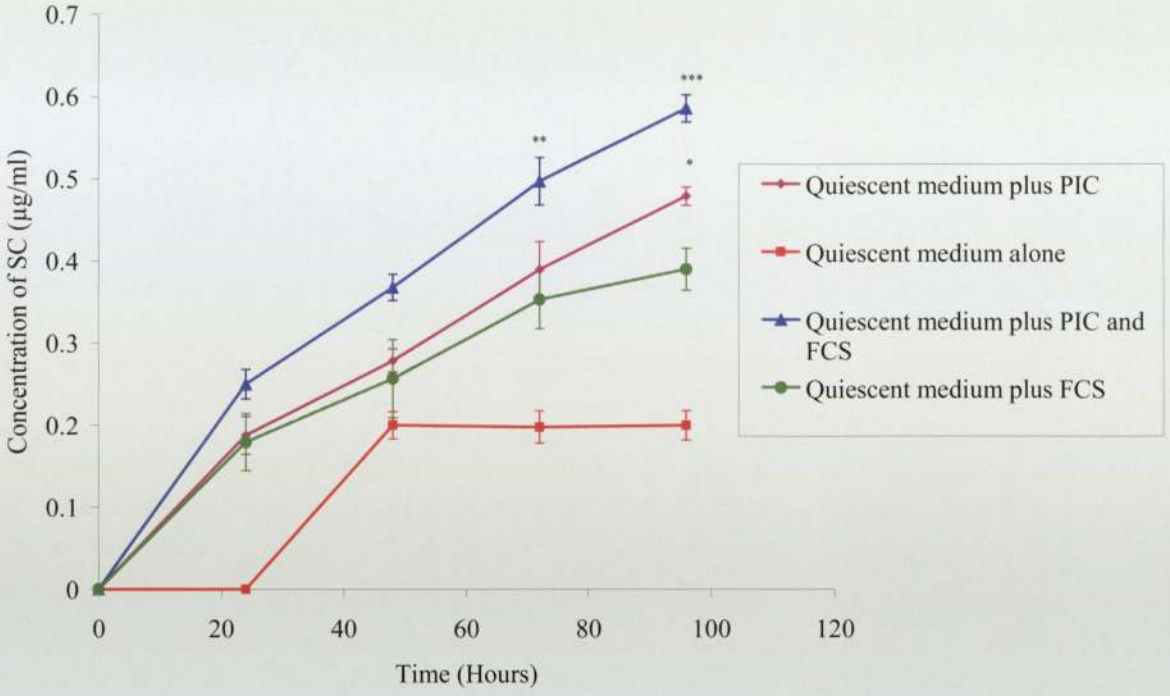

Figure 6.2 The effect of PIC and/or 10 % FCS on production of SC by CALU-3 cells. Cleared supernatants were analysed by ELISA. Data are expressed as mean ± SD of three independent experiments with three replicates. * = p • 0.05 ** = p • 0.01, *** = p • 0.001 vs. SC release from CALU-3 cells maintained in quiescent medium only.

The data presented in figure 6.2 show that when cell cultures were maintained in quiescent medium (without PIC or FCS), SC was detected at 48 hours at 0.20 ± 0.016 µg/ml (2 pg/cell). The concentration of SC detected at 72 hours was 1.9 ± 0.020 µg/ml (1.9 pg/cell) and the concentration of SC detected at 96 hours was 0.19 ± 0.018 µg/ml (1.9 pg/cell; figure 6.2).

When cell cultures were incubated with FCS, SC was detected at 24 hours at 0.19 ± 0.035 µg/ml (1.8 pg/cell) and the concentration of SC detected continued to increase up until the 96 hour time point (figure 6.2). Following incubation with FCS for 48 hours the mean concentration of SC detected was 0.25 ± 0.048 µg/ml (2.5 pg/cell; figure 6.2). After 72 hours the mean concentration of SC detected was 0.35 ± 0.036 µg/ml (1.9 pg/cell) and following incubation with FCS for 96 hours the mean concentration of SC detected was 0.39 ± 0.026 µg/ml (1.95 pg/cell). Even though there was increased production of SC in comparison to

cultures maintained in quiescent medium, the difference was not statistically significant (p = 0.07).

SC was detected in the supernatants of cell cultures that were incubated with PIC for 24 hours at a mean concentration of 0.18 ± 0.017 μg/ml (1.8 pg/cell; figure 6.2). The concentration of SC detected continued to increase as time increased. Following incubation with PIC for 48 hours the mean concentration of SC detected was 0.28 ± 0.016 μg/ml (2.8 pg/cell). This increased to 0.39 ± 0.029 μg/ml (3.9 pg/cell) at 72 hours and 0.48 ± 0.020 μg/ml (4.8 pg/cell; figure 6.2) after 96 hours. The concentration of SC detected at 96 hours was significantly higher in cultures incubated with PIC than the concentration of SC detected in the supernatants of cultures maintained in quiescent medium only (p • 0.05; figure 6.2).

In cell cultures that were incubated with FCS and PIC for 24 hours, SC was detected at a mean concentration of 0.25 ± 0.023 μg/ml (2.8 pg/cell) and the concentration of SC detected increased as time increased. Overall the concentration of SC detected was higher in cultures that were incubated with FCS and PIC compared to the concentration of SC detected in the supernatants from cultures that were treated with FCS or PIC and cultures that were maintained in quiescent medium only (figure 6.2). Following incubation with FCS and PIC for 48 hours the mean concentration of SC detected was 0.36 ± 0.014 μg/ml (3 pg/cell; figure 6.2). After 72 hours incubation the mean concentration of SC detected was 0.5 ± 0.034 μg/ml (2.5 pg/cell; figure 6.2). Following incubation for 96 hours the mean concentration of SC detected was 0.6 ± 0.011 μg/ml (2.7 pg/cell; figure 6.2). At 72 and 96 hours the concentration of SC detected was significantly higher in cultures incubated with both FCS and PIC, compared to cultures in quiescent medium alone (p • 0.01 and 0.001 respectively).

6.3.2 The Effect of Conditioned Medium on the Integrity of SC

Western blot analysis can provide information on to the molecular weight(s) of a protein, as fragmented or degraded proteins would be separated from the intact species during electrophoretic separation. In order to investigate whether there was protease activity in cell cultures it was decided to incubate SC with conditioned medium prior to separating by SDS PAGE. If SC had been cleaved, the fragments could be detected when the SC was immobilised on a nitrocellulose membrane and incubated with a polyclonal anti-SC antibody. The results of the Western blot analysis are shown in figure 6.3.

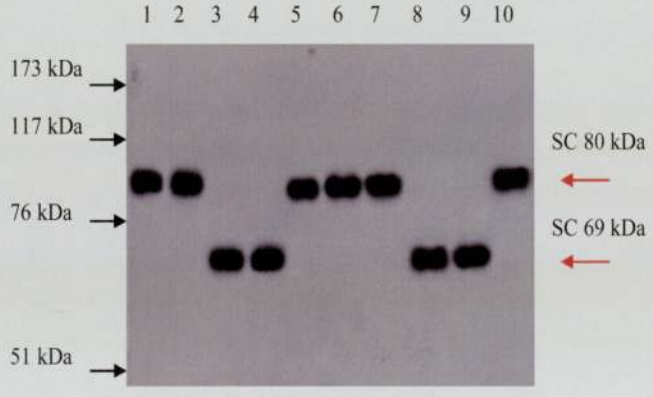

Figure 6.3 Scanned image of Nordic Immunology standard SC preparation separated using SDS PAGE on a 7.5 % polyacrylamide gel. Positions of molecular weight markers are shown to the left of the image. The expected positions of SC are indicated by the arrows to the right of the image. The image is representative of three independent experiments. Lane 1: Standard SC boiled in reducing sample buffer, lane 2: Standard SC boiled in non reducing sample buffer, lane 3: SC incubated with trypsin, boiled in reducing sample buffer, lane 4: SC incubated with trypsin, boiled in non reducing sample buffer, lane 5: SC incubated with trypsin and PIC boiled in non reducing sample buffer, lane 6: SC incubated with PIC boiled in non reducing sample buffer, lane 7: Conditioned medium only boiled in non reducing sample buffer, lane 8: SC incubated with conditioned media, boiled in reducing sample buffer, lane 9: SC incubated with conditioned medium, boiled in non reducing sample buffer, lane 10: SC incubated with conditioned medium and PIC, boiled in non reducing sample buffer

The image shown in figure 6.3 shows that when standard SC was loaded onto the gel (lanes one and two), SC was detected at 80 kDa, which indicates that reducing does not alter the size of SC. When SC was incubated with trypsin, (lane three), SC detected was at 69 kDa (figure

6.3). This was also true when SC that had been incubated with conditioned medium (lane four) was loaded onto the gel (figure 6.3). When SC that had been incubated with trypsin and PIC (lane five) was separated on the gel, SC was detected at a molecular weight of 80 kDa (figure 6.3). The findings in lanes three, four and five indicate that SC is not resistant to proteolytic degradation, when SC was incubated with trypsin it was degraded unless it had been incubated with PIC also. SC at 80 kDa was detected when SC that had been incubated with PIC was electrophoretically separated (lane six; figure 6.3). When conditioned medium (quiescent medium from CALU-3 cells after 48 hours culture) was separated, SC at 80 kDa was detected (lane seven; figure 6.3). SC at 69 kDa SC was detected in lane eight where a preparation of SC was incubated with conditioned medium had been loaded (figure 6.3). When SC was incubated with conditioned medium and separated on the gel (lane nine) SC at 69 kDa was detected (figure 6.3). SC at 80 kDa was detected when SC was incubated with conditioned medium and PIC (lane 10; figure 6.3).

6.4 Discussion

In chapter 6, two hypotheses were tested; the first was that CALU-3 cell proliferation would result in increased secretion of SC. The second was that the addition of protease inhibitors to the cell culture model would maintain intact SC in culture supernatants. The first hypothesis was proved correct as it was shown that when cells were incubated with FCS, cell proliferation occurred and there were an increased number of cells secreting SC. Quiescent medium, unlike FCS, does not contain growth factors and therefore cell proliferation is prevented. The data presented in figure 6.1 shows that the addition of FCS into cell cultures results in cell proliferation. The number of cells per ml was significantly higher ($p \bullet 0.01$) in cultures that were incubated for 72 and 96 hours with FCS than cultures that were not incubated with FCS. It is shown in figure 6.2 that there was an increase in the mean concentration of SC detected in the supernatants of cells treated with FCS. It was observed that the concentration of SC detected in supernatants of cells incubated with FCS was higher than the concentration of SC detected in cultures maintained in quiescent medium. At 24 hours the cell numbers were the same for cultures that were maintained in quiescent medium and for cultures that were incubated with FCS, however the concentration of SC detected in cultures incubated with FCS was higher than the concentration of SC detected in culture maintained in quiescent medium. These observations suggest that FCS was exerting anti-proteolytic properties that were preventing the degradation of SC and enabling its detection. It was calculated that the concentration of SC detected per cell for cultures incubated with both FCS and PIC or FCS was 2.7 ± 0.3 pg/cell and 2.2 ± 0.3 pg/cell, respectively. However when cells were incubated with PIC there was no cell proliferation but the concentration of SC detected following 24 incubation was 1.8 pg/cell and this increased to 4.8 pg/cell after 96 hours incubation (figure 6.1). The higher concentration of SC detected in the supernatants of

cells incubated with FCS suggests that FCS does not contain proteases as the concentration of SC continues to increase and also that it does exert a protease inhibitor effect. However given that the concentration of SC detected per cell density was lower in cells incubated with FCS than in cells incubated with PIC, it is probable that the data presented in figure 6.2 showing a higher concentration of SC detected in the supernatants of cells incubated with FCS is higher because there are more cells secreting SC as opposed to FCS maintaining intact SC in culture medium. It is also possible that some cleavage of SC was still occurring in cultures incubated with FCS. Although there was an overall higher concentration of SC detected in the culture supernatants of cells incubated with FCS compared to the concentration of SC detected in supernatants of cells maintained in quiescent medium, the concentration of SC per cell was lower than that observed in cultures incubated with PIC. This interesting observation suggests that even though there were a higher number of cells secreting SC, it is possible that some of that SC was still being cleaved. It is known that when cells are passaged, FCS must be removed as it inhibits the action of trypsin, however it is possible that there were proteases present in FCS that were also degrading SC.

It is important to note that as mentioned previously when SC production per cell was calculated it was apparent that PIC was preventing degradation of SC as there was a higher concentration of SC detected per cell in cultures that were incubated with PIC compared to the concentration of SC detected per cell in cultures maintained in quiescent medium or incubated with FCS (figure 6.2). Therefore it is possible that the SC was cleaved by HAT or another membrane anchored serine protease expressed by CALU-3 cells. The earlier detection of SC at 24 hours and the increase in the concentration of SC as time progresses in cultures incubated with PIC and/or FCS can be explained by the presence of leupeptin in PIC, which inhibits the action of serine proteases.

Another interesting observation made was that when cells were incubated with both FCS and PIC, there was not a dramatic difference in the concentration of SC compared to the concentration of SC detected in culture supernatants of cells incubated with either FCS or PIC (figure 6.2). Notably the concentration of SC produced per cell was lower in supernatants from cells that had been incubated with FCS compared to cells incubated with PIC. These findings were unusual as one would expect to see a dramatic increase in SC concentration when cells are actively proliferating and proteolytic degradation is prevented. It could be hypothesised that an increase in cell number would result in an increase in membrane anchored proteases, if this were the case then it could be reasonably be suggested that the PIC within the culture medium was exhausted and thus no dramatic difference in the concentration of SC was seen.

Western Blot analysis showed that when SC was incubated with either conditioned medium or trypsin a lower molecular weight fragment of SC was detected at 69 kDa in comparison to 80 kDa band that was detected in the standard preparation of SC (figure 6.3). It was also shown that when SC was incubated with PIC in addition to conditioned medium or trypsin, prior to electrophoretic separation, the SC detected was at its expected molecular weight of 80 kDa (figure 6.3). These findings indicated that there was protease activity in conditioned medium as it was apparent that intact SC had been cleaved, resulting in this lower molecular weight fragment. It is possible that the protease activity was due to either membrane bound trypsin or HAT as the molecular weight of the SC fragment detected in SC that was pre incubated with exogenous trypsin was also 69 kDa. This suggests that when SC was incubated with either conditioned medium or trypsin the SC was cleaved at the same position of its amino acid sequence. It is also interesting to note that Pilette *et al.*, (2003) detected SC at a molecular weight of 70 kDa following cleavage of SC with the serine protease neutrophil elastase. As anti-infective and anti-inflammatory roles for SC have been proposed *in vitro*

and, *in vivo* the cleavage of SC by serine proteases could be pathologically significant. Pilette *et al.*, (2003), also demonstrated that serine proteases cleave SC from SIgA, therefore it is possible that HAT would do the same. Even though the cleavage of SC from SIgA is not relevant in this system, *in vivo,* increased cleavage of SC from SIgA could result in the degradation of IgA as it is known that SC confers a degree of proteolytic resistance to IgA Brown *et al.*, (1970), Lindh, (1975) and Underdown and Dorrington, (1974). Pilette *et al.,* (2003) also showed that even though free SC was degraded by neutrophil elastase, when in complex with IgA, SC conferred resistance to IgA, the degradation of IgA *in vivo* could have implications in humoral defence.

Taken together these data indicate that both hypotheses were correct. Cell proliferation does result in a higher number of CALU-3 cells secreting SC and therefore there will be an increase the concentration of SC detected in culture supernatants. In addition protease activity in cell culture supernatants reduces the concentration of intact SC in CALU-3 culture medium. The findings that SC is detected at 24 hours and that SC continues to accumulate up to 96 hours following incubation with either FCS or PIC is now more in keeping with the findings of Loman *et al.*, (1997) and (1999) and Godding *et al.,* (1998).

7 Discussion

The data presented in this thesis shows that SC release by CALU-3 cells in culture is up regulated by LPS and LTA. Initially it was necessary to develop analytical techniques to enable the analysis and quantification of SC released from CALU-3 cells. Western blotting was used to analyse the form of SC and a capture ELISA was developed to quantify the concentration of SC released. It was shown that Western blotting using either monoclonal or polyclonal antibodies to SC detected SC to a lower limit of 0.3 μg/ml. In comparison the data presented in chapter 4 show that the ELISA developed using a monoclonal capture antibody with polyclonal detection antibody detected SC to a lower limit of 0.17 μg/ml. The difference in the sensitivity of these techniques could be explained by the fact that the denaturing detergent SDS is used during electrophoretic separation on polyacrylamide gels. It is possible that SDS could block the native epitopes that are required for recognition of SC by the antibody and this would prevent antibody binding. It can be concluded that Western blotting is useful for detecting SC at concentrations of no less than 0.3 μg/ml of SC, whereas the ELISA detects SC at concentrations no less than 0.17 μg/ml. However, Western blotting has the advantage over ELISA in that it is possible to obtain information on the size of the protein and whether it was intact. The ELISA was effective at measuring lower concentrations of SC, which would be beneficial when investigating the secretion of free SC by cells in culture.

It is shown in this thesis that when LPS or LTA are used to stimulate the CALU-3 cells there is an up regulation of SC expression. In summary it was observed that the maximum concentration of SC detected when cells were stimulated with *E. coli* LPS was 2.14 ± 0.09 μg/ml when analysed using an ELISA. This maximal response was observed when cells were stimulated with 1 μg/ml *E. coli* LPS for 48 hours, it was calculated that the endotoxin

concentration of this sample was 0.09 EU/ml. The maximum concentration of SC detected when cells were stimulated with *S. aureus* LTA was 1.41 ± 0.09 μg/ml when analysed using an ELISA. This maximal response was observed following stimulation with 10 μg/ml *S. aureus* LTA for 48 hours, the endotoxin concentration of this sample was found to be 0.1 ± 0.051 EU/ml. The maximum concentration of SC detected when cells were stimulated with LPS from *B. cepacia* was 2.10 ± 0.09 μg/ml when analysed using an ELISA. This maximal response was observed when cells were stimulated with 10 μg/ml *B. cepacia* LPS for six hours, the endotoxin concentration of this sample was found to be 0.85 ± 0.064 EU/ml. The maximum concentration of SC detected when cells were stimulated with LPS from *P. aeruginosa* was 1.40 ± 0.07 when analysed using an ELISA. This maximal response was observed when cells were stimulated with 10 μg/ml *P. aeruginosa* LPS for 96 hours, the endotoxin concentration of this sample was found to be 0.82 ± 0.032. It was suggested in chapter 5 that in the cell culture model presented in this thesis, the expression of SC was regulated by LPS or LTA through Toll-like receptor signalling and by the upregulation of inflammatory cytokines. However so far only IL-4, IFN• Loman *et al.*, (1997) and (1999) and Godding *et al.*, (1998) and potentially IL-1• Pilette *et al.*, (2003) have been found to up regulate SC expression in airway epithelial cells. There is no conclusive evidence that IL-4 and INF• are up regulated by LPS or LTA and it was suggested in chapter 5 that this needs to be investigated further.

Experiments conducted on the human colonic carcinoma cell line HT-29 cells *in vitro* showed that TNF-• Kvale *et al.*, (1988), Schjerven *et al.*, (2001) and Liu *et al.*,(2007), IFN• Sollid *et al.*, (1987), Phillips *et al.*, (1990) and Ackermann *et al.*, (1999), IL-4 Phillips *et al.*, (1990), Ackermann *et al.*, (1999) and Schjerven *et al.*, (2000) and IL-1 Hayashi *et al.*, (1997) up regulated SC expression. It is known that LPS stimulates the release of IL-1 and TNF-• *in vivo*. These observations are interesting and although this particular cell line was not used in

this thesis, these findings suggest the possibility that TNF-• and IL-1 were responsible for the up regulation of SC in CALU-3 cell culture model used in this thesis following LPS or LTA stimulation. Of course this theory must be viewed with caution as potentially there are intrinsic differences between intestinal and airway epithelial cells. However, Pilette *et al.,* (2003) observed that IL-1• increased the expression of SC in airway epithelial cells. It was shown by Bahl and Foreman (1994) that LPS stimulated the release of IL-1• from peritoneal mouse macrophages *in vitro* and therefore it is possible that IL-1• stimulated the up regulation of SC in CALU-3 cells following incubation with LPS or LTA.

It is important to consider the concentration of SC *in vivo,* when examining its proposed anti-inflammatory and anti-infective role. Marshall *et al.* (2004) found that there was a higher concentration of SC in sputum samples from asthmatic and CF subjects (24.5 ± 5.8 μg/ml and 40.32 ± 4.1 μg/ml respectively) compared to the concentration of 7.84 ± 2.1 μg/ml of SC in the sputum samples of normal subjects. These findings are important as they show that the concentration of SC is increased during chronic inflammation and potentially chronic infection. These data are supported by the findings of the data from the *in vitro* CALU-3 model presented in this thesis. SC expression in CALU-3 cells was up regulated following stimulation with bacterial stimuli compared to constitutive release of SC. It should be taken into consideration however that glucocorticoids are a commonly inhaled medication used by asthmatic patients have been shown to up regulate expression of SC in rat hepatocytes Wira and Colby, (1985). Therefore it is possible that the combination of inflammatory mediators and inhaled glucocorticoids were responsible for the increased concentration of SC observed in asthmatic and CF subjects Marshall *et al.,* (2004). However glucocorticoids were not present in the CALU-3 cell model used in this thesis which suggests that the upregulation of SC was a result of stimulation from pro inflammatory agents.

Another interesting observation made during this work was that when LPS from *E. coli* or *B. cepacia* was used to stimulate cells SC expression was rapidly up regulated, but as the concentration of LPS used to stimulate cells increased and the period of incubation increased the expression of SC decreased. When LPS from *P. aeruginosa* or LTA from *S. aureus* was used to stimulate cells an up regulation in SC expression was observed, but only when cells were stimulated for longer time periods with higher concentrations of bacterial stimuli. In addition it was observed that unlike when cells were stimulated with higher concentrations of LPS from *E. coli* or *B. cepacia* for longer incubation periods, the concentration of SC detected did not decrease. It was observed during the work presented in this thesis that stimulating cells with 10 μg/ml *E. coli* LPS for over 24 hours, 1 μg/ml *B. cepacia* LPS for over 24 hours or 10 μg/ml *B. cepacia* LPS for over 6 hours resulted in cell death. These observations explain why there was a decrease in the concentration of SC detected in the CALU-3 cell model used in the thesis. Physiologically, it is known that during bacterial infection with gram negative organisms, low plasma concentrations of LPS (approximately 34 pg/ml) elicit an immune response that results in fever and raised blood pressure, whereas higher plasma concentrations of LPS (approximately 61 pg/ml) are toxic and result in severe systemic inflammation Behre *et al.,* (1992). It is possible that the differences in the up regulation of SC expression between different types and concentrations of LPS, between LPS and LTA and between different incubation times *in vitro* can be explained by the physiological action of LPS and LTA. It was found that the endotoxin contamination in the 10 μg/ml LTA preparation used in this thesis was 0.1 ± 0.051 EU/ml, and therefore it must be taken into consideration that endotoxin was stimulating the upregulation of SC as opposed to LTA. However assuming that LTA and not endotoxin contamination was responsible for the increase in SC expression, it is known that LTA is approximately 100 fold less active than LPS *in vitro* Grunfeld *et al.*, (1999) and this could also explain why there was delayed upregulation of SC observed in this assay.

Another explanation for the differences observed in SC up regulation could be that the *P. aeruginosa* LPS is not considered as potent an inflammatory activator as either *E. coli* 0111 or *B. cepacia* J2315 LPS as it does not initiate as intense an inflammatory response as the latter two Zughaier *et al.*, (1999). This could therefore explain why there was a delayed SC up regulation and why no cell death was observed in cells that were stimulated with *P. aeruginosa* LPS.

The constitutive release of SC is also examined in this thesis and it was observed that SC did not accumulate in cell culture supernatants between 48 and 96 hours. This observation was surprising as Huang *et al.*, (1976) found that the secretion of SC in a colonic carcinoma cell line was the result of *de novo* synthesis of SC. Therefore one would expect *de novo* synthesis of SC to continue for as long as the cells were viable. It was hypothesised that either the maintenance of cells in quiescent medium prevented cell proliferation resulting in no accumulation of SC or that SC was being degraded by HAT or another membrane anchored serine protease expressed by CALU-3 cells. Indeed it was observed that when cells proliferated, the concentration of SC detected increased as the period of incubation increased. However, per cell number the concentration of SC detected in proliferating cells was lower than the concentration of SC detected per cell when cells were incubated with protease inhibitors. These findings suggest that SC was being degraded by serine proteases expressed by CALU-3 cells. Intact SC expressed by CALU-3 cells was detected using the Western blotting methods described in this thesis however it appeared that degraded SC was not detected. Western Blot analysis using a polyclonal antibody to SC showed that when SC was incubated with conditioned medium a lower molecular weight fragment of SC was detected at 69 kDa in comparison to 80 kDa band that was detected in the standard preparation of SC. Polyclonal anti-SC was derived from many different B cell lines in response to the SC and as

such each Ig molecule recognises a different epitope, which enabled the detection of degraded SC. It was also shown that when SC was incubated with PIC in addition to conditioned medium, the SC detected was at its expected molecular weight of 80 kDa. These findings indicated that there were proteases that cleave SC in conditioned medium as it was apparent that intact SC had been cleaved, resulting in this lower molecular weight fragment. It is possible that the suggested protease activity was due to either membrane bound trypsin or HAT as the molecular weight of the SC fragment detected in SC that was pre incubated with exogenous trypsin was also 69 kDa. This could imply that when SC was incubated with either conditioned medium or trypsin the SC was cleaved at the same position of its amino acid sequence. It is also interesting to note that Pilette *et al.*, (2003) detected SC at a molecular weight of 70 kDa following cleavage of SC with the serine protease, neutrophil elastase *in vitro*. Pilette *et al.*, (2003), also demonstrated that serine proteases cleave SC from SIgA; therefore it is possible that membrane bound trypsin or HAT would do the same. Even though the cleavage of SC from SIgA is not relevant in the cell culture system presented in this thesis, *in vivo,* increased cleavage of SC from SIgA could result in an increase in the release of free SC. Marshall *et al.,* (2004), found that in the sputa of CF subjects, SC was detected using Western blotting at molecular weights ranging from 62 kDa to 500 Da, which indicated that the SC was degraded. It is known that there are high levels of the neutrophil chemoattractant IL-8 in the lungs of CF patients Dean *et al.,* (1993). Since Pilette *et al.*, (2003) showed that SC is cleaved by neutrophil elastase this could provide an explanation as to why the SC in CF subject's sputa was degraded. Interestingly it was shown that this degraded SC bound significantly less IL-8 than the SC from normal subjects. IL-8 is important in inflammatory mediation as it is a neutrophil chemoattractant. Therefore the fact that degraded SC in CF subjects sputa does not bind IL-8 as efficiently as SC from normal subjects could provide an explanation as to why there is chronic inflammation seen in the lungs of CF patients.

From the work presented in this thesis it has been shown that SC expression is up regulated *in vitro* following stimulation with LPS or LTA and that SC is readily cleaved by serine proteases. The fact that LPS and LTA stimulate the release of pro inflammatory cytokines *in vivo* and that LPS and LTA have been shown here to up regulate SC expression suggests that there is a link between the two. The regulation of the expression, form and function of SC following bacterial stimulation now requires further investigation, as does the function of degraded SC. Further investigation of SC will provide a more comprehensive insight into its proposed anti-inflammatory and anti-infective properties which could a basis for the development of novel anti infective and anti inflammatory drugs.

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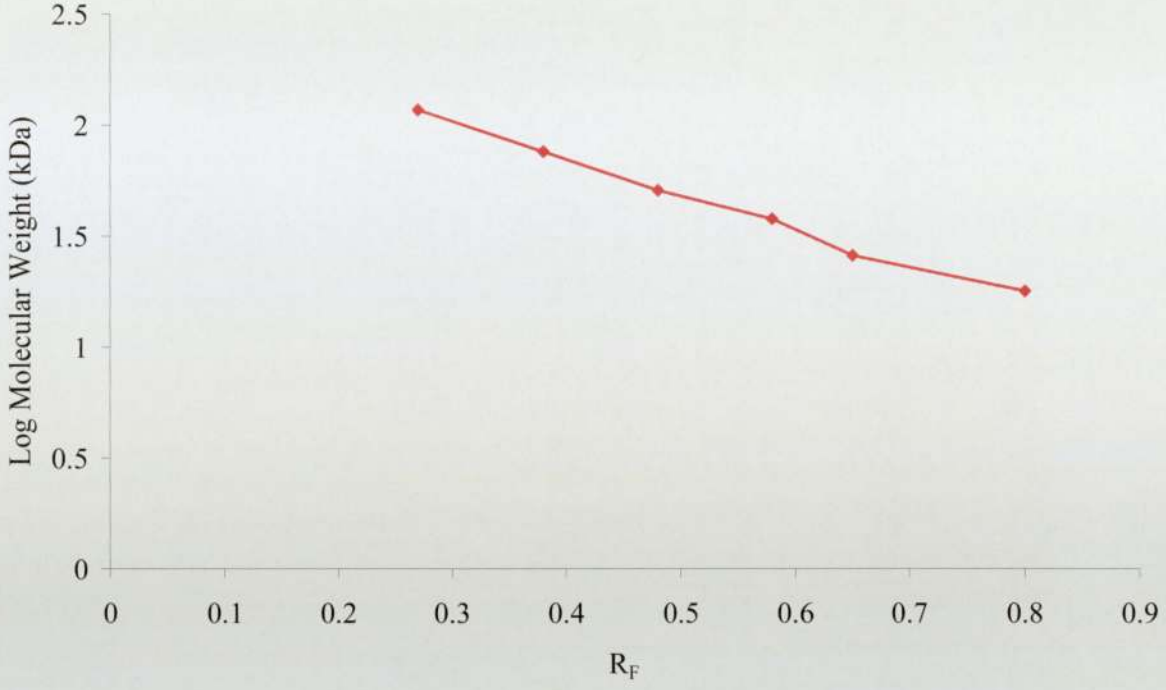
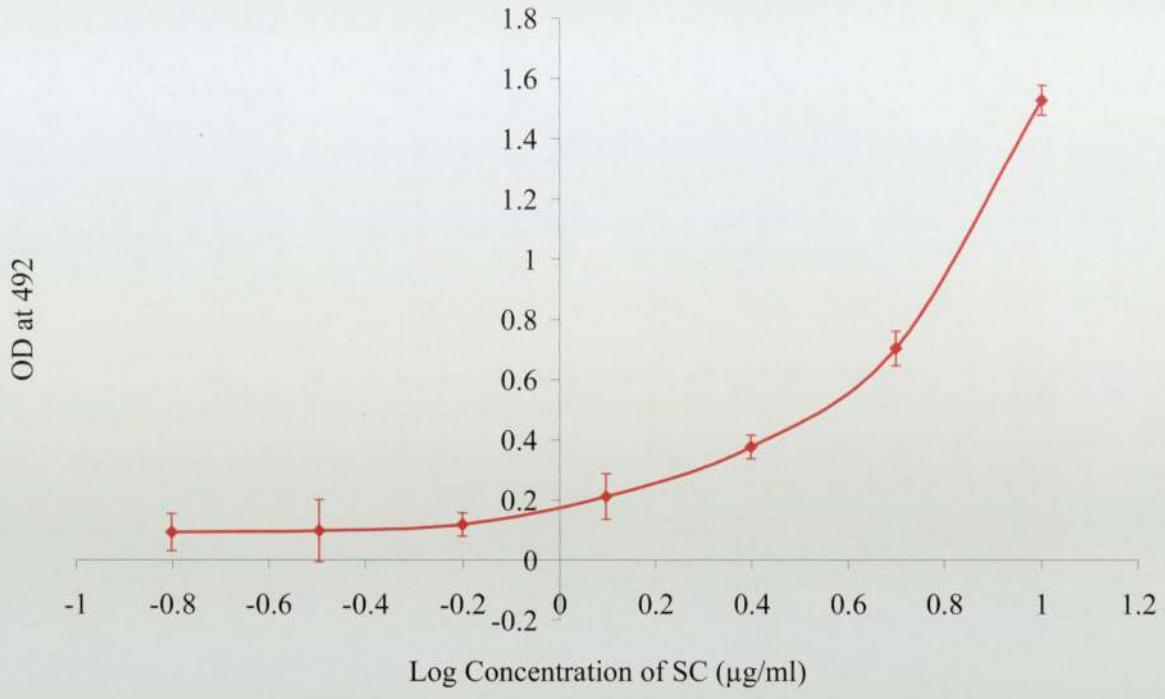
Appendix 1



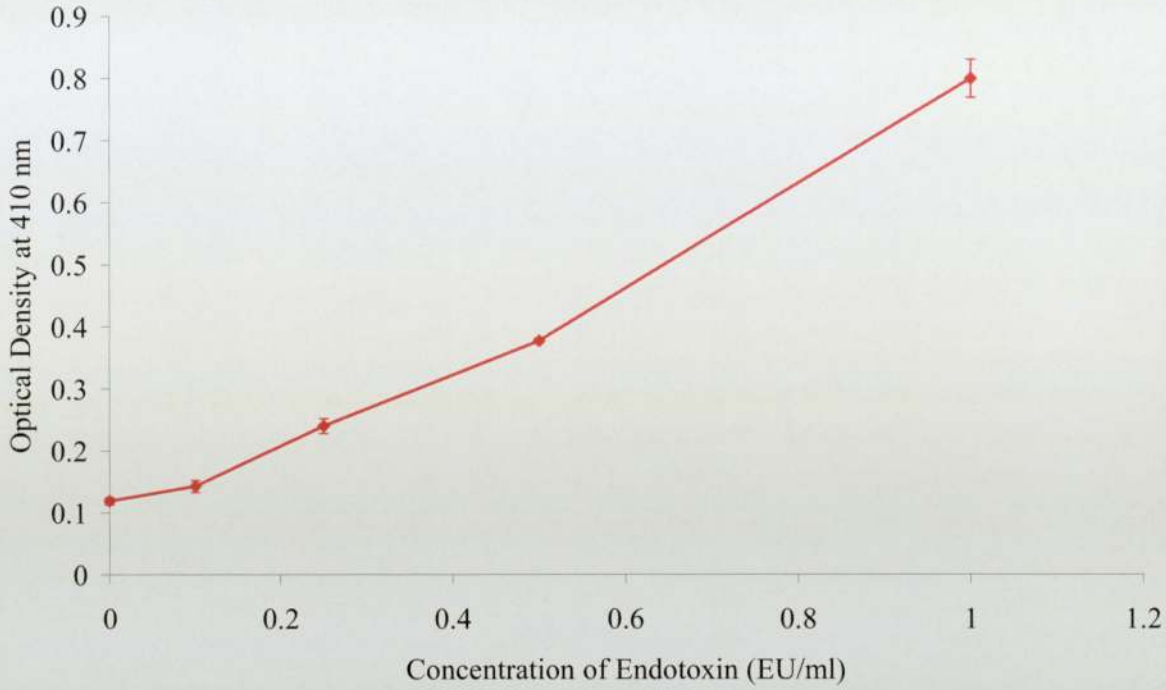
The R_F value in relation to the molecular weight of standard molecular weight markers.

Appendix 2



The mean OD measurements in relation to the concentration of SC as measured by an ELISA using monoclonal anti-SC to capture and polyclonal anti-SC to detect. This figure is representative of 16 independent experiments.

Appendix 3



The mean OD measurements in relation to the concentration of endotoxin as measured by the LAL assay. This figure is representative of 1 experiment with 3 replicates.