Studies on the role of the pyrogenic staphylococcal toxins in sudden infant death syndrome

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Declaration

All of the work presented in this thesis was carried out by, or under the direct supervision of the author.

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Contents

Abbreviations

Abstract 1

Chapter 1 Introduction

1.1 Historical background 4

1.2 Diagnosis and definition 5

1.3 Pathological findings in SIDS victims at autopsy 6

1.4 Risk factors associated with SIDS

1.4.1 Smoking 9

1.4.2 Young maternal age 9

1.4.3 Gender 10

1.4.4 Prone sleeping position 10

1.4.5 Socio-economic background 11

1.4.6 Low birth weight 11

1.4.7 Excess heating 12

1.5 Current SIDS hypotheses

1.5.1 Prenatal/developmental

1.5.1.1 Metabolic deficiencies 14

1.5.1.2 Developmental anomalies of the lungs 15

1.5.2 Perinatal

1.5.2.1 Temperature regulation and heat stress 15

1.5.2.2 Delayed development of the "adult-like" temperature pattern 16

1.6 Similarities between SIDS and infections of the respiratory tract 17

1.6.1 Evidence from pathological observations 18

1.6.2 Epidemiological evidence for infection in SIDS 20

1.7 Secretor status and the Lewis blood group antigens

1.7.1 Genetic control of secretor status 22

1.7.2 Biochemistry of the Lewis system 22

1.8 Micro-organisms associated with SIDS

1.8.1 Viruses 26

1.8.2 Bacteria 27

1.8.2.1 Factors enhancing frequency and density of colonisation of infants by pathogenic bacteria 30

1.9 The possible role of bacterial toxins in sudden infant death syndrome 33

1.9.1 Synergy between infectious agents

1.9.1.1 Synergy between bacterial toxins and viruses 37

1.9.1.2 Synergy between pyrogenic toxins and endotoxin 38

1.9.1.3 Synergy between pyrogenic toxins 40

1.9.1.4 Synergy between products of cigarette smoke and bacterial toxins 40

1.9.2 The staphylococcal enterotoxins as superantigens

1.9.2.1 Mode of action of superantigens 41

1.9.2.2 Structure of the staphylococcal superantigens 41

1.9.2.3 Pathological response to staphylococcal superantigens 45

1.9.3 How could common bacterial toxins cause a SIDS death? 46

1.9.3.1 A possible role for inflammatory mediators in SIDS deaths 47

1.10 Aims of this study 52

Chapter 2 General materials and methods

2.1 Toxins and anti-toxins 54

2.2 Enzyme linked immunosorbent assays (ELISA)

2.2.1 ELISA reagents

2.2.1.1 Coating buffer 56

2.2.1.2 Phosphate buffered saline (PBS) 56

2.2.1.3 Washing buffer 56

2.2.1.4 Blocking buffer 57

2.2.1.5 Phosphate citrate buffer 57

2.2.1.6 Substrate 57

2.2.1.7 Stopping solution 57

2.3 Tissue culture: culture media, supplements and cells

2.3.1 Dulbecco's modified Eagle's medium 58

2.3.2 Human peripheral blood monocytes 58

2.3.2 L-929 cells 59

2.4 Monocyte challenge assay

2.4.1 Toxin challenge 59

2.5 Nitrite assay

2.5.1 Nitrite detection method 60

2.5.2 Greiss reagent 61

2.5.3 Nitrite standard 61

2.6 Bioassay for detection of TNF-α 61

2.6.1 TNF-α standard 62

2.7 Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE)

2.7.1 SDS PAGE buffers and solutions 63

2.7.1.1 Electrode buffer 63

2.7.1.2 Separating gel buffer 63

2.7.1.3 Stacking gel buffer 64

2.7.1.4 Acrylamide stock solution 64

2.7.1.5 Separating and stacking gels 64

2.7.2 SDS PAGE method 65

2.7.3 Preparation of samples for polyacrylamide gel electrophoresis 67

2.7.3.1 Sample buffer 67

2.7.4 Coomassie blue staining of polyacrylamide gels for protein 67

2.7.4.1 Staining solution 1 68

2.7.4.2 Staining solution 2 68

2.7.4.3 Staining solution 3 68

2.7.4.4 Decolourising solution 1 68

2.7.4.5 Decolourising solution 2 69

Chapter 3 Detection of microbial surface antigens that bind Lewis[a]

3.1 Introduction 70

3.2 Materials and methods

3.2.1 Monoclonal antibodies 73

3.2.2 Determination of protein concentration 74

3.2.3 Preparation of immunogen 74

3.2.4 Immunisation procedure 76

3.2.5 Detection of anti-idiotypic antibodies 76

3.2.6 Purification of the anti-idiotypic antibody against anti-Lewis[a] 77

3.2.7 Separation of the anti-idiotypic antibody from non-immunoglobulin material on protein A Sepharose beads 79

3.2.8 Agglutination assay to detect the anti-idiotypic antibody on protein A Sepharose beads 79

3.2.9 Co-agglutination assay 80

3.2.10 Micro-organisms 80

3.3 Results

3.3.1 Detection of the anti-idiotypic antibody in immune mouse sera 82

3.3.2 Specificity of the protein A Sepharose reagent 83

3.3.3 Co-agglutination assay using the anti-idiotypic reagent 86

3.3.3.1 *S. aureus* 86

3.3.3.2 *N. gonorrhoeae* 86

3.3.3.3 *N. meningitidis* 88

3.3.3.4 *H. influenzae* 88

3.3.3.5 *H. pylori* 88

3.3.3.6 *C. albicans* 92

3.4 Discussion 92

Chapter 4 Lewis antigen expression on human monocytes and binding of pyrogenic toxins

4.1 Introduction 98

4.2 Materials and methods

4.2.1 Toxins and antibodies 101

4.2.2 Collection of monocytes 101

4.2.3 Detection of Lewis^a on monocytes 103

4.2.4 Detection of toxins bound to monocytes 104

4.2.5 Binding of toxins to secretor and non-secretor monocytes 105

4.2.6 Binding inhibition assay 105

4.2.7 Monocyte challenge assay 106

4.2.8 The effect of pre-treatment with the Lewis antigens on TSST-1 induced nitric oxide production 107

4.2.9 Assay for nitric oxide production 107

4.2.10 Flow cytometry 108

4.2.11 Statistical methods 108

4.3 Results

4.3.1 Detection of Lewis^a on human monocytes 109

4.3.2 Binding of staphylococcal toxins to human monocytes 109

4.3.3 Binding of SEB to secretor and non-secretor monocytes 113

4.3.4 Inhibition of toxin binding by antibodies to the Lewis antigens 116

4.3.5 Production of nitric oxide in response to the staphylococcal toxins 116

4.3.6 Effect of Lewis antigens on induction of nitric oxide by TSST-1 122

4.4 Discussion

4.4.1 Utilisation of the Lewis antigens as binding sites by the staphylococcal enterotoxins 122

4.4.2 Induction of nitric oxide from monocytes by pyrogenic toxins 123

4.4.3 Effect of the Lewis antigens on induction of nitric oxide by TSST-1 124

Chapter 5 Antigenic cross-reactivity among bacterial toxins and the protective effect of infant immunisation

5.1 Introduction 126

5.2 Materials and methods

5.2.1 ELISA reagents 128

5.2.2 Direct ELISA for detection of toxins 128

5.2.3 Sandwich ELISA for detection of toxins 129

5.2.4 Polyacrylamide gel electrophoresis 130

5.2.5 Direct ELISA for cross-reactivity among toxins 131

5.2.6 Detection of cross-reactivity among toxins by the sandwich ELISA system 132

5.2.7 Production of rabbit anti-DPT serum 133

5.2.8 Detection of antibodies to DPT by ELISA 133

5.2.9 Whole cell ELISA with *B. pertussis* 134

5.2.10 Binding of *B. pertussis* typing serum to PT and the staphylococcal toxins 135

5.2.11 Neutralisation of toxin bioactivity using antibodies to TSST-1 and the DPT vaccine 136

5.2.12 Statistical methods 137

5.3 Results

5.3.1 Assessment of toxin purity 137

5.3.2 Binding of antisera to toxins assessed by ELISA 138

5.3.3 Sandwich ELISA 141

5.3.4 Binding of anti-DPT antiserum to the DPT vaccine and the staphylococcal toxins 141

5.3.5 Binding of staphylococcal anti-toxins to *B. pertussis* 143

5.3.6 Binding of the *B. pertussis* typing serum to PT and the staphylococcal toxins 143

5.3.7 Neutralisation of the staphylococcal enterotoxins with anti-TSST-1 147

5.3.8 Neutralisation of the staphylococcal enterotoxins with anti-DPT 147

5.3.9 Age distribution of SIDS infants following changes in the DPT immunisation schedule 148

5.4 Discussion

5.4.1 Cross-reactivity between toxins 153

5.4.2 Comparison of ELISA techniques 154

5.4.3 Induction of cross-reactive antibodies by DPT immunisation 155

5.4.4 Neutralisation of staphylococcal toxin bioactivity by antisera to TSST-1 and the DPT vaccine 156

5.4.5 Age distribution of SIDS cases following changes to the DPT immunisation schedule 157

Chapter 6 Synergy between bacterial toxins in induction of inflammatory response from human monocytes

6.1 Introduction 159

6.2 Materials and methods

6.2.1 Monocyte purification 162

6.2.2 Monocyte challenge assay 162

6.2.3 Detection of inflammatory mediators 163

6.3 Results

6.3.1 The effect of endotoxin concentration on nitric oxide production 163

6.3.2 Induction of nitric oxide production by combinations of toxins 164

6.3.3 The effect of time on TNF-α production 165

6.3.4 The effect of toxin concentration on TNF-α production 170

6.3.5 Induction of TNF-α production by combinations of toxins 172

6.4 Discussion 175

Chapter 7 The effects of cigarette smoke and viral infection on inflammatory responses of human monocytes

7.1 Introduction 179

7.2 Materials and methods

7.2.1 Production of cigarette smoke extract 182

7.2.2 Dose response to CSE 184

7.2.3 Preparation of RSV infected monocytes 184

7.2.4 Dose response of RSV infected and non-infected human peripheral blood monocytes to CSE and TSST-1 186

7.2.5 Determination of TNF-α content 187

7.2.6 Determination of nitric oxide content 187

7.2.7 Statistical methods 187

7.3 Results

7.3.1 Nitrite production in response to CSE 188

7.3.2 Dose response to interferon-γ, nicotine and cotinine 188

7.3.3 The effect of IFN-γ, nicotine and cotinine on release of TNF-α and nitric oxide from human monocytes 191

7.3.4 The effect of CSE, RSV and/or TSST-1 on TNF-α release from human monocytes 194

7.3.5 The effect of CSE, RSV and/or TSST-1 on NO release from human monocytes 196

7.3.6 Variability of TNF-α and NO responses of individual donors 201

7.4 Discussion 206

Chapter 8 General discussion 211

8.1 Detection of microbial surface antigens that bind Lewis[a] 214

8.2 Lewis antigen expression on human monocytes and binding of pyrogenic toxins 215

8.3 Antigenic cross-reactivity among bacterial toxins and the protective effect of infant immunisation 216

8.4 Synergy between bacterial toxins in induction of inflammatory response from human monocytes 218

8.5 The effects of cigarette smoke and viral infection on inflammatory responses of human monocytes 220

8.6 Possible applications of this study 222

8.7 Future work 224

References 226

Appendix

Abbreviations

BHI	brain heart infusion broth
BSA	bovine serum albumin
CSE	cigarette smoke infusion
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
FITC	flourescein isothiocyanate
HRP	horseradish peroxidase
Ig	immunoglobulin
IL	interleukin

IFN-γ	interferon gamma
KLH	keyhole limpet haemocyanin
Le^a	$Lewis^a$
Le^b	$Lewis^b$
Le^x	$Lewis^x$, CD15
LPS	lipopolysaccharide
NO	nitric oxide
OPD	ortho-phenylenediamine dihydrochloride
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
RSV	respiratory syncytial virus

SDS	sodium dodecyl sulphate
SE	staphylococcal enterotoxin
SIDS	sudden infant death syndrome
TNF-α	tumour necrosis factor alpha
Tris	Tris(hydroxymethyl)aminomethane
TSST-1	toxic shock syndrome toxin-1

Abstract

Sudden infant death syndrome is the largest single cause of death of infants between 1 week and 12 months of age in this country, and although public awareness of the risk factors involved has increased over the last few years, the incidence of SIDS still stands at approximately 0.7 per 1000 live births in Scotland. The aim of this study was to examine how infection might play a role in SIDS in relation to developmental and environmental risk factors identified in epidemiological studies by examining the binding of bacterial toxins to human cells and their effects, singly and in combination on induction of inflammatory mediators.

There is a correlation between the incidence of SIDS, isolation of *Staphylococcus aureus* and expression of the Lewis^a blood group antigen in the 2 to 4 month age range. Lewis^a has previously been shown to act as a surface receptor for certain bacteria on human epithelial cells, the first stage of this study was to develop a screening method to detect adhesins that bind Lewis^a on toxigenic strains of *S. aureus* and other bacteria isolated from SIDS infants. Additional experiments indicated that some of the superantigenic toxins of *S. aureus* utilise this antigen as a receptor on monocytes and are capable of stimulating the production of inflammatory mediators from these cells.

The pertussis toxin of *B. pertussis* is capable of binding to Lewis^{a} and Lewis^{x} and it has been suggested that asymptomatic whooping cough is one cause of SIDS. Changes in the age range of SIDS infants were observed between 1988 and 1994, after the DPT immunisation schedule was changed from 3 months to 2 months of age in October 1990. The protective effect of DPT immunisation suggested in several large epidemiological studies was examined with reference to antigenic cross reactivity between the DPT vaccine and the staphylococcal toxins. Results obtained in enzyme linked immunosorbent assays (ELISA) indicated that antibodies cross-reactive with the staphylococcal toxins were produced by rabbits in response to DPT immunisation. Assays for nitric oxide production by human monocytes in response to the toxins indicated that the antibodies were able to neutralise partially some of the inflammatory activities of the toxins.

In animal models the combined effects of nicotine, endotoxin and virus infection enhanced the lethal effects of the staphylococcal toxins. These agents, separately and in combination, were examined for their ability to induce TNF-α and nitric oxide from human monocytes. Synergy between toxins in induction of these mediators was shown to occur with several toxin combinations, particularly SEB plus TSST-1 or endotoxin.

Incubation of human monocytes infected with respiratory syncytial virus (RSV) or with a water soluble cigarette smoke extract (CSE) was found to alter the

inflammatory responses of these cells to TSST-1, generally by induction of increased TNF-α levels and decrease of the nitric oxide response.

These studies help to explain the effects of some of the developmental and environmental risk factors associated with SIDS which could contribute to the series of events that lead to sudden death in some infants.

Chapter 1

Introduction

1.1 Historical background.

The sudden and unexpected death of an infant is not a new phenomenon, though from the earliest available reference in the Bible, dating to approximately 500 BC until the 19th century such deaths appear to have been attributed solely to overlaying. Asphyxiation resulting from the obstruction of breathing by bed clothes or the infants' carer was a feasible hypothesis given that the incidence of bed sharing was then far higher than that which occurs in present day western society. In the early 1830s this view was superseded by another single cause hypothesis, that of status thymo-lymphaticus [reviewed by Valdes-Dapena, 1995] or airway obstruction caused by a pathologically enlarged thymus causing compression of the trachea. This hypothesis was also widely accepted and not disputed until the mid 1940s when the first steps to examine the problem of cot death systematically were made.

Given the scarcity of evidence available, the hypotheses put forward to explain cot deaths have been remarkably varied and many have been found to be inaccurate. Theories as diverse as allergic reaction to aspirated cow's milk [Parish *et al.*, 1960] and failures of the immune system (hypogammaglobulinaemia) [Spain *et al.*, 1954] have been put forward and have generally been disproved. Several of the current hypotheses are discussed below (1.5). During the 1960s and 1970s public awareness of the problem of cot death increased and there was a corresponding increase in cot death research, both of its pathology and its epidemiology. The epidemiological data gained from studies carried out in several different countries identified many of the predisposing factors, such as socio-economic class, birth weight and race which guide current research on the problem of sudden infant death syndrome (SIDS).

1.2 Diagnosis and definition.

SIDS is the largest cause of death in infants in industrialised countries between the ages of 1 week and 12 months, accounting for approximately 50% of the total number of deaths in this age range and having an incidence of approximately 0.7 per 1000 live births in Scotland during 1995 [Brooke *et al.*, 1997]. The current working definition of SIDS as proposed by Beckwith in 1970 is:

> "*The sudden death of an infant or young child which is unexpected by history, and in a thorough post-mortem examination fails to demonstrate an adequate cause of death.*"

This definition, although there are currently calls to modify it, demonstrates our lack of understanding of why certain infants die within the first months of life. Entering a verdict of SIDS in a post-mortem report is not a diagnosis but rather the only conclusion left after all other possible explanations for the infants' death have been found inadequate. This does not, however mean that all SIDS infants appear totally normal at autopsy. Several pathological findings are commonly associated with the syndrome.

1.3 Pathological findings in SIDS victims at autopsy.

Petechial haemorrhages, small areas of blood in the mucous membranes, are commonly present throughout the lungs, along the coronary arteries and the thoracic lobe of the thymus. Occasionally they are present beneath the epicardium (Table 1.1).

Diffuse interstitial oedema in the lungs is one of the most common findings in SIDS victims at autopsy. Pathological changes in the upper respiratory tract are also frequently observed, including inflammatory cell infiltration in the nasopharynx,

larynx and trachea and thickening of the basement membrane of the epithelium in the larynx and trachea [Keeling, 19??].

Pathological changes in the brain are also noted at autopsy, including brain stem astrocytic gliosis involving the nucleus ambiguus and the dorsal motor nucleus of the vagus [Takashima *et al.*, 1978] which may be the result of infection or a hypoxic episode but could also have produced hypoxia by affecting the normal regulation of breathing. Detachment of ependymal cells from the ventricular lining of the brain is occasionally observed. This too can be indicative of hypoxia/ischaemia or a minor infection [Keeling, 19??].

Table 1.1 Typical postmortem findings in SIDS [Berry and Keeling, 1989].

External:
Well nourished, normally developed baby
Frothy blood-tinged fluid around the nose (50%)
Hypostatic staining often anterior indicating face-down position
Cyanosis of the lips and nailbeds
Internal:
"Large" thymus with petechiae (80%)
Petechiae beneath visceral pleura
Epicardial petechiae
Full expansion of the lungs
Liquid heart blood (80%)
Prominent lymph nodes and Peyer's patches
Empty bladder (50%)
Microscopic:
Pulmonary congestion and oedema
Mild inflammation of the upper respiratory tract
Focal fibrinoid necrosis of the vocal cords
Persistent haemopoiesis in the liver

1.4 Risk factors associated with SIDS.

Risk factors associated with SIDS are summarised in table 1.2.

1.4.1 Smoking.

Maternal smoking during pregnancy has long been accepted as a modifiable risk factor in SIDS with several studies showing a dose-dependent relationship between smoking and increased risk of SIDS. An approximate sevenfold increase in risk was noted in one study when the mother smoked more than twenty cigarettes per day [Murphy *et al.*, 1982]. Other studies have found similarly high increases in risk which remain significant after controlling for potentially confounding factors such as maternal age and socio-economic class [Poets *et al.*, 1995]. It has, however, only been recently that postnatal exposure to cigarette smoke from either the mother or other family members has been addressed as a risk factor in its own right [Mitchell *et al.*, 1993; Schoendorf and Kiely, 1992; Blair *et al.*, 1996; Brooke *et al.*, 1997].

1.4.2 Young maternal age.

SIDS deaths are more common in infants of mothers under the age of 25 and the risk increases with increasing birth order. The combination of these two factors increases the risk of a SIDS death by a far higher factor than would be expected if this were

merely an additive effect, such that a second born child of a mother aged under 20 would be seven times more likely to die of SIDS than that born to a mother aged 25 or over [Gibson, 1992].

1.4.3 Gender.

The male/female ratio of SIDS deaths is approximately 1.6:1. This disparity is not seen in non-SIDS infant deaths and may be due to later development of physiological control mechanisms in males [Gibson, 1992] or an increased susceptibility of male infants to respiratory tract infections [Ruuskanen and Ogra, 1993].

1.4.4 Prone sleeping position.

The prone sleeping position is acknowledged as a strong risk factor for SIDS which has resulted in educational campaigns to discourage the placing of infants in this position in many countries worldwide with a corresponding decrease in SIDS incidence [Willinger *et al.*,1996; Bacon *et al.*, 1996; Øyen *et al.*, 1996]. It is, however, still common in SIDS deaths [Bacon *et al.*, 1996; Brooke *et al.*, 1997].

1.4.5 Socio-economic background.

The confidential enquiry into stillbirths and deaths in infancy (CESDI) (England) case-control study (SIDS n=195, controls n=780) found the risk of SIDS to be greater in families of a lower socio-economic background. Factors studied included unemployment, housing conditions and parental education [Bensley *et al.*, 1996]. These findings are consistent with those of Brooke *et al.* [1997] (Scotland), Taylor and Sanderson, [1995] (U.S.A) and Daltveit *et al.* [1996] (Scandinavia), however, it is difficult to isolate any one of the socio-economic markers as a risk factor for SIDS as the relationship between them is so complex.

1.4.6 Low birth weight.

Analysis of suspected risk factors in several long-term epidemiological studies has provided conflicting results which appear to depend to a large extent on country, ethnic group and the method of statistical analysis used in the study. By univariate analysis, low birth weight (<2,500 g) was identified as a significant risk factor in the Scottish study of Brooke *et al.*, [1997] (SIDS n=147, controls n=276) and in an American study by Li and Daling, [1991] (SIDS n=916, controls n=3,704). Subsequent multivariate analysis of the Scottish study, correcting for gestational age, showed this risk factor to be non-significant. Although multivariate analysis of the

American data was not performed, comparison of the various ethnic groups represented in the study showed that low birth weight was only a significant risk factor among whites, American Indians and blacks, not among Asians or Hispanics. Gestational age was not controlled for in this study, however, as a number of ethnic groups were studied, low birth weight was defined as being below the population mean minus 1.96 standard deviations.

1.4.7 Excess heating.

The studies of Fleming *et al.*, [1990] and Brooke *et al.*, [1997] found that a combined thermal rating (tog value) of bedding and clothing greater than 10 was a significant risk factor by univariate analysis. Over-heating is thought to play a role in enhancing the effect of infection to precipitate a SIDS death in certain infants [Gilbert *et al.*, 1992].

Table 1.2 Developmental and environmental risk factors for SIDS.

Risk factor	**Reference**
Peak incidence in 2-4 month age range	Gibson, 1992 Brooke *et al.*, 1997
Mild respiratory tract infection	Williams *et al.*, 1984 Gilbert *et al.*, 1992
Exposure to cigarette smoke	Mitchell, 1996 Blair *et al.*, 1996 Brooke *et al.*, 1997
Prone sleeping position	Fleming *et al.*, 1990 Beal, 1995 Blair *et al.*, 1996 Brooke *et al.*, 1997
Bottle rather than breast fed	Ford *et al.*, 1993
No immunisation or late immunisation	Hofman *et al.*, 1987 Walker *et al.*, 1987

1.5 Hypotheses to explain SIDS.

1.5.1 Prenatal/developmental.

1.5.1.1 Metabolic deficiencies.

It is estimated that approximately 10% of SIDS cases result from a metabolic deficiency [Bonham and Downing, 1992], but this figure is uncertain as such deficiencies are not commonly tested for on post-mortem derived material. Studies into the role of metabolic deficiencies in SIDS have concentrated on two main areas: defects in glucose homeostasis and defects in fatty acid metabolism.

Abnormal development of the enzymes involved in gluconeogenesis or glycogen metabolism will result in functional immaturity of the mechanisms which control glucose homeostasis thus rendering the infant susceptible to potentially fatal episodes of hypoglycaemia. The likelihood of such episodes occurring can be greatly exacerbated by environmental factors such as low calorie intake and minor clinical or sub-clinical infections [Burchell *et al.*, 1992]. Failure of mitochondrial fatty acid oxidation limits the amount of ketone bodies available to the brain and muscles as an alternative source of energy in the event of depletion of glycogen stores, again resulting in potentially fatal hypoglycaemic episodes.

1.5.1.2 Developmental anomalies of the lungs.

It has been hypothesised that a percentage of SIDS deaths may be caused by failure of lung development *in utero*, thus reducing the surface area available for gaseous exchange and increasing the risk of hypoxic episodes. A recent study [Beech *et al.*, 1996] found that the total number of terminal ducts was significantly reduced in low birth weight infants (both controls and SIDS cases) and in normal birth weight SIDS cases. The gas exchange surface density was also found to be significantly reduced in normal and low birth weight SIDS cases compared to normal birth weight controls.

1.5.2 Perinatal.

1.5.2.1 Temperature regulation and heat stress.

An increase in body heat and metabolic rate in response to bacterial or viral infection might lead to a fatal event if heat loss is prevented by excessive amount of clothing. As the brain accounts for 40% of the total oxygen usage in young infants [Gilbert *et al.*, 1992], the head is a major site of heat production. It is also the most effective area of heat loss [Nelson *et al.*, 1989; Wailoo *et al.*, 1989]. Any interference with heat loss from the head, *e.g.* bed covers or a hat, could lead to heat stress in the brain even without a concomitant increase in core temperature [Gilbert *et al.*, 1992];

however, the combination of infection and overwrapping is only associated with risk of death in infants over 10 weeks old, possibly due to these infants being less vulnerable, and therefore, requiring more risk factors to cause a fatality. Alternatively, the higher levels of body fat and increased metabolic rate to surface area ratio render these infants more susceptible to thermal stress than younger infants in whom other temperature regulation mechanisms may be important [Gilbert *et al.*, 1992]. Such failures of temperature regulation have been associated with an increase in apnoeic episodes [Ford *et al.*, 1996] as well as enhancement of the inflammatory response to infection.

1.5.2.2 Delayed development of the "adult-like" temperature pattern.

Infants' night time temperature patterns switch to an "adult-like" pattern at about 11 weeks of age (range=7 to 22 weeks), and elevation of night-time temperature can occur in response to immunisation and minor infection [Peterson *et al.*, 1996]. Other physiological changes are associated with the change in night-time temperature pattern, the decrease in weight of the adrenal glands in the first 2 months of life is associated with decreased plasma levels of cortisol [Wittekind *et al.*, 1993]. Circadian variations in cortisol secretion have been observed in adults with the lowest secretion rates occurring in the early hours of the morning when the majority of SIDS deaths are thought to occur. This drop in night-time cortisol secretion occurs

in the week following the switch to the adult-like body temperature rhythm [Peterson and Wailoo, 1994]. As cortisol and the other glucocorticoids play a major role in the control of inflammatory responses [Reichlin, 1993], reduced levels of these hormones during a period when both maternal and infant antibody levels are insufficient to neutralise infectious agents and toxins could result in a period of susceptibility to the detrimental effects of inflammatory mediators. If the switch in temperature pattern occurs after the infant's antibody response has developed, the likelihood of sudden death due to an uncontrolled inflammatory response will be reduced. This view is supported by evidence that Asian infants, in whom the switch in temperature pattern occurs significantly later than in white infants have a lower incidence of SIDS [Peterson and Wailoo, 1992].

1.6 Similarities between SIDS and bacterial infections of the respiratory tract.

Research into the cause of sudden infant death syndrome over the last thirty years has frequently examined the involvement of infectious agents. The literature contains many reports of the isolation of various toxigenic bacteria and viruses from SIDS victims at autopsy, and several hypotheses have been put forward as to how these infectious agents could have caused death. As the only sources of this data are post mortem reports and epidemiological studies, there is no direct evidence that these

microorganisms actually caused the death of the infant. The diagnosis of SIDS precludes any findings of fatal septicaemia or viraemia, and it is the lack of a set of recognisable signs at autopsy attributable to a specific pathology which presents one of the major problems in SIDS research, "where to start"? This question is best addressed by comparison of SIDS autopsy findings and epidemiological data with those of more easily recognisable pathologies.

1.6.1 Evidence from pathological observations.

The role of micro-organisms in SIDS deaths is unclear, pathological changes attributed to bacteria and viruses observed at autopsy cannot, under the current definition, appear to be sufficient to cause the death of a previously healthy infant. There is, however, indirect evidence that micro-organisms may be involved as minor inflammation of the respiratory tract is observed in about 60% of SIDS cases [Berry, 1992]. These pathological findings are consistent and indicate activation of the infant's inflammatory or immune responses (Table 1.3).

Table 1.3 Evidence for inflammatory or immune responses in SIDS.

Nervous system:	Brainstem gliosis	Naeye, 1976
	IFN-α in brain	Howatson *et al.*, 1992
	↑ IL-6 in spinal fluid	Vege *et al.*, 1996
Respiratory tract:	↑ IgM cells in trachea	Stoltenberg *et al.*, 1992
	Inflammatory infiltrate	Emery and Dinsdale, 1974
	Mast cell degranulation	Harrison *et al.*, 1992
	Pulmonary oedema	Herbert and Andrews, 1979
	Petechiae	Beckwith, 1988
Gut:	↑ IgA cells in duodenum	Stoltenberg *et al.*, 1992
Blood:	↓Maternal IgG to LPS core	Oppenheim *et al.*, 1994
	↑ Infant IgM to LPS core	Oppenheim *et al.*, 1994
	↑ Serum mast cell tryptase	Holgate *et al.*, 1994
	↑ Acute phase reactants	Kilpatrick *et al.*, 1997
	Cross-linked fibrin degradation products	Goldwater *et al.*, 1990

1.6.2 Epidemiological evidence for infection in SIDS.

The factors associated with susceptibility to infection, particularly respiratory tract infection, are similar to those associated with SIDS [Gibson, 1992]. During the 2 to 4 month age range in which the majority of SIDS cases occur there is a window of susceptibility to infection caused by the decrease in levels of maternal transplacentally acquired immunoglobulins occurring prior to the infants' development of active immunity to a variety of infectious agents.

The seasonality of SIDS, though questioned recently for certain geographical locations [Fleming *et al.*, 1996; Kiberd, 1996], still appears to hold in most countries with the majority of SIDS cases occurring during the winter months when there is a higher incidence of respiratory tract infections [Williams *et al.*, 1984].

The incidences of both SIDS [Scragg *et al.* 1993] and respiratory tract infections [Bonham *et al.*, 1981; Pershagen, 1986] is higher in the infants of smokers compared with non-smokers. Other recognised risk factors for SIDS are also associated with susceptibility to infection including bottle feeding [Arnon, 1984, Ford *et al.*, 1993]; the risk of SIDS is reduced in breast fed infants which are protected to a certain extent from respiratory and gastrointestinal pathogens by components of colostrum and breast milk including serum, leucocytes and antibodies. Infants born to families from the lower socioeconomic groups have been identified as being at risk from

SIDS by several studies though this could reflect the influence of several other risk factors such as maternal smoking, bottle feeding, poorer housing and lower levels of health care also linked with lower socioeconomic class.

Susceptibility to infection and possibly to SIDS can be affected by genetic and developmental factors as well as environmental ones. Epidemiological studies have found associations between blood group and susceptibility to several diseases of both infectious and non-infectious aetiology. Epidemiological evidence of the association between the ABO blood group antigens and secretor status and infectious diseases was reviewed by Blackwell [1989a]. Individuals who are unable to secrete the glycoprotein forms of the ABO blood group antigens in body fluids (non-secretors) have been found to be more susceptible than secretors to a wide range of bacterial and fungal diseases. This increased susceptibility has been demonstrated in infectious diseases including bacterial meningitis [Blackwell *et al.*, 1986] and also auto-immune conditions such as rheumatic fever [Haverkorn and Goslings, 1969], thyrotoxicosis [Collier *et al.*, 1988] and insulin dependent diabetes mellitus [Blackwell *et al.*, 1989; Aly *et al.*, 1991; 1992a,b].

1.7 Secretor status and the Lewis blood group antigens.

1.7.1 Genetic control of secretor status.

Secretion of the ABO blood group antigens is controlled by allelic genes, *Se* and *se*, on chromosome 19 [Watkins, 1980] and is inherited as a Mendelian dominant character. An individual homozygous (*SeSe*) or heterozygous (*Sese*) for the secretor gene is termed a "secretor" whereas those homozygous for the silent allele (*sese*) are termed "non-secretors". Among the European population approximately 75 to 80% are secretors and 20 to 25% are non secretors, the ratio of secretors to non-secretors varies between different ethnic groups [Race and Sanger, 1975].

Secretor status can be determined by detection of the ABO blood group antigens in the saliva using a haemagglutination inhibition test or by determination of the Lewis blood group antigens as the secretor gene regulates expression of the Lewis phenotype [Race and Sanger, 1975].

1.7.2 Biochemistry of the Lewis system.

The product of the Lewis gene (*Le*) is an α-4/3-L-fucosyltransferase that adds an α-L-fucosyl residue to the subterminal N-acetylglucosamine of the H or Type 1

precursor chains. The Lewis^a (Le^a) determinant contains L-fucose joined at the C-4 position of the subterminal N-acetylglucosamine of a Type 1 precursor chain. The Lewis^b (Le^b) determinant has two fucosyl residues attached to adjacent sugars, one linked to the N-acetylglucosamine as in the Lewis^a determinant and the other linked to the terminal β-galactosyl residue by the fucosyl transferase coded for by the secretor gene [Watkins, 1980] (Figure 1.1).

Unlike the ABO antigens which are synthesised endogenously by erythrocytes, the Lewis blood group antigens are produced at a distant and as yet unknown site and released into the plasma as glycosphingolipids carried by high- and low-density lipoproteins [Hammar *et al.*, 1981; reviewed in Henry *et al.*, 1995] from where they are adsorbed into the cells membrane. The Lewis^a antigen (Le^a) is found predominantly on the cells of non-secretors whereas secretors primarily express the Lewis^b antigen (Le^b), although they are capable of expressing varying quantities of Lewis^a [Issit, 1985]. During early neonatal development, however, the fucosyl transferase coded for by the Lewis gene reaches functional maturity faster than that of the secretor gene. This allows the enzyme coded for by the Lewis gene to add fucose to the subterminal sugar of the Type 1 precursor chain first thus rendering the substrate unavailable to the enzyme coded for by the secretor gene. This results in the majority of infants in the 2 to 4 month age range expressing large quantities of the Lewis^a antigen which will drop to adult levels by two years of age (Figure 1.2).

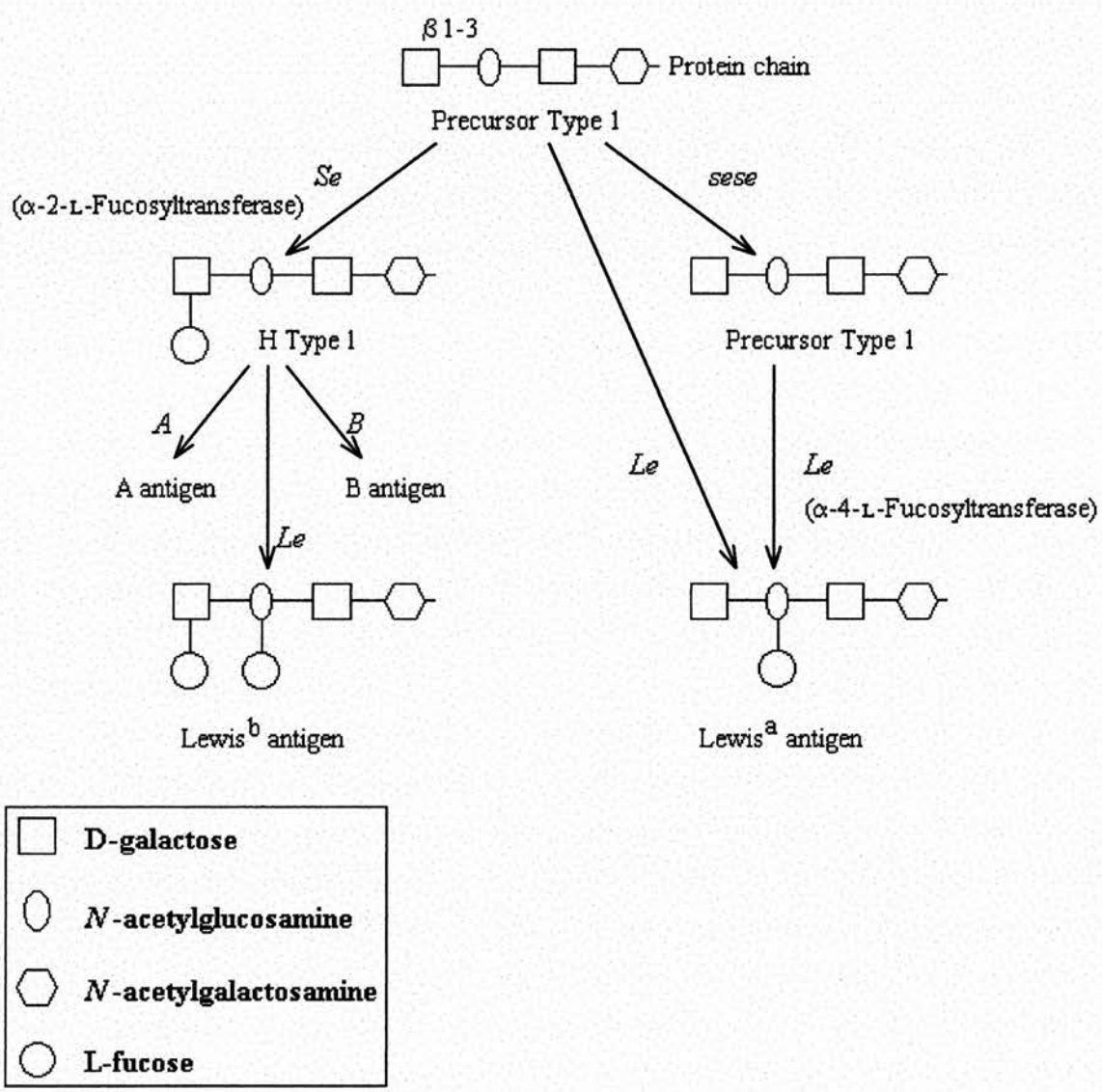


Figure 1.1 Biosynthesis of the Lewis antigens.

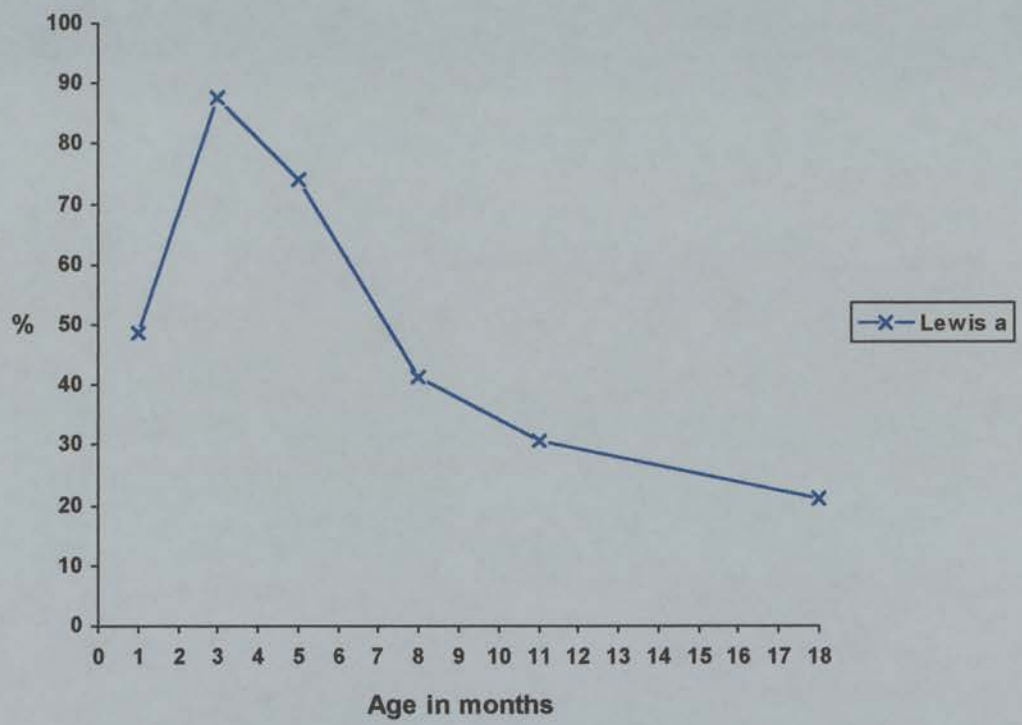


Figure 1.2 Expression of the Lewis[a] antigen in the first 18 months of life.

1.8 Micro-organisms associated with SIDS.

1.8.1 Viruses.

The epidemiological evidence for involvement of respiratory viruses in SIDS cases is inconsistent [Carpenter and Gardner, 1990; Uren *et al.*, 1980]. No studies have shown a systemic viraemia or elevated levels of interferon-γ, nor has a single predominant virus or family of viruses been identified in studies of SIDS victims [Ford *et al.*, 1990; Nelson *et al.*, 1975; Fleming, 1992]. Those isolated from SIDS cases include influenza [Zink *et al.*, 1987], respiratory syncytial virus (RSV) [Williams *et al.*, 1984], rhinovirus [Las Heras and Swanson, 1983], rotavirus [Yolken and Murphy, 1982], and adenoviruses [An *et al.*, 1993]. Given the technical difficulties of isolating viruses from post-mortem samples the extent of viral involvement in SIDS may have been underestimated in the past [Flemming, 1992]; however, a more recent survey found no differences in isolation of viruses from SIDS cases and controls matched for age, time of year and geographic area [Gilbert *et al.*, 1992].

One study has provided a possible link between the mild respiratory tract infection commonly noted in SIDS victims the week prior to death [Stanton *et al.*, 1978] and the actual fatal event. Bruhn *et al.* [1977] reported an association between RSV infection and prolonged sleep apnoea.

1.8.2 Bacteria.

There are two main ways in which bacteria can cause acute disease:

(1) Invasion of areas of the body which are normally sterile e.g. meningitis;

(2) Production of toxins by bacteria on mucosal surfaces e.g. diphtheria and pertussis.

Of these, the invasive bacteria can be immediately ruled out as a cause of SIDS as they leave clear indications of their presence. Given that the pathological effects of endotoxin are usually associated with invasive bacterial diseases many of the studies on bacterial involvement in SIDS have concentrated on species which colonise mucosal surfaces and produce diffusible toxins which enter the bloodstream. Both enteric and respiratory bacteria have been isolated from SIDS cases (Table 1.4), but the pathology and epidemiology of SIDS has more in common with that of the respiratory bacteria than those found in the gastrointestinal tract.

Two of the toxigenic bacteria which colonise the respiratory tract have been implicated in SIDS: *Bordetella pertussis*, the causative organism of whooping cough [Nicoll and Gardner, 1988; Lindgren *et al.*, 1997], and *Staphylococcus aureus* [Morris *et al.*, 1987; Newbould *et al.*, 1989; Telford *et al.*, 1989; Malam *et al.*,

1992]. *B. pertussis* has not been isolated from a SIDS case though its potential involvement has not been ruled out given the difficulty of isolating this organism, even from infants with symptomatic whooping cough [Davis *et al.*, 1990]. Epidemiological studies on SIDS during periods of unusually high whooping cough prevalence have indicated a correlation between infection with *B. pertussis* and SIDS [Nicoll and Gardner, 1988; Lindgren *et al.*, 1997]. Toxigenic strains of *S. aureus* have been isolated from SIDS victims in several studies [Telford *et al.*, 1989; Murrell *et al.*, 1993].

Table 1.4 Toxigenic bacteria isolated from SIDS victims.

Species	Toxin	Superantigen	Reference
S. aureus	enterotoxins, TSST-1	yes	Newbould *et al.*, 1989 Malam *et al.*, 1992 Murrell *et al.*, 1993
B. pertussis	pertussis toxin endotoxin	no yes	Nicoll and Gardner, 1988 Lindgren *et al.*, 1997
Haemophilus influenzae	endotoxin	yes	Telford *et al.*, 1989 Oppenheim *et al.*, 1994
Clostridium perfringens	enterotoxin A	yes	Murrell *et al.*, 1987 Lindsay *et al.*, 1993
Streptococcus pyogenes	pyrogenic toxins A&B	yes	Morris *et al.*, 1987
E. coli	enterotoxins, verotoxins, endotoxin	? yes	Bettleheim *et al.*, 1989;1990 Oppenheim *et al.*, 1994

1.8.2.1 Factors enhancing frequency or density of colonisation of infants by pathogenic bacteria.

1. Frequency of colonisation:

The frequency of isolation of potentially pathogenic bacteria is higher in people who smoke compared with non-smokers [Blackwell *et al.*, 1990; 1992]. An increased frequency of isolation is also observed during the winter months which parallels SIDS, this may be due to the higher incidence of respiratory viral infections during this season.

2. Density of colonisation:

The density of bacterial colonisation of mucosal surfaces is an important factor in the initiation of both invasive and toxigenic bacterial diseases [Beachey, 1981]. Several risk factors enhance the ability of bacteria implicated in SIDS to bind to epithelial cells:

The Lewis^a antigen acts as an epithelial cell surface receptor for certain bacterial adhesin molecules, including those expressed by *S. aureus* [Saadi *et al.*, 1993] and *B. pertussis* [Saadi *et al.*, 1996]. The Lewis^a antigen is expressed by 80 to 90% of infants in the 2 to 4 month age range and as can be seen in figure 1.3 this peak in

expression correlates with the highest rate of isolation of *S. aureus* and incidence of SIDS. Infection of the human epithelial cell line Hep-2 with respiratory syncytial virus results in a significant increase in the binding of pertussis, staphylococci and haemophilus over uninfected Hep-2 cells [Saadi *et al.*, 1993; Saadi *et al.*, 1996; Raza *et al.*, 1993]. Epithelial cells sampled from smokers bind greater numbers of staphylococci, pertussis and *H. influenzae* than those taken from non-smokers [Saadi *et al.*, 1996; El-Ahmer *et al.*, 1994].

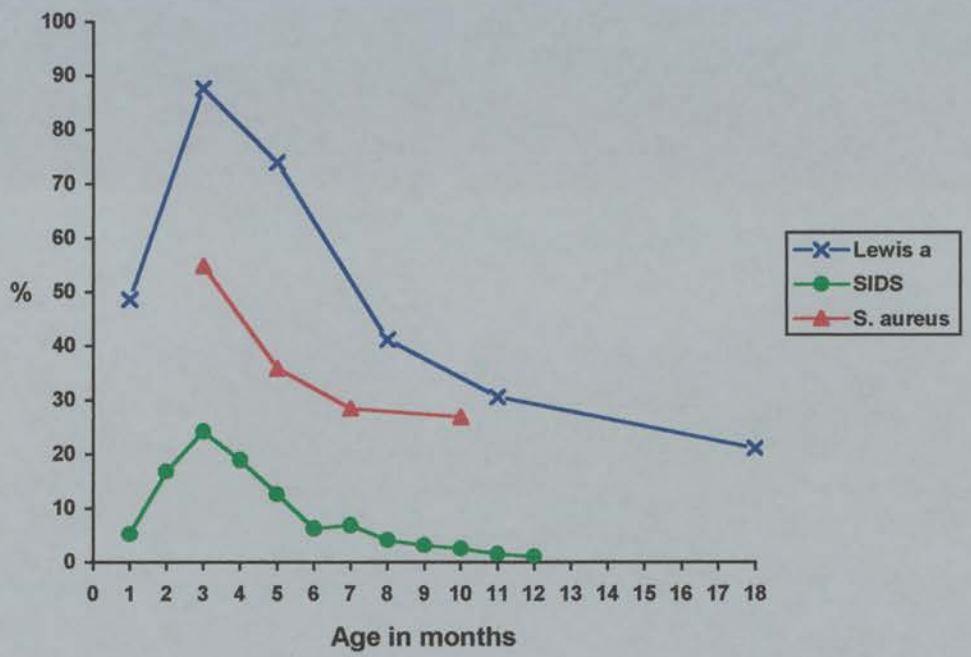


Figure 1.3 Comparison of Lewis[a] expression, SIDS incidence and isolation of *S. aureus* in Scotland by age.

1.9 The possible role of bacterial toxins in sudden infant death syndrome.

The hypothesis examined in this work is that the toxins produced by certain common bacteria can, under some conditions, cause the death of a previously healthy infant via uncontrolled production of inflammatory mediators.

The role of *S. aureus* in relation to SIDS was examined in this study as it is a toxigenic bacterium which fits closely the mathematical model of bacterial infection in SIDS proposed by Morris *et al.* [1987] which predicts that the infant population will encounter the bacteria involved in precipitating the series of events culminating in SIDS within the first 50 days of life. There is a correlation between the incidence of isolation of *S. aureus* and that of SIDS (Figure 1.3) and studies by Newbould *et al.* [1989] and Malam *et al.* [1992] have demonstrated the presence of staphylococcal enterotoxin C (SEC) and toxic shock syndrome toxin (TSST-1) in tissue samples of SIDS victims. Factors associated with SIDS such as concomitant viral infection and exposure to tobacco smoke have been shown to enhance the binding of *S. aureus* to human cells. While certain of these factors have also been shown to enhance binding of *B. pertussis*, the role of this organism in SIDS is more speculative as it has not been isolated from SIDS cases [Saadi *et al.*, 1993; Saadi *et al.*, 1996a].

Although *S. aureus* is isolated from over 50% of healthy infants in the 2 to 4 month age range, not all of them become SIDS victims. Certain factors must be present to produce a fatal outcome from a minor infection. In this age range the susceptibility of an infant to colonisation by potentially pathogenic bacteria will be influenced by the factors mentioned above: Lewis[a] antigen expression; exposure to tobacco smoke; underlying viral infection; and low maternal and infant antibody levels. Once colonisation of the upper respiratory tract has occurred whether the bacteria will grow and produce toxins will be determined by whether or not the colonising strain possesses the genes for toxin production and if the permissive temperature range in which the toxins are induced is reached. The pyrogenic toxins of *S. aureus* are only produced between 37 and 40°C. This is higher than the usual temperature of the upper respiratory mucosal surfaces which are cooled by the passage of air as the infant breathes. The temperature of this area could however, be increased, for example by:

1. overwrapping - excessive amounts of clothing or blankets may raise body temperature and reduce exposed surface area for heat loss;

2. the prone sleeping position - heat loss from the face is reduced, the cooling effect of the passage of air over the mucosal surfaces is reduced, and rebreathing of warm, recently exhaled air will occur;

3. mild viral infection - blocking one or both nostrils with catarrh reducing air flow and induction of pyrexia.

These factors could elevate the temperature of the mucosal surfaces thus creating a suitable micro-environment for the production of toxins (see Figure 1.4). The pyrogenic effect of the toxins themselves would also act to maintain the elevated temperature, prolonging their production. Recent evidence indicates that in older children (3-8 years) the prone position significantly increases the temperature of the nasal passages [Moloney *et al.*, submitted for publication].

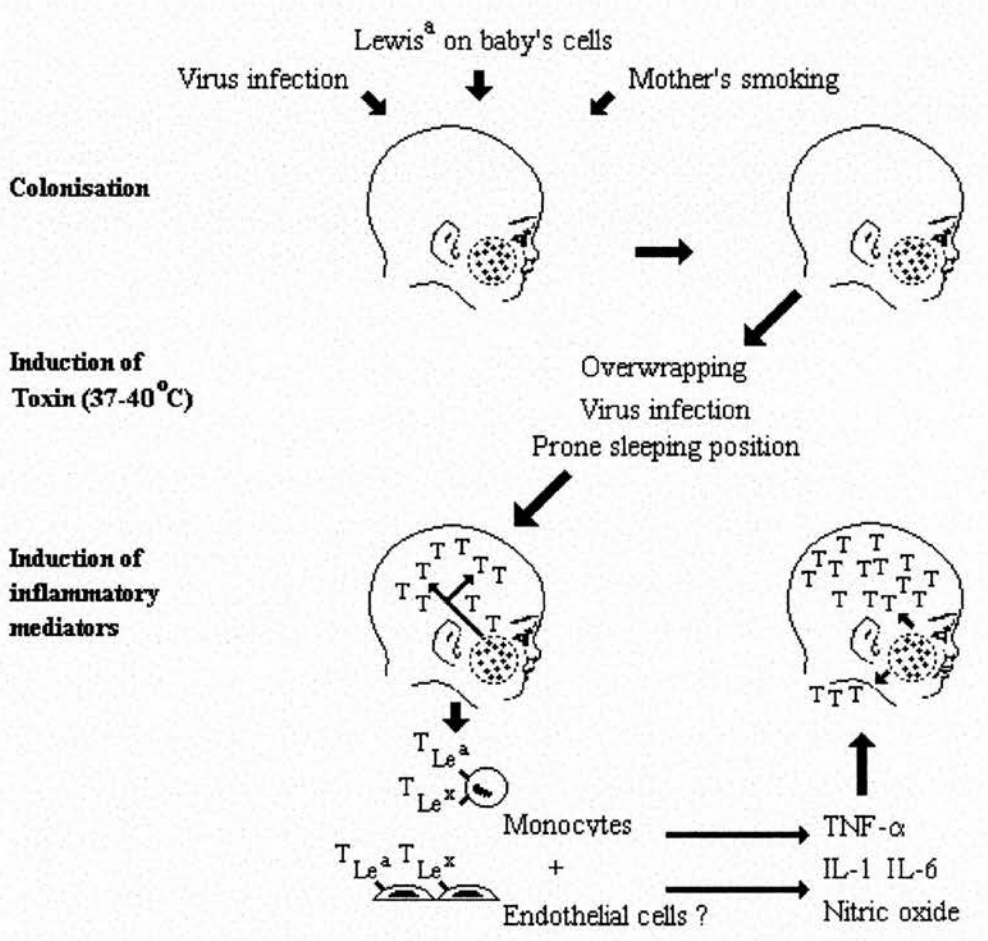


Figure 1.4 Factors leading to colonisation of infants by potentially pathogenic bacteria and induction of pyrogenic toxins.

1.9.1 Synergy between infectious agents.

1.9.1.1 Synergy between bacterial toxins and viruses.

Several groups have examined interactions between bacterial toxins and viruses. Animal studies using neonatal ferrets have shown that infection with the influenza virus can enhance the animals response to staphylococcal α and γ toxins, as well as to endotoxin and diphtheria toxin by factors ranging from 3 to 84 [Jakeman *et al.*, 1991]. Similar results were observed by Lundemose *et al.* [1993] in a study of cytokine production by human leucocytes in response to influenza virus and endotoxin from *E coli*. *In vivo* studies using mice showed that sub-clinical infection with lymphocytic choriomeningitis virus had a similar effect on enhancing the lethality of staphylococcal enterotoxin B (SEB) at normally tolerable levels. Partial protection was afforded by neutralisation of TNF-α by soluble receptor proteins and blockade of nitric oxide production by aminoguanidine. Neutralisation of interferon-γ by monoclonal antibodies had no effect on survival rates [Sarawar *et al.*, 1994]. This enhanced effect may result from an auto-amplification of the cytokine responses to the different infectious agents. Production of tumour necrosis factor-α by monocytes/macrophages in response to extracellular bacteria can be enhanced by the action of interferon-γ which is produced in response to viral infection [Matic and Simon, 1992], while the lymphocyte stimulating activity of TNF-α can enhance IFN-γ production [Stevens *et al.*, 1993]. The very high levels of cytokines which could

result from such a positive feedback loop could result in the death of an infant by multiple organ failure [Stevens *et al.*, 1993] (Figure 1.5)

1.9.1.2 Synergy between pyrogenic toxins and endotoxin.

The endotoxins (lipopolysaccharide, LPS) of Gram negative bacteria are potent stimulators of B cells and mononuclear phagocytes on their own, capable of causing fatal septic shock mediated by TNF-α at high concentrations. When present in low concentrations, LPS causes stimulation of mononuclear phagocytes, thus augmenting their cytokine response to other toxins. This lethal synergistic effect between bacterial exotoxins and LPS from different species has been demonstrated in the chick embryo model [Drucker *et al.*, 1992; Sayers *et al.*, 1995a]. Further evidence of a role for LPS in SIDS includes the recent observations that some SIDS infants had significantly lower IgG antibodies and elevated IgM antibodies to endotoxin core [Oppenheim *et al.*, 1994], and the fact that staphylococci are often isolated along with Gram negative bacteria such as *H. influenzae* and *E. coli* from SIDS victims at autopsy.

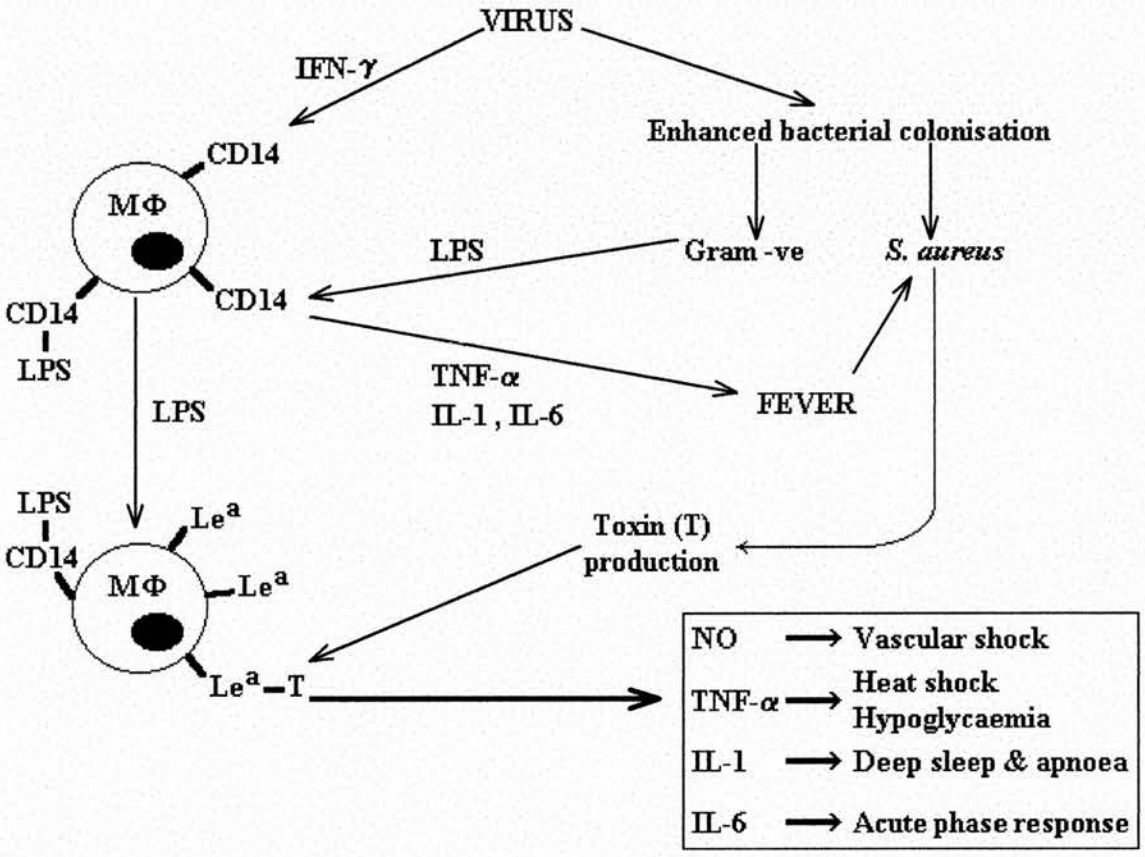


Figure 1.5 Synergy between viral infections, endotoxins and pyrogenic toxins in induction of inflammatory mediators (Mφ = macrophage).

1.9.1.3 Synergy between pyrogenic toxins.

Many of the toxigenic strains of *S. aureus* produce more than one type of toxin. Of those which colonise humans, it is common to find isolates producing TSST-1 and SEC_1. Previous studies have shown that the toxicity of this combination is greater than that of TSST-1 alone or in combination with any of the other staphylococcal enterotoxins [Crass and Bergdoll, 1986]. *S. aureus* isolates producing TSST-1 and SEC have been implicated in SIDS [Newbould *et al.*, 1989; Malam *et al.*, 1992].

1.9.1.4 Synergy between products of cigarette smoke and bacterial toxins.

The work of Sayers *et al.* [1995b], using the chick embryo model, demonstrated that the lethality of small amounts of toxins produced by bacteria isolated from SIDS cases could be greatly enhanced by the addition of a concentration of nicotine equal to that produced in man by 0.05 cigarettes. This enhancement of lethality by nicotine was further increased when toxins were present in combination.

1.9.2 The staphylococcal toxins as superantigens.

1.9.2.1 Mode of action of superantigens.

The staphylococcal enterotoxins and TSST-1 are termed superantigens because of their ability to stimulate a large percentage of T cells resulting in high levels of cytokines which can cause shock. Superantigens cause non-antigen-specific proliferation of T cells by binding outwith the antigen binding groove of MHC class II molecules on antigen presenting cells and cross-linking with the β chain variable (Vβ) region of the T cell antigen receptor (TcR). For example, Vβ2 is associated with TSST-1 and Vβ8 with SEB [Kappler *et al.*, 1989]. In this way all T cells bearing a specific Vβ sequence in their TcR are stimulated (Figure 1.6).

1.9.2.2 Structure of the staphylococcal superantigens.

The toxins produced by *S. aureus* are a group of structurally related single chain proteins of relatively low molecular mass. They are serologically classified into five groups, SEA, B, C, D and E, though SEC contains three subtypes. A further staphylococcal toxin, originally named SEF was later renamed toxic shock syndrome toxin (TSST-1). The toxins share the physical characteristics of being charged and heat and protease stable. There are also structural and sequence similarities, a

centrally located disulphide loop is present in all of the toxins except TSST-1 and all are rich in serine, threonine, and aspartic acid residues. Similarities in primary sequence are most clearly seen between SEA and SEE which exhibit approximately 90% sequence homology [Callahan *et al.*, 1990] though SEB and the SEC subtypes also appear to be related, showing approximately 65% homology at the amino acid level [Singh *et al.*, 1988]. These similarities continue at the secondary structure level, circular dichroism (CD) analysis has shown that the staphylococcal enterotoxins have a low α helix and high β pleated sheet content (Table 1.5).

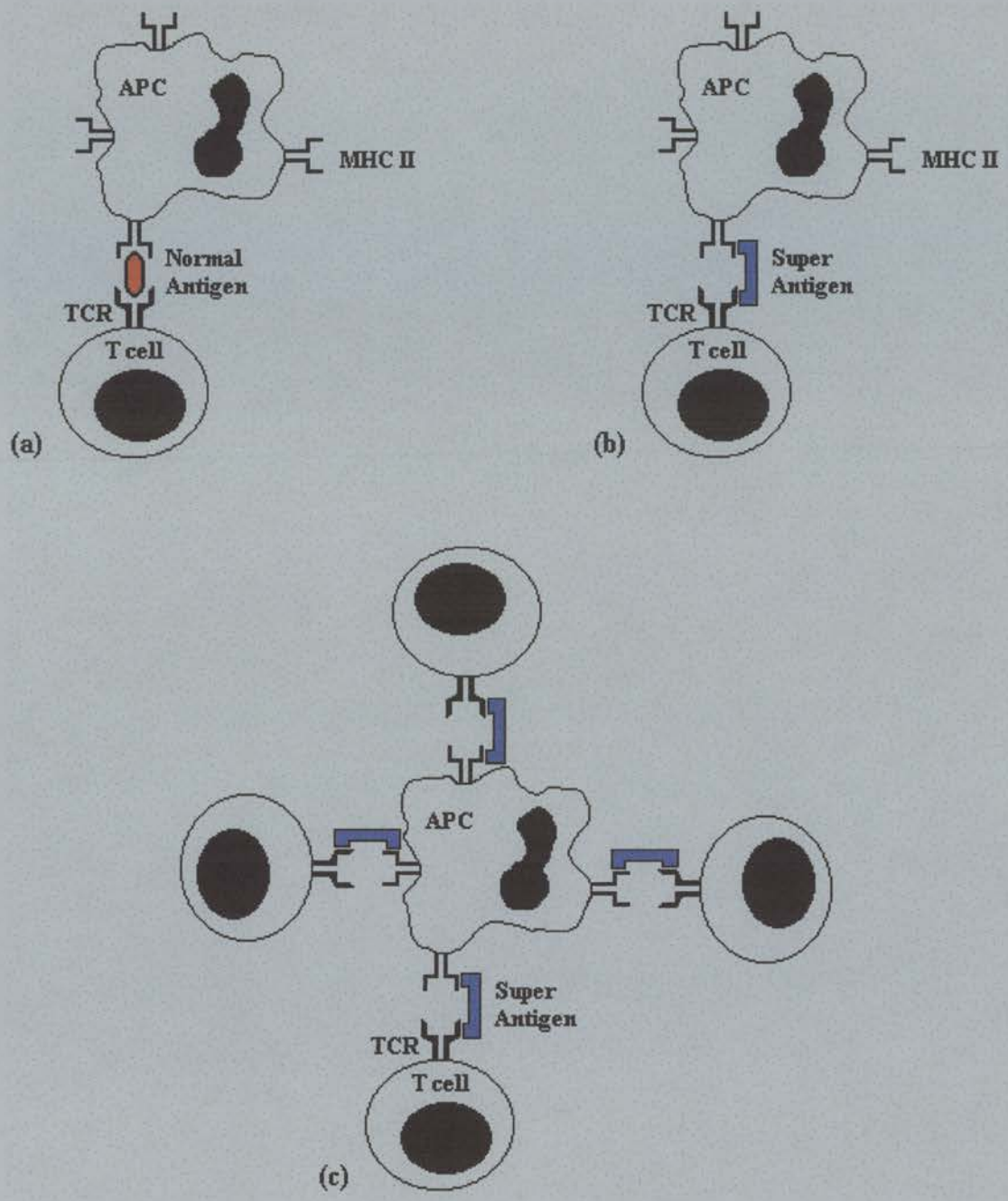


Figure 1.6 Comparison of conventional and superantigen binding: (a) Conventional antigen recognition. (b) Superantigen recognition outwith the antigen binding groove of the MHC II molecule. (c) Binding and stimulation of large numbers of non-antigen specific T cells.

Table 1.5 Characteristics of the staphylococcal enterotoxins.

Toxin	Molecular Mass (kDa)	Isoelectric Point (pI)	CD Structure (%)	
			α helix	β sheet
SEA	27.8	7.3	11	36
SEB	28.3	8.6	10	55
SEC_1	26.0	8.6	15	38
TSST-1	24.0	7.2	6	51

(Adapted from Johnson *et al.*, 1991).

1.9.2.3 Pathological response to staphylococcal superantigens.

The staphylococcal toxins were recognised in the early 1960s as a cause of food poisoning, inducing weakness, pyrexia and vomiting, not as a result of damage to the intestinal tissues but by the excessive production of interleukin-2 (IL-2) from proliferating T cells. Superantigen binding can induce autoimmunity, in which T cells recognising self-antigens proliferate. This can also depress immunity, directly by non-specific suppression of immunoglobulin synthesis [Poindexter and Schlievert, 1986], and indirectly when the T cells die off following superantigen stimulation and are therefore unavailable to respond to any subsequent infection. Staphylococcal toxins are known to cause toxic shock syndrome and have been implicated in Kawasaki disease by enhancement of clones bearing the Vβ2 sequence on their TcR [Leung *et al.*, 1995].

Possibly the most pertinent effect of the staphylococcal enterotoxin superantigens in relation to sudden infant death syndrome is their ability to enhance lethal endotoxin shock. In a rabbit model the pyrogenic toxins were shown to enhance the lethality of endotoxin by up to 100,000 fold [Sugiyama *et al.*, 1964; Schlievert, 1982]. This is thought to be due to alteration of liver clearance function by blockade of the reticuloendothelial system [Fujikawa *et al.*, 1986]. Similar enhancement of endotoxin effect is seen with TSST-1 in the renal tubular cells of rat kidneys [Keane *et al.*, 1986].

Although the majority of research into superantigen function has concentrated on TcR binding and T cell stimulation, studies on the effect of MHC class II engagement by these antigens on a variety of human cells, particularly macrophages, has shown similarly high levels of stimulation and production of cytokines including interleukin-1 (IL-1) [Ikejima *et al.*, 1984] and tumour necrosis factor α (TNF-α) [Fast *et al.*, 1988; Parsonnet and Gillis, 1988].

1.9.3 How could common bacterial toxins cause a SIDS death?

In the first few months of life when maternal antibody levels have waned initial protection from infectious agents is provided by the acute inflammatory response. It is possible that a certain percentage of SIDS deaths result from pathophysiological inflammatory responses to microbial products, both bacterial and viral, possibly augmented by environmental factors such as exposure to cigarette smoke, occurring during a time when cytokine controls are not sufficiently developed to damp down the production and effects of inflammatory mediators and endocrine responses cannot cope with the effects of these mediators on glucose homeostasis.

The cytokine response which results in inflammation is also involved in the process of septic shock in which the cytokines rather than the bacterial products cause most of the clinical signs [Parrillo, 1993].

The early stages of septic shock in adults may be asymptomatic. In some infants SIDS could be the result of septic shock which has been fatal before the histopathological signs of an inflammatory response have had time to develop. This outcome has been termed "pre-morbid shock" [Stevens *et al.*, 1993].

Production of TNF-α from mononuclear phagocytes can be elicited by both endotoxin and the pyrogenic exotoxins and initiates a cytokine cascade including IL-1, IL-6, TNF-α itself, and low molecular weight inflammatory cytokines of the IL-8 family, which play important roles in mediating septic shock [Newbould *et al.*, 1989; Parrillo 1993]. It is the co-stimulatory and synergistic effects of these and other inflammatory mediators that lead to shock and death. IFN-γ produced in response to viral infection augments production and many of the potentially lethal actions of TNF-α, as well as enhancing nitric oxide synthase activity [Stevens *et al.*, 1993].

1.9.3.1 A possible role for inflammatory mediators in SIDS deaths.

Work on the induction of septic shock in a rabbit model by TSST-1 has shown that many of the clinical observations associated with a shock state are due to the production of TNF-α and IL-1 [Ikejima *et al.*, 1988]. The researchers noted that the major organ affected by TSST-1 was the lungs which showed diffuse petechial haemorrhages similar to those of SIDS victims. On administration of a combination

of TNF-α and IL-1 haemorrhage was again noted, and in addition, proteinaceous fluid and immune cells were found in the alveolar spaces [Okusawa *et al.*, 1988].

There are several possible means by which an excessive cytokine response to a minor toxic stimulus could trigger a fatal event in a susceptible infant:

1. Reduction of blood pressure and tissue perfusion: TNF-α and nitric oxide are both capable of causing a significant reduction in blood pressure. TNF-α reduces myocardial contractility and causes relaxation of vascular smooth muscle tone both directly and indirectly by stimulating the production of vasodilators such as NO and prostacyclin from the vascular endothelium. Nitric oxide is thought to further lower blood pressure by altering endothelial integrity thus causing vascular leakage [Ochoa *et al.*, 1992]. Tissue perfusion can be decreased by high levels of TNF-α causing the formation of intravascular thromboses via endothelial alterations promoting coagulation and vascular plugging by activated neutrophils [Stevens *et al.*, 1993].

2. Hyperthermia: TNF-α and IL-1 induce fever via the production of prostaglandins from cytokine-stimulated hypothalamic cells which would account for the very high temperatures recorded for some SIDS infants [Sunderland and Emery, 1981].

3. Disturbance of glucose homeostasis: Systemic TNF-α causes severe metabolic disturbances including a potentially fatal drop in blood glucose levels [Stevens *et al.*, 1993].

4. Sleep apnoea: The increased frequency of sleep apnoeas during respiratory infections [Steinschneider, 1972] are thought to be due to IL-1 and muramyl peptide from bacterial cell walls inducing periods of deep sleep [Guntheroth, 1989]. Nitric oxide is also capable of producing hypoxic events when produced in high local concentrations by cytokine stimulated airway epithelial cells, probably by increasing bronchial blood flow and plasma exudation into the airways thus obstructing breathing [Kuo *et al.*, 1992; Alving *et al.*, 1993].

5. Uncontrolled stimulation of inflammatory cells: IL-1 has a stimulatory activity on effector cells of the immune and inflammatory responses. Along with TNF-α it acts on macrophages to produce further IL-1 and induce synthesis of IL-6, termed the acute phase response, which results in rapid and drastic changes in plasma protein composition thought to enhance the efficacy of the inflammatory response. Control of IL-1 is by a naturally occurring inhibitor, a biologically inactive structural homologue, produced by macrophages stimulated by antibody/antigen complexes [diGiovine and Duff, 1990]. Given that the infants antibody levels at this stage of development will be minimal or absent, inhibition of IL-1 by this method will not be efficient.

6. Synergistic effect of cytokines: Interferon-γ (IFN-γ) produced by T cells and natural killer (NK) cells in response to viruses and TSST-1[Jupin *et al.*, 1988] is a potent activator of macrophages which not only enhances TNF-α synthesis at the transcriptional level but also augments many of the effects of TNF-α on endothelial cells. Both TNF-α and IL-1 cause an up-regulation of IFN-γ receptors on monocytes, presumably to increase the capacity of IFN-γ to activate the cell [Krakauer and Oppenheim, 1993]. IL-1, though incapable of causing tissue damage itself, potentiates the harmful effects of TNF-α (Figure 1.7).

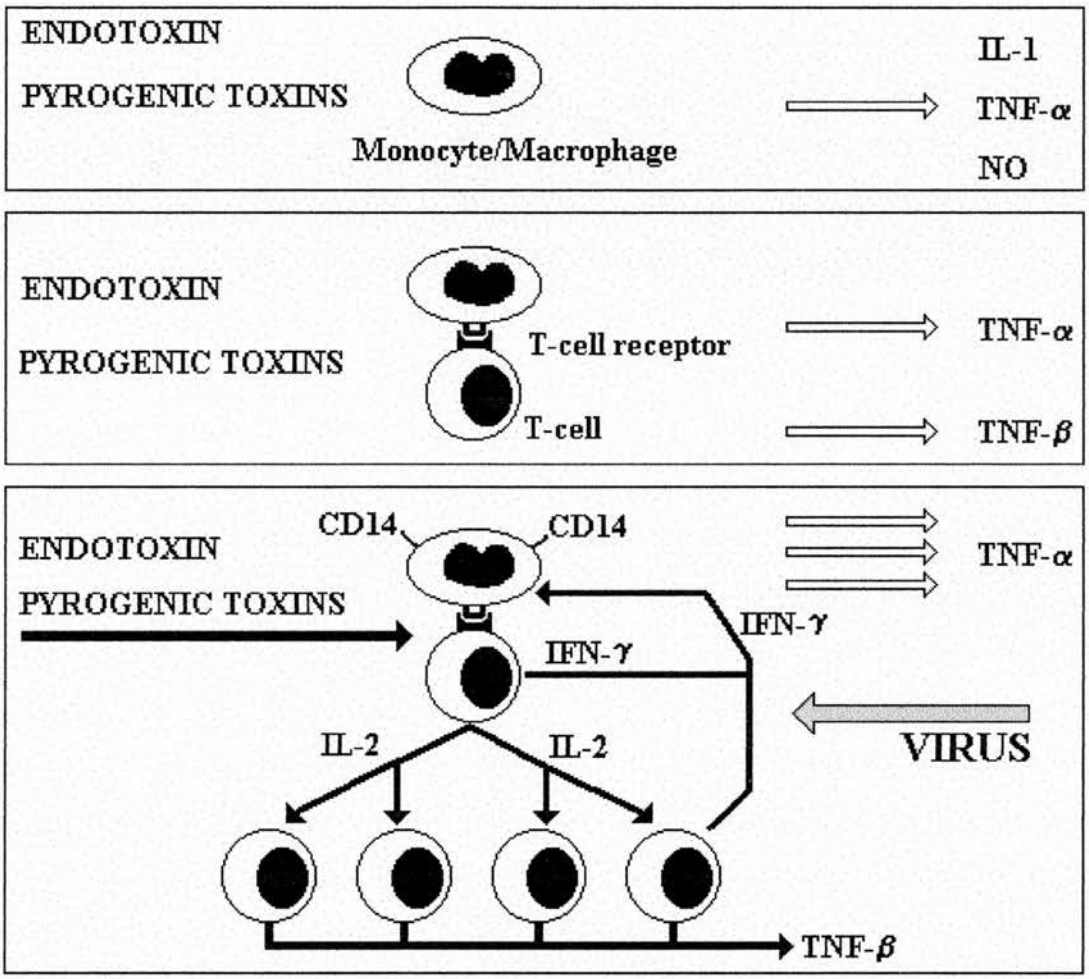


Figure 1.7 Synergistic effects between cytokines.

1.10 Aims of this study.

The aim of this study was to examine the role of developmental and environmental risk factors associated with SIDS in relation to the hypothesis that common bacterial toxins are involved in precipitating some of these infant deaths.

The principal objectives were:

1. to assess the ability of various bacterial strains to bind to the Lewis^a antigen which is known to act as a cell surface receptor for some bacterial species and bacterial toxins and is expressed by the majority of infants in the 0 to 3 month age range;

2. to determine if, as is the case for the pertussis toxin, the enterotoxins of *Staphylococcus aureus* can bind to the Lewis^a antigen on human peripheral blood mononuclear cells;

3. to assess the level of antigenic cross-reactivity between the staphylococcal enterotoxins and other common bacterial toxins to examine the possible protective effect reported for diphtheria-pertussis-tetanus vaccination;

4. to examine additive or synergistic effects between the staphylococcal enterotoxins and lipopolysaccharide from *E. coli* in induction of the inflammatory mediators nitric oxide and tumour necrosis factor-α from human peripheral blood mononuclear cells;

5. to examine the role of cigarette smoke components and virus infection on induction and enhancement of inflammatory responses to bacterial and viral products.

CHAPTER 2

General materials and methods

All chemicals were reagent grade and obtained from BDH unless otherwise specified.

2.1 Toxins and anti-toxins.

The toxins and anti-toxins used in these studies are listed in Tables 2.1 and 2.2.

Table 2.1 Toxins and suppliers.

Toxin	Source	Supplier
Staphylococcal enterotoxin A	*S. aureus*	Toxin Technology Inc.
Staphylococcal enterotoxin B	*S. aureus*	Toxin Technology Inc. Sigma
Staphylococcal enterotoxin C_1	*S. aureus*	Toxin Technology Inc.
Toxic shock syndrome toxin-1	*S. aureus*	Toxin Technology Inc.
Pertussis toxin	*B. pertussis*	Sigma

Table 2.2 Antibodies and suppliers.

Antibody	Source	Supplier
Anti-SEA	Rabbit	Toxin Technology Inc.
Anti-SEB	Rabbit	Toxin Technology Inc. Sigma
Anti-SEC_1	Rabbit	Toxin Technology Inc.
Anti-TSST-1	Rabbit	Toxin Technology Inc.
HRP anti-SEA	Rabbit	Toxin Technology Inc.
HRP anti-SEB	Rabbit	Toxin Technology Inc.
HRP anti-SEC_1	Rabbit	Toxin Technology Inc.
HRP anti-TSST-1	Rabbit	Toxin Technology Inc.
HRP anti-rabbit IgG	Sheep	Scottish Antibody Production Unit (SAPU)

2.2 Enzyme linked immunosorbent assays (ELISA).

2.2.1 ELISA reagents.

2.2.1.1 Coating buffer.

The coating buffer contained $NaHCO_3$ 1.59 g, NaH_2CO_3 2.93 g and NaN_3 (Sigma) 0.2 g in 1 litre of distilled water, pH 9.6.

2.2.1.2 Phosphate buffered saline (PBS).

Phosphate buffered saline contained Na_2HPO_4 (Fisons) 4.08 g, NaH_2PO_4 2.0 g and NaCl (Sigma) 3.04 g in 1 litre of distilled water, pH 7.2.

2.2.1.3 Washing buffer.

The washing buffer contained PBS with 0.1% bovine serum albumin (BSA) (w/v) (Sigma) and 0.02% Tween 20 (v/v).

2.2.1.4 Blocking buffer.

Phosphate buffered saline plus 1% BSA (w/v).

2.2.1.5 Phosphate citrate buffer.

Phosphate citrate buffer contained of 21 g of citric acid and 14.2 g of Na_2HPO_4 in 1 litre of distilled water, pH 5

2.2.1.6 Substrate.

Ortho-phenylenediamine dihydrochloride (OPD) (Sigma) (0.04 g) added to 100 ml of phosphate citrate buffer and activated with 40 µl H_2O_2 (Sigma) (30%, v/v) immediately prior to use.

2.2.1.7 Stopping solution.

The enzymatic reaction was stopped with 12.5% (v/v) H_2SO_4 in distilled water.

2.3 Tissue culture: culture media, supplements and cells.

2.3.1 Dulbecco's modified Eagle's medium.

Dulbecco's modified Eagle medium (DMEM) (Sigma) was used as the basis of the culture media for both human peripheral blood monocytes and L-929 cells and is referred to throughout as DMEM with no additives. Culture media consisted of DMEM with L-glutamine (Gibco) (2 mM), penicillin (Gibco) (100 IU ml^{-1}) and streptomycin (Gibco) (100 $\mu g\ ml^{-1}$), referred to throughout as DMEM with additives.

2.3.2 Human peripheral blood monocytes.

Monocytes were collected from buffy coats supplied by the Scottish National Blood Transfusion Service (SNBTS) using a Ficoll-hypaque gradient (Sigma tissue culture grade Histopaque-1077 Hybri-Max) by the following method. The buffy coats were diluted 1/2 in sterile PBS to a volume of 40 ml and layered onto 8 ml of Histopaque in a 50 ml conical centrifuge tube (Greiner). The tubes were centrifuged at 400 x g for 30 min at room temperature and the interface layer containing the monocytes removed. The monocytes were washed twice in sterile PBS at 150 x g for 10 min and the supernatant discarded. The cells were resuspended in 20 ml of DMEM with additives plus 15% (v/v) fetal calf serum (FCS) (Sigma), transferred to a 75 cm^2

tissue culture flask (Greiner) and incubated at 37 °C for 30 min to separate monocytes from non-adherent blood cells. The medium containing non-adherent cells was poured off and the monocytes harvested by gentle scraping with a cell scraper in 20 ml of fresh DMEM with additives plus 15% FCS. A viable count was performed using the Trypan blue dye exclusion method and the concentration of monocytes adjusted to 2 x 10^6 cells ml^{-1} in DMEM with additives plus 15% FCS.

2.3.3 L929 cells.

The tumourigenic murine fibroblast cell line L-929 was obtained from the European Collection of Animal Cell Cultures (ECACC) (Porton Down, Salisbury, England). Cells were cultured in DMEM with additives plus 10% (v/v) FCS at 37°C in a 5% CO_2 atmosphere until they had reached a semi-confluent state.

2.4 Monocyte challenge assay.

2.4.1 Toxin challenge.

The monocyte suspensions were placed in the wells of a sterile 24 well tissue culture plate (Greiner) in 0.5 ml volumes (1 x 10^6 cells/well) and the toxins added as 0.5 ml

volumes of twice the desired final concentration for single toxin assays or 0.25 ml volumes of four times final concentration for synergy or blocking studies involving either 2 toxins or toxin and antibody respectively. The plate was incubated for 3 days at 37°C in a 5% CO_2 atmosphere. The supernatants were extracted from the wells and centrifuged at 1,250 x g to remove cellular debris and stored at -20°C prior to assaying for nitric oxide and tumour necrosis factor-α content.

2.5 Nitrite assay.

2.5.1 Nitrite detection method.

Nitric oxide production was assayed by measurement of the stable breakdown product nitrite in the culture supernatant which reacts with the Greiss reagent to form a purple azo dye, the method was adapted from Zhang et al., [1994]. The supernatants (400 µl) were placed in 4 ml test tubes (Greiner) and mixed with an equal volume of Greiss reagent. The tubes were incubated for 10 min at room temperature and read at 570 nm on a Jenway 6100 spectrophotometer.

2.5.2 Greiss reagent.

Naphthylethylenediamine dihydrocloride (Sigma) 0.3% w/v in distilled water mixed 1:1, immediately prior to use with sulfanilamide (Sigma) 1% w/v in 5% conc. H_2PO_4. The components of the Greiss reagent were prepared fresh for each experiment.

2.5.3 Nitrite standard.

Dilutions of $NaNO_2$ (Sigma) in distilled water were used as the nitrite standard and included in each experiment.

2.6 Bioassay for detection of TNF-α.

L929 mouse fibroblast cells were grown to confluence in 75 cm^2 tissue culture flasks (Greiner), one day old monolayers were trypsinised, washed and adjusted to 3 x 10^5 viable cells ml^{-1} in Dulbecco's modified Eagle's medium (DMEM) plus 10% FCS before being distributed in 100 µl volumes into sterile 96 well flat bottom tissue culture plates. The plates were incubated overnight at 37°C in a 5% CO_2 atmosphere.

The medium was replaced with 50 μl volumes of the cell supernatant or TNF-α standards. The wells were made up to 100 μl with fresh DMEM plus 10% FCS containing 2 μg ml^{-1} actinomycin D (Sigma) and the plates incubated overnight as above.

The supernatants were removed from the wells, the plates blotted dry and 50 μl volumes of 0.5% crystal violet (w/v) in 20% methanol (v/v) in distilled water were added and incubated at room temperature for 2 minutes. The excess stain was washed off with tap water and the plates dried using a hair dryer. Acetic acid (20%, v/v in distilled water) was added to the wells and the optical density at 570 nm determined with a Dynatech MR 700 plate reader.

2.6.1 TNF-α standard.

Natural human tumour necrosis factor-α (88/786) was obtained from the National Institute for Biological Standards and Control (NIBSC) and a range of dilutions from 0 to 100 IU ml^{-1} included in each experiment.

2.7 Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE).

2.7.1 SDS PAGE buffers and solutions.

All solutions were made up in distilled water. Gloves were worn during the preparation and subsequent handling of acrylamide solutions.

2.7.1.1 Electrode buffer.

The electrode buffer contained 0.025 M Tris, 0.192 M glycine and 0.1% (w/v) SDS, pH 8.3.

2.7.1.2 Separating gel buffer.

The separating gel buffer contained 0.75 M Tris-HCl at pH 8.8 plus SDS (0.2% w/v).

2.7.1.3 Stacking gel buffer.

The stacking gel buffer contained 0.25 M Tris-HCl at pH 6.8 plus 0.2% w/v SDS.

2.7.1.4 Acrylamide stock solution.

Acrylamide stock solution (40% w/v) contained 100 g acrylamide (BDH Electrophoresis Grade) and 2.7 g methylene bis acrylamide (BDH Electrophoresis Grade) in 250 ml of distilled water.

2.7.1.5 Separating and stacking gels.

The separating and stacking gels were prepared as described in Table 2.3. The gel solutions were prepared in thoroughly cleaned filtration flasks and de-gased by applying a controlled negative pressure to the flask using a vacuum pump prior to addition of TEMED (NNN'N'-tetramethyl-1,2-diaminoethane) and ammonium persulphate.

2.7.2 SDS PAGE method.

An electrophoresis cassette was made, consisting of 2 glass plates (160 mm x 125 mm x 2mm) thoroughly cleaned with methanol separated by 1.5 mm plastic spacers and clamped together using 2 bulldog clips per spacer. The sides and bottom of the cassette were sealed with molten Vaseline.

A 12% separating gel (Table 2.3) was poured into the cassette using a 20 ml syringe and 19g needle to within 4 cm of the top of the cassette and allowed to set. A 4% stacking gel (Table 2.3) was poured onto the separating gel and a comb to form the sample wells inserted. After the stacking gel had set, the comb was removed, the cassette placed in an electrophoresis tank (Jencons Scientific Ltd.) and electrode buffer (2.7.1.1) added.

Heat denatured samples (2.7.3) were added to the wells in 20 µl volumes and electrophoresed through the stacking gel at 60 V constant voltage and through the separating gel at 150 V constant voltage until the dye front was within 30 mm of the bottom of the cassette. The cassette was then removed from the electrophoresis tank and the glass plates separated to allow removal of the gel for staining.

Table 2.3 Preparation of polyacrylamide gels.

	12% separating gel	4% stacking gel
Distilled water	5.2 ml	3.5 ml
Separating buffer	17.5 ml	-
Stacking buffer	-	5.0 ml
Acrylamide solution	10.5 ml	1.0 ml
TEMED	0.05 ml	0.02 ml
Ammonium persulphate	1.75 ml	0.5 ml

2.7.3 Preparation of samples for polyacrylamide gel electrophoresis.

Protein samples for PAGE were adjusted to 100 μg ml^{-1} in distilled water and mixed 1:1 with sample buffer. The samples were then heated at 100 °C in a water bath to denature the protein.

2.7.3.1 Sample buffer.

Sample buffer contained Tris (4% w/v), SDS (20% v/v), glycerol (2% v/v), 2-mercaptoethanol (1% v/v) and bromophenol blue (0.002% v/v), pH 6.8.

2.7.4 Coomassie blue staining of polyacrylamide gels for protein.

The polyacrylamide gels were stained for protein using the method of Hancock and Poxton (1988). The gels are incubated in a series of 3 staining and 2 decolourising solutions. The gels are incubated overnight in solution 1 and then sequentially in solutions 2 to 5, each for 1 h at room temperature with gentle shaking in an orbital incubator (Gallenkamp) at 30 rpm.

2.7.4.1 Staining solution 1.

Staining solution 1 contained propan-2-ol (25% v/v), acetic acid (10% v/v) and Coomassie brilliant blue R-250 (0.05% w/v).

2.7.4.2 Staining solution 2.

Staining solution 2 contained propan-2-ol (10% v/v), acetic acid (10% v/v) and Coomassie blue (0.005%).

2.7.4.3 Staining solution 3.

Staining solution 3 contained acetic acid (10% v/v) and Coomassie blue (0.0025%).

2.7.4.4 Decolourising solution 1.

Decolourising solution 1 contained methanol (40% v/v) and acetic acid (10% v/v).

2.7.4.5 Decolourising solution 2.

Decolourising solution 2 contained acetic acid (10 % v/v).

CHAPTER 3

Detection of microbial surface antigens that bind Lewis[a]

3.1 Introduction

Individuals who are non-secretors of their ABO blood group antigens are over represented among patients with certain infectious diseases or carriers of potentially pathogenic micro-organisms: rheumatic fever and carriers of group A *Streptococcus pyogenes* [Glynn *et al.*, 1956, Haverkorn and Goslings, 1969]; meningococcal disease [Blackwell *et al.*, 1986a]; invasive disease due to type b *Haemophilus influenzae* [Blackwell *et al.*, 1986b]; oral and vaginal yeast infections [Thom *et al.*, 1989, Aly *et al.*, 1991] and oral carriers of yeasts [Burford-Mason *et al.*, 1988, Blackwell *et al.*, 1989b, Aly *et al.*, 1992]; patients with peptic ulcers resulting from infection with *Helicobacter pylori* [Mourant *et al.*, 1978]. One hypothesis put

forward to explain these epidemiological findings is that some strains of micro-organisms have adhesins that bind to the Lewis^a antigen which is usually expressed in greater quantities on epithelial cells of non-secretors compared with secretors [Blackwell, 1989a; Blackwell, 1989b; Blackwell *et al.*, 1992].

Among older children and adults, the proportion of individuals whose erythrocytes are agglutinated by anti-Lewis^a is generally 20 to 25%. Between the ages of 2 to 4 months, approximately 80 to 90% of infants express the antigen on their erythrocytes [Issit, 1985], but this declines to the levels expected in adults by 18 to 24 months. Lewis^a antigen on epithelial cells is adsorbed from secretions and Lewis^a is readily detectable in the body fluids of infants [Blackwell *et al.*, 1992; Raza *et al.*, 1991]. Among infants who had died of SIDS the Lewis^a antigen was identified in the respiratory secretions of 71% [Blackwell *et al.*, 1992; Saadi *et al.*, 1993].

It has been observed that binding of *Staphylococcus aureus* to epithelial cells was positively correlated with the amount of Lewis^a detected on the epithelial cells of individual donors by flow cytometry. Binding of the strains could be significantly reduced by pre-treating the epithelial cells with the monoclonal anti-Lewis^a used to detect the antigen in flow cytometric assays [Saadi *et al.*, 1993]. A protein from outer membrane preparations of a toxigenic staphylococcal strain has been isolated by affinity purification methods using synthetic Lewis^a [Saadi *et al.* 1994a]. Lewis^a,

Lewis^{b} and H type 2 antigen have also been identified as receptors for adhesins on *Candida albicans* [Cameron and Douglas, 1996] and *H. pylori* [Alkout *et al.*, 1997].

Studies in which antibodies to host cell epitopes were used to inhibit bacterial binding have been criticised on the grounds that the antibody is binding not to the receptor for the bacteria but to an antigen in close proximity to the proposed receptor. The inhibition observed is due to stearic hindrance. One approach to dealing with these criticisms is production of anti-idiotypic antibodies to the antibodies used in the binding inhibition assays. Anti-idiotypic antibodies are raised to the antigen binding portion of a primary antibody, the Fab portion. The Fab of the anti-idiotypic antibody has a conformation which is a mirror image of that of the Fab portion of the primary antibody therefore the Fab portion of the anti-idiotypic antibody will resemble the structure of the antigen to which the monoclonal antibody binds [Koprowski *et al.*, 1984].

The objectives of this part of the project were:

1. To produce an anti-idiotypic antibody to monoclonal anti-Lewis^{a}.

2. To determine if the anti-idiotypic antibodies could bind to strains of micro-organisms that express surface components which bind Lewis^{a}.

3. To develop a reagent to determine if the anti-idiotypic antibodies could be used in a rapid screening method to detect surface components on pathogenic micro-organisms that bind to Lewis^a.

3.2 Materials and methods.

3.2.1 Monoclonal antibodies.

Monoclonal anti-Lewis^a antibodies LM 112/161.8, LM 112/159.1, LM 112/160.4 and anti-Lewis^b antibodies LM 137/264.2 and LM 137/268 were kindly provided by Dr. Robin Frazer of SAPU, Carluke, Lanarkshire. The monoclonal and polyclonal anti-Lewis antibodies used for cell typing and antibodies to the blood group antigens were obtained from SNBTS (Table 3.1).

LM 112/161.8 was purified by affinity chromatography with synthetic Lewis^a conjugated to Synsorb beads (Chembiomed). The bound antibody was eluted by 2% (w/v) ammonia in 0.9% (w/v) saline (pH 11) at room temperature. After dialysis to remove the ammonia, the final protein concentration was estimated spectrophotometrically and adjusted to 40 $\mu g\ ml^{-1}$.

3.2.2 Determination of protein concentration.

The method of Bradford (1976) was used to determine the concentration of the eluted antibody. The antibody solution (100 μl) was made up to 5 ml in protein reagent (Coomassie Brilliant Blue G-250 (0.01%, w/v), ethanol (4.7%, v/v) and phosphoric acid (8.5%, w/v) in 1 L distilled H_2O). The two solutions were mixed by repeated inversion for 2 min. and left to stand for 30 min. to allow the dye to bind to the protein. The sample was then read at 595 nm on a Jenway 6100 spectrophotometer, the protein weight read against a standard curve of known weights of bovine serum albumin and the concentration adjusted to 40 μg ml^{-1}.

3.2.3 Preparation of immunogen.

Purified anti-Lewis^a (LM 112/161.8) (2 ml of a solution containing 40 μg ml^{-1}) was conjugated with keyhole limpet haemocyanin (KLH) by adding 0.1 ml of a 1 in 5 dilution of a stock solution of glutaraldehyde (6.5%, v/v) and 0.1 ml of KLH (10 mg ml^{-1}). After incubation for 1 hr at room temperature, 0.1 ml of 1M lysine was added and the solution dialysed overnight against phosphate buffered saline (PBS) (2.2.1.2).

Table 3.1 Monoclonal antibodies to blood group antigens.

Antibody	Source	Ig class	Supplier
Anti-Le^a 112/159.1	Mouse	IgM	SAPU
Anti-Le^a 112/160.4	Mouse	IgM	SAPU
Anti-Le^a 112/161.8	Mouse	IgM	SAPU
Anti-Le^b 137/264.2	Mouse	IgM	SAPU
Anti-Le^b 137/268	Mouse	IgM	SAPU
HRP anti-mouse IgG	Rabbit	IgG	SAPU
Anti-Le^a	Mouse	IgM	SNBTS
Anti-Le^b	Mouse	IgM	SNBTS
Anti-A	Mouse	IgG	SNBTS
Anti-B	Mouse	IgG	SNBTS
Anti-M	Mouse	IgG	SNBTS
Anti-N	Mouse	IgG	SNBTS

3.2.4 Immunisation procedure.

Female BALB/c mice (n=3) were injected subcutaneously with the KLH-antibody conjugate (5 μg anti-Lewis^a) emulsified in Freund's complete adjuvant (0.5 ml) followed 1 week later by 5 μg of anti-Lewis^a conjugated to KLH in incomplete adjuvant (0.5 ml). After 6 weeks the mice were immunised with a further 5 μg of the antibody conjugate in PBS (0.5 ml). The mice were bled 7 days later and the serum separated by centrifugation at 100 x *g* for 15 min.

3.2.5 Detection of anti-idiotypic antibodies.

An ELISA to detect the anti-idiotypic IgG against anti-Lewis^a was adapted from that used in previous studies [Zorgani *et al.* 1992]. Two-fold dilutions of the affinity purified anti-Lewis^a used to immunise the mice were prepared (1/10 to 1/80) in coating buffer (2.2.1.1). The dilutions (100 μl) were used to coat 96 well flat bottom microtitre plates (Immulon II, Dynatech) and the plates incubated overnight at 4°C. The antibody was removed from the plates and they were washed three times with washing buffer (2.2.1.3) with a microtitre plate washer (Dynatech). Blocking buffer (2.2.1.4) (100 μl), was added to all wells for 30 min at room temperature then removed. Similar plates were prepared using monoclonal anti-Lewis^b (1/10 to 1/80) to coat the plates.

Normal mouse serum and the immune sera were diluted 1/50, 1/250 and 1/500 in blocking buffer and 100 µl added to the plates. After 45 min at 37°C in an orbital shaking incubator, the plates were washed three times and horseradish peroxidase (HRP) conjugated anti-mouse IgG (SAPU) (100 µl) diluted 1/500 in blocking buffer was added to the plates. The plates were incubated at 4°C overnight then washed as before. The substrate (2.2.1.6) (100 µl) was added to each well. The plates were incubated at room temperature in the dark for 15 min to allow the colour to develop and the reaction stopped with 12.5% (v/v) H_2SO_4 (50 µl). The colour change was measured at 492 nm on a Dynatech MR700 plate reader. Negative controls included wells to which only coating buffer or blocking buffer but no test or control serum was added.

3.2.6 Purification of the anti-idiotypic antibody against anti-Lewis^a.

Sepharose 4B beads (Pharmacia) were reconstituted and washed twice in 10^{-3} M HCl. The supernatant was discarded, coupling buffer ($NaHCO_3$ 8.7 g and NaCl 29.2 g in 1 L distilled H_2O) was added to the Sepharose and mixed on a rotary mixer for 10 min before centrifugation at 40 x *g*. This process was then repeated and the supernatant discarded after each wash. Affinity purified monoclonal anti-Lewis^a was diluted 1/2 to a volume of 4 ml in coupling buffer and added to the Sepharose beads. The Sepharose was incubated on a rotary mixer at room temperature for 2 h then

centrifuged at 40 x *g* for 10 min to remove unbound antibody. The Sepharose was washed in coupling buffer by rotary mixing for 10 min and centrifuged at 40 x *g* for 10 min. Ethanolamine (1 M) (5 ml) was added to the Sepharose to block any remaining active groups and incubated on a rotary mixer at room temperature for 2 h before centrifugation at 40 x *g* for 10 min. The supernatant was discarded and the Sepharose washed 3 times by centrifugation at 40 x *g* for 10 min with alternate buffers, acetate (10 ml 1M acetic acid plus 2.92 g NaCl in 1 L distilled H_2O, pH 4) and bicarbonate ($NaHCO_3$ 8.4 g plus NaCl 58.4 g in 1 L distilled H_2O, pH 8). The Sepharose 4B coupled to anti-Lewis^a was stored at 4°C in NET buffer (NaCl 8.7 g, EDTA 14.88 g and Tris 4.8 g in 1 L distilled H_2O).

The immune mouse serum was diluted 1/100 and 2 ml added to the Sepharose beads in a plastic universal container and incubated overnight at 4°C on a rotary mixer. Unbound serum components were removed by centrifugation at 40 x *g* for 10 min. The Sepharose was washed twice in NET buffer for 10 min on a rotary mixer and centrifuged at 40 x *g* for 10 min. The supernatant was retained for further cycles of purification. The anti-idiotypic antibodies were eluted from the Sepharose by mixing with 1 ml of 1 M acetic acid (pH 4) for 10 min and centrifugation at 40 x *g*. The supernatant was removed and neutralised with an equal volume of 1 M Tris (pH 10).

3.2.7 Separation of the anti-idiotypic antibody from non-immunoglobulin material on protein A conjugated Sepharose 4B.

Protein A conjugated Sepharose beads (Pharmacia) were reconstituted as described above and stored in NET buffer. The supernatant obtained by affinity purification with the anti-Lewis^a conjugated to Sepharose 4B was added to the protein A conjugated Sepharose and incubated for 2 days at 4°C on a rotary mixer. The non-immunoglobulin material was removed from the protein A Sepharose by washing five times in NET buffer by centrifugation as above (3.2.5). The protein A Sepharose linked to the anti-idiotypic antibody was stored in 2 ml of NET buffer at 4°C.

3.2.8 Agglutination assay to detect the anti-idiotypic antibody on the protein A Sepharose beads.

The specificity of the anti-idiotypic antibody bound to the protein A Sepharose beads was assessed by an agglutination assay against anti-mouse IgG, anti-Lewis^a, anti-Lewis^b (SAPU) and monoclonal typing antibodies against the blood group antigens A, B, M and N (SNBTS). The Sepharose beads were placed on slides (30 µl) and mixed with antibody (10 µl) diluted 1/4 in PBS. The slides were incubated at room temperature for 10 min and any agglutination of the Sepharose beads noted. The test slides were compared with negative controls containing the anti-idiotypic reagent

plus PBS and unconjugated Sepharose 4B with anti-Lewis^{a}, anti-Lewis^{b} and the typing antibodies.

3.2.9 Co-agglutination assay.

The co-agglutination assay was developed using the anti-idiotypic antibodies conjugated to the protein A Sepharose beads to screen bacteria and yeast isolates for surface antigens that bind Lewis^{a}. A heavy suspension of bacteria or yeasts (10 µl) was mixed with the protein A Sepharose bound to the anti-idiotypic antibody (30 µl) on slides and incubated at room temperature for 10 min. Agglutination of test samples was assessed by eye and compared with the control slides (3.2.7) with the inclusion of an unconjugated protein A Sepharose control. Bacteria and yeasts that agglutinated were tested for auto-agglutination by incubation with PBS.

3.2.10 Micro-organisms.

S. aureus strains NCTC 8531, NCTC 10652, NCTC 10654, NCTC 10655, NCTC 11965 were purchased from the National Collection of Type Cultures; 41206 was kindly provided by Dr. J. Medcroft, Public Health Laboratory Service, Reading,

Berkshire; and the Oxford strain was obtained from the departmental teaching collection (Table 3.2) all were grown on nutrient agar.

Neisseria meningitidis (18 strains) were from the laboratory culture collection. Those with prefixes "AK" or "I" were obtained from patients and carriers in Greece; the remainder were isolated from patients and carriers in Scotland. The antigenic phenotypes were confirmed by Dr. R. J. Fallon, Meningococcal Reference Laboratory (Scotland), Ruchill Hospital, Glasgow (Table 3.4). For *Neisseria gonorrhoeae* 2 strains were tested, a serum sensitive strain (8865) with "complex" lipo-oligosaccharide (LOS) type and serum resistant strain (757) with "simple" LOS [Winstanley *et al.*, 1983] (Table 3.3). All neisseriae isolates were grown on modified New York City medium [Young, 1978].

There were 13 isolates of *Haemophilus influenzae* (Table 3.5) those with the "H" prefix were patient strains obtained from Dr. Kristin Jonsdottir, Department of Microbiology, University of Iceland. The remainder were carrier isolates kindly supplied by Dr. Marina Barbour, Oxfordshire Department of Public Health. These were cultured on boiled blood agar. They were serotyped by agglutination with type specific serum (Wellcome Diagnostics).

Three isolates of *Helicobacter pylori* (table 3.6) obtained from patients with peptic ulcers were supplied by Dr. A. F. Mentis, Department of Microbiology, Hellenic Institute Pasteur, Athens, Greece. The strains were maintained on Skirrow medium in a microaerophillic environment.

Two isolates of *Candida albicans* (Table 3.7) one with an adhesin inhibitable by fucose (GDH 2346) and one with an adhesin inhibitable by N-acetyl galactosamine (GDH 2023) [Critchley and Douglas, 1987] were kindly provided by Dr. Julia Douglas, Department of Microbiology, University of Glasgow. These were grown on malt agar.

3.3 Results.

3.3.1 Detection of the anti-idiotypic antibody in immune mouse sera.

The ELISA results showed a pattern of decreasing optical density relative to increasing dilution in 2 of the immune sera (L1R0 and L0R1) tested against monoclonal anti-Lewis^a and also to a small extent in the normal mouse serum (NMS), subsequent haemagglutination assays using monoclonal antibodies to the Lewis antigens indicated the presence of an antigen cross-reactive with the anti-

Lewis antibodies on lysed mouse erythrocytes present in normal mouse serum (Figure 3.1). A similar pattern was observed with plates coated with anti-Lewis^b antibody but at lower levels in both the immune and normal mouse sera (Figure 3.2). Serum L0R1 gave the highest O.D. readings with anti-Lewis^a but values similar to that of the non-immunised mouse serum with anti-Lewis^b. Serum L1R0 gave the highest O.D. readings with anti-Lewis^b but values slightly higher than the non-immune mouse serum with anti-Lewis^a. The L0R1 serum was used in the preparation of the Sepharose beads for agglutination studies.

3.3.2 Specificity of the protein A Sepharose reagent.

The anti-idiotypic antibody bound to the protein A Sepharose beads was strongly agglutinated by anti-mouse IgG antibodies, the monoclonal anti-Lewis^a which was used for the immunisation and 2 additional monoclonal anti-Lewis^a antibodies tested. Weak agglutination indicated that there was cross-reactivity with anti-Lewis^b but no agglutination was observed with the antibodies to other blood groups (Table 3.2). The results indicate that the component bound to the protein A Sepharose beads was an IgG which is specific for anti-Lewis^a and the shared structure in anti-Lewis^b; the paratope of the Fab portion of the anti-idiotypic antibody appeared to have a structure similar to that of the Lewis^a antigen.

Figure 3.1 Detection by ELISA of binding of immune and normal mouse sera (NMS) to monoclonal anti-Lewis[a].

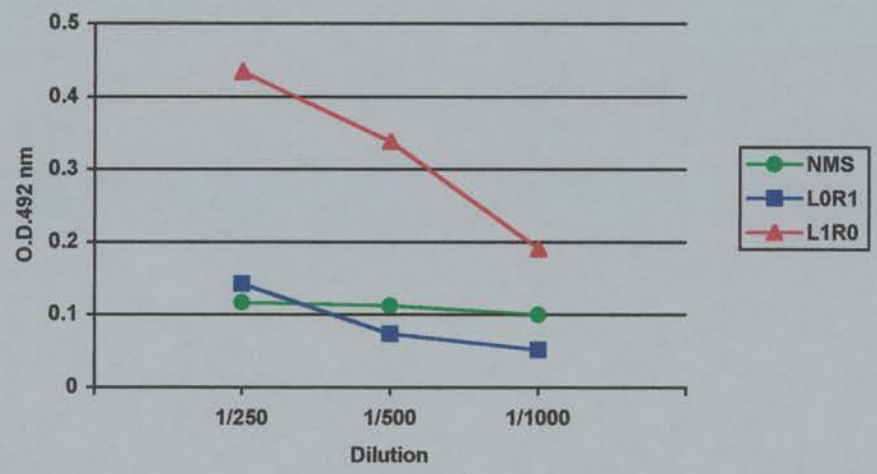


Figure 3.2 Detection by ELISA of binding of immune and normal mouse sera (NMS) to monoclonal anti-Lewis[b].

Table 3.2 Agglutination of the anti-idiotypic antibody on Sepharose beads by blood grouping antibodies and anti-mouse IgG.

Antibody	**Agglutination**
Anti-mouse IgG	+++
Anti-Lewis^a 159.1	+++
Anti-Lewis^a 160.4	+++
Anti-Lewis^a 264.2	++
Anti-Lewis^b	+
Anti-A	-
Anti-B	-
Anti-M	-
Anti-N	-

3.3.3 Co-agglutination assay using the anti-idiotypic reagent.

3.3.3.1 *S. aureus.*

The strongest agglutination observed was for strains of *S. aureus*. All strains, including 4 toxigenic isolates were agglutinated (Table 3.3). None of the bacteria tested caused agglutination of the unconjugated protein A Sepharose control. A protein A negative strain was not available for testing; therefore the possibility that the agglutination was non-specific cannot be eliminated.

3.3.3.2 *N. gonorrhoeae.*

For gonococci, the serum sensitive strain 8865 was agglutinated but the serum resistant strain 757 was not (Table 3.4).

Table 3.3 Agglutination of *S. aureus* isolates by the anti-idiotypic reagent.

Strain	Toxin	Agglutination
NCTC 8532	-	++
NCTC 10652	SEA	+
NCTC 10654	SEB	++
NCTC 10655	SEC	+++
NCTC 11956	SEA, TSST-1	++
41206	SEB	+
Oxford	-	++

Table 3.4 Agglutination of *N. gonorrhoeae* isolates by the anti-idiotypic reagent.

Strain	LOS type	Agglutination
757	simple	-
8865	complex	++

3.3.3.3 *N. meningitidis.*

Of the 18 strains of meningococci examined, 10 (56%) were agglutinable (Table 3.5). Agglutination by the anti-idiotypic reagent was not associated with serogroup, serotype or subtype of the bacteria; 3 carrier strains were autoagglutinable (4691, I 156 and I 642).

3.3.3.4 *H. influenzae.*

Of the 9 serotype b isolates of *H. influenzae* examined, 6 (67%) were agglutinable, as were 1 of the 2 type e and 1 of the 2 type f isolates tested (Table 3.6).

3.3.3.5 *H. pylori.*

Of the 3 isolates of *H. pylori* only 1 was agglutinated by the reagent (Table 3.7).

Table 3.5 Agglutination of *N. meningitidis* isolates by the anti-idiotypic reagent (* indicates autoagglutinable strain).

Source	Strain	Phenotype	Agglutination
Greece	AK 2	B:2b:P1.10	+
	AK 8	B:2b:P1.10	+
	AK 11	B:NT:P1.9	++
	AK 15	P:2b:P1.10	++
	AK 22	B:2b:P1.10	+
	AK 27	B:14:P1.10	-
	AK 31	B:2b:P1.10	-
	I 31	B:-:P1.6	-
	I 65	NG:14:P1.2	-
	I 66	B:14:P1.2	+
	I 156	Y:-:-	++ *
	I 545	B:2b:P1.2	-
	I 642	B:4:P1.15	++ *
Scotland	A 14	C:2b:P1.2	-
	A 43	B:4:P1.15	-
	4315	B:-:-	-
	4691	NG:-:-	+ *
	4813	B:-:-	++

Table 3.6 Agglutination of *H. influenzae* isolates by the anti-idiotypic reagent.

Source	Strain	Type	Agglutination
Iceland	H 14	b	+
	H 25	b	+
England	7786	b	+
	8216	b	+
	7654	b	-
	8214	b	+
	8215	b	+
	7985	b	-
	8195	b	-
	7306	e	-
	8119	e	+
	8186	f	+
	8184	f	-

Table 3.7 Agglutination of *H. pylori* isolates by the anti-idiotypic reagent.

Source	Strain	Agglutination
Greece	H 203a	-
	H 205a	+
	H 206a	-

Table 3.8 Agglutination of *C. albicans* isolates by the anti-idiotypic reagent.

Source	Strain	Agglutination
Scotland	2023	-
	2346	++

3.3.3.6 *C. albicans.*

The strain of *C. albicans* with the fucose inhibitable adhesin was agglutinated but the other strain with the adhesin inhibited by N-acetyl galactosamine was not (Table 3.8).

3.4 Discussion.

One of the difficulties in identification of host cell receptors for microbial adhesins is the possibility that the adhesin is binding to an antigen that is adjacent to or co-purified with the component under investigation. Even if binding of the micro-organism can be inhibited with monoclonal antibodies to the putative receptor, there is always the possibility that the inhibition results from stearic hindrance of the actual receptor which may be situated very close to the epitope to which the antibody binds. A more direct method would be to use purified antigen, but at the time the study was carried out this approach was limited by two practical problems. Commercially available purified preparations of Lewis^a antigen were very expensive; therefore, it was not feasible to use these for screening large numbers of strains. While the antigen can be obtained from secretions by affinity purification with anti-Lewis^a, it is attached to proteins and lipids which may obscure or enhance interactions with the bacteria.

The conformation of the antigen binding region of the anti-idiotypic antibodies will be very similar to that part of the antigen recognised by the monoclonal antibody. The specificity of the anti-idiotypic antibodies can be confirmed by comparison of agglutination assays with the monoclonal antibody to which it was produced with results obtained with monoclonal antibodies to other epitopes on the host cell surface. The only cross-reactivity observed was with the monoclonal anti-Lewis^b antibody tested. This was expected as the Lewis^a and Lewis^b antigens are constructed on the same precursor chain and the structure of Lewis^a is contained within Lewis^b.

The anti-idiotypic reagent agglutinated a strain of *S. aureus* and one of *C. albicans* which are known to possess surface components that bind to the Lewis^a antigen. *C. albicans* GDH 2346 expresses an adhesin that binds fucose, the immunodominant sugar of Lewis^a; and by ELISA, it has been found to bind highly purified Lewis^a antigen [Aly, 1992a,b] and more recently synthetic Lewis^a, Lewis^b and H type 2 conjugated to biotin [Blackwell, unpublished observations]. Co-agglutination assays were consistent with this data; strain GDH 2346 was agglutinated but strain GDH 2023, which expresses an adhesin that binds N-acetyl galactosamine [Critchley and Douglas, 1987] was not.

A major band has been obtained by Synsorb Lewis^a affinity purification of outer membrane preparations of *S. aureus* 10655 which was strongly agglutinated by the anti-idiotypic reagent [Saadi *et al.*, 1994a]. While the other *S. aureus* isolates were

also agglutinated by the anti-idiotypic reagent, the possibility that the protein A on these bacteria was responsible for the observed agglutination of the IgG cannot be eliminated. More recent studies by other members of the research group have demonstrated inhibition of binding of *S. aureus* 10655 using synthetic Lewis^a [Saadi *et al.*, 1996].

The third objective was to assess the anti-idiotypic antibodies for screening species for which epidemiological studies have found non-secretors are more susceptible. The co-agglutination assay was simple and reproducible. The results were useful as a pilot study to test the hypothesis that anti-idiotypic antibodies might be of value in overcoming some of the problems associated with specificity of identification of host cell receptors for micro-organisms and development of new screening reagents for epidemiological studies. This might be particularly useful for development of new typing reagents for meningococci. The majority of isolates from patients and carriers from Greece were not serotypable with the reagents developed for Northwest Europe and America [Tzanakaki *et al.*, 1993]; but 8/12 of the Greek isolates tested in this study were agglutinated by the anti-idiotypic reagent. *H. influenzae*, both typable and non-typable strains have been isolated from SIDS infants [Telford *et al.*, 1989]. The results obtained here indicate some strains have adhesins that bind Lewis^a, however, studies on frequency of isolation of bacterial species from healthy infants indicate that *H. influenzae* is found more frequently in older infants ($\geq$ 6 months of age) [Mackenzie *et al.*, 1996; Blackwell *et al.*, submitted for publication].

Production of monoclonal anti-idiotypic antibodies will be necessary for the quantities needed for large scale screening and development of more sensitive detection assays such as ELISA or flow cytometry methods. New methods for the synthesis of blood group oligosaccharides and production of biotinylated derivatives at reasonable costs have made the development of anti-idiotypic reagents such as this less attractive; however, it was useful in complementing the studies indicating the role of the Lewis^a antigen as a receptor for certain micro-organisms.

The bacterial co-agglutination assay suggests that molecules that bind Lewis^a are present on strains of several bacterial species to which non-secretors appear more susceptible than secretors. The anti-idiotypic reagent will be useful in epidemiological studies to determine if those strains that express the Lewis^a adhesin are isolated more frequently from patients or carriers who are non-secretors or from those secretors who express large amounts of Lewis^a [Blackwell *et al.*, 1992, Saadi *et al.*, 1993]. Isolation and characterisation of these adhesins and assessment of their immunogenicity might lead to new approaches to investigation of pathogenesis of some of these diseases and their prevention.

In relation to bacterial species isolated from SIDS infants, recent epidemiological studies have assessed normal nasopharyngeal flora of healthy infants and SIDS victims [Mackenzie *et al.*, 1996]. The predominant isolate among infants in the 0 to 3 month age range was *S. aureus* (57%) compared with *Haemophilus influenzae*

(22%), *Streptococcus pyogenes* (13%), and *Neisseria lactamica* (6%). Isolation of staphylococci was not associated with socio-economic class, maternal smoking or symptoms of virus infection. Isolation of *S. aureus* in the Scottish infants and in Swedish infants [Anainsson *et al.*, 1992] paralleled the peak in expression of the Lewis^a antigen (Figure 1.3). The isolation rates of these bacteria are mirrored in the agglutination study: all of the strains of *S. aureus* tested were agglutinable with the anti-idiotypic antibody to anti-Lewis^a whereas only 61% of the *H. influenzae* strains appeared to bind this antigen.

These observations suggest a parallel with the vulnerability of calves to colonisation by the enterotoxigenic K99 strains of *Escherichia coli*. The receptor most avidly recognised by the adhesins on the *E. coli* K99, the oligosaccharide N-glycolyl neuraminic acid α 2-3 galactose, is expressed abundantly in the first weeks of life during which the calves are susceptible to the diarrhoeal disease caused by these bacteria. As the expression of the antigen declines, so does the susceptibility to colonisation and disease [Mouricout *et al.*, 1990].

Currently 60% of SIDS deaths now occur in Britain in the 0 to 3 month age range. While 56% of healthy infants in this age range were carriers of *S. aureus*, these bacteria were isolated from 86.4% of 37 local SIDS infants under 3 months of age during the period of the studies on healthy infants. Identification of pyrogenic toxins of *S. aureus* in over half of the SIDS infants examined [Zorgani *et al.*, 1997]

complements these epidemiological studies and indicates that further investigation of the role of toxigenic *S. aureus* in the events leading to SIDS was warranted.

CHAPTER 4

Lewis antigen expression on human monocytes and binding of pyrogenic toxins

4.1 Introduction

There is evidence that Lewis^a and the structurally related Lewis^x (CD15) act as receptors on monocytes for the pertussis toxin of *B. pertussis* [van t'Wout *et al.*, 1992], another toxigenic bacterium implicated by epidemiological studies as a cause of SIDS [Wennegren *et al.*, 1987; Nicoll and Gardner, 1988; Lindgren *et al.*, 1997]. Although there is no evidence that pertussis toxin is present in SIDS infants, both SEC_1 and TSST-1 have been identified [Newbould *et al.*, 1989; Malam *et al.*, 1992; Zorgani *et al.*, 1997]. These pyrogenic toxins are superantigens which have significant physiological effects such as induction of fever (>38.5°C), possibly through direct action on the hypothalamus or through their induction of tumour

necrosis factor alpha (TNF-α) or interleukin 1 (IL-1). The bacteria produce these toxins in the temperature range 37 to 40°C, and the amount of toxin produced increases with the increasing temperature [Bohach *et al.*, 1990]. The actual cause of a SIDS death might be heat shock reflected by the unusually high temperatures recorded for some of these infants at autopsy [Sunderland and Emery, 1981]. The increased frequency or duration and depth of sleep due to induction of IL-1 has also been proposed as a link between respiratory infection and fatal sleep apnoea [Guntheroth, 1989]. In an animal model it has been demonstrated that sub-clinical infection with murine lymphocytic choriomeningitis virus can enhance lethality of SEB, induction of nitric oxide and TNF-α have been implicated in the lethal responses to the pyrogenic staphylococcal toxins [Sarawar *et al*, 1994]. Nitric oxide is associated with profound hypotension refractory to vasoconstrictor therapy and TNF-α with induction of fever via increased prostaglandin synthesis and reduced myocardial contractility.

The pathological effects of superantigens have been attributed to non-specific activation of T cells and antigen presenting cells. The proposed mode of action of the toxins is that they bind to MHC class II antigens of the antigen presenting cells on a site external to the antigen binding groove and to specific Vβ sequences on the T cell receptor thereby cross-linking the two cell types [Janeway *et al.*, 1989; White *et al.*, 1989]. This study examined the effect of the staphylococcal enterotoxins on

peripheral blood mononuclear cells rather than T cells to assess the ability of these toxins to bind to the Lewis^a antigen.

Adherence of *B. pertussis* to monocytes by the pertussis toxin (PT) can be reduced by capping of surface components with some monoclonal antibodies specific for Lewis^a and the structurally related Lewis^x. The toxin appears to bind to these fucosylated polylactosamines, increasing intracellular calcium and acting as a C type lectin [van t'Wout *et al.*, 1992]. Work by Saadi *et al.* [1993] indicates Lewis^a is a receptor for toxigenic staphylococci; further evidence for this was obtained in chapter 3.

Oligosaccharides in human milk have been demonstrated to reduce bacterial binding to epithelial cells [Saadi *et al.*, 1996]. Breast feeding is suggested to have a protective effect against SIDS [Ford *et al.*, 1993]; however, there have been no studies on the effects these oligosaccharides might have on the staphylococcal toxins implicated in SIDS, such as TSST-1 [Newbould *et al.*, 1989] and SEC_1 [Malam *et al.*, 1992].

The objectives for this part of the project were to determine:

1. if the Lewis antigens act as receptors on monocytes for the pyrogenic staphylococcal toxins;

2. if the toxins elicit production of nitric oxide which has been implicated in animal studies on the lethality of staphylococcal enterotoxin B.

3. if the oligosaccharides of Lewis^a and Lewis^b can reduce the activity of the toxins.

4.2 Materials and methods

4.2.1 Toxins and antibodies.

The toxins and antibodies used in these experiments are listed in Tables 2.1 and 2.2. Antibodies used to detect host cell antigens and fluorescein isothiocyanate (FITC) conjugated antibodies are listed in Table 4.1.

4.2.2 Collection of monocytes.

Human peripheral blood monocytes were purified from buffy coats as described in 2.3.2.

Table 4.1 Antibodies to host cell antigens and FITC-conjugated antibodies.

Antibody	**Animal source**	**Ig class**	**Supplier**
Anti-CD14	Mouse	IgG	Scottish Antibody Production Unit (SAPU)
Anti-Lewis[a]	Mouse	IgM	
Anti-Lewis[b]	Mouse	IgM	
FITC anti-mouse IgG	Rabbit	IgG	
FITC anti-mouse IgM	Rabbit	IgG	
FITC anti rabbit IgG	Donkey	IgG	

4.2.3 Detection of Lewis^a on monocytes.

Blood samples (20 ml) were obtained from healthy adult volunteers of known secretor status and the monocytes collected as described in section 2.3.2. To prevent internalisation of antibodies or toxin the monocytes were diluted to 5 x 10^5 cells ml^{-1} and fixed in 1% paraformaldehyde at room temperature for 1 h. The cells were then washed twice in PBS at 375 x *g* for 10 min. prior to addition of murine monoclonal anti-Lewis^a (1/5) (0.5 ml) and incubated at 37°C for 1 h in an orbital shaking incubator. The cells were washed twice as above and the supernatant discarded. The monocytes were incubated with FITC conjugated rabbit anti-mouse IgM (1/200) (200 µl) at 37°C for 30 min. as above and washed three times to remove unbound FITC labelled antibody. The cells were resuspended in 1 ml of 1% buffered paraformaldehyde and stored in the dark at 4°C prior to reading on an EPICS XL (Coulter) flow cytometer (4.2.10).

Control samples labelled with anti-CD14 (SAPU) were used to localise the monocyte population on the flow cytometer bitmap as small numbers of other cell types are present after purification. Non-specific binding of the FITC labelled antibody to the monocyte surface was controlled for using samples containing monocytes, PBS and FITC conjugated anti-mouse IgG or FITC conjugated anti-mouse IgM.

4.2.4 Detection of toxins bound to monocytes.

Monocytes (5 x 10^5) fixed with 1% paraformaldehyde at room temperature for 1 h were incubated with varying dilutions of the staphylococcal enterotoxins at 37°C for 45 min in an orbital shaker. The cells were washed twice, resuspended in 0.5 ml of PBS and incubated with 200 µl volumes of homologous rabbit anti-toxin at a dilution of 1/200 for 1 h at 37°C. This dilution was used as preliminary studies had shown that anti-toxin concentrations above 1/200 resulted in high levels of non-specific binding. The cells were washed three times and resuspended as above and incubated for 30 min at 37°C with 200 µl volumes of FITC conjugated donkey anti-rabbit IgG (1/250) (SAPU).

After washing three times as above, the cells were resuspended in 0.1% buffered paraformaldehyde (500 µl) and stored in the dark at 4°C until analysed by flow cytometry on an EPICS XL. Non-specific interaction of rabbit IgG and Fc receptors on the monocyte surface was controlled by using samples containing monocytes, PBS and FITC conjugated anti-rabbit IgG.

4.2.5 Binding of toxins to secretor and non-secretor monocytes.

Human peripheral blood monocytes were isolated from heparinised venous blood samples (20 ml) taken from healthy adult volunteers within the department, one secretor and one non-secretor. The monocytes (1 x 10^6 cells ml^{-1}) were fixed in 1% buffered paraformaldehyde and incubated for 1 h at 37°C in an orbital shaking incubator with SEB (Sigma) diluted to 400 and 800 μg ml^{-1} and the binding assay outlined above followed. Toxin binding to the two cell populations was assessed by flow cytometry on an EPICS XL.

4.2.6 Binding inhibition assay.

The following murine monoclonal antibodies were assessed for their ability to inhibit binding of the staphylococcal toxins to monocytes: anti-Lewis^a (1/5) and anti-Lewis^b (1/5). The fixed monocytes were incubated with 200 μl volumes of antibody for 60 min at 37°C. The cells were washed twice to remove unbound antibody and the binding assay for the toxins outlined above followed. The cells were then assessed by flow cytometry.

4.2.7 Monocyte challenge assay.

The monocyte suspension (0.5 ml of 2 x 10^6 cells ml^{-1}) in DMEM with additives plus 15% FCS (2.3.1) was added to the wells of a 24 well tissue culture plate (Greiner). A time course experiment was carried out with monocytes challenged with varying concentrations of SEB to determine the optimum incubation time for production of nitric oxide. The culture supernatants were sampled at 24 hour intervals over a period of 4 days and assayed for nitrite content.

Preliminary dose response experiments were carried out to ascertain the optimum toxin concentration for induction of nitric oxide. The staphylococcal toxins SEA, SEB, SEC_1 and TSST-1 were diluted in DMEM without additives to concentrations of 0.05, 0.25, 0.5, and 2.5 $\mu g\ ml^{-1}$. The monocytes were then challenged with 0.5 ml of dilutions of the staphylococcal toxins (2.4.1). The cells were incubated for 72 h (Table 4.3) at 37°C in 5% CO_2. The culture supernatants were removed and centrifuged at 1,250 x *g* for 10 mins to remove any remaining cells prior to determination of nitrite content.

4.2.8 The effect of pre-treatment with the Lewis antigens on TSST-1 induced nitric oxide production.

TSST-1 was diluted in DMEM with no additives to give a working concentration of 2 μg ml^{-1} and incubated overnight at 37°C in an orbital shaker with equal volumes of either synthetic $Lewis^a$ or $Lewis^b$ (50 μg ml^{-1}) (Dextra Labs), Lewis antigen concentrations were set in excess of those used in other blocking studies [Saadi, personal communication] to ensure saturation of binding sites on TSST-1. The toxin/Lewis antigen mixtures were then used in the monocyte challenge assay to determine whether or not the Lewis antigens were capable of blocking the bioactivity of TSST-1. The toxin/Lewis antigen mixtures (0.5 ml) were added to the monocyte suspension (0.5 ml) and incubated as described above.

4.2.9 Assay for nitric oxide production.

This assay, modified from Zhang *et al.*, [1994], was used to determine the amount of nitric oxide produced by monocytes in response to toxin challenge by measuring the levels of its stable breakdown compound nitrite (2.5.1).

4.2.10 Flow cytometry.

Flow cytometric analysis of cells was performed using an EPICS XL flow cytometer (Coulter). Cell populations were selected from the bitmap to exclude fragmented cells and any contaminating (non-monocyte) cell types remaining after Ficoll-hypaque separation of monocytes from buffy coats. The bitmap displays the sample population as forward angle light scatter versus 90° light scatter allowing differentiation of cell size and relative granularity among the population. Background fluorescence was blanked using control samples containing untreated cells plus fluorescent antibody. The percentage of cells in test samples showing fluorescence greater than background was assessed as a single parameter histogram measuring fluorescence on a logarithmic scale. Mean channel fluorescence values of the positive cells were multiplied by percentage of positive cells to give the binding index (BI).

4.2.11 Statistical methods.

Data are represented as the mean and standard deviation of the replicate experiments.

4.3 Results

4.3.1 Detection of Lewis^a on human monocytes.

The binding indices for anti-Lewis^a to fixed monocytes from secretors (S) (n=3) and non-secretors (NS) (n=3) are shown in figure 4.1.The average binding index observed of anti-Lewis^a to non-secretor cells was 3 times that of secretor cells.

4.3.2 Binding of staphylococcal toxins to human monocytes.

Figures 4.2 to 4.5 show the binding of dilutions of the staphylococcal enterotoxins SEA, SEB, SEC_1 and TSST-1 to human monocytes. All of the toxins bound to the monocytes in a dose dependant manner. The data were used to determine the optimum toxin concentration of SEA 0.5 $\mu g\ ml^{-1}$, SEB 1 $\mu g\ ml^{-1}$, SEC_1 20 $\mu g\ ml^{-1}$ and TSST-1 20 $\mu g\ ml^{-1}$ for use in the binding inhibition experiments with antibodies to the Lewis antigens. All samples tested were from secretors expressing Lewis^a and Lewis^b, determined by agglutination of erythrocytes by blood grouping antibodies to Lewis^a and Lewis^b prior to purification of the monocytes (n=4) (data represents the mean of 2 experiments with 4 donors).

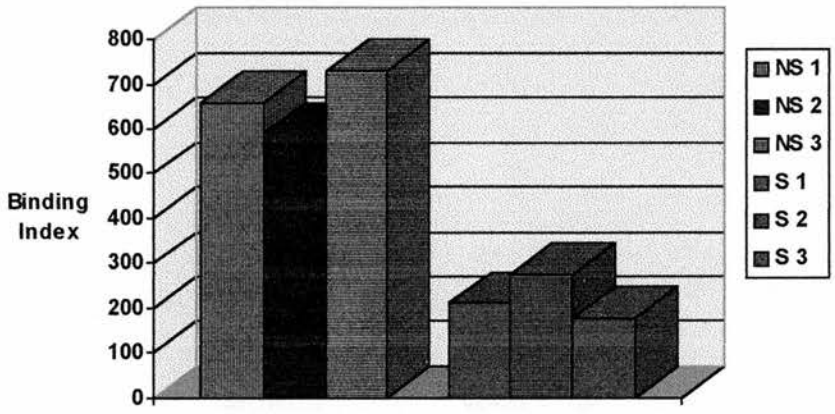


Figure 4.1 Binding of anti-Lewis[a] to secretor (S) and non-secretor (NS) monocytes.

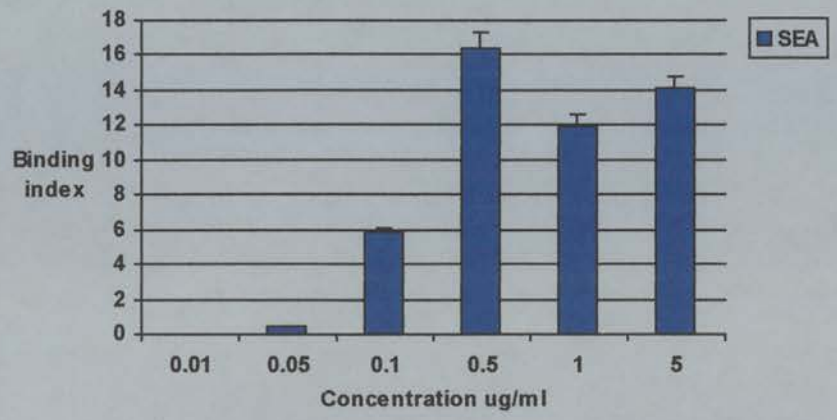


Figure 4.2 Binding of SEA to monocytes.

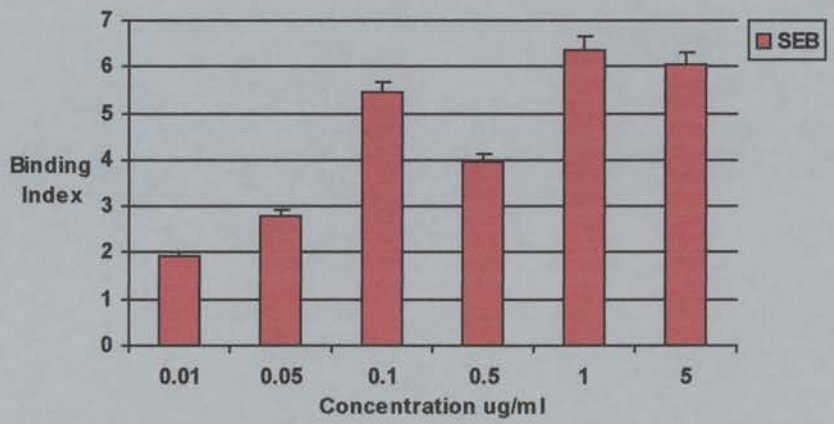


Figure 4.3 Binding of SEB to monocytes.

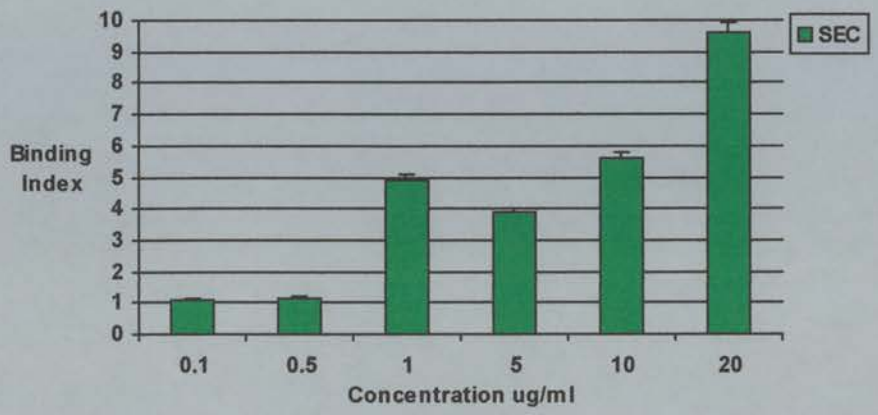


Figure 4.4 Binding of SEC_1 to monocytes.

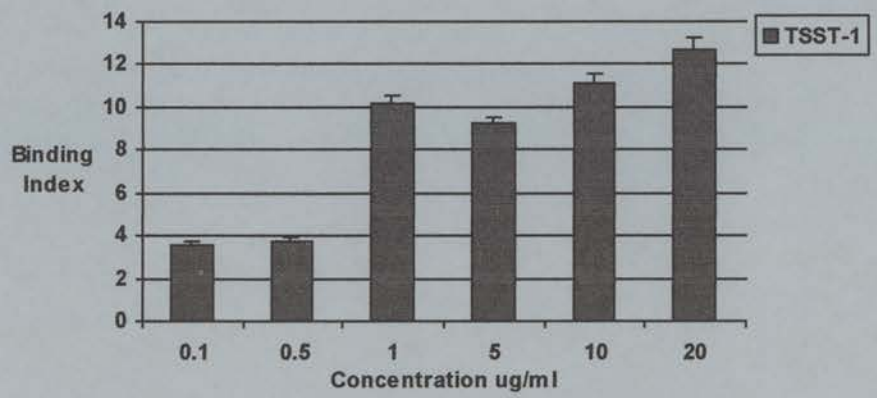


Figure 4.5 Binding of TSST-1 to monocytes.

4.3.3 Binding of SEB to secretor and non-secretor monocytes.

To elicit a clear difference in toxin binding levels to secretor and non-secretor monocytes the concentration of toxin used had to be greatly increased. Given the relatively small amounts of SEA, SEC_1 and TSST-1 available and their cost, it was only feasible to carry out this part of the project using SEB supplied by Sigma. In 2 experiments with 1 non-secretor donor and 1 secretor donor the toxin showed increased binding to the non-secretor fixed cell population (NS) (n=1) (binding index for anti-Lewis^a=729.8) which expressed higher levels of the Lewis^a antigen than the secretor (S) (n=1) (binding index for anti-Lewis^a=175.4) population at both 400 and 800 $\mu g\ ml^{-1}$, an increase of 114.37% and 133.38% respectively (Figure 4.6). Although the increase in binding of toxin to non-secretor cells compared with secretor cells is not as marked as the increase in binding of anti-Lewis^a. There was a similar pattern in binding of monoclonal anti-Lewis^a and detection of toxin bound to the monocytes.

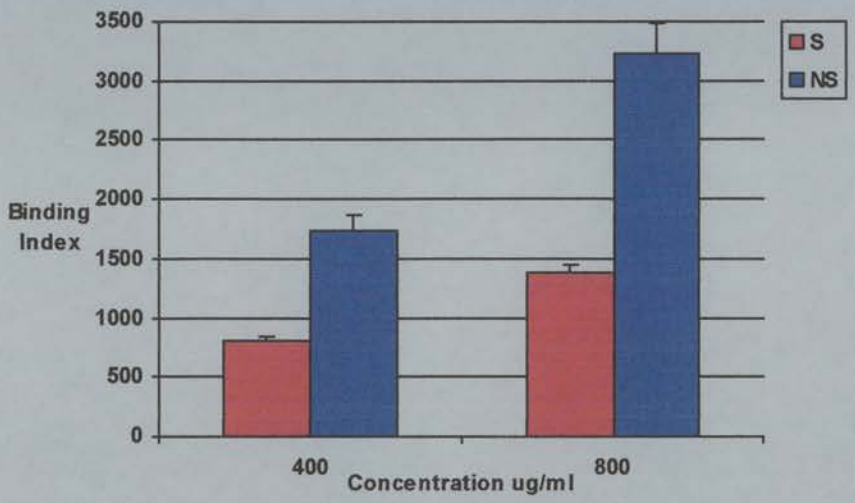


Figure 4.6 Binding of SEB to monocytes from a secretor (S) and a non-secretor (NS) donor (data are mean of 2 experiments).

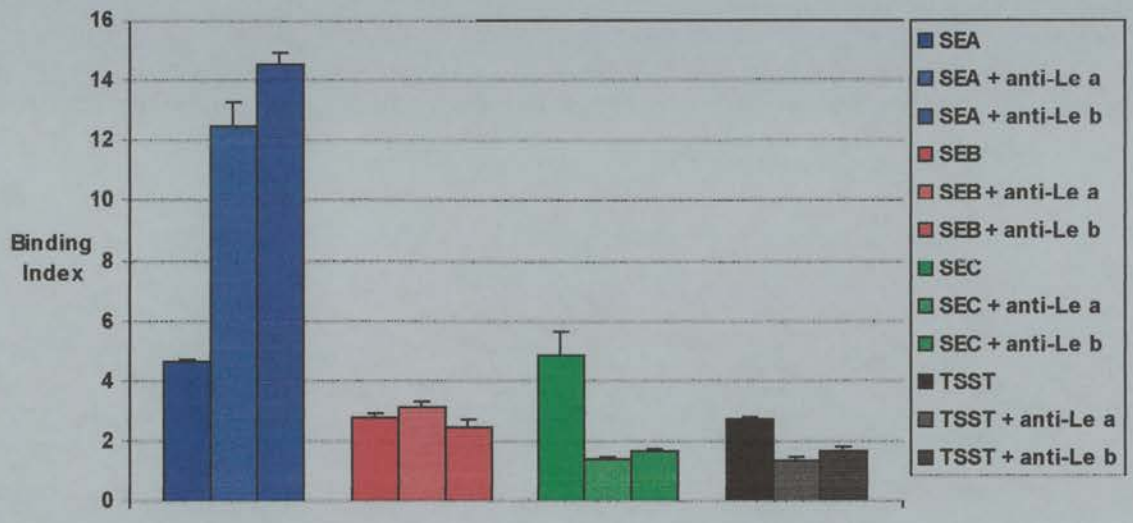


Figure 4.7 Binding of staphylococcal toxins to monocytes pre-treated with antibodies to the Lewis antigens.

4.3.4 Inhibition of toxin binding by antibodies to the Lewis antigens.

In 4 experiments using fixed cells, pre-treatment of monocytes with anti-Lewis^a or anti-Lewis^b caused the greatest reduction in binding of SEC_1 (20 μg ml^{-1}) (70.93% and 66.21% respectively), the level of TSST-1 (20 μg ml^{-1}) binding was also reduced, though to a lesser extent (50.94% and 39.63% respectively). Binding of SEA (0.5 μg ml^{-1}) was greatly increased on the monocytes incubated with anti-Lewis^a and anti-Lewis^b (168.43% and 213.21% respectively), the binding index for SEB (1 μg ml^{-1}) remained similar in both control and test samples (non-significant), this result does not support the findings of the binding assay for SEB to secretor and non-secretor monocytes. All samples tested were from secretor donors (n=8) expressing both Lewis^a and Lewis^b (Figure 4.7).

4.3.5 Production of nitric oxide in response to staphylococcal toxins.

Increased nitric oxide levels were detectable in all of the SEB challenged samples except the most concentrated (10 μg ml^{-1}) by 48 h. All samples showed moderate to high nitric oxide production by 72 h, the 96 h sample results were similar to those at 72 h. The incubation time for further experiments was 3 days. The peak of nitric oxide production from the monocytes probably occurs between 2 and 3 days with the higher levels in the 72 h sample indicating accumulation of nitrite molecules in the

culture supernatant rather than a delayed response as nitric oxide levels had reached a plateau by 96 h (Table 4.3).

The monocyte challenge assay was used with a range of dilutions of the 4 staphylococcal toxins to determine the optimum concentration of each toxin for induction of nitric oxide. The nitrite levels in test samples were obtained from the optical density of the sample read from the standard curve controls for each experiment (Figure 4.8). Challenge with the staphylococcal toxins was shown to induce production of nitric oxide from human monocytes in a dose dependent manner though the higher toxin concentrations (above 1 $\mu g\ ml^{-1}$) showed lower levels of nitric oxide production. This appeared to be due to the cells dying as cell viability was found to be reduced in these wells. All of the toxins induced similar levels of nitric oxide though the actual amounts produced varied with each monocyte sample. The data in table 4.4 represents the results of experiments with monocytes from 10 different donors.

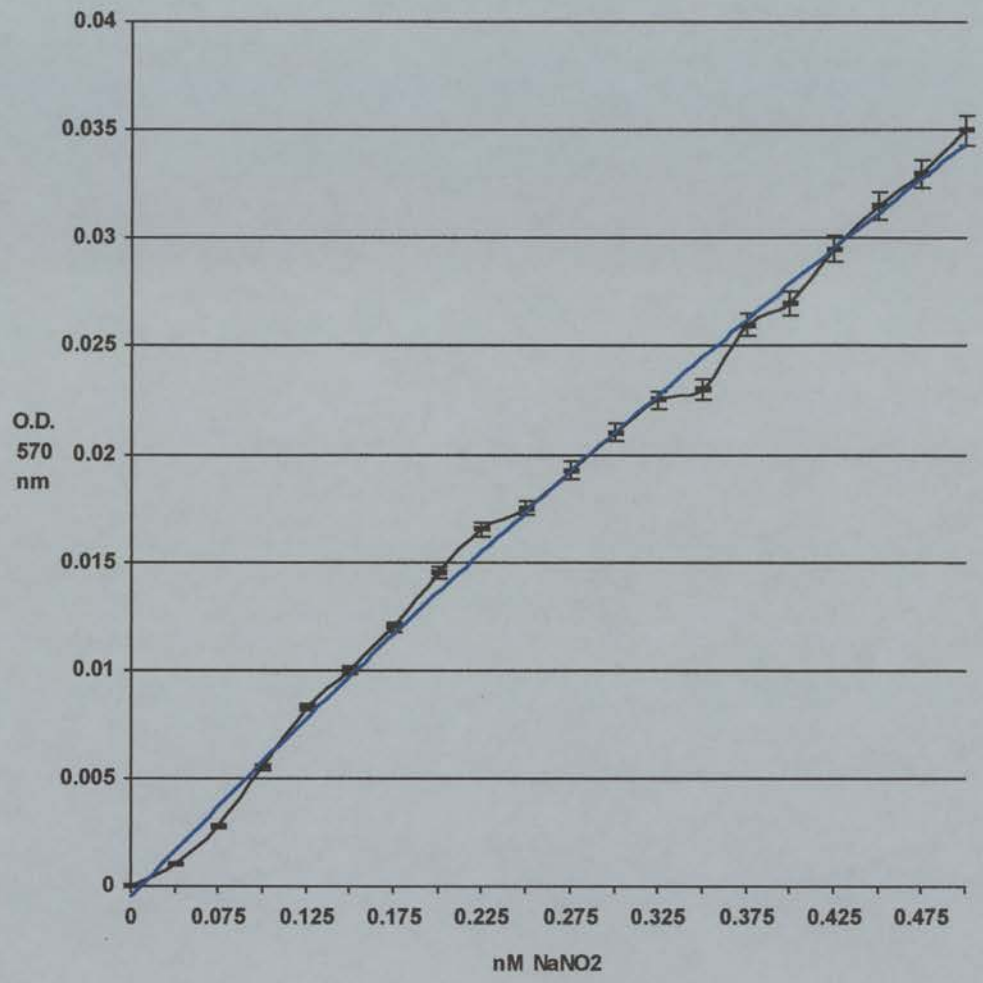


Figure 4.8 $NaNO_2$ standard curve.

Table 4.3 nM NO response to staphylococcal enterotoxin B challenge by monocytes over a 96 hour time course. (Standard deviation figures in brackets. Data are the mean of 4 experiments).

SEB	24 hours	48 hours	72 hours	96 hours
10 μg ml^{-1}	0.000 (0.0000)	0.000 (0.0000)	0.135 (0.0129)	0.135 (0.0071)
5 μg ml^{-1}	0.000 (0.0000)	0.021 (0.0071)	0.146 (0.0057)	0.149 (0.0057)
1 μg ml^{-1}	0.007 (0.0071)	0.014 (0.0000)	0.236 (0.0086)	0.236 (0.0071)
0.5 μg ml^{-1}	0.001 (0.0000)	0.064 (0.0071)	0.311 (0.0186)	0.300 (0.0143)
0.1 μg ml^{-1}	0.000 (0.0000)	0.050 (0.0129)	0.240 (0.0186)	0.250 (0.0114)
0.05 μg ml^{-1}	0.000 (0.0000)	0.007 (0.0071)	0.211 (0.0057)	0.200 (0.0157)

Table 4.4 Nitrite production at 72 hours in response to increasing concentrations of SEA, SEB, SEC_1 and TSST-1. (Mean of 10 donors).

Toxin	Mean nM NO	S.D.
SEA		
2.5 μg ml^{-1}	0.187	0.0143
0.5 μg ml^{-1}	0.232	0.0229
0.25 μg ml^{-1}	0.199	0.0172
0.05 μg ml^{-1}	0.164	0.0143
SEB		
2.5 μg ml^{-1}	0.160	0.0172
0.5 μg ml^{-1}	0.257	0.0143
0.25 μg ml^{-1}	0.199	0.0257
0.05 μg ml^{-1}	0.166	0.0200
SEC_1		
2.5 μg ml^{-1}	0.212	0.0300
0.5 μg ml^{-1}	0.259	0.0386
0.25 μg ml^{-1}	0.194	0.0358
0.05 μg ml^{-1}	0.160	0.0314
TSST-1		
2.5 μg ml^{-1}	0.186	0.0172
0.5 μg ml^{-1}	0.217	0.0272
0.25 μg ml^{-1}	0.204	0.0229
0.05 μg ml^{-1}	0.187	0.0329

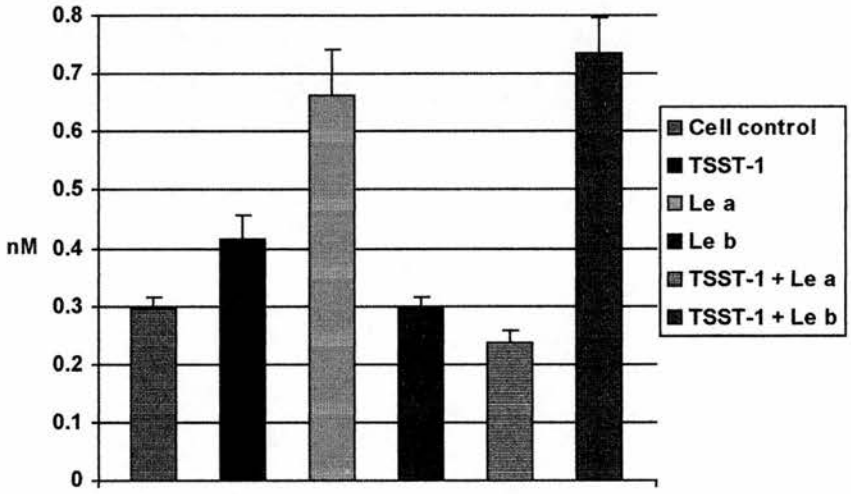


Figure 4.9 Nitric oxide induction by TSST-1 in the presence and absence of synthetic Lewis[a] and Lewis[b] (data expressed as nM NO produced).

4.3.6 Effect of Lewis antigens on induction of nitric oxide by TSST-1.

In experiments with monocytes from 8 different donors, the ability of TSST-1 (0.5 $\mu g\ ml^{-1}$) to induce production of nitric oxide from monocytes was reduced by pre-treatment of the toxin with the Lewis^a antigen (12.5 $\mu g\ ml^{-1}$) (42.36% reduction from TSST-1 control level), the structurally related Lewis^b antigen caused an increase in TSST-1 bioactivity of 78.47%. The Lewis^a control stimulated a higher level of nitric oxide production than the TSST-1 control (an increase of 59.27%) (figure 4.9).

4.4 Discussion

4.4.1 Utilisation of the Lewis antigens as binding sites by the staphylococcal enterotoxins.

It is known that the pertussis toxin of *B. pertussis* uses the Lewis^a and structurally related Lewis^x antigens as cell surface receptors on human monocytes [van t'Wout *et al.*, 1992]. The first objective of this part of the study was to determine if the staphylococcal enterotoxins could utilise the Lewis antigens in a similar way. The flow cytometric assays for binding and inhibition of binding of the toxins by monoclonal antibodies to the Lewis antigens showed that all of the toxins bound to the surface of monocytes, and at the concentrations used, the binding of SEC_1 and

TSST-1 could be reduced by the antibodies to the Lewis antigens, the increased binding of SEA to anti-Lewis^{a} and anti-Lewis^{b} treated cells may be the result of either a conformational change in cell surface receptors caused by binding of the antibodies or an epitope of the antibodies themselves providing a binding site for the toxin. The apparent correlation between binding of SEB at high concentrations (400 and 800 $\mu g\ ml^{-1}$) and expression of Lewis^{a} was not observed in the inhibition assay when the cells were treated with anti-Lewis^{a} and anti-Lewis^{b}, this may have been due to the greatly reduced quantities of toxin used (1 $\mu g\ ml^{-1}$). Studies by Malam *et al.*, 1992 and Newbould *et al.*, 1989, both using fixed human kidney samples, found SEC and TSST-1 in significantly higher percentages of SIDS victims compared with controls (SEC 36%, TSST-1 18%, both SEC and TSST-1 8%). SEA, SEB and SED were not found in the kidney samples [Malam *et al.*, 1992]. Co-production of SEC and TSST-1 is common in strains of *S. aureus* affecting humans [Bohach *et al.*, 1990].

4.4.2 Induction of nitric oxide from monocytes by pyrogenic toxins.

The staphylococcal toxins caused induction of nitric oxide from monocytes in a dose dependant manner though a decrease in NO production was observed at the highest toxin concentration, this could have resulted from the cells dying prior to mounting an inflammatory response.

4.4.3 Effect of the Lewis antigens on induction of nitric oxide by TSST-1.

The third objective was to determine if induction of nitric oxide from monocytes by TSST-1 was affected by reducing the number of available Lewis antigen binding sites on the toxin by pre-treatment with synthetic oligosaccharides of Lewis^a and Lewis^b. Although the synthetic oligosaccharides were capable of stimulating production of nitric oxide on their own, a marked decrease was observed in the ability of TSST-1 to induce nitric oxide production when the toxin was treated with synthetic Lewis^a. This could be interpreted as additional evidence that the toxin is capable of using this antigen as a binding site. Despite sterile conditions and aseptic procedure used in the preparation of the TSST-1/Lewis antigen mixtures, the increase in nitric oxide production in response to the synthetic Lewis^a antigen could have been due to contamination of the sample, though this was not seen in the cells treated with both Lewis^a and TSST-1. Another explanation for this is that binding of the TSST-1 to the synthetic Lewis^a covered the stimulatory epitope on the oligosaccharide. Synthetic Lewis^b appeared to enhance the effect of TSST-1. Further work is required to compare the effects of natural and synthetic oligosaccharides of the Lewis antigens on the staphylococcal toxins to clarify this point.

Because there appeared to be sites on the pyrogenic toxins that bind Lewis^{a}, the possibility of cross-reactivity with the pertussis toxin was considered further in the following chapter.

CHAPTER 5

Antigenic cross-reactivity among bacterial toxins and the protective effect of infant immunisation

5.1 Introduction

Results in chapter 4 indicated that some of the pyrogenic staphylococcal toxins might, like the pertussis toxin, use Lewis^a as a receptor on monocytes. This led to the hypothesis that the apparent protective effect of immunisation with the diphtheria, pertussis, tetanus (DPT) vaccine in relation to SIDS could be due to the presence of similar epitopes on the staphylococcal toxins and pertussis toxin.

Although it was suggested that DPT immunisation was associated with SIDS [Baraff *et al.*, 1983], epidemiological studies in the United States demonstrated that

unimmunised infants or infants immunised late were at increased risk of SIDS [Hoffman *et al.*, 1987; Walker *et al.*, 1987], an increase in SIDS deaths was also noted in Sweden when pertussis immunisation was discontinued [Wennegren *et al.*, 1987]. It has been suggested that some SIDS deaths could be due to asymptomatic whooping cough, if so immunisation would help reduce these deaths.

Although the decline in cot deaths in Britain has been attributed to the campaign started in October 1991 to reduce the prone sleeping position, there was a major change in infant health care practice that affected all infants and predated the "back to sleep" campaign by a year. In October 1990 the infant immunisation schedule was changed to begin at 2 months rather than 3 months. This change appears to coincide with the beginning of the decline in SIDS deaths in Britain. If DPT immunisation played a role in reducing SIDS deaths, it was predicted that there would be a shift in the age range with a decline in the proportion of SIDS deaths occurring in the post-immunisation group (>2 months).

The objectives of the study were:

1. to assess cross-reactivity between the staphylococcal enterotoxins and PT of *B. pertussis* using polyclonal antisera to the enterotoxins;

2. to determine if immunisation with the DPT vaccine induced antibodies that bound to staphylococcal enterotoxins;

3. to determine if pre-incubation with polyclonal anti-DPT could reduce the inflammatory effects of the staphylococcal toxins on human monocytes;

4. to assess the age distribution of SIDS infants before and after the changes in infant immunisation schedules.

5.2 Materials and Methods

5.2.1 ELISA reagents.

The toxins, antitoxins and reagents for the direct and sandwich ELISA protocols are listed in 2.1 and 2.2.

5.2.2 Direct ELISA for detection of toxins.

The direct ELISA to detect the toxins was a two step method in which the toxins were diluted in PBS (2.2.1.2) at pH 8 and bound directly to the wells of a 96 well

ELISA plate (Immulon II, Dynatech). The plate was incubated overnight at 4°C, washed six times with washing buffer (2.2.1.3) on a Dynatech microtitre plate washer, dried and blocked with blocking buffer (2.2.1.4) to reduce non-specific binding. The anti-toxins were diluted in blocking buffer, added to the plate and incubated at 37°C for 45 min. The plate was washed six times and dried. Horseradish peroxidase (HRP) conjugated anti-IgG specific for the species in which the anti-toxin was produced was diluted in blocking buffer and added to the wells (100 µl). The plate was incubated at 37°C for 45 min as above, washed eight times and dried. The OPD substrate (2.2.1.6) (100 µl) activated with hydrogen peroxide was added to all of the wells and the plate incubated in the dark for 15 minutes to allow colour to develop. The enzymatic reaction was stopped with of 12.5% (v/v) sulphuric acid (50µl) and the plate read at 492 nm on a Dynatech MR 700 plate reader. Negative control wells contained PBS, blocking buffer and HRP conjugated antibody.

5.2.3 Sandwich ELISA for detection of toxins.

The polyclonal capture antibody was diluted in coating buffer and distributed in 100 µl volumes into a 96 well ELISA plate. The plate was incubated at room temperature for 2 hours or at 4°C overnight and the primary antibody removed The plate was washed three times and dried. Blocking buffer (100 µl) was added to the wells and the plate incubated at room temperature for 30 min before removing the blocking

buffer and blotting dry. The toxins were diluted in blocking buffer and added to the appropriate wells (100 µl). The plate was incubated at 37°C in an orbital incubator for 45 min before being washed three times to remove unbound antigen and blotted dry. The HRP conjugated antitoxin was diluted in blocking buffer and added to the wells (100 µl). The plate was incubated at room temperature for 2 h or at 4°C overnight before being washed six times and dried as above. The OPD substrate was added and the experiment carried out as in 5.2.2.

5.2.4 Polyacrylamide gel electrophoresis.

The purity of the toxins obtained from Toxin Technology Inc. and Sigma was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) using the method of Laemmli [1970] (2.7.2) to ensure that any cross-reactivity was not the result of any other toxin or toxin fragment being co-purified with the named toxin.

Toxin samples were diluted to 200 µg ml^{-1} except for the pertussis toxin (Sigma) which was left undiluted as the supplied stock concentration was only 50 µg ml^{-1}. The toxin samples were mixed 1:1 with sample buffer (2.7.3.1) and denatured by heating to 100 °C for 10 min. The samples were then placed in the 4% stacking gel (2.7.1.5) wells in 20 µl volumes and run at a constant 60 V until they reached the

interface with the 12% separating gel (2.7.1.5). The constant voltage was increased to 150 V until the dye front had migrated to within 30 mm of the bottom of the gel. The gel was then removed and the toxins stained for using the Coomassie blue staining procedure (2.7.4).

5.2.5 Direct ELISA for cross-reactivity among toxins.

The polyclonal antisera above and an antiserum to SEB (Sigma) at a dilution of 1/100 in blocking buffer were used in an ELISA to assess the degree of cross-reactivity among several bacterial toxins. The following toxins were bound to 96 well microtitre plates in PBS (pH 8) overnight at 4°C: SEB (Sigma),SEA, SEC and TSST-1 (Toxin Technology) (0.5 μg ml^{-1}); PT (Sigma) (2μg ml^{-1}); endotoxin of *E. coli* (026:B6) (Sigma) (1μg ml^{-1}). The plates were blocked with blocking buffer for 30 min at room temperature. The antisera (100μl) were added and the plate incubated at 37°C in an orbital shaker for 1 h. The plates were washed three times with washing buffer, and HRP conjugated sheep anti-rabbit IgG (1/200) was added and incubated overnight at 4°C. The plates were washed six times, the OPD substrate added and the plates read as described above (5.2.2). Negative controls were as above (5.2.2).

Cross-reactivity with heterogeneous toxins was expressed as a percentage of the optical density value of each toxin with its homologous antitoxin (100%). Values ≥ 100 were judged to be evidence of cross-reactivity.

5.2.6 Detection of cross-reactivity among toxins by the sandwich ELISA system.

The results of the direct ELISA system (5.2.4) were compared with those of a sandwich ELISA. The plates were coated overnight at 4°C with rabbit polyclonal antisera to SEA, SEB, SEC and TSST-1 diluted 1/100 in coating buffer. The plates were blocked with blocking buffer for 1 h at room temperature. The toxins, SEA, SEB, SEC, TSST-1(Toxin Technology), PT (Sigma) (0.5 μg ml^{-1}) or LPS (Sigma) (2.5 μg ml^{-1}) were added and the plates incubated at room temperature for 90 min. The plates were washed three times and HRP conjugated rabbit anti-SEA, SEB, SEC, and TSST-1 IgG (1/100) was added. The plates were incubated for 2 h at 37°C and washed six times prior to the addition of the OPD substrate. The plates were read as before and the results expressed as in 5.2.4. Negative control wells contained the capture antisera, blocking buffer and the HRP conjugated antisera.

5.2.7 Production of rabbit anti-DPT serum.

A female New Zealand White (NZW) rabbit was inoculated sub-cutaneously with 5 doses (0.5 ml) of DPT vaccine (Evans) at 1 month intervals. The vaccine contains toxoids of diphtheria, pertussis and tetanus and chemically killed whole cell *B. pertussis.* The rabbit was bled 7 days after the final boost and the serum separated by centrifugation at 1,250 x g for 20 min. The immune serum was stored at -20°C prior to use. Non-immune serum was obtained from the rabbit prior to immunisation and stored under the same conditions.

5.2.8 Detection of antibodies to DPT by ELISA.

Normal rabbit serum and serum from the immunised rabbit were tested in an ELISA system in which 96 well microtitre plates were coated with DPT vaccine diluted 1/40 in coating buffer overnight at 4°C. The plates were washed three times and blocked for 30 min at room temperature with blocking buffer. The serum from the immunised rabbit was diluted in blocking buffer (1/20, 1/50, 1/100, 1/200, 1/400), added to the plate and incubated at 37°C in an orbital shaker for 1 h. After washing three times, HRP conjugated sheep anti-rabbit IgG (1/200) in blocking buffer was added, the plates were incubated overnight at 4°C and washed six times prior to addition of OPD for 10 min in the dark at room temperature. The reaction was

stopped with 12.5% H_2SO_4 and read at 492 nm. The test was compared with identical dilutions of serum obtained from the rabbit prior to immunisation and normal rabbit serum from SAPU. Negative control wells contained the test or control sera plus HRP conjugated sheep anti-rabbit IgG.

5.2.9 Whole cell ELISA with *B. pertussis*.

The whole cell ELISA method described by Abdillahi and Poolman [1988] for typing *N. meningitidis* was adapted for this study. A fimbriate type 1,2 strain of *B. pertussis* 8002 (supplied by Dr. N.W. Preston, Manchester University) was used in these experiments. Wells of ELISA plates were coated with 100 µl volumes of *B. pertussis* (4×10^9 ml^{-1}) in coating buffer for 48 h at 4°C. The plates were washed three times and blocked with blocking buffer for 30 min at room temperature. The polyclonal antisera to the staphylococcal enterotoxins were diluted (1/50, 1/100, 1/200 and 1/250) in blocking buffer, added to the plates and incubated at 37°C for 1 h. The plates were washed three times prior to overnight incubation at 4°C with HRP conjugated anti-rabbit IgG. The plates were washed six times and the substrate added to the plates for 10 min. The reaction was stopped with 12.5% H_2SO_4 and the plates read at 492 nm. Control wells contained antisera to the staphylococcal toxins without bacteria and bacteria with only HRP conjugated anti-rabbit IgG.

5.2.10 Binding of *B. pertussis* typing serum to PT and the staphylococcal toxins.

The direct ELISA for detection of toxins (5.2.2) was modified to assess the binding of *B. pertussis* typing serum (Difco) to PT and the staphylococcal toxins. The plates were coated overnight at 4°C with the toxins diluted to 0.5 μg ml^{-1} in PBS (pH 8). The plates were blocked with blocking buffer for 30 min. at room temperature. Doubling dilutions of the *B. pertussis* typing serum (1/50 to 1/400) were added to the wells and the plates incubated at 37°C for 45 min. The plates were washed six times and dried. HRP conjugated anti-rabbit IgG diluted to 1/100 in blocking buffer (100 μl) was added to the wells and the plate incubated at 37°C for 45 min. as above. The plates was washed eight times and dried prior to addition of the substrate. The enzymatic reaction was stopped after 15 min. with 50 μl of 12.5% sulphuric acid and the plate read at 492 nm as above (5.2.2). Negative control wells contained either toxin, blocking buffer and HRP conjugated anti-rabbit IgG or blocking buffer, typing serum and HRP conjugated anti-rabbit IgG.

5.2.11 Neutralisation of toxin bioactivity using antibodies to TSST-1 and the DPT vaccine.

The monocyte challenge assay (2.4) was modified to assess the ability of rabbit polyclonal anti-TSST-1 (Toxin Technology) and the polyclonal antiserum raised to the DPT vaccine to neutralise the bioactivity of the staphylococcal enterotoxins. The toxins were diluted in DMEM with no additives to a working concentration of 4 μg ml^{-1} and incubated overnight at 37°C with equal volumes of polyclonal anti-TSST-1 (1/100) in DMEM or serum from the rabbit immunised with DPT (1/50) in DMEM. Pre-bleed serum (1/50) in DMEM was used as a negative control. The toxin/antibody mixtures were added in 0.5 ml volumes to the wells of a 24 well tissue culture plate containing 1 x 10^6 monocytes in 0.5 ml of DMEM with additives plus 15% FCS to give a final dilution of 1 μg ml^{-1} toxin and 1/400 anti-TSST-1. The plates were incubated for 3 days at 37°C in a 5% CO_2 atmosphere. The culture supernatants were centrifuged at 1,250 x *g* for 10 min to remove cellular debris and stored at -20°C prior to determination of nitric oxide content.

The level of neutralisation was calculated as the percentage decrease in NO production in toxin plus antibody samples compared with the toxin plus DMEM control.

5.2.12 Statistical methods.

The data are represented as the mean and standard deviation of the replicate experiments. The χ^2 test for trend was used to assess changes in age distribution among SIDS infants between 1988 and 1994. Data for the South East of Scotland was provided by the Scottish Cot Death Trust.

5.3 Results

5.3.1 Assessment of toxin purity.

The SDS polyacrylamide gels run to assess the purity of these toxin samples showed clear major bands for all four of the toxins. Very faint secondary bands were observed just below the SEB and SEC_1 bands. Only one band was visible in the SEA and TSST-1 samples. Molecular mass markers were not run with the gels as the masses of the staphylococcal enterotoxins are well documented [Johnson *et al.* 1991]; however, the relative positions on the gel of the toxin bands conformed to their relative molecular masses: SEA (27.8 kDa); SEB (28.3 kDa); SEC_1 (26.0 kDa); TSST-1 (24.0 kDa). Pertussis toxin (Sigma) was also run but at a lower concentration (25 $\mu g\ ml^{-1}$) due to the concentration of the original stock solution

being less than that of the Toxin Technology samples (100 μg ml^{-1}). The pertussis toxin sample produced 3 very faint bands, the middle band being of a similar molecular mass to TSST-1 (24 kDa) (figure 5.1).

5.3.2 Binding of antisera to toxins assessed by ELISA.

The results of 6 experiments in which the toxins were bound directly to the ELISA plate are summarized in Table 5.1. Anti-SEA showed significant cross-reactivity with TSST-1. Anti-SEC_1 showed significant cross-reactivity with SEB. No significant cross-reactivity was observed with other heterologous toxin/antitoxin combinations. None of the anti-toxins produced values greater than 100 in assays with endotoxin. For PT the only anti-toxin with O.D. values similar to those for the homologous toxin was anti-TSST-1.

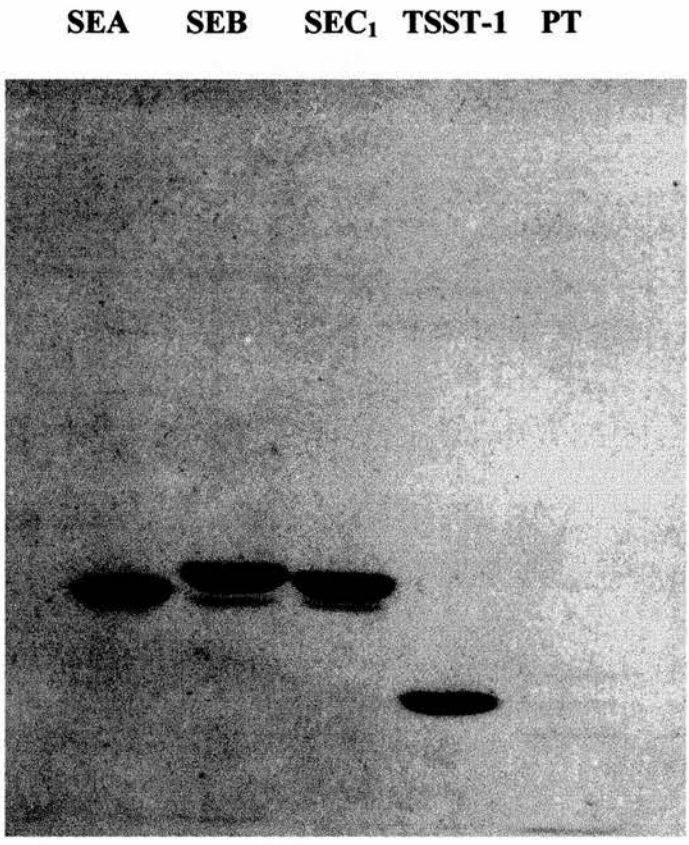


Figure 5.1 SDS polyacrylamide gel electrophoresis to ascertain the purity of the staphylococcal enterotoxins and pertussis toxin stained with Coomassie brilliant blue.

Table 5.1 Cross-reactivity of the staphylococcal enterotoxins, pertussis toxin and lipopolysaccharide expressed as % O.D. of toxin plus homologous anti-toxin. (Data are mean of six experiments).

Anti-toxin	Toxin	Mean %	Range	S.D.
Anti-SEA	SEA	100.0	-	-
	SEB	**107.1**	52.6 - 211.1	55.7
	SEC	73.9	45.7 - 96.4	22.7
	TSST-1	**114.2**	96.4 - 125.6	**12.1**
	PT	63.3	17.2 - 92.3	28.1
	LPS	39.2	24.4 - 99.8	26.3
Anti-SEB	SEB	100.0	-	-
	SEA	70.0	44.4 - 140.0	37.9
	SEC	71.8	22.9 - 157.5	47.6
	TSST-1	78.9	44.2 - 167.5	47.3
	PT	46.3	17.7 - 89.0	27.5
	LPS	49.7	26.2 - 99.1	26.5
Anti SEC	SEC	100.0	-	-
	SEA	97.6	70.0 - 107.3	38.3
	SEB	**245.0**	65.0 - 339.8	**114.6**
	TSST-1	**124.9**	87.3 - 168.6	30.5
	PT	77.6	33.3 - 125.0	33.7
	LPS	80.4	26.2 - 140.0	39.7
Anti-TSST-1	TSST-1	100.0	-	-
	SEA	**114.8**	32.7 - 188.0	59.1
	SEB	**120.5**	47.1 - 187.0	49.1
	SEC	82.7	40.4 - 148.0	38.8
	PT	**108.2**	62.0 - 164.0	45.4
	LPS	79.6	32.0 - 172.0	50.4

5.3.3 Sandwich ELISA.

The results of 6 experiments using the sandwich ELISA system are summarised in Table 5.2. With this method no cross-reactivity was detected. The only antibodies binding a non-homologous toxin to any degree were anti-SEB with SEC_1 (37.18%) and anti-SEC_1 with SEB (5.78%), for which the greatest binding was observed in the direct ELISA. No reaction was observed between pertussis toxin or LPS and any of the antisera to the staphylococcal enterotoxins.

5.3.4 Binding of anti-DPT antiserum to the DPT vaccine and the staphylococcal toxins.

The OD value obtained for anti-DPT at a dilution of 1/100 and the vaccine used to immunize the rabbit was taken as 100%. Binding of non-immune rabbit sera to the toxins was minimal with OD readings similar to that of the wells containing coating buffer alone (1.2 to 4.5%). Figure 5.2 summarizes the results as the means of 6 experiments. OD values for binding of the immune rabbit serum to the staphylococcal toxins was at least 60% that of binding to the vaccine at a dilution of 1/100 and the highest binding was observed for TSST-1 (104.19% and 53.25% at antibody dilutions of 1/100 and 1/200 respectively).

Table 5.2 Cross-reactivity of the staphylococcal enterotoxins, pertussis toxin and LPS by sandwich ELISA. (Data are mean of 6 experiments expressed as % O.D. of toxin plus homologous anti-toxin).

	SEA	SEB	SEC_1	TSST-1	PT	LPS
Anti-SEA	100.0	-	-	-	-	-
Anti-SEB	-	100.0	37.18	-	-	-
Anti-SEC_1	-	5.78	100.0	-	-	-
Anti-TSST-1	-	-	-	100.0	-	-

5.3.5 Binding of staphylococcal anti-toxins to *B. pertussis*.

The antisera to the staphylococcal toxins were examined in the whole cell ELISA with *B. pertussis* to determine if binding to the DPT vaccine was due to cross-reactive antigens on the surface of *B. pertussis*. The OD value obtained with the *B. pertussis* typing serum at a dilution of 1/50 was taken as 100%. Figure 5.3 summarizes the results of 6 experiments. Among the antisera to the staphylococcal toxins, the antiserum to SEC_1 showed the greatest binding (86.8% at 1/200); the other antisera also showed moderate to high levels of binding. There was a steady decrease in binding of anti-TSST-1 to the bacteria with increasing dilution of the antiserum.

5.3.6 Binding of the *B. pertussis* typing serum to PT and staphylococcal toxins.

Figure 5.4 summarizes the results of 5 experiments. The O.D value at 492 nm obtained for binding of the typing serum at a dilution of 1/50 to PT was taken as 100%. At a dilution of 1/50, the highest binding of the typing serum was obtained with the PT. At higher dilutions, binding of anti-*B. pertussis* typing serum was greatest with SEA at 1/100 (72.07%) and with SEB at dilutions of 1/200 and 1/400 (69.81% and 96.98% respectively).

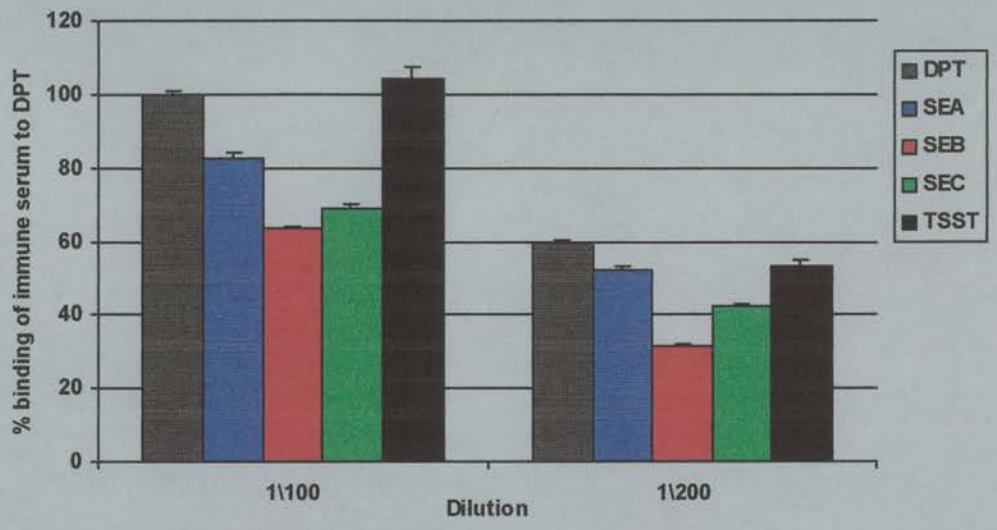


Figure 5.2 Binding of serum from a rabbit immunised with DPT to the staphylococcal toxins and the DPT vaccine.

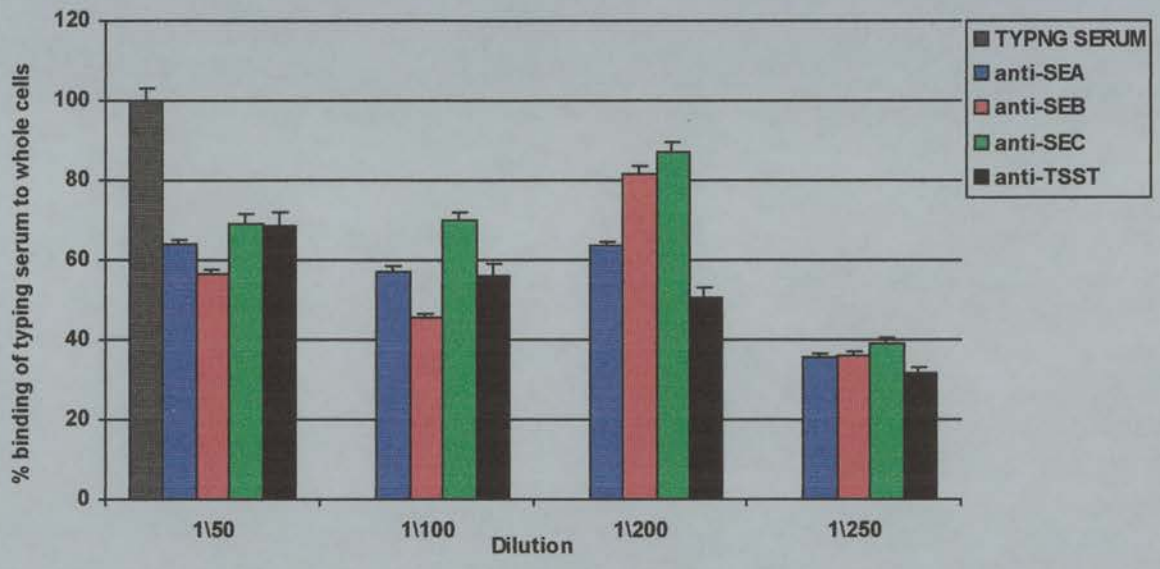


Figure 5.3 Binding of antiserum to the staphylococcal toxins to whole cell *B. pertussis*. Typing serum to *B. pertussis* was used as the positive control (100%).

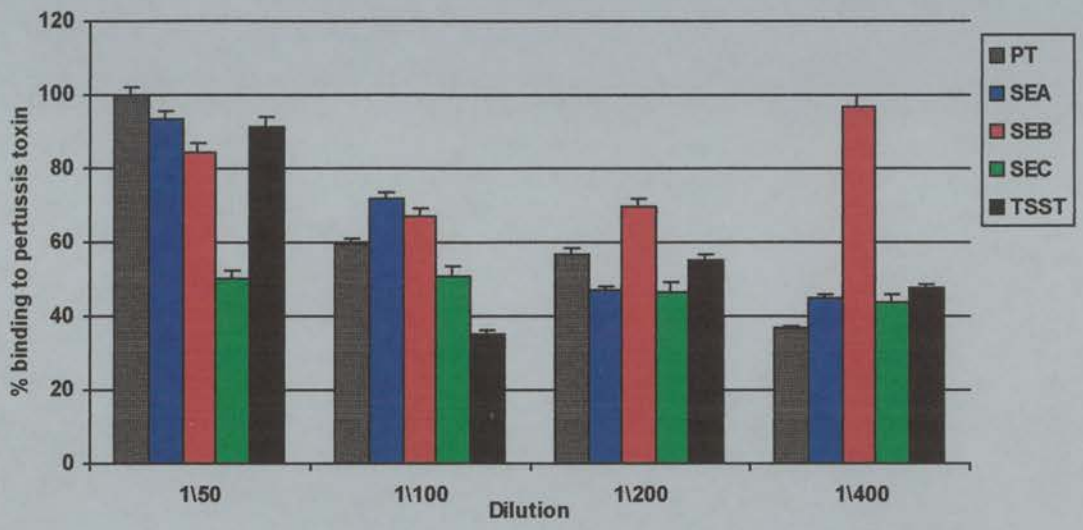


Figure 5.4 Binding of *B. pertussis* typing serum to pertussis toxin and the staphylococcal toxins. Binding of the serum to pertussis toxin was used as the positive control (100%).

5.3.7 Neutralisation of the staphylococcal enterotoxins with anti-TSST-1.

The results of 6 experiments are summarised in figure 5.5. The level of nitrite obtained with toxin alone was taken as 100%. The levels of nitrite obtained with the toxins treated with anti-TSST-1 (1/400) were expressed as the percentage of their respective controls. With the exception of SEC_1 all of the staphylococcal enterotoxins showed a reduction in their ability to induce production of nitric oxide from human monocytes when pre-incubated with polyclonal anti-TSST-1. The neutralising effect of the antibody was greatest with its homologous toxin (26.29% reduction) though it showed only a minor reduction in the bioactivity of SEC_1 (1.05% reduction). SEA and SEB activities were reduced by 16.39% and 23.40% respectively.

5.3.8 Neutralisation of the staphylococcal enterotoxins with anti-DPT.

The results of 4 experiments are summarised in figure 5.6. The polyclonal antiserum to the DPT vaccine was used at a dilution of 1/50, rather than 1/100 as used in the ELISA to maximise any neutralising effect on the toxins, it reduced the ability of the toxins to induce nitric oxide production. The greatest reduction was again observed with TSST-1 (52.65%) though all of the toxins showed higher levels of neutralisation

than with anti-TSST-1, SEA (17.12%), SEB (36.61%) and SEC_1 (30.84%). The normal rabbit serum showed no ability to neutralise the effects of the staphylococcal toxins. The antibody alone controls (monocytes plus anti-TSST-1 and monocytes plus anti-DPT) showed very little activity above baseline nitric oxide levels produced by monocytes in the medium alone.

5.3.9 Age distribution of SIDS infants following changes in the DPT immunisation schedule.

The number of SIDS deaths in Scotland from 1980-1994 is shown in figure 5.7 (data supplied by the Scottish Cot Death Trust). The incidence showed little fluctuation over the decade until 1990-91 when there was a sharp drop in the number of cot deaths. Table 5.3 shows the age distribution of SIDS cases in southeast Scotland (Lothian, Borders and Fife regions). Although there has been a decrease in the total number of SIDS deaths since 1990, the proportion of infants aged 2 months or less has increased from 11% in 1988 to 56% in 1994 ($P<0.001$).

Figure 5.5 Neutralisation of the staphylococcal toxins using monoclonal anti-TSST-1 (data shown as % of toxin alone control).

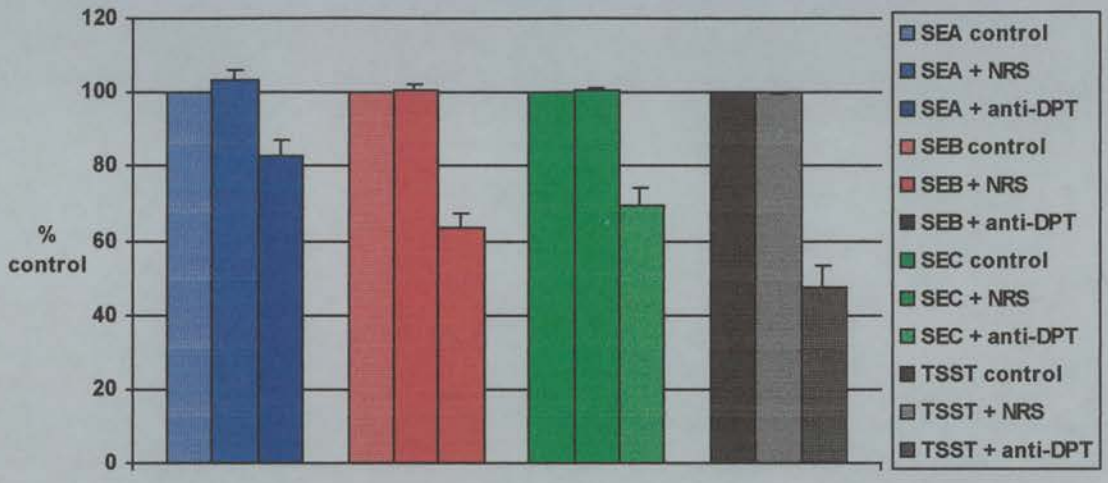


Figure 5.6 Neutralisation of the staphylococcal toxins using a rabbit polyclonal antiserum to the DPT vaccine (data shown as % of control levels).

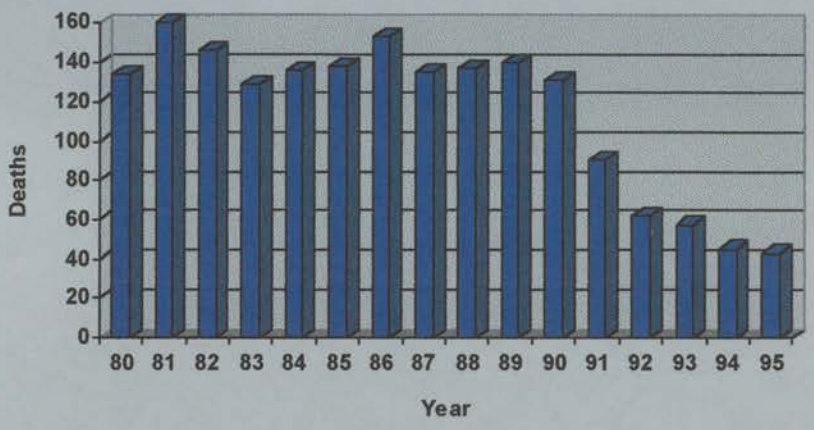


Figure 5.7 SIDS incidence in Scotland, 1980-1995.

Table 5.3 Age distribution of SIDS cases in southeast Scotland.

Year	Total	≤ 2 Months No. (%)	> 2 Months No. (%)
1988	28	3 (10.7)	25 (89.3)
1989	38	6 (15.8)	32 (84.2)
1990	40	12 (30)	28 (70)
1991	17	4 (23.5)	13 (76.5)
1992	13	5 (38.5)	8 (61.5)
1993	17	7 (41.2)	10 (58.8)
1994	16	9 (56.3)	7 (43.6)

χ^2 test for trend = 14.37, P<0.001.

5.4 Discussion

5.4.1 Cross-reactivity between toxins.

The first objective of the study was to assess cross-reactivity among staphylococcal toxins and pertussis toxin as it and the staphylococcal toxins appear to have epitopes that bind the Lewis^a antigen [van t'Wout *et al.*, 1992; chapter 4]. The findings of cross-reactivity in the direct ELISA between the staphylococcal enterotoxins are supported by evidence of similarities in their sequence and structure which indicates that they fall into two groups: SEA, SED and SEE make up one group; SEB, SEC_1 and SEC_3 form the other [Marrack and Kappler, 1990]. It is known that the members of both groups share a high amino acid sequence homology: SEA, D, E >90% [Marrack and Kappler, 1990]; SEB, SEC_1 65% [Johnson *et al.*, 1991]. TSST-1 exhibits a secondary structure similar to that of the other staphylococcal enterotoxins (low α-helix and high β-pleated sheet content) [Chou and Fasman, 1973], the published three-dimensional structures of SEB and TSST-1 [Swaminathan *et al.*, 1992 and Sridhar Prasad *et al.*, 1993] also show similarities, though TSST-1 shares little primary sequence with the other staphylococcal superantigens (40-45%) [Micusan and Thibodeau, 1992].

The degree to which polyclonal anti-*B. pertussis* binds epitopes on the staphylococcal enterotoxins appears to depend on the concentration of the antibody. Its binding most to SEB at higher dilutions suggests that it is most cross-reactive with this toxin rather than with SEA; however, it is possible that different components of the antiserum are recognising different epitopes on the toxins. Throughout the range of dilutions, the antiserum to SEC_1 was found consistently to produce the highest O.D. values of the antisera to the staphylococcal toxins with whole cell *B. pertussis* (Figure 5.3).

5.4.2 Comparison of ELISA techniques.

The differences found between the direct and sandwich ELISA techniques for detection of antibody cross reactivity do not agree with the findings of Fey *et al.* [1984] whose comparisons of different ELISA systems for the detection of single staphylococcal toxins in food samples found the double-antibody sandwich technique to be superior to other versions of the ELISA. The higher levels of cross-reactivity detected by the direct ELISA is not an artifact of non-specific binding as negative control levels were similarly low in both systems. Agreement was seen between the two ELISA systems in showing cross reactivity between SEB and SEC_1 (Tables 5.1 and 5.2). The results of the direct ELISA are also supported by the observed neutralisation of the staphylococcal toxins by anti-TSST-1 and the

antiserum to DPT (Table 5.1 and Figures 5.4 and 5.5). Because of its higher specificity, the sandwich ELISA has been applied successfully to other studies for detection of staphylococcal toxins in tissues from SIDS infants [Zorgani *et al.*, 1997].

5.4.3 Induction of cross-reactive antibodies by DPT immunisation.

Reports from the early 1980's suggested that immunisation for DPT might be associated with SIDS [Baraff *et al.*, 1983]; however, extensive epidemiological investigations found that immunisation reduced the risk of SIDS [Walker et al., 1987; Hofmann *et al.*, 1987]. The second objective of this study was to investigate how DPT immunisation could reduce the risk of SIDS.

As *B. pertussis* is not commonly isolated from infants and there are no reports of it being found in SIDS cases [Mackenzie *et al.*, 1996], it is possible that induction of antibodies cross-reactive with the staphylococcal toxins is the cause of the observed protective effect of DPT immunisation. In this study, the rabbit polyclonal anti-DPT was far more reactive with TSST-1 than with any of the other staphylococcal enterotoxins. Anti-TSST-1 was the only one of the antisera to the enterotoxins which appeared to bind as well to PT as to its homologous antigen (Table 5.1) indicating the presence of several shared epitopes on these toxins. The antiserum to SEC_1 demonstrated the highest levels of binding to whole cell *B. pertussis*. The vaccine

appears to have epitopes cross-reactive with the two staphylococcal enterotoxins most commonly isolated from SIDS cases [Malam *et al.*, 1992; Newbould *et al.*, 1989]. Antibodies to the staphylococcal toxins might be induced by cross-reactive epitopes on the pertussis, tetanus or diphtheria toxoids. Alternatively, they might be induced by non-specific stimulation of B cells by the endotoxin in the whole *B. pertussis*. Experiments in which individual toxoids, acellular pertussis vaccine and cells of *B. pertussis* are used as individual immunogens are needed to answer these questions.

5.4.4 Neutralisation of staphylococcal toxin bioactivity by antisera to TSST-1 and the DPT vaccine.

Neutralisation of SEA and SEB but not SEC_1 by the antisera to TSST-1 (Figure 5.5) supports the results of the direct ELISA (Table 5.1). The evidence gained from the neutralisation assays showed that the polyclonal anti-DPT partially neutralises the ability of all of the pyrogenic staphylococcal enterotoxins tested to elicit nitric oxide production from monocytes, this was not observed with samples treated with normal rabbit serum (Figure 5.4).

5.4.5 Age distribution of SIDS cases following changes in the DPT immunisation schedule.

Although the decline in SIDS has been attributed to the campaign to discourage the prone sleeping position, the decrease in SIDS in Scotland began before the "back to sleep" campaign was initiated in England and Wales in the autumn of 1991 [Gibson *et al.*, 1991]. The major change in infant care practices in all areas of Britain occurred in October 1990, initiation of immunisation for DPT at the age of 2 months instead of 3. This preceded the back to sleep campaign by a year. There have also been increases in the proportion of infants immunized for pertussis and a decrease in attendance at infant welfare clinics which acted as major opportunities for cross infection [Southall *et al.*, 1993].

There has been a decrease in the total number of SIDS cases in Southeast Scotland; but, the proportion of SIDS cases aged less than or equal to 2 months has significantly increased from 11% in 1988 to 56% in 1994 (χ^2 = 14.37, P<0.001). These results indicate that the older age group is now better protected against SIDS. The contributions of immunisation and the back to sleep campaigns need to be assessed in detail in relation to reduction in SIDS cases.

The earlier immunisation of infants may prevent a small number of deaths by asymptomatic pertussis suggested by epidemiological studies [Nicoll and Gardner,

1988; Lindgren *et al.*, 1997]; however, as there are no reports of isolation of *B. pertussis* from SIDS infants or identification of pertussis toxin in their tissues, it is possible that the protective effect of DPT vaccination on SIDS is due to induction of antibodies which are capable of partially neutralising the effects of the pyrogenic staphylococcal toxins which have been identified in tissues of SIDS victims.

CHAPTER 6

Synergy between bacterial toxins in induction of inflammatory response from human monocytes

6.1 Introduction

If a single micro-organism were responsible for sudden infant death syndrome, there should be temporal or geographical clusters of these infant deaths associated with outbreaks of the disease due to the organism in the community [Golding *et al.*, 1985]. Except for an association between the presence of respiratory syncytial virus infection among pre-school children [Williams *et al.*, 1984] and studies in Norway in which there is an association with pertussis infection in the community [Lindgren *et al.*, 1997], there is no strong epidemiological evidence for the involvement of a single infectious agent in SIDS.

The work of Oppenheim *et al.* [1994] has implicated Gram-negative bacteria by demonstrating raised levels of IgM to endotoxin core in SIDS infants, particularly in infants under 3 months of age. Studies in which the chick embryo model was used to examine the effect of bacterial toxins on viability indicated that there might be synergistic effects between products of *S. aureus* and *E. coli* [Drucker *et al.*, 1992; Sayers *et al.*, 1995a]. In a rabbit model the pyrogenic toxins of staphylococci have been observed to enhance the effect of endotoxin by 100,000 fold [Bohach *et al.*, 1990]. Both staphylococci and *H. influenzae* are often isolated from SIDS infants [Telford et al., 1989] and also from healthy infants and their mothers in studies of factors influencing bacterial carriage [Mackenzie *et al.*, 1996]. It has been observed that strains of *S. aureus* which colonise humans commonly produce two or more toxins, particularly TSST-1 and SEC_1 [Bohach *et al.*, 1990].

Evidence from animal models indicates that TNF-α and nitric oxide are involved in killing of virus infected mice by sub-lethal concentrations of staphylococcal enterotoxin B and that a substantial degree of protection is afforded by treatment with either dimeric soluble recombinant human TNF receptor-Fc fusion protein or the nitric oxide synthase inhibitor aminoguanidine. This protective effect was not observed with concomitant injection of anti-recombinant human interferon-γ [Sarawar *et al.*, 1994].

It has been suggested that some cases of SIDS might be due to induction of nitric oxide or TNF-α from host cells by pyrogenic toxins or the effect of these toxins being enhanced by other pathogenic substances including viruses, cigarette smoke and small amounts of endotoxin [Blackwell *et al.*, 1992; Blackwell *et al.*, 1995].

The objectives of this part of the project were:

1. To assess production of nitric oxide and TNF-α from human monocytes following exposure to pyrogenic toxins or LPS;

2. To assess the effects of combinations of these toxins in order to test the hypothesis that there are additive or synergistic effects between the toxins similar to those observed with the chick embryo and mouse models.

6.2 Materials and Methods

6.2.1 Monocyte purification.

Human peripheral blood monocytes were purified from buffy coats as described in section 2.3.2.

6.2.2 Monocyte challenge assay.

Monocyte suspensions (0.5 ml) were added to the wells of a 24 well tissue culture plate and incubated at 37°C for 15 min. The staphylococcal enterotoxins SEA, SEB, SEC_1 and TSST-1 or lipopolysaccharide (LPS) from *E. coli* serotype 026:B6 were diluted in DMEM without additives to give a final dilution at the optimum concentration for each toxin assessed by dose response (Tables 4.3 And 6.4). The monocytes were challenged with either 0.5 ml volumes of single staphylococcal toxins (1 μg ml^{-1}), 0.25 ml volumes of paired staphylococcal toxins (2 μg ml^{-1}) or 0.25 ml volumes of staphylococcal toxins (2 μg ml^{-1}) plus 0.25 ml volumes of LPS (0.4 μg ml^{-1}) (2.4.1). The cells were incubated at 37°C in 5% CO_2 and sampled at various intervals. The culture supernatants were removed and centrifuged at 1,250 x

g for 10 min to remove any remaining cells prior to determination of nitrite and TNF-α content.

The time course experiment using SEB (4.2.7) was repeated with sampling of the culture supernatants at 16, 20, 24 and 40 h for detection of TNF-α.

6.2.3 Detection of inflammatory mediators.

Nitric oxide production in response to the toxins was measured by the nitrite assay (2.5.1) and TNF-α by the bioassay with L-929 cells (2.6).

6.3 Results

6.3.1 The effect of endotoxin concentration on nitric oxide production.

Preliminary dose response experiments, similar to those for the staphylococcal toxins, were carried out to ascertain the optimum LPS concentration for induction of nitric oxide from human monocytes (Table 4.3). All of the staphylococcal toxins showed the greatest stimulation of nitric oxide production at a concentration of 0.5 μg ml^{-1} The optimum concentration of LPS was found to be 0.1 μg ml^{-1} (Table 6.1).

6.3.2 Induction of nitric oxide production by combinations of toxins.

When incubated with the staphylococcal enterotoxins and LPS, the levels of nitric oxide production were greater than that of monocytes challenged with the enterotoxins alone (Table 6.2). The effect of the staphylococcal toxin alone was taken as 100% and the effect of addition of LPS (0.1 μg ml^{-1}) expressed as a percentage of the results with the staphylococcal toxin. The greatest increases were seen in the response of monocytes challenged with SEB and SEC_1, an increase of 146.5% and 85.2% respectively. These results show synergy between SEB and LPS, and to a lesser extent between SEC_1 and LPS. A slight (>30%) additive effect was observed between SEA and TSST-1 and LPS. All of these increases were significant according to the standard deviations.

The staphylococcal enterotoxins were also observed to provoke a greater production of nitric oxide when present in combination with other staphylococcal enterotoxins (Table 6.3). TSST-1 appeared to significantly enhance the effects of all of the other toxins based on the standard deviations. This effect was most pronounced with SEB, causing an increase in nitric oxide production of 58.6% over that of SEB alone. Significant enhancement of activity was also observed with combinations of SEA and SEC_1.

6.3.3 The effect of time on TNF-α production.

Initial time course experiments were carried out with SEB. The time course for production of TNF-α was considerably shorter than that for nitric oxide with the highest levels seen after 20 h. The results in Table 6.4 are expressed as the percentage cytotoxicity of the culture supernatants for L-929 cells compared with unchallenged cells. Figure 6.1 shows the TNF-α standard curve produced using a range of dilutions of natural human TNF-α. Below 1 IU ml^{-1} there was little cytotoxic effect on L-929 cells. The significant decrease in active TNF-α levels observed across the concentration range, with the exception of 5 and 1 μg ml^{-1}, from 20 to 24 hours may be due to release into the medium of soluble TNF-α receptors rendering the molecule biologically inactive or the negative feedback effect of interleukin-6 (IL-6) on TNF-α production [Aderka *et al.*, 1989].

Table 6.1 Effect of concentration of endotoxin on release of nitric oxide. (means of 4 experiments).

LPS	Mean nM nitrite	S.D.
1.0 μg ml^{-1}	0.139	0.0114
0.5 μg ml^{-1}	0.182	0.0272
0.1 μg ml^{-1}	0.247	0.0114
0.05 μg ml^{-1}	0.200	0.0172
0.01 μg ml^{-1}	0.129	0.0257

Table 6.2 NO production by monocytes challenged with enterotoxins and enterotoxins plus LPS. (Data are mean of 6 expts.)

Toxin	Control %	Toxin + LPS %	S.D.
SEA	100	128.0 (126.5-130.0)	1.4800
SEB	100	246.5 (216.6-282.9)	21.0735
SEC_1	100	185.2 (154.4-215.8)	30.2812
TSST-1	100	116.7 (111.4-121.3)	4.1751

Table 6.3 nM nitrite production by human monocytes in response to challenge with staphylococcal enterotoxins and combinations of enterotoxins.

(S.D. in brackets Data are mean of 4 experiments).

	SEA	**SEB**	**SEC**	**TSST-1**
SEA	0.250 (0.0143)	0.260 (0.0071)	0.325 (0.0071)	0.300 (0.0143)
SEB		0.200 (0.0071)	0.225 (0.0071)	0.350 (0.0000)
SEC			0.225 (0.0214)	0.325 (0.0500)
TSST-1				0.260 (0.0214)

Table 6.4 TNF-α response to staphylococcal enterotoxin B challenge by monocytes over a 40 hour time course. Data expressed as % killing of L929 cells compared with controls to which no toxin was added. (S.D. in brackets. Data are mean of 3 experiments).

SEB	16 hours	20 hours	24 hours	40 hours
10 μg ml^{-1}	0.463 (0.4374)	13.00 (2.0127)	8.19 (2.1496)	1.71 (1.7136)
5 μg ml^{-1}	3.01 (0.8935)	13.52 (1.8451)	13.29 (1.4103)	4.87 (1.2938)
1 μg ml^{-1}	2.08 (0.4818)	22.43 (2.3853)	21.07 (0.7332)	2.47 (0.4489)
0.5 μg ml^{-1}	4.65 (0.4786)	44.41 (2.8313)	36.53 (3.0842)	4.35 (1.4824)
0.1 μg ml^{-1}	3.67 (0.4391)	36.15 (4.5424)	25.89 (2.7094)	6.49 (1.1268)
0.05 μg ml^{-1}	3.52 (0.5206)	21.85 (0.6923)	14.37 (3.0491)	1.00 (0.1746)

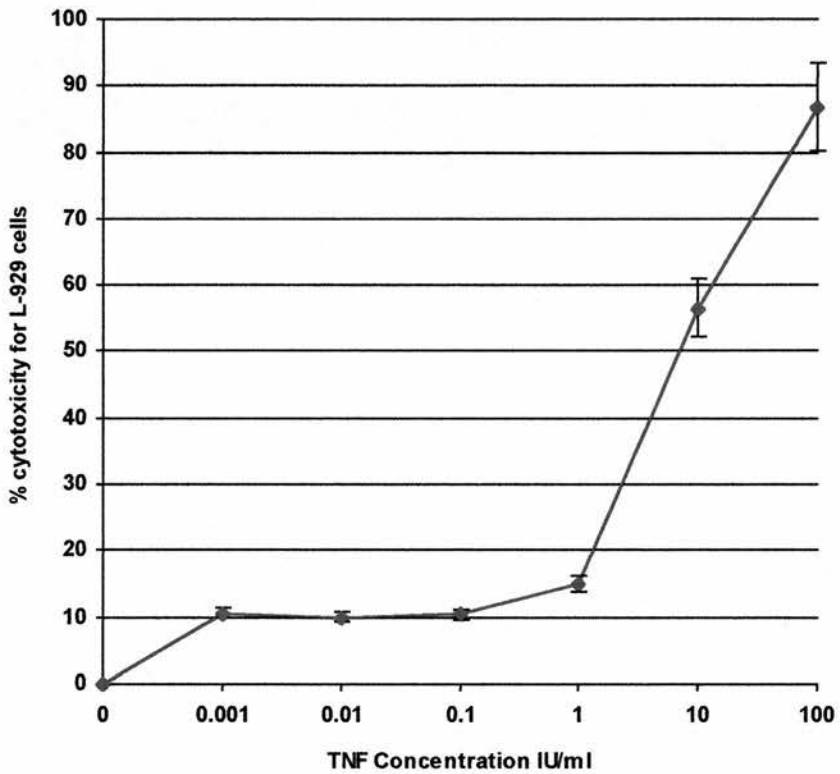


Figure 6.1 TNF-α standard curve at 20 h (mean and standard deviation of 4 experiments).

6.3.4 The effect of toxin concentration on TNF-α production.

The pattern of production of TNF-α from monocytes in relation to dose varied with different staphylococcal toxins. The data in Table 6.5 represents the means of 3 experiments and is expressed as the percentage cytotoxicity for L-929 cells of supernatant from monocytes treated with toxin compared with unchallenged monocytes. SEA, SEB and SEC_1 showed a decrease in induction of TNF-α with increasing dose across the concentration range tested, only TSST-1 showed an increase with dose. For SEB and SEC_1 the greatest cytotoxicity was observed at a concentration of 0.5 μg ml^{-1} as in the nitric oxide studies. For SEA the maximum effect was observed at 1 μg ml^{-1}; but for TSST-1 the percentage killing was greatest at 5 μg ml^{-1}. There were far greater variations in the raw data for TNF-α than in that for nitric oxide production. The dose response to LPS (Table 6.6) was also less clear than that obtained for nitric oxide with the maximum killing observed with 0.1 μg ml^{-1}. The highest levels of TNF-α production were seen in response to challenge with TSST-1.

Table 6.5 Effect of concentration of staphylococcal enterotoxins on induction of TNF-α production. (Data expressed as average % cytotoxicity for L929 cells and IU ml^{-1} from the standard curve compared with control to which no toxin was added. Data are mean of 3 experiments).

Toxin	Mean % cytotoxicity (range)	S.D.	IU ml^{-1}
SEA			
10 μg ml^{-1}	21.3 (13.68-27.35)	5.6901	3.3
5 μg ml^{-1}	24.97 (21.25-28.06)	2.8156	4.1
1 μg ml^{-1}	50.67 (40.05-59.60)	8.0695	8.0
0.5 μg ml^{-1}	36.97 (29.60-42.10)	5.3432	6.5
0.1 μg ml^{-1}	34.99 (31.52-37.60)	2.5555	5.4
SEB			
10 μg ml^{-1}	11.95 (9.85-13.07)	1.4838	>1
5 μg ml^{-1}	13.52 (10.26-19.25)	4.0645	>1
1 μg ml^{-1}	17.07 (12.85-21.08)	3.3634	1.5
0.5 μg ml^{-1}	36.42 (30.32-42.13)	4.8296	5.7
0.1 μg ml^{-1}	29.89 (24.05-34.25)	4.2936	4.3
SEC_1			
10 μg ml^{-1}	3.44 (1.25-3.97)	1.6158	>1
5 μg ml^{-1}	9.83 (5.25-15.89)	4.4680	>1
1 μg ml^{-1}	10.43 (7.28-13.80)	2.6666	>1
0.5 μg ml^{-1}	38.24 (15.86-51.62)	15.9238	6.1
0.1 μg ml^{-1}	39.08 (19.21-52.35)	14.3116	6.5
TSST-1			
10 μg ml^{-1}	74.39 (65.83-81.05)	6.3571	64.1
5 μg ml^{-1}	78.52 (72.12-84.51)	5.0662	77.0
1 μg ml^{-1}	68.78 (63.55-79.83)	7.1724	47.9
0.5 μg ml^{-1}	57.04 (51.08-62.15)	4.5591	13.1
0.1 μg ml^{-1}	57.72 (46.12-65.86)	8.4233	15.0

6.3.5 Induction of TNF-α production by combinations of toxins.

Table 6.7 represents the results of 4 experiments. An increase in TNF-α levels was observed in monocytes challenged with enterotoxins plus LPS compared with those challenged with the enterotoxins alone (100%). Although the increases were not as great as those observed for nitric oxide, there were significant increases observed with SEB plus LPS (80.3%) and TSST-1 plus LPS (25.2%). Non significant increases were observed for the other enterotoxins plus LPS.

In 4 experiments, production of TNF-α in response to SEB was significantly increased when in combination with SEC_1 and TSST-1. Other combinations, SEA\SEB, SEA\TSST-1, and SEC_1\TSST-1, showed a significant decrease in the observed percentage of killing (Table 6.8).

Table 6.6 Effect of LPS concentration on induction of TNF-α production expressed as average % cytotoxicity for L-929 cells above cell controls. (Data are mean of 3 experiments).

LPS	Mean % cytotoxicity (range)	S.D.	IU ml^{-1}
1.0 μg ml^{-1}	25.83 (15.21-38.91)	6.9235	4.5
0.5 μg ml^{-1}	32.17 (14.11-48.33)	12.3248	5.0
0.1 μg ml^{-1}	33.91 (20.79-42.37)	7.2560	5.2
0.05 μg ml^{-1}	15.85 (3.31-35.86)	10.9381	>1
0.01 μg ml^{-1}	28.76 (12.54-42.59)	11.2517	4.8

Table 6.7 TNF-α production by monocytes challenged with enterotoxins and enterotoxins plus LPS.

Toxin	Control %	Toxin + LPS %	S.D.
SEA	100	104.6	10.1304
SEB	100	**180.3**	**27.3415**
SEC_1	100	101.7	19.2861
TSST-1	100	**125.2**	**7.4375**

Table 6.8 TNF-α production by human monocytes challenged with staphylococcal enterotoxins and combinations of enterotoxins expressed as % cytotoxicity for L929 cells compared with controls to which no toxin was added. (S.D. in brackets. Data are mean of 4 experiments).

	SEA	**SEB**	**SEC**	**TSST-1**
SEA	47.69 (12.1497)	22.27 (6.9020)	43.21 (17.2001)	22.11 (7.4354)
SEB		18.73 (7.0440)	41.07 (3.2796)	55.09 (17.7167)
SEC			47.79 (6.9930)	16.75 (4.6708)
TSST-1				55.92 (11.5554)

6.4 Discussion

The role of monocytes, especially nitric oxide production and the cytokine cascade responses involving tumour necrosis factor-α (TNF-α), interleukin 1 (IL-1) and interleukin 6 (IL-6), are of great importance in combating bacterial infections; however, when not adequately controlled, these inflammatory responses can have potentially fatal effects. Certain conditions may be present that result in an uncontrolled cytokine response leading to the death of the infant.

This study provides evidence that the staphylococcal enterotoxins can induce nitric oxide and TNF-α production from human peripheral monocytes and that they can act with each other and with LPS to produce greater amounts of these inflammatory mediators. These results are consistent with those of studies using the chick embryo model of SIDS [Drucker *et al.*, 1992; Sayers *et al.*, 1995a; 1996] showing similar patterns of synergy between the toxins and LPS; however, use of human peripheral blood monocytes and examination of the inflammatory mediators which animal models suggest are involved is more relevant to SIDS in humans. LPS from *E. coli* was used in this study as epidemiological studies have shown lower levels of IgG and higher levels of IgM to endotoxin core in SIDS infants aged below 3 months [Oppenheim *et al.*, 1994]. This organism is more common in bottle fed infants [Bettleheim, 1990] in the SIDS age range and low concentrations of LPS have previously been shown to enhance the lethality of the staphylococcal toxins in animal

models [Lee *et al.*, 1987; Drucker *et al.*, 1992]. Sayers *et al.* [1995a] showed simultaneous isolation of both staphylococci and enterobacteria from nasopharyngeal swabs of SIDS infants at a ratio of 7:1 compared with healthy infants.

The mean quantities of TNF-α present in the culture supernatants were calculated as their percentage cytotoxicity for L-929 cells. Figures for IU ml^{-1} were derived from the standard curve of the experiments. Results for the percentage cytotoxicity were included in the tables to demonstrate the individual variations in response.

Given that the combination of TSST-1 and SEB provoked such a large increase in nitric oxide production over that of either toxin alone, it is fortunate that co-production of these two toxins is mutually exclusive as SEB producers do not possess the TSST-1 structural gene (*tst*) [Kreiswirth, *et al.*, 1989]. This does not, however, rule out the possibility of two different strains of *S. aureus* being present, one producing TSST-1 and the other producing SEB. The non-significant change in NO production in response to SEA when present with SEB and the very slight rise when present with TSST-1 are also worthy of note as studies on the competitive binding of the staphylococcal toxins using murine fibroblasts have shown that SEA can completely inhibit the binding of SEB [Fraser, 1989] and TSST-1 [Chintagumpala *et al.*, 1991]. It is possible that bound SEA either covers residues on the MHC class II molecule which are essential for SEB/TSST-1 binding or that its binding causes conformational changes in the MHC molecule thus obscuring the SEB/TSST-1

binding sites (Figure 6.2). SEB and TSST-1 have little effect on SEA binding, this suggests the presence of multiple binding sites for SEA on MHC class II molecules. Similar studies have shown that neither SEB or TSST-1 compete with the other toxin for binding sites [Scholl *et al.*, 1989; Chintagumpala *et al.*, 1991] (Figure 6.2). The increased nitric oxide production in response to the SEB/TSST-1 combination is consistent with these findings.

These studies addressed the mechanisms by which combinations of staphylococcal toxins and staphylococcal toxins with endotoxin could produce the results observed in the chick embryo model. The next stage in the study was to assess the effects of virus infection and exposure to cigarette smoke, since nicotine had been shown in the chick embryo model to enhance the lethality of the bacterial toxins.

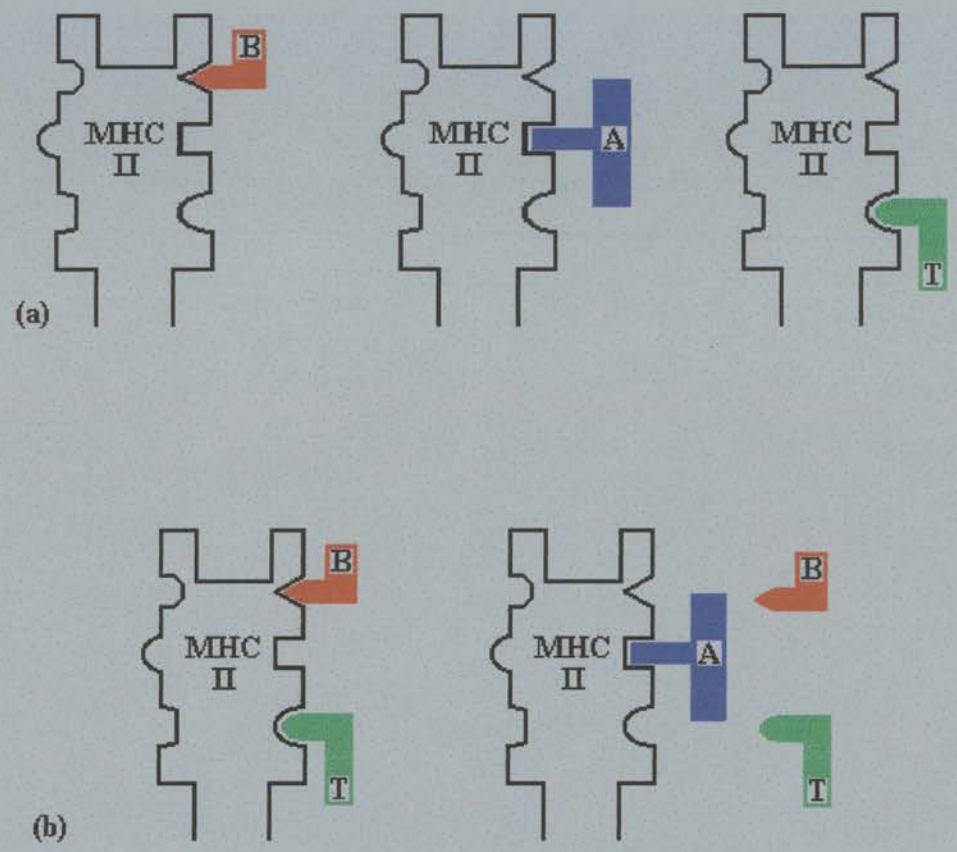


Figure 6.2 Competitive binding of the staphylococcal toxins SEA, SEB and TSST-1: (a) Single toxins appear to bind to discrete sites on the MHC II molecule. (b) When present in combination with SEA binding of SEB and TSST-1 is completely inhibited.

Chapter 7

The effects of cigarette smoke and viral infection on inflammatory responses of human monocytes

7.1 Introduction

The effect of maternal smoking has been identified as one of the major risk factors associated with SIDS but its actual significance has proved difficult to judge in epidemiological studies as it is linked with lower socioeconomic class [Stephenson *et al.*, 1997]. Infants of lower income families are at a higher risk of SIDS for a number of reasons other than the high percentage of mothers who smoke relative to higher income families. The risk factors commonly associated with lower socioeconomic class include poorer housing conditions, larger numbers of siblings, shorter pregnancy interval, younger and unmarried mothers, lower levels of prenatal

care and poorer nutrition. Many of these factors are also found among groups such as native Americans [Gibson, 1995] and Australian Aborigines [Alessandri *et al.*, 1996] in which there are higher levels of bacterial carriage and infection [Todd *et al.*, 1985; Leach *et al.*, 1994].

Evidence for the role of maternal smoking in relation to infection and SIDS can be gained from studying cultures in which smoking is not linked with socioeconomic class. In Greece approximately one third of the female population smoke irrespective of class and educational level. Kremastinou *et al.* [1994] found that there was a significant correlation between smoking by mothers and other household members involved in child care and carriage of meningococci among Greek school children. Smoking could lead to colonisation by potentially pathogenic bacteria by enhancing the incidence of respiratory virus infection. Isolation of pathogenic bacteria such as staphylococci [Musher and Fainstein, 1981] and meningococci [Blackwell *et al.*, 1992] is also higher from smokers themselves, thus providing a reservoir for infection which could be transmitted to their children.

Another problem in determining the role that smoking plays in SIDS is that most of the evidence comes from epidemiological studies in which the data is generally collected retrospectively. This presents the problem of obtaining accurate information on the extent of smoking in the household due to under reporting of the number of cigarettes smoked per day and the fact that it is usually only maternal

smoking habits that are investigated. The presence of other smokers in the immediate or extended family can have a great effect on the amount of cigarette smoke to which an infant is exposed.

Work by Sayers *et al.* [1995b] assessed the effect of nicotine on the lethality of bacterial toxins isolated from SIDS infants in a chick embryo model. Their results showed that even very low concentrations of nicotine could enhance the effects of non-lethal toxin mixtures which resulted in killing of the chick embryos. Their study, as with most experimental models of the effects of tobacco smoke, used purified nicotine which although undoubtedly very toxic, is only one of the many compounds present in tobacco smoke. Assessing the interactions of all of the components of tobacco smoke to enhance or detract from the toxicity of nicotine would provide a more accurate model.

Studies by Sarawar *et al.* [1994], using a mouse model, and Lundemose *et al.* [1993] using human peripheral blood leukocytes indicated that virus infection is also capable of enhancing the effects of endotoxin and the pyrogenic toxins. Both studies found evidence of increased TNF-α production which, when blocked with a soluble TNF-α receptor in the mouse model reduced the lethality of the virus/toxin combination.

The aim of this part of the study was to assess interactions between an infusion of the soluble components of cigarette smoke, the staphylococcal toxin TSST-1 and respiratory syncytial virus (RSV) on induction of TNF-α and nitric oxide, inflammatory mediators implicated in the mouse model [Sarawar *et al.*, 1994].

7.2 Materials and methods

7.2.1 Production of cigarette smoke extract (CSE).

A hand operated vacuum pump was used to draw cigarette smoke through DMEM with no additives or serum. The apparatus consisted of a 250 ml Duran bottle with two holes drilled in the cap. Through one of these holes was placed a length of sterilised tubing with a connection to a piece of larger bore tubing which held the cigarette. Another length of tubing was run from the other hole to the vacuum pump. The cigarette smoke was drawn through the medium by the change in pressure caused by extracting the air from the upper part of the bottle (Figure 7.1). The smoke from 10 cigarettes (Regal King Size) was infused into 100 ml of the medium at a rate of approximately 1 cigarette per 10 min to provide a stock solution of 0.1 cigarette/ml. The medium was filter sterilised using a 0.2 μm Millipore syringe filter and stored at -20°C prior to use.

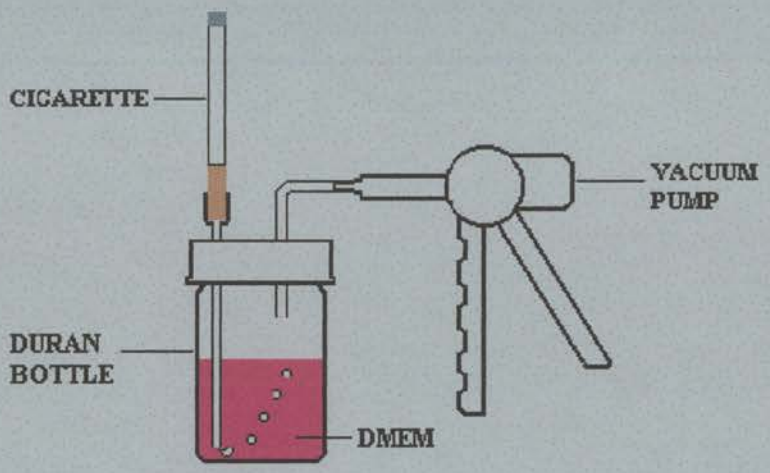


Figure 7.1 Apparatus used in the production of the cigarette smoke extract.

7.2.2 Dose response to CSE.

The monocyte challenge assay (2.4.1) was used to determine the optimum concentration of CSE for use in the study. Human peripheral blood monocytes were purified from buffy coats as described previously (2.3.2), their viability assessed by Trypan blue exclusion, and cultured at 2 x 10^6 cells ml^{-1} (0.5 ml) in 24 well tissue culture plates at 37°C and 5% CO_2. Doubling dilutions of CSE (1/1 to 1/512) were made in DMEM with no additives (0.5 ml) and added to the wells. The plates were then incubated for 72 h at 37°C in a 5% CO_2 atmosphere. The culture supernatants were removed and centrifuged at 1,250 x *g* to remove cellular debris and stored at -20°C prior to determination of nitric oxide content. The optimum concentration of TSST-1 had been determined previously (Table 4.3).

Dose response experiments were also carried out with IFN-γ (Sigma) (25, 50, 100 and 200 IU ml^{-1}), nicotine (Sigma) (50, 100, 200 and 400 μg ml^{-1}) and the nicotine metabolite cotinine (Sigma) (50, 100, 200 and 400 μg ml^{-1}).

7.2.3 Preparation of RSV infected monocytes.

The Edinburgh strain of the respiratory syncytial virus (RSV) (subgroup A) was grown by Dr. M. Raza in 24 h sub-confluent monolayers of HEp-2 cells incubated

with RSV at a multiplicity of infection (MOI) of 2 infectious particles per cell for 1 h. The monolayers were maintained in Eagle's minimum essential medium (Gibco) (pH 7.4) containing $NaHCO_3$ (0.85 g l^{-1}), L-glutamine (2 mM), penicillin (100 IU ml^{-1}), streptomycin (100 μg ml^{-1}) and 10% FCS (v/v) for 48 h. The virus was harvested by freezing at -70 °C and thawing to lyse the cells. The suspension was centrifuged at 700 x *g* for 10 min to remove cellular debris, resuspended in DMEM with additives plus 10% FCS (v/v) and adjusted to 2 x 10^6 plaque forming units ml^{-1}.

Human monocytes obtained from buffy coats (2.3.2) were distributed into the wells of a 24 well tissue culture plate as 1 x 10^6 cells per well in DMEM with additives plus 10% FCS (v/v) (0.5 ml). DMEM with no additives (0.5 ml), RSV (MOI=2) (0.5 ml), or a combination of RSV (MOI=2) and cigarette smoke extract (0.5 ml) were then added to the wells.

The proportion of monocytes infected with RSV in each sample was determined by an indirect immunofluorescence technique using a monoclonal antibody to glycoprotein G of RSV (kindly supplied by Prof. P.J. Watt, Southampton University). Monocyte suspensions (1 x 10^6 cells ml^{-1}) (200 μl) were incubated with anti-glycoprotein G (1/100) (100 μl) for 30 min at 37 °C. The cells were washed 3 times in PBS by centrifugation at 680 x *g* for 7 min. and resuspended in PBS (200 μl). FITC-conjugated anti-mouse IgG (1/200) (100 μl) was added to the suspensions and the cells incubated at 37°C for 30 min prior to washing 3 times in PBS as above. The

cells were resuspended in 200 µl of PBS and fixed with 100 µl of 1% buffered paraformaldehyde. The proportion of fluorescent cells was determined by flow cytometry.

7.2.4 Dose response of RSV infected and non-infected human peripheral blood monocytes to CSE and TSST-1.

The monocyte challenge assay (2.4.1) was used to test the ability of CSE and TSST-1 to induce production of nitric oxide and tumour necrosis factor-α from normal human monocytes and monocytes infected with RSV. Dilutions of CSE and TSST-1 were made in DMEM with no additives. The monocyte suspensions (1 x 10^6 cells/well) were cultured in 24 well tissue culture plates at 37°C in 5% CO_2. After incubation for 24 h, TSST-1 (10 µl at 5 µg ml^{-1}) and/or CSE (100 µl at 0.1 cigarette ml^{-1}) were added to the appropriate test wells by withdrawing 200 µl of the culture supernatant and adding the TSST-1 and/or CSE prior to returning the supernatant to the well. This procedure was carried out to avoid abrupt contact of concentrated toxin with the cells and to ensure a uniform toxin concentration throughout the well. Samples were collected from each well after a further 20 h for TNF-α detection and 72 h for nitric oxide detection, the optimum sampling times having already been determined (Tables 4.3 and 6.4). Negative controls containing no monocytes included DMEM plus CSE and/or RSV. The samples were centrifuged at 1,250 x *g*

to remove cellular debris and stored at -20°C prior to determination of TNF-α and NO content.

7.2.5 Determination of TNF-α content.

The TNF-α activity of the samples was estimated as described in section 2.6. The results are expressed as the percentage cytotoxicity for L-929 cells.

7.2.6 Determination of nitric oxide content.

Nitric oxide production was measured by the nitrite assay (2.5).

7.2.7 Statistical methods.

The data obtained from the test samples were compared with controls containing only medium or other test groups using paired *t* tests.

7.3 Results

7.3.1 Nitrite production in response to CSE.

Dose response experiments found CSE to contain significant levels of nitrites. To discriminate between CSE nitrites and evidence of cellular NO production, a 1/10 dilution of CSE (0.01 cigarettes ml^{-1}) was used in the subsequent experiments (Figure 7.2).

7.3.2 Dose response to interferon-γ, nicotine and cotinine.

In 2 experiments with 4 donors no dose response to IFN-γ, nicotine or cotinine was observed in the concentration levels tested (Table 7.1). Concentrations of IFN-γ 50 IU ml^{-1}, nicotine and cotinine 0.1 μg ml^{-1} were used in the subsequent experiments.

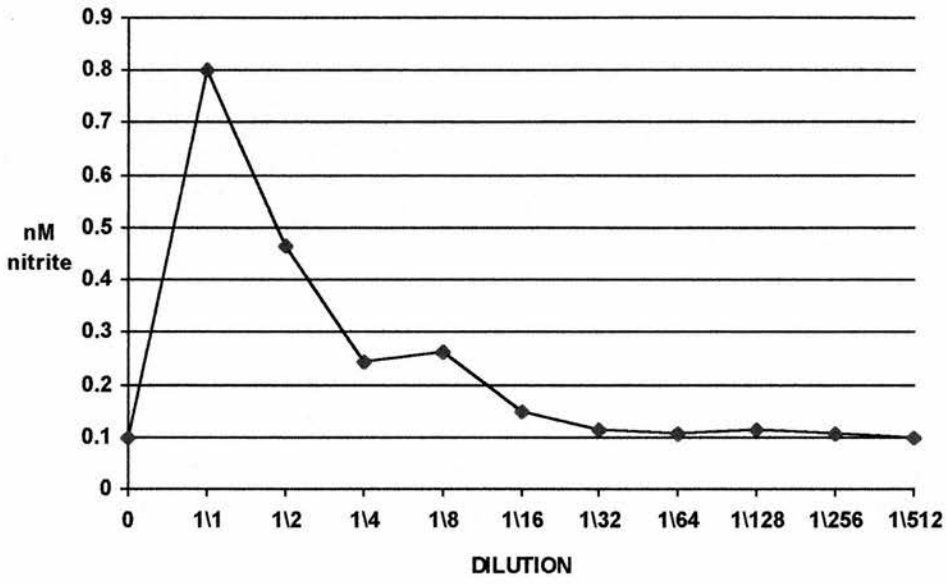


Figure 7.2 Dose response to dilutions of cigarette infusion measured by nitric oxide production from human peripheral blood monocytes. Starting concentration (1\1) is equal to 0.1 cigarettes per ml of medium (mean of 2 experiments with 2 donors).

Table 7.1 Dose response of monocytes to interferon-γ, nicotine and cotinine by nitric oxide and TNF-α production (mean of 2 experiments).

Sample		nM nitrite	% cytotoxicity for L-929 cells
Monocyte Control		0.0000	53
IFN- γ	200 IU	0.0429	60
	100 IU	0.0143	24
	50 IU	0.0429	60
	25 IU	0.0000	56
NICOTINE	0.4 μg ml^{-1}	0.0429	47
	0.2 μg ml^{-1}	0.0000	47
	0.1 μg ml^{-1}	0.0000	36
	0.05 μg ml^{-1}	0.0000	35
COTININE	0.4 μg ml^{-1}	0.0858	54
	0.2 μg ml^{-1}	0.0429	56
	0.1 μg ml^{-1}	0.0715	44
	0.05 μg ml^{-1}	0.0715	41

7.3.3 The effect of IFN-γ, nicotine and cotinine on release of TNF-α and nitric oxide from human monocytes.

The effects of IFN-γ, nicotine and cotinine were assessed with cells from 6 donors. There was no significant increase in the percentage killing of L-929 cells by medium from cells exposed to nicotine (mean 34%, SE 8.7) or cotinine (mean 39%, SE 7.2) compared with cells incubated with DMEM alone (mean 32%, SE 7.2). Incubation with IFN-γ did, however, result in a significant increase in TNF-α levels (mean 51%, SE 3.0, CI 6.2, 19.0, P=0.012) (Figure 7.3)

Production of NO was not significantly increased compared with control levels (0.1 nM ml^{-1}, SE 0.03) in the presence of IFN-γ (mean 0.15 nM ml^{-1}, SE 0.03) or nicotine (mean 0.13 nM ml^{-1}, SE 0.03) but was significantly increased by the presence of cotinine (mean 0.26 nM ml^{-1}, SE 0.03, 95% CI 0.03, 0.15, P=0.018) (Figure 7.4).

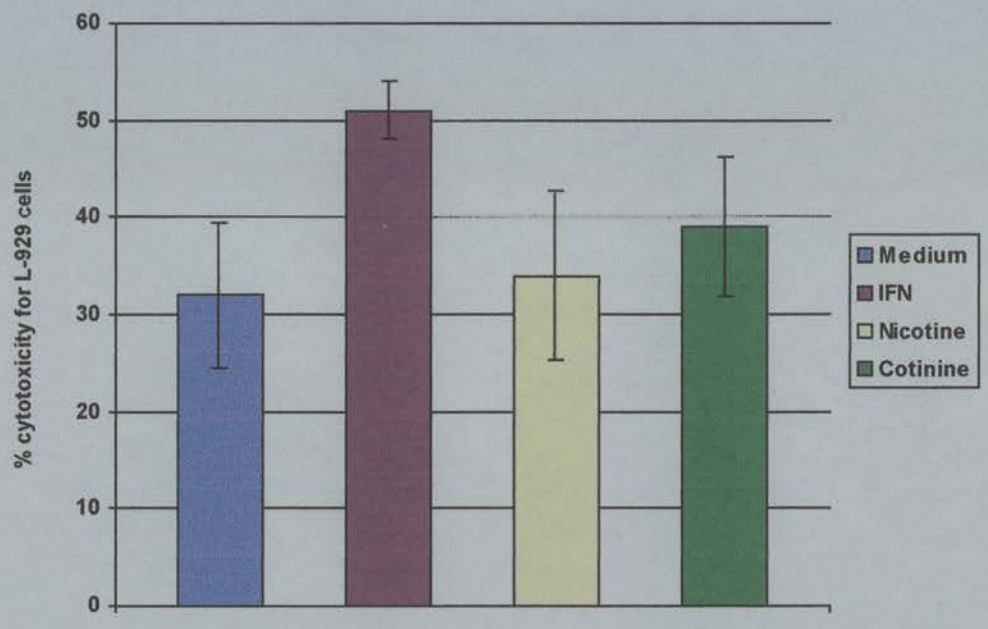


Figure 7.3 TNF-α activity elicited from human monocytes in response to IFN-γ, nicotine or cotinine (data expressed as % cytotoxicity for L-929 cells).

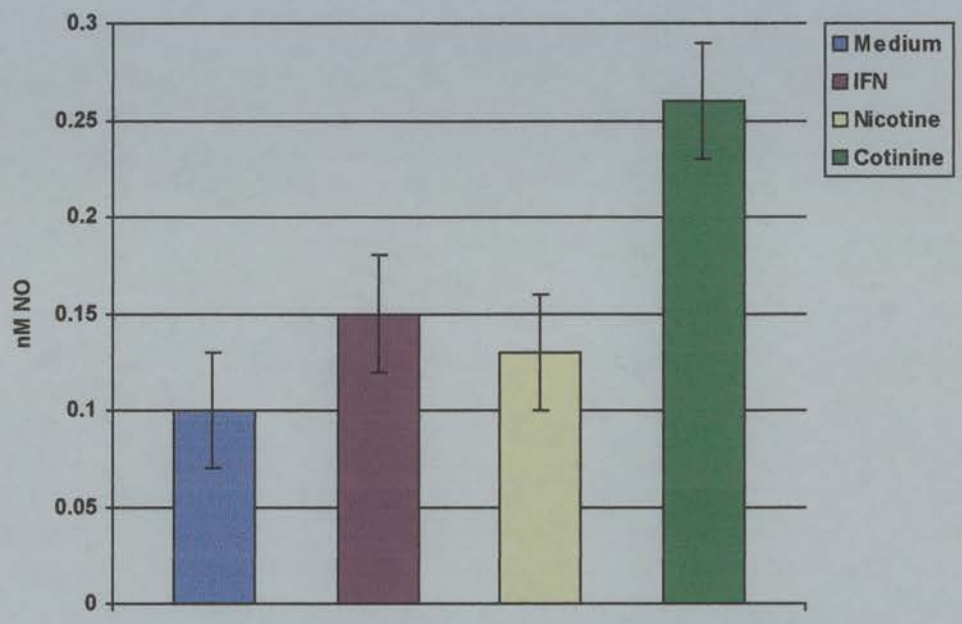


Figure 7.4 Release of nitric oxide from monocytes in response to IFN-γ, nicotine or cotinine.

7.3.4 The effect of CSE, RSV and/or TSST-1 on TNF-α release from human monocytes.

The TNF-α activity observed in experiments with monocytes from 31 donors is expressed as cytotoxicity for L-929 cells (Figure 7.5). In experiments with monocytes from 24 donors, compared with supernatants from cells incubated with medium alone (mean 45%, SE 4.5), supernatants from cells incubated with CSE had significantly increased levels of TNF-α (mean 60%, SE 5.3, 95%CI 3.8, 19.7, P = 0.006), as did the supernatants from RSV infected cells (mean 68%, SE 4.5, 95%CI 16.4, 34.1, P = <0.001). In experiments with monocytes from 31 donors, compared with supernatants from cells incubated with medium alone (mean 48%, SE 4.5), supernatants of cells incubated with TSST-1 had significantly increased levels of TNF-α activity (mean 63%, SE 4.3, 95%CI 6.2, 24.3, P = 0.002).

The effects of combinations of TSST-1 with CSE, RSV or both on of TNF-α activity were examined in experiments with cells from 20 donors. Compared with TNF-α activity detected in supernatants from cells exposed to medium alone (mean 41%, SE 5.5), a non-significant increase was observed in supernatants from cells incubated with CSE and TSST-1 (mean 51%, SE 7.1). Compared with controls, a significant increase was observed in supernatants from cells incubated with RSV and TSST-1 (mean 76%, SE 3.5, 95%CI 25.7, 42.4, P = <0.001) and in supernatants incubated with RSV, CSE and TSST-1 (mean 71%, SE 6, 95%CI 19.9, 44.9, P = <0.001).

Compared with TNF-α activity in supernatants from cells exposed to CSE alone (mean 61%, SE 7.2), there was decreased activity in supernatants of cells incubated with a combination of CSE and TSST-1 (mean 51%, SE 7.4, NS), but the levels observed for combination of CSE, RSV and TSST-1 were significantly higher (mean 71%, SE 6.5, 95%CI 11.7, 40.0, P = 0.002). Compared with TNF-α activity in supernatants from cells incubated with RSV alone (mean 68%, SE 5.6), supernatants from cells incubated with RSV and TSST-1 had increased levels of activity (mean 76%, SE 3.6, 95%CI 3.1,14.3, P = 0.001), as did the supernatants from cells incubated with all three agents (mean 71%, SE 6.5) but the difference was not significant.

The mean paired increases in percentage of killing of L-929 cells due to various combinations of agents compared with controls is summarised in Figures 7.6a, b, and c. CSE increased the TNF-α response from RSV infected cells (P = 0.001), decreased the response from cells exposed to TSST-1 (non-significant) and did not affect RSV-infected cells incubated with TSST-1. TSST-1 decreased TNF-α response from cells incubated with CSE (non-significant) but stimulated the activity when cells were infected with RSV (P = 0.001). TSST-1 had no effect on TNF-α response from RSV infected cells incubated with CSE. RSV increased TNF-α production from cells incubated with CSE, TSST-1 or both (P = 0.003, 0.003 and 0.03 respectively).

7.3.5 The effect of CSE, RSV infection and/or TSST-1 on NO release from human monocytes.

The supernatants from cells of 31 donors were examined for nitrite levels induced in response to CSE, RSV or TSST-1 (Figure7.7). Compared with supernatants from cells incubated with medium only (mean 0.36 nM ml^{-1}, SE 0.05), supernatants from cells incubated with CSE had lower levels of NO (mean 0.33 nM ml^{-1}, SE 0.05, 95%CI -0.16,-0.03, P = 0.009). RSV infection did not significantly affect the levels of NO detected (mean 0.34 nM ml^{-1}, SE 0.57). Supernatants from cells incubated with TSST-1 alone or combinations of agents including TSST-1 had levels of nitrite that did not differ significantly from the controls.

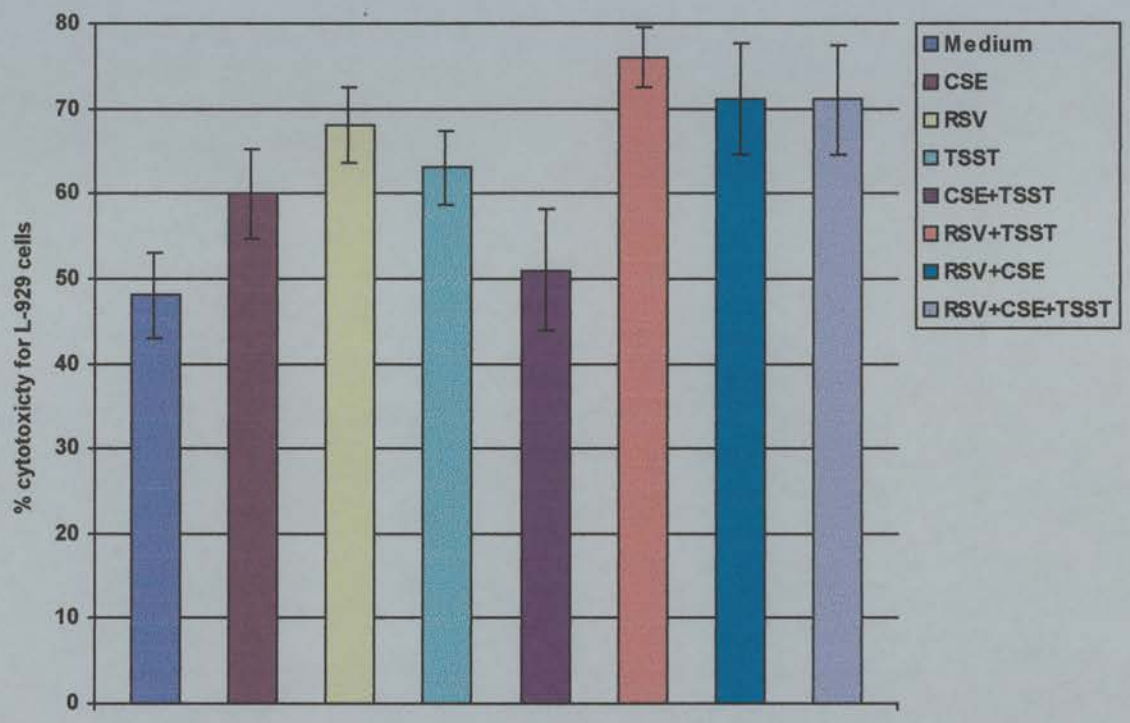
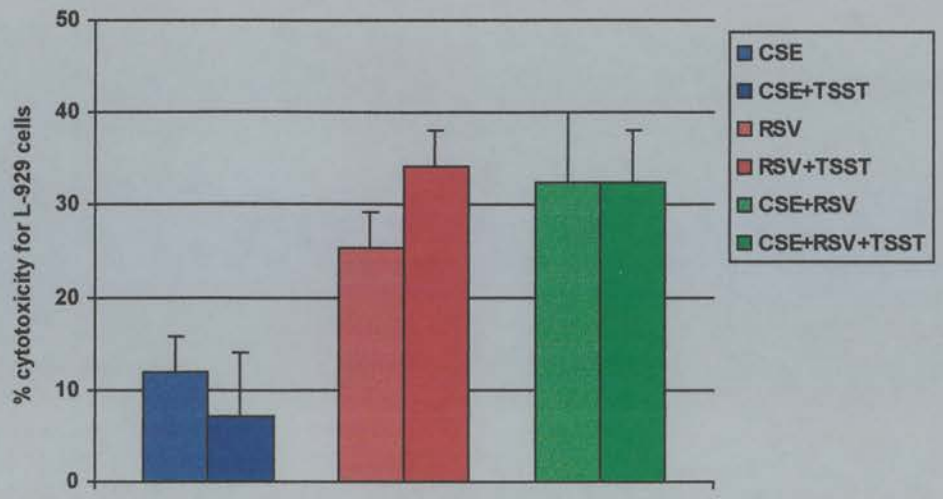
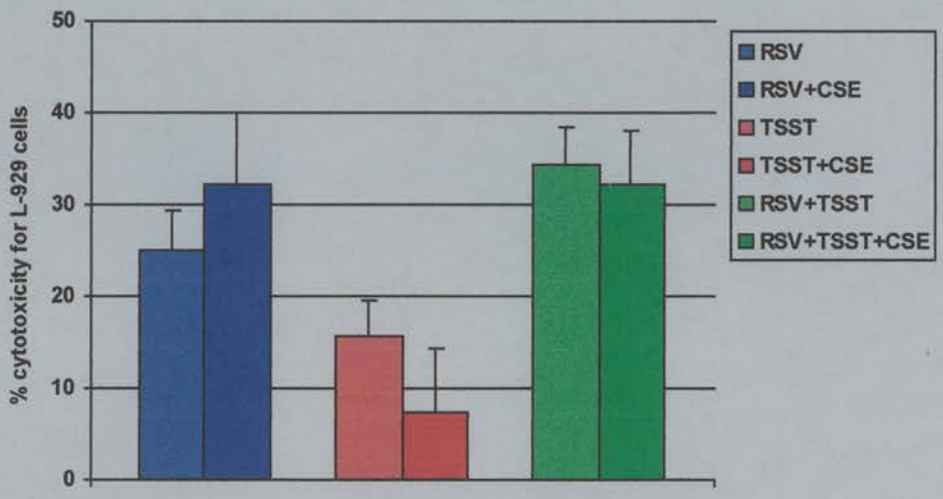


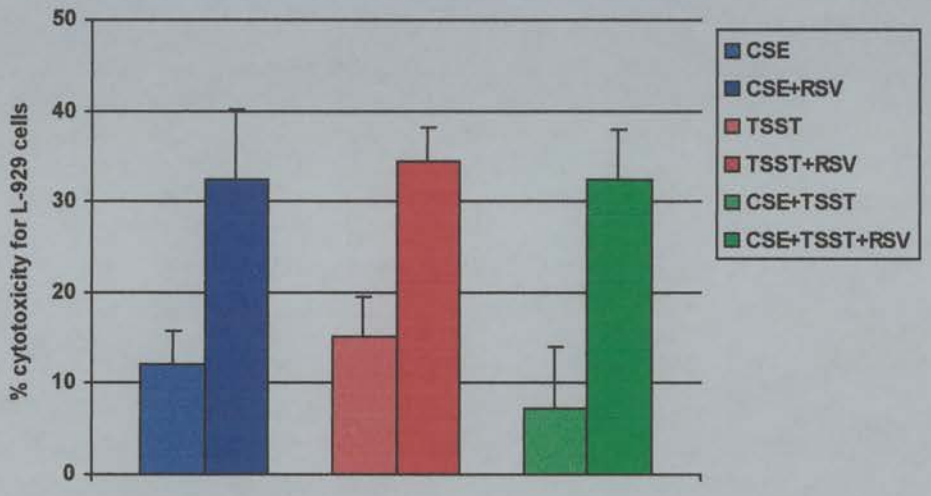
Figure 7.5 TNF-α activity elicited from human monocytes in response to combinations of CSE, RSV and TSST-1.


% cytotoxicity for L-929 cells
50
40
30
20
10
0
CSE
CSE+TSST
RSV
RSV+TSST
CSE+RSV
CSE+RSV+TSST

(7.6a)


% cytotoxicity for L-929 cells
50
40
30
20
10
0
RSV
RSV+CSE
TSST
TSST+CSE
RSV+TSST
RSV+TSST+CSE

(7.6b)



(7.6c)

Figure 7.6 (a, b, and c) Paired differences in production of TNF-α in response to combinations of CSE, RSV, and TSST-1 by percentage killing of L-929 cells.

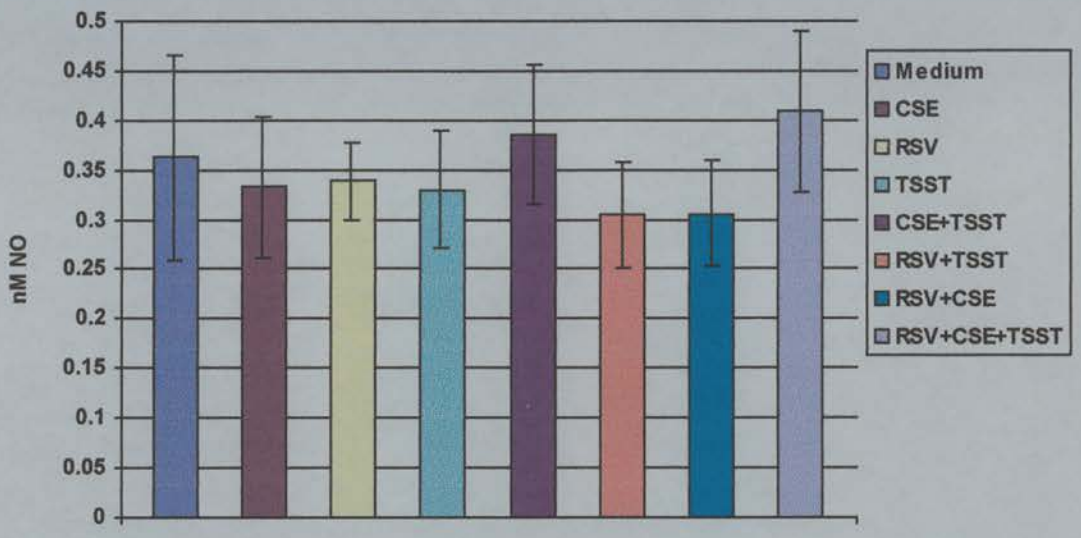


Figure 7.7 Production of nitric oxide by human monocytes in response to combinations of RSV, CSE, and TSST-1.

7.3.6 Variability of TNF-α and NO responses of individual donors.

Each set of experiments was carried out with cells from different donors. Individual TNF-α and NO responses to CSE, RSV infection and TSST-1 separately and in combination, are summarised in Table 7.2. TNF-α and NO responses of the test samples were classified as very high if the levels of killing of L-929 cells or levels of nitrite were more than twice the value of the controls in which monocytes were cultured with medium alone. Responses were classified as very low if the levels of the test samples were less than half those of the control. The most common pattern observed in the experiments was increased TNF-α production and decreased NO production.

Compared with the controls, exposure to CSE increased TNF-α activity in 75% of experiments and NO levels in 33%. The remainder showed varying degrees of inhibition in comparison to the control. Very high levels of TNF-α activity were observed in 12% of experiments but none showed very high levels of NO production in response to CSE. Very low levels of TNF-α in response to CSE were not observed, but in 12% of experiments, the NO response of the test was less than half that of the control. In response to CSE, cells from 25% of the donors showed increases in both TNF-α activity and release of NO and 25% showed a decrease in both. Increased levels of TNF-α activity and decreased NO levels were observed in 42% of

experiments. Among these, monocytes from 4% of donors showed extreme responses (Table 7.2).

RSV infection resulted in increased TNF-α activity in 92% of experiments and increased NO release in 50%. Compared with uninfected controls, RSV infection resulted in very high levels of TNF-α activity in 25% of experiments and very high levels of NO in 25 %. Compared with uninfected controls, RSV infection resulted in very low levels of TNF-α activity in 4% of experiments and of NO in 21%. Both TNF-α and NO responses were increased in 50% of experiments and decreased in 8%. RSV infected monocytes exhibited increased TNF-α activity and reduced production of NO in 33% of experiments; 4% showed extreme responses.

Exposure to TSST-1 increased TNF-α activity in 81% of experiments and NO production in 58%. Compared with the controls, very high TNF-α activity was observed in 26% of experiments and NO in 6% of experiments. Compared with the controls, TSST-1 resulted in very low levels of TNF-α activity in 3% of experiments and of NO in 12%. Both TNF-α activity and levels of NO were increased in 52% of experiments and decreased in 13%. TNF-α activity was increased and NO decreased in 29% of experiments. None showed extreme responses.

The effects of CSE and RSV were assessed with monocytes from 15 donors. RSV and CSE resulted in increased levels of TNF-α activity in 87% of experiments and

increased NO in 13%. Compared with controls, exposure to RSV and CSE induced very high TNF-α activity in 48% of experiments and very high levels of NO in 7%. Compared with controls, very low levels of TNF-α activity and NO were observed in 7% and 48% of experiments respectively. Levels of both TNF-α activity and NO were increased from control levels in 13% of experiments and decreased in 13%. RSV infected monocytes challenged with CSE exhibited increased TNF-α activity and decreased NO production in 67% of experiments with 20% showing extreme responses.

The effects of CSE and TSST-1 were assessed with monocytes from 20 donors. Compared with the controls, exposure to CSE and TSST-1 increased TNF-α activity in 65% of experiments and NO levels in 45%.Very high levels of TNF-α activity were observed in 30% of experiments but none showed very high levels of NO production in response to CSE and TSST-1. Very low levels of TNF-α activity in response to these agents were observed in 5% of experiments and of NO in 20% of experiments. In response to CSE and TSST-1, cells from 25% of the donors showed increased TNF-α activity and NO production and 15% showed a decrease in both. Increased levels of TNF-α activity and decreased NO levels were observed in 40% of experiments. No extreme responses were observed.

The effects of RSV and TSST-1 were assessed with monocytes from 20 donors. RSV and TSST-1 resulted in increased levels of TNF-α activity in 95% of experiments

and increased NO release in 30%. Compared with controls, RSV and TSST-1 resulted in very high levels of TNF-α activity in 40% of experiments and very high levels of NO in 20 %. Compared with controls, very low levels of TNF-α activity in response to RSV and TSST-1 were not observed, but in 30% of experiments the levels of NO were classified as very low. Both TNF-α and NO responses were increased in 30% of experiments and decreased in 5%. RSV infected monocytes challenged with TSST-1 exhibited increased TNF-α activity and reduced production of NO in 50% of experiments. Again no extreme responses were observed.

Exposure to CSE, RSV and TSST-1 increased TNF-α activity in 95% of experiments and NO production in 35%. Compared with the controls, very high levels of TNF-α activity were observed in 60% of experiments and of NO in 5% of experiments in response to the three agents. Compared with the controls, these agents resulted in very low levels of TNF-α activity in 5% of experiments and of NO in 25%. Both TNF-α activity and NO production was increased in 35% of experiments and decreased in 6%. TNF-α activity was increased and NO production decreased in 55% of experiments; 20% showed extreme responses.

Table 7.2 Response of individual donors to challenge with combinations of RSV, CSE, and TSST-1 by production of TNF-α and nitric oxide.

		% donors				
Challenge		**Increased**	**Decreased**	**Very High**	**Very Low**	**↑ TNF-α ↓ NO (extreme)**
CSE	**TNF-α**	75	25	12	0	42 (4)
(n=24)	**NO**	33	63	0	12	
	TNF-α+ NO	25	25	0	0	
RSV	**TNF-α**	92	8	25	4	33 (4)
(n=24)	**NO**	50	46	25	21	
	TNF-α+ NO	50	8	0	4	
TSST	**TNF-α**	81	19	26	3	29 (0)
(n=31)	**NO**	58	42	6	12	
	TNF-α+ NO	52	13	0	0	
CSE+RSV	**TNF-α**	87	13	48	7	67 (20)
(n=15)	**NO**	13	87	7	48	
	TNF-α+NO	13	13	0	0	
CSE+TSST	**TNF-α**	65	35	30	5	40 (0)
(n=20)	**NO**	45	50	0	20	
	TNF-α+ NO	25	15	0	0	
RSV+TSST	**TNF-α**	95	5	40	0	50 (0)
(n=20)	**NO**	30	60	20	30	
	TNF-α+ NO	30	5	0	0	
RSV+CSE+	**TNF-α**	95	5	60	5	55 (20)
TSST	**NO**	35	60	5	25	
(n=20)	**TNF-α+ NO**	35	6	0	0	

7.4 Discussion.

While there is evidence that viral infection and cigarette smoke might enhance bacterial colonisation of infants [Saadi *et al.*, 1996; Musher and Fainstein, 1981], the effects of these agents in relation to TSST-1 on induction of inflammatory mediators had not been examined. Evidence that sub-lethal amounts of SEB can kill virus infected mice has been shown by Sarawar *et al.* [1994] implicating the production and interaction of the inflammatory mediators TNF-α and IFN-γ. The study by Sayers *et al.* [1995b] with toxins produced by bacteria isolated from SIDS cases showed an increased lethality of tolerable quantities of *S. aureus* and *Klebsiella pneumoniae* toxins when the toxins were present in combination (9% killing at a dilution of 1/8) which was increased by the addition of nicotine (800 ng ml^{-1} neat solution) (82% killing at 1/8 to 46% killing at 1/32). Combinations of toxins from *S. aureus* and *E. coli* showed an even greater response to nicotine (toxin combination = 9% killing at 1/4, toxin combination + nicotine = 82% killing at 1/4 to 73% killing at 1/32). On the basis of such evidence the activities of cigarette smoke and viral infection were assessed for their effect on the ability of TSST-1 to induce inflammatory mediators from human monocytes.

Individual variations observed in the inflammatory response to toxic stimuli and RSV infection, though controlled for using unchallenged monocytes, could reflect stimulation of the cells prior to collection from the donors. As the buffy coats

obtained from the SNBTS were from anonymous donors there was no way to ascertain if the donors were suffering from infection. As a safeguard against the use of stimulated monocytes from unwell blood donors, buffy coats containing unusually high numbers of monocytes (>4 x 10^7 monocytes per 50 ml buffy coat) were routinely discarded. The level of production of inflammatory mediators has been observed to vary with blood group, cells from donors of blood group O produced significantly greater quantities of TNF-α, IL-6 and nitric oxide than those from donors of blood group A in response to *H. pylori* antigens [Alkout, 1997], for this reason all of the buffy coat samples used throughout this work were from O rhesus positive donors. Research into a potential role for blood group as a risk factor in SIDS appears to be limited to studies on maternal blood group, with one study identifying type O and two studies identifying type B as being associated with an increased incidence of SIDS [reviewed by Kelly and Shannon, 1982].

The increased production of TNF-α by virus infected monocytes in response to TSST-1 and/or CSE was not generally accompanied by an increase in NO production. For the majority of experiments, production of NO was decreased. The production of NO by macrophages and alveolar lining cells in the airways is known to have a protective effect by counteracting bronchoconstriction induced by inflammatory mediators such as leukotriene D_4 (LTD_4) [Hogman *et al.*, 1993]. It would appear that the combinations of toxin, virus and CSE used in this study are capable of reducing this protective mechanism.

The evidence for an additive, if not synergistic, effect between RSV, CSE and TSST-1 supports the hypothesis that the interactions of infectious and environmental agents on production of inflammatory mediators could contribute to the death of a susceptible infant. These findings with partially purified toxins and human cells are consistent with those of Sayers *et al.* [1995b] with the chick embryo model. The toxin extraction method of Sayers *et al.* [1995a,b] employed isolates of bacteria from SIDS cases cultured overnight on a dialysis membrane over agar, the bacterial growth was harvested in Hank's balanced salt solution which was then centrifuged and filter sterilised for use as the toxin preparation. The toxicity of these crude preparations relative to the commercially available purified toxins, might have more powerful effects such as those observed with nicotine and cotinine compared with the water soluble smoke extract. The crude toxin extracts could contain substances such as peptidoglycan and lipoteichoic acid which are capable of acting synergistically to elicit powerful inflammatory responses [De Kimpe *et al.*, 1995].

Further work is also required to clarify the mechanisms underlying the effect of tobacco smoke on the responses of monocytes to bacterial toxins. Development of a more extensive cytokine profile from *in vitro* studies would provide guidance as to which cytokines should be sought in SIDS victims.

Although *S. aureus* is present in over 50% of infants in the 0 to 3 month age range, production of the pyrogenic toxins is probably induced only on rare occasions. The

toxins are produced only at temperatures between 37 and 40°C [Bohach *et al.*, 1990] which is higher than the normal temperature of the nasopharynx (approximately 35.6°C) [Molony *et al.*, 1996]. Many SIDS victims have a mild respiratory infection in the week prior to their deaths. This could result in fever and blockage of the nasal passage which could increase the temperature of the nasopharyngeal mucosa to the permissive temperature for toxin induction. Recent studies in young children indicate that the prone position results in significantly higher temperatures in the nose, some of which reached 37°C [Molony *et al.*, submitted for publication].

The resulting local toxin concentration would stimulate immune cells to produce the cytokines TNF-α and IL-1 which would sustain the elevated temperature allowing further toxin production. The prolonged toxin-cytokine production would form a positive feedback loop resulting in a systemic TNF-α response. Studies in both human and animal models have shown that elevated TNF-α levels produce effects commonly associated with severe toxaemia [Parrillo, 1993].

Further evidence for the role of TNF-α in SIDS comes from a study by Ikejima *et al.* [1988] on the induction of shock-like states in rabbits by administration of TNF-α and IL-1. Their findings showed a synergistic effect between TNF-α and IL-1 with the major target organ being the lungs. This shock state resulted in an accumulation

of proteinaceous fluid and immune cells in the alveolar spaces and petechiae, both common findings in SIDS victims at autopsy [Howat *et al.* 1994].

A possible link between the night time prevalence of SIDS cases and control of the inflammatory response by cortisol [Reichlin, 1993] and other glucocorticoids has been suggested [Blackwell *et al.*, 1995]. As the adrenal gland decreases in weight in the first two months of life, there is a concomitant decrease in plasma cortisol levels [Wittekind *et al.*, 1993]. In adults, circadian variations in cortisol production have been reported, secretion rates drop in the early hours of the morning when the majority of SIDS cases are thought to occur. In adults, the ability to control TNF responses to endotoxin are significantly reduced after midnight [Pollmacher *et al.*, 1996]. During the infant's development, cortisol levels drop dramatically in the week following the switch to "adult-like" body temperature rhythms. This period following this switch could be when infants are more susceptible to the pathological effects of these inflammatory mediators. If the infection and toxin challenge were to occur outwith this time period, either while levels of maternal transplacental antibodies are high or afterwards when the infants own immune system has started to produce antibodies to the toxins, the risk of SIDS is likely to be reduced.

Chapter 8

General discussion

Detailed discussion of results is given at the end of each chapter. The purpose of this chapter is to give an overview of the project findings, to assess the implications of the work for further reducing the incidence of SIDS, to examine ways in which experimental techniques could be improved and to propose further work to clarify the findings of the study.

The general aim of this study was to examine how common bacteria might play a role in sudden infant death syndrome. It has long been recognised that a certain number of SIDS deaths might be due to bacterial colonisation; however, very few of the studies attempting to test this hypothesis have postulated how such bacteria could cause the death of an infant in a way which would be consistent with the epidemiological and post-mortem findings commonly associated with SIDS. Given the highly probable multi-factorial nature of a problem as complex as SIDS, it is

unlikely that any one hypothesis will ever explain the cause of more than a subset of the deaths attributed to it. The remit of this project was to examine the way in which the toxins of common bacteria could trigger a series of events leading to a SIDS death in the context of developmental and environmental factors identified in epidemiological studies.

The Lewis^{a} blood group antigen acts as a receptor on epithelial cells for certain bacterial species, including *S. aureus*. The increased proportion of infants expressing this antigen during the age range in which the majority of SIDS cases occur could contribute to density of colonisation by toxigenic bacteria such as *S. aureus* or some Gram-negative bacteria often isolated from SIDS infants such as *H. influenzae* (Chapter 3). Colonisation would not be sufficient to cause the death of an infant as *S. aureus* only produces toxins at temperatures of 37 to 40°C and normal local temperature is lower than this. Some other factors must raise the temperature of the nasopharyngeal mucosa to this level. This effect could be achieved by environmental causes such as overwrapping, an excessively warm room, placing the infant in the prone position, or by a concomitant infectious agent such as a minor respiratory tract infection (commonly observed in SIDS infants in the week prior to death). Once toxin production has begun, this elevated temperature will be maintained by the pyrogenic effect of the toxins themselves and also by the effects of the proinflammatory cytokines produced by the infant in response to the toxins. At this stage decreased night-time levels of cortisol associated with developmental changes

could result in a period when an infant is more vulnerable to the detrimental effects of inflammatory mediators. Toxin production and an uncontrolled systemic cytokine response might lead to "pre-morbid toxic shock" and death. For the effect to occur, the toxins would have to be present in very high concentrations, or acting in synergy with small amounts of endotoxin, viral induced cytotoxins or toxic substances such as cigarette smoke in the environment. The prone sleeping position could enhance density of colonisation by toxigenic bacteria; however, if the toxins were to act synergistically, death could possibly result from a sparse colonisation or from infection with bacterial strains which produce only minor amounts of toxins such as *S. epidermidis* which would not be seen as being sufficiently significant to cause death.

Epidemiological studies, as well as identifying factors which increase the risk of SIDS, have identified certain factors which appear to decrease its likelihood. One such factor is immunisation against DPT. This apparent protective effect was observed in southeast Scotland when the infant immunisation schedule for DPT was changed from 3 to 2 months of age. There was a downward shift in the age distribution of SIDS cases. This effect was studied in relation to the hypothesis that antibodies produced by the infant in response to the DPT vaccine could bind to shared epitopes on the staphylococcal toxins resulting in partial protection from these toxins. Since *B. pertussis* has not been isolated from SIDS victims and isolation of *S. aureus* parallels the age distribution of SIDS, the effect of DPT immunisation is

more likely to be induction of antibodies cross-reactive with the staphylococcal toxins.

8.1 Detection of microbial surface antigens that bind Lewis^a.

The first stage of the project was to determine which bacteria were capable of using the Lewis^a antigen as a receptor. At the time of this study synthetic oligosaccharides of the Lewis antigens were not commercially available and affinity purified Lewis antigens from donor samples are usually bound to lipids and proteins. The problem was addressed by the production of an anti-idiotypic antibody to anti-Lewis^a, the Fab portion of which would have the same spatial configuration as the Lewis^a antigen. This method provided a sufficiently high yield of anti-idiotypic antibody to screen large numbers of bacterial isolates for their ability to bind to Lewis^a. The anti-idiotypic antibody was left attached to the protein A Sepharose beads used in the final stage of purification to avoid excessive dilution of the antibody and to enhance the clarity of reactions in the bacterial agglutination assay. This provided a rapid and reproducible agglutination assay which was easy to read by eye, and the controls included with each test ensured that no false positives were recorded due to bacteria binding to the protein A Sepharose beads or autoagglutination. As synthetic blood group oligosaccharides are now available at reasonable costs the value of this assay as a screening tool is somewhat reduced, however; it was useful in the context of this

study to determine which bacteria would be expected to play a role in SIDS during the age range when infants are at greatest risk.

8.2 Lewis antigen expression on human monocytes and binding of pyrogenic toxins.

The majority of work on superantigens has concentrated on their interactions with MHC class II molecules; however, as it has been shown that the pertussis toxin of *B. pertussis* can utilise the Lewis^a antigen as a receptor [van t'Wout *et al.*, 1992], the ability of the staphylococcal toxins to bind to this antigen was examined. Although all four of the staphylococcal toxins tested bound to the surface of human monocytes, binding of SEA and SEB could not be reduced by pre-treatment with antibodies to Lewis^a or Lewis^b indicating that they are binding to other cell surface antigens. The toxins for which binding was significantly reduced by pre-treatment with the anti-Lewis antibodies, SEC_1 and TSST-1, have been shown by two studies to be significantly more common in SIDS victims than controls [Newbould *et al.*, 1989; Malam *et al.*, 1992].

Further studies using MHC II depleted cell lines or monocytes pre-treated with a selection of anti-MHC II antibodies onto which purified Lewis^a has been passively

adsorbed could be developed to further clarify the role of this antigen as a receptor for the staphylococcal toxins.

The reasons underlying the observed induction of nitric oxide production in response to synthetic Lewis^a are as yet unknown. It is possible that this is a reaction to residual levels of preservatives or other compounds used in the production of this synthetic oligosaccharide and that purified natural Lewis^a would not have the same effect. Repetition of this work using the staphylococcal toxins that were not inhibited by anti-Lewis^a to determine if the Lewis antigens are capable of blocking their ability to induce inflammatory mediators, including NO and TNF-α, would also be useful to complement the flow cytometry studies.

8.3 Antigenic cross-reactivity among bacterial toxins and the protective effect of infant immunisation.

The findings of antigenic cross-reactivity between several of the staphylococcal toxins were expected from reported similarities in their amino acid sequence and secondary structure [Chou *et al.*, 1973; Marrack and Kappler, 1990; Johnson *et al.*, 1991]. Several groups have previously studied cross-reactivity between the staphylococcal enterotoxins, A, B, and C (sub-groups 1, 2, and 3), by immunodiffusion [Lee *et al.*, 1980], radioimmunoassay [Johnson *et al.*, 1972; Spero

et al., 1978 and 1979] and immunoblot [Thompson *et al.*, 1984] of whole and tryptic fragments of the toxins. In general their results showed reciprocated heterologous reactions between SEB and SEC, this contrasts with the results of this study in which the heterologous reaction of anti-SEC_1 with SEB was not reciprocated by the anti-SEB antibody and SEC_1. One study [Thompson *et al.*, 1984] using a range of antitoxins prepared in different rabbits found that out of four antitoxins prepared against SEB only one reacted with SEB and SEC_1, this provides a likely explanation for the lack of heterologous reaction of anti-SEB observed in this study. Although no previous work appears to have been carried out on the cross reactivity of TSST-1 with the other staphylococcal toxins, reports on the similarities present in the three-dimensional structures of TSST-1 and SEB [Swaminathan *et al.*, 1992] support the findings presented here.

Comparison of the direct and sandwich ELISA methods gave results which, in this application, were contradictory to the findings of Fey *et al.* [1984] who found the double antibody sandwich method to be superior to other ELISA techniques. The results observed with the direct ELISA, although a simple assay, are in agreement with those of the studies referred to above which used more complex and time-consuming methods. In this application, the lack of cross-reactivity observed in the sandwich ELISA may be due to toxin epitopes being obscured by binding to the polyclonal capture antibody which remain available to the secondary polyclonal antibody when the toxins are bound directly to the plate. This point could be clarified

by using a monoclonal antibody as the capture antibody followed by the secondary polyclonal antibody.

The reactions observed between rabbit polyclonal antibodies to the DPT vaccine and the two most commonly isolated staphylococcal toxins from SIDS cases, SEC and TSST-1, indicate the presence of cross-reactive epitopes. This could help explain the epidemiological evidence that DPT immunisation reduces the risk of SIDS, and might contribute to the observed shift in the age range of local SIDS cases following changes in the infant immunisation schedule. Further evidence was obtained by the ability of the antiserum to DPT to partially neutralise the toxin-induced nitric oxide response, particularly with TSST-1. Unfortunately, it does not indicate which component of the vaccine is responsible for this effect. Repetition of this work using antibodies raised to the individual components of the vaccine, tetanus, pertussis and diphtheria toxoids and both cellular and acellular pertussis vaccines will be needed to answer this question.

8.4 Synergy between bacterial toxins in induction of inflammatory response from human monocytes.

The ability of small amounts of bacterial toxins to act synergistically in induction of an inflammatory response has been studied by several groups in relation to SIDS

using animal models [Drucker *et al.*, 1992; Sayers *et al.*, 1995]. One problem with such an approach is that although synergy between bacterial toxins can be clearly demonstrated in animal systems, these experiments give little information as to how such an effect could cause death in a human infant. Other animal studies, mostly on synergy between toxins and viral products [Sarawar *et al.*, 1994], examined cytokine release but this still does not necessarily represent what is occurring in a human system. These studies have been useful as preliminary work, but assays using human cells, such as those of Lundemose *et al.* [1993] are necessary to validate their results in relation to SIDS.

Direct comparison of the results from this study and those of Drucker *et al.* [1992] and Sayers *et al.* [1995a] was not possible as they had used crude preparations of mixed toxins prepared from SIDS bacterial isolates rather than purified single toxins. The findings of synergy between the staphylococcal toxins and LPS [Drucker *et al.*, 1992; Sayers *et al.*, 1995a] and murine lymphocytic choriomeningitis virus (LCMV) [Sarawar *et al.*, 1994] are consistent with the increased levels of cytokine production observed in this study. Further supporting evidence for the ability of TNF-α and NO to produce a lethal outcome in response to toxin synergy is obtained from the work of Sarawar *et al.* [1994]. The survival of mice with an asymptomatic viral infection subsequently challenged with a non-lethal quantity of SEB was enhanced by blocking the effect of TNF-α, using a soluble dimeric TNF receptor-Fc fusion protein, and NO, using the nitric oxide synthase inhibitor aminoguanidine. The

possibility of a synergistic effect between LPS, the staphylococcal enterotoxins and non-toxigenic strains of staphylococci such as *S. epidermidis* needs to be examined. The work by Sayers *et al.* [1995a] demonstrated synergy between LPS and staphylococcal strains which produced no detectable toxins. This could be due to interactions between LPS and peptidoglycan or lipoteichoic acid which are present in larger amounts in Gram positive bacteria. Extending the range of inflammatory mediators examined to include IL-1, TNF-β and IFN-γ might also provide clues to how the interactions of cytokines could give rise to SIDS.

8.5 The effects of cigarette smoke and viral infection on inflammatory responses of human monocytes.

Exposure to cigarette smoke has been identified as possibly the most important modifiable risk factor in relation to SIDS by epidemiological studies in many countries. Its significance is not appreciably diminished by controlling for possible confounding factors commonly observed in the population of smokers such as lower socio-economic class, nor is it affected by the ethnicity of the population studied. The results of these studies have indicated that eliminating both prenatal and postnatal exposure to tobacco smoke could reduced the total number of SIDS deaths by between 12 [Malloy *et al.*, 1988] and 66% [Brooke *et al.*, 1997].

Several hypotheses have been proposed to explain the pathological role of tobacco smoke components in SIDS. These can separated into two groups:

(1) prenatal effects including reduction of birth weight [Elwood *et al.*, 1987; Brooke *et al.*, 1989], chronic fetal hypoxia [Bulterys *et al.*, 1990], disturbance of normal lung development [Martinez, 1991] and damage to the fetal brainstem [Krous *et al.*, 1981];

(2) effects of smoking in the infant's first year of life, passive inhalation of smoke increases an infant's susceptibility to respiratory tract infections [Fergusson *et al.*, 1980; Taylor and Wadsworth, 1987] and reduction of peripheral arterial chemoreceptor ability to detect lowered blood oxygen levels [Holgert *et al.*, 1995].

Despite the large number of noxious compounds present in tobacco smoke such as tar and CO, practically every study in the literature has concentrated on the effects of nicotine alone. For the purposes of this study it was decided that water soluble components of cigarette smoke would give a more accurate picture of the ability of passive smoke inhalation to stimulate an inflammatory response as it would contain the majority of the components capable of being absorbed into the blood stream.

Peripheral blood monocytes were used in this study rather than alveolar macrophages as they are easily obtainable in sufficiently large quantities. They are also less likely

to have been stimulated by inhaled antigens prior to the experiments. The L-929 bioassay for the detection of TNF-α was used throughout this study as, unlike ELISA systems, it measures the amount of biologically active TNF-α present in a sample rather than total TNF-α, a percentage of which will be rendered inactive either by binding to soluble TNF-α receptors or partial degradation by monocytes.

Increased production of TNF-α was expected in response to the virus, toxin and cigarette smoke extract challenges; however, the observed reduction in nitric oxide levels, as previously noted by Kharitonov *et al.* [1995] in studies involving both habitual and episodic smokers, could have serious consequences if they occur in the lungs of an infant with a respiratory tract infection. It is suggested that NO plays a protective role in asthmatic reactions by counteracting bronchoconstriction induced by inflammatory mediators [Barnes and Liew, 1995], a view which is supported by evidence of a small bronchodilatory response observed in asthmatic patients inhaling high concentrations of NO. Removal of this protective mechanism and a concomitant increase in TNF-α levels could result in severe bronchoconstriction and hypoxia.

8.6 Possible applications of this study.

The results of this study suggest possible intervention strategies which could be useful in further reducing the number of SIDS deaths:

(1) Development of vaccines against the staphylococcal toxins identified in SIDS. As whole cell vaccines against *S. aureus* do not appear to be particularly effective, even though antibody titres were raised incidence of infection was not reduced [Poole-Warren *et al.*, 1991]. This is an effect observed in natural repeated exposure to *S. aureus* [Wergeland *et al.*, 1989]. Development of a vaccine to the superantigen SEA is currently underway using genetically attenuated mutants of the toxin in which the MHC class II binding site of the toxin is altered. This prevents it from binding outwith the antigen binding groove and allows it to be processed as a normal antigen [Bavari *et al.*, 1996]. This has provided complete protection from challenge with the wild type toxin in mice. Further development of such vaccines and identification of shared epitopes on the toxins might eventually provide a vaccine capable of protecting humans from several, if not all, of the staphylococcal toxins.

(2) Identification of epitopes in the DPT vaccine that induce antibodies cross-reactive with the staphylococcal toxins would allow future development of DPT vaccines to optimise their protective effects against the staphylococcal toxins.

(3) A program of immunisation with non-toxic fragments of SEC and TSST-1 may prevent the observed decrease in antibodies to these toxins in women in the third trimester of pregnancy possibly resulting in higher levels of transplacentally acquired immunoglobulins to these toxins in the infant.

The use of such intervention strategies could protect younger infants and those identified as being at risk from SIDS during the window of susceptibility caused by waning maternal antibody levels in the second month after birth.

8.7 Future work

Further work is required to clarify the mechanisms underlying some of the observations made in this study and to provide extra information for the development of intervention strategies to further reduce SIDS. Extending the range of inflammatory cytokines examined to include the other important mediators of the innate immune response such as IL-1, IFN-γ, TNF-β and those of the acute phase response would allow development of a cytokine profile of response to combinations of bacterial toxins and virus infection. This could be of use in further elucidating the role of infection in SIDS.

Assessment of the levels of antibodies to the staphylococcal toxins and LPS required to reduce the effect of these toxins on inflammatory cells would be helpful for the development of a passive immunisation strategy for infants identified as being at risk of SIDS. The contribution of physiological changes to a window of susceptibility to the effects of inflammatory mediators could also be examined using cortisol or other

glucocorticoids in the monocyte challenge assay to examine their ability to reduce inflammatory responses to toxic stimuli.

Repetition of this work using other bacterial antigens such as endotoxins of different species and extracts of less toxigenic staphylococci and other inflammatory cells, particularly mast cells, could identify other combinations of infectious agents and host responses capable of triggering a potentially fatal outcome.

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Appendix

Suppliers of chemicals, reagents and plasticware.

BDH	**Poole, Dorset, England.**
Dynatech	**Billingshurst, Sussex, England.**
Evans Medical Ltd.	**Leatherhead, England.**
Fisher Scientific (Fisons)	**Loughborough, England.**
Gibco	**Paisley, Scotland.**
Greiner	**Dursley, England.**
Pharmacia LKB Biotechnology	**St. Albans, England.**
Scottish Antibody Production Unit (SAPU)	**Law Hospital, Carluke, Scotland.**

Sigma	**Poole, Dorset, England.**
Toxin Technology Inc.	**Sarasota, Florida, USA.**