Bacterial toxins in Sudden Unexpected Nocturnal Death

Dr. Osama M. Almadani

PhD.

University of Edinburgh

1999

Declaration

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

University of Edinburgh

Osama Almadani

I dedicated this work to my parents, my wife Samia and my daughters Hala,Zean and Rand.

Acknowledgement

I would like to thank my supervisors, Prof. Busuttil and Dr. Blackwell for their support especially Dr. Blackwell for the long hours of patient discussions she spent with me which created a good atmosphere for the research to be accomplished.

I would like to thank my colleagues for their assistance and support, particularly Dr. Alkout and Dr. Raza for their assistance with flow cytometry. Many thanks also to Valerie James for her assistance in the Infection and Immunity Laboratory.

I am grateful to the ministry of health in Saudi Arabia, which funded the research and particularly to Dr. Osama Shobokshi for his encouragement and support.

Finally, I express gratitude to my wife and my family at home for all their support, encouragement over the years.

Contents

Abbreviation

DMEM	Dolbecco's modified Eagle's medium
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
FITC	flourescein isothiocyanate
HPA	Hypothalamic-pituitary-adrenal
HRP	horseradish peroxidase
Ig	imuonoglobulin
IL	interleukin
OPD	ortho-phenylenediamine dihydrochloride
PBS	phosphate buffered saline
SUND	sudden adult nocturnal death
SIDS	sudden infant death syndrome
SE	staphylococcal enterotoxin
TNF-α	tumour necrosis factor alpha
TSST-1	toxic shock syndrome toxin-1

Abstract

Sudden Unexpected Nocturnal deaths (SUND) occur in young immigrant workers, mainly from Southeast Asia, employed in countries such as Singapore and Saudi Arabia. Pyrogenic toxins of *Staphylococcus aureus* have been identified in two cases of sudden unexpected death in adults in Britain, and it has been suggested that these toxins or others with superantigen properties might induce strong inflammatory responses leading to SUND. Inflammatory responses are less well controlled at night when most of these deaths occur. Although SUND is a significant problem in Saudi Arabia it is difficult to carry out post mortem examinations because of cultural objections in relation to disfiguring of the body.

The objectives of the present study were: 1) to develop a method of tissue sampling that dose not disfigure the body; 2) to develop a method for detection of toxins in tissues and body fluids; 3) to assess in a model system the effect on inflammatory responses to TSST-1 of cortisol levels comparable to those present at night, during the day and under conditions of physiological stress; 4) to assess the levels of antibodies to pyrogenic staphylococcal toxins in relation to age range affected and predominance of male deaths; 5) to assess the levels of IgG to the toxins needed to reduce the production of inflammatory mediators in a model system.

A needle biopsy method for obtaining tissue samples was tested.

Enzyme linked immunosorbent assays (ELISA) and a flow cytometry method were developed to screen body fluids and tissues for toxic shock syndrome (TSST-1) and staphylococcal enterotoxins A (SEA), B (SEB) and C (SEC). Both frozen and formalin fixed tissues were obtained from infants who died suddenly and

unexpectedly in Scotland, France or Australia. The flow cytometry method detected toxin in both frozen and fixed tissues, but ELISA was suitable only for frozen tissues or those fixed less than 12 months. TSST-1 and enterotoxins were identified in over half the specimens from infants. Of the 4 samples from SUND victims, one was borderline positive for TSST-1; however the bodies had been stored for extended period prior to autopsy.

Human buffy coats were used to examine the effect of IgG to the toxins for neutralising activity and the effect of cortisol. Tumour necrosis factor α (TNF-α) was detected by bioassay with L929 cells, interleukin-6 (IL-6) and interleukin-10 (IL-10) were measured by ELISA. IL-6 and TNF-α elicited by the toxins were not reduced by night time level of cortisol (5-10 μg dl^{-1}) levels. Day time levels of cortisol (10-20 μg dl^{-1}) significantly inhibited IL-6 production but not TNF-α in responses. Stress levels of cortisol (40-80 μg dl^{-1}) significantly reduced all three cytokines earlier than the normal day time levels.

ELISA was used to assess levels of IgG to TSST-1, SEA and SEC. The majority of the population tested had sufficient antibodies to reduce TNF-α and IL-6 responses elicited by TSST-1 and SEC in the model system. In the age range in which most SUND cases occur (20-39 years), males had significantly lower levels of IgG to TSST-1 compared with females.

The methods developed for collection of tissue specimens, detection of toxins and quantitative IgG assays for antitoxins can be applied to investigation of SUND victims to test the hypothesis that some of these deaths are precipitated by pyrogenic staphylococcal toxins.

Chapter 1

General introduction

Sudden Unexpected Nocturnal Death (SUND)

Sudden and unexpected death of young adults during sleep is a regional phenomenon within Southeast Asia and in other countries such as the Middle East, and occurs in populations that are culturally and genetically distinct from the local population. It also is said to occur sporadically in most countries of the world.

These deaths are not anticipated by history, and a thorough *post mortem* examination including histology, toxicology and microbiology fails to demonstrate an adequate cause of death.

1.1 Prevalence of unexpected deaths in different populations

Sudden Unexpected Nocturnal Death (SUND) was reported as early as 1915 in young men in Manila, (Aponte, 1960) and in 1959 in Japan (Sugai, 1959) where it is known as 'bangungut' (nightmare death) and 'pokkuri' (sudden death), respectively. It was suggested that migrants from affected populations in Asia carry with them the

susceptibility to sudden death in sleep. (Munger, 1987). This syndrome had been known for a long time as 'lai-tai' (night death) among the indigenous population in north-eastern Thailand and Laos, but it was only documented when southeast Asian refugees died suddenly and unexpectedly in a mysterious manner in the USA (US Centers for Disease Control, 1981). SUND was subsequently reported to occur among immigrants from the Philippines, Thailand, and Bangladesh in countries such as Singapore (Goh *et al.*, 1990) and also among southeast Asian refugees (Baron *et al.*, 1983). Victims of SUND are usually employed in moderately heavy manual jobs such as labourers, construction workers, handy men or gardeners. Most of these deaths take place during the first year of residence in their adopted country and usually occur at night (Blackwell *et al.*, 1994).

Southeast Asian refugees in Thailand have the highest known rate of sudden death during sleep, but the risk declines greatly after immigration to the United States (US Centres for Disease Control, 1988).

In Singapore, most of these deaths (88.9%) occurred among young men from provinces of northeast Thailand (Goh *et al.*, 1993). A survey by the Thai Public Health Service found that unexpected deaths among men between the ages of 20-59 was highest in the north-east provinces (32/100,000) compared with their incidence in central (9.42/100.000), southern (18.26/100.000) or northern (20.9/100.000) provinces (Blackwell *et al.*, 1994). The victims were aged 21-54. The median interval between arrival in Singapore and death was 8 month, most of the victims

have been healthy before retiring to bed. When deaths were witnessed, roommates were alerted or awakened by noisy breathing, gasping or groaning. Attempts at resuscitation by chest massage were useless, and death occurred soon afterwards. In some cases the body was rigid and its fists clenched, and there was urinary incontinence.

Very few abnormal findings were observed at autopsy, which is routine for all sudden unexplained deaths in Singapore. In most cases, the heart was not enlarged and coronary arteries were normal. In 64% of victims, there was moderate to severe intra-alveolar haemorrhage (Goh *et al.*, 1993). Histopathological examination revealed myocarditis or pneumonitis in some, but extensive microbiological, biochemical, and toxicological investigations were inconclusive. The autopsy findings of pulmonary haemorrhage in more than half of the victims in Singapore resemble the findings in Filipino cases (Aponte, 1960). This was higher than those in southeast Asian refugees who died in a similar manner in the USA (Baron *et al.*, 1983).

Preliminary results of serial sections of the hearts of some cases indicate evidence of anomalies in the cardiac conduction system, as previously noted in Japanese men (Okada & Kawai, 1983) and in Southeast Asian refugees in the USA (Kirschner *et al.*, 1986). The functional significance of these pathological defects is not known, but it was thought that it could be related to sleep-related cardiac arrhythmias. (Kirschner

et al., 1986). No changes, which were pathognomonic to the conjoint, were identified.

For cultural reasons, autopsies are not carried out on unexpected deaths in Thailand; therefore, no comparisons can be made between the deaths in native and immigrant populations, and deaths of preens former the same geographical regions occurring in foreign countries. Familial aggregation of SUND has been observed among Southeast Asian refugees in the USA (Goh *et al.*, 1993) which suggests there might be a genetic contribution to susceptibility to these deaths.

Surveillance for sudden deaths was conducted among Laotian-Hmong refugees in the Ban Vinai refugee camp in northeastern Thailand to determine if similar deaths occurred there. Sixteen sudden and unexpected deaths associated with sleep were found that were similar to the sudden deaths noted among southeast Asian refugees in the United States. The victims of sudden deaths among Hmong refugees were predominantly males. The median age and length of stay in the USA before death were 33 years and 17 month respectively. They were all healthy and died during sleep (Adler, 1995). The rate of SUND from 1981-1982 among 25-44 years old Hmong men was 92/100,000 (Baron *et al.*, 1983).

Unexpected deaths in New Mexico from 1977 to 1988 were reviewed, and from this group 650 cases were obtained. The crude rate of sudden, unexpected death among New Mexico residents (5 to 39) years old during the study period was 6.6/100,000

persons. Males were affected approximately twice as often as females, and the incidence among American Indians (17.5/100,000) and Blacks (18.5/100,000) was relatively higher than for Caucasians (7.7/100,000) (Anderson *et al.*, 1994).

During the period between 1980-1989, 58 cases of SUND were recorded in the Eastern Province of Saudi Arabia (Faris & Fathalla, 1992), and it is suggested that incidence of such cases are on the increase during this period. Between 1993 to 1996, approximately 150 cases were recorded in the eastern province of Saudi Arabia. The majority of these deaths take place at night, during the early hours of the morning or during an afternoon nap. Most of the victims are young males from Thailand and Bangladesh (Al Madani, personal unpublished observation). In contrast, to Singapore autopsies are not performed routinely in Saudi Arabia on persons dying suddenly and unexpectedly unless the circumstances indicate the death was non-accidental. This had limited the depth of the investigations into the causes of these deaths.

1.2 Hypotheses to explain the causes of SUND

The epidemiological and pathological features of SUND deaths have stimulated a number of hypotheses as to their causes; however, as yet there is no satisfactory explanation for these deaths. Autopsies usually yield no significant pathological or toxicological findings.

1.2.1 Cardiac abnormalities

Ventricular fibrillation has been postulated as the ultimate cause of these deaths (Kirschner *et al.*, 1986). Electrocardiograph (ECG) surveys were carried to determined if ECG abnormalities are more common among refugees in Thailand at high risk of SUND compared with Asian and other groups in the USA who are at lower risk. They found that in men heart-rate-corrected QT interval was significantly greater among Laotian refugees in Thailand at high risk compared with Laotian refugees in the USA at lower risk and US residents at negligible risk (Munger *et al.*, 1991).

Among refugees in Thailand, prolonged QT interval was associated with poor thiamine status and a history of seizure-like episodes in sleep. Thiamine deficiency has been postulated as a cause of prolonged QT interval and sudden death in this region. (Munger *et al.*, 1991). Diurnal fluctuations in QT interval may be pronounced and related to changes in autonomic tone. The QT interval of humans is longer when asleep than when awake, possibly because of increased parasympathetic tone, decreased sympathetic tone, or both, during sleep, (Browne *et al.*, 1983).

1.2.2 Diet

Thiamine deficiency is widespread among the poor of Southeast Asia and can produce cardiac and neurological effects, which are widespread. These include

cardiac conduction and repolarisation abnormalities, arrhythmias, autonomic dysfunction and in cases in which Wernicke's encephalopathy has resulted even in sudden death. Sudden death during sleep has been observed in thiamine-deficient dogs. (Read & Harrington, 1981). Starvation studies with young male volunteers in Minnesota in the 1940s found that the ECG effects of pure thiamine deficiency included significant lengthening of the QT interval, low QRS voltage, and bradycardia (Munger *et al.*, 1991). The QT interval returned to normal after administration of thiamine.

Isolated thiamine deficiency is rare and other dietary factors may be important. Raw, dried, and fermented fish products form a substantial part of the southeast Asian diet and contain potent anti-thiamine compounds (Munger *et al.*, 1991).

Studies on the diet of Laotian refugees among whom similar deaths occur did not substantiate the hypothesis. The results could not account for the predominance of males among the victims as both men and women shared the same diet. The recent studies by (Goh *et al.*, 1993) found no evidence to support this hypothesis.

1.2.3 Hypokalaemia

Potassium is one of the most abundant intracellular cations and is involved in many vital cellular functions. Alteration of potassium concentrations is arrhythmogenic and

causes not only ventricular ectopic beats, tachycardia and fibrillation but also induces muscle weakness, paralysis, and rhabdomyolysis (Helfant, 1986).

Several factors have been postulated precipitating causes of SUND (Thai Ministry of Public Health Report, 1990). They include a large meal of glutenous rice or carbohydrate on the night of death and physical or mental stress. All these factors could lower serum potassium by stimulating the shift of potassium into cells (Williams *et al.*, 1985).

Hypokaleamic periodic paralysis (HPP) and endemic distal renal tubular acidosis are endemic in the northeast region of Thailand, and can present as sudden onset of muscle paralysis with potentially lethal cardiac arrhythmias and respiratory failure occurring late at night or the early hours of the morning (Nimmannit *et al.*, 1991). Since distal renal tubular acidosis is three times more prevalent in females than in males (Nilwarangkur *et al.*, 1990), it is difficult to attribute chronic potassium deficiency as a cause of SUND which involves mainly males.

1.2.4 Stress

Mental and emotional problems such as homesickness are thought to contribute to a significant proportion of SUND cases among Thai immigrants. Mental stress is known to induce cardiac arrhythmia and is associated with increased susceptibility to viral infections of the upper respiratory tract (Lown, 1982). Stress is thought to

influence immune function through autonomic nerves innervating lymphoid tissue (Felten & Olschowka, 1987) or hormone-mediated alteration of immune cells (Rabin *et al.*, 1989). Psychological stress was associated in a dose-response manner with an increased risk of acute infectious respiratory illness, and this risk was attributable to increased rates of infection rather than to an increased frequency of symptoms after infection (Cohen *et al.*, 1991). Stress may also alter immune responses through the adoption of coping behaviours such as increased smoking and alcohol consumption (Kiecolt-Glaser & Glaser, 1988).

1.2.5 Smoking

Smoking has been suggested to be associated with SUND. Studies by the Thai Ministry of Public Health found a higher proportion of smokers (70%) among men who died unexpectedly in Thailand compared with the general population (50%). Smoking might contribute to the predominance of males among SUND victims as smoking is more prevalent among Thai men (50%) than among Thai women (<10%) (Blackwell *et al.*, 1994).

A significant proportion of SUND victims are smokers or even if they did not smoke personally, they died in an environment in which they were passively exposed to cigarette smoke.

1.2.6. Asymptomatic infection

1.2.6.1 Melioidosis

Epidemiological investigations showed that a family history of similar deaths and serological evidence of current or recent infection with *Burkholderia (Pseudomonas) pseudomallei* were significantly associated with SUND. Because the victims were all men from villages in northeastern Thailand, melioidosis was investigated as a possible cause. The disease is endemic in the region and predominantly affects men. Melioidosis is caused by the Gram-negative organism which is found in the soil and surface water of rice fields, of southeast Asia (Goh *et al.*, 1993). The spectrum of diseases produced by *B. pseudomallei* is not well known, but most infections are thought to be symptomless (Kanai & Dejsirilert, 1988; Wongwanich *et al.*, 1996).

1.2.6.2 Pertussis toxin

It has been suggested from epidemiological evidence that some cases of SIDS might be due to asymptomatic infection by *Bordetella pertussis* (Nicoll & Gardner, 1988). The area of northeast Thailand from which most of the SUND victims in Singapore originated has the highest incidence of whooping cough in the country. Until 1978, there was no childhood vaccination programmes; therefore, the age group affected by SUND might be vulnerable to this infection (Blackwell *et al.*, 1994). No studies have been carried out to attempt to isolate the bacteria or their toxins in these individuals.

1.3 Toxigenic bacteria SIDS and SUND

Several pre-disposing factors associated with SUND are similar to those observed for sudden infant death syndrome (SIDS) (Blackwell *et al.*, 1994). A hypothesis that toxigenic bacteria might contribute to some cases of SUND has evolved from studies on the role of bacterial toxins in SIDS. The absence of invasive micro-organisms at autopsy, or significant prodromal illness other than mild respiratory tract infection are common in both (Blackwell *et al.*, 1994).

The idea that commonly occurring bacterial toxins have a pathogenic role in some cases of SIDS is gaining support. It is consistent with the epidemiological features of SIDS, including the winter excess of cases, the characteristic age distribution, and the association with a prone sleeping position (Morris *et al.*, 1987; Morris, 1999). The role of toxigenic bacteria and parallel between risk factors for SIDS and SUND are assessed in the following sections.

Pyrogenic toxins of *S. aureus* have been identified in tissues of two cases of sudden unexpected death in adults (Zorgani *et al.*, 1997) and in 6 year old child who died unexpectedly following parainfluenza infection (Bentley *et al.*, 1997).

1.3.1 Toxigenic bacteria

Bacteria implicated in SIDS are listed in Table 1.1. Many of the toxins of these bacteria can act as superantigens by binding to major histocompatibility complex (MHC) class II proteins, causing extensive T-cell proliferation and cytokine release (Marrack & Kappler, 1990). These cytokines mediate toxic shock syndrome the pathophysiology of which mimics that of endotoxin shock (Bohach *et al.*, 1990; Bone, 1994). The systemic effects of pyrogenic toxins are manifested in rapid onset of high fever, shock, capillary leakage, and multi-organ dysfunction.

Table 1.1 Bacteria implicated in SIDS or SUND

species	toxin	superantigen	references
Staphylococcal aureus	Enterotoxins, TSST-1	yes	(Newbould *et al.*, 1989; Malam *et al.*, 1992)
Bordetella pertussis	Pertussis toxin/endotoxin	No/yes	(Nicoll & 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.*, 1993)
Streptococcus pyogenes	Pyrogenic toxins A and B	yes	(Telford *et al.*, 1989)
Escherichia coli	Enterotoxin, verotoxins	?	(Bettelheim *et al.*, 1990; Bettelheim *et al.*, 1995;
	Endotoxin	yes	Oppenheim *et al.*, 1994)
Pseudomonas aeruginosa	Exotoxin	?	(Rumbaugh *et al.*, 1999)
Burkholderia cepacia	Endotoxin	yes	(Kanai & Dejsirilert, 1988; Wongwanich *et al.*, 1996;
			Zughaier *et al.*, 1999)

1.3.2 Toxic shock syndrome (TSS)

Toxic shock syndrome (TSS) was first suggested as specific pathological entity in 1980-1981, when numerous cases were identified among women using tampons (Chesney *et al.*, 1981). The toxic shock syndrome toxin (TSST-1) accounts for more than 90% of cases that are associated with menstruation. Other enterotoxins account for 50% of cases unrelated to menstruation. Non-menstrual cases have increased recently and now account for one third of all cases. Patients with non-menstrual TSS have higher mortality rates than those with menstrual TSS (Wergeland *et al.*, 1989). Colonisation by *S. aureus* and TSST-1 has also been observed following influenza or influenza-like illnesses in adults (MacDonald *et al.*, 1987).

1.3.3 Pyrogenic toxins

Staphylococci that produce pyrogenic toxins including TSST-1, have been isolated from SIDS infants (Telford *et al.*, 1989). Pyrogenic staphylococcal toxins have been identified in SIDS infants (Newbould *et al.*, 1989; Malam *et al.*, 1992; Murrell *et al.*, 1993). These superantigens exert a powerful effect on the immune system, stimulating T-cells, which subsequently induce the formation of large amounts of cytokines. Generation of an overwhelming inflammatory response may lead to death by shock (Lindsay *et al.*, 1994). These toxins cause fever (>38.5^{o} C), are mitogenic for lymphocytes, induce release of tumour necrosis factor (TNF-α), interleukin 1 (IL-1) and nitric oxide (Blackwell *et al.*, 1995). The pyrogenic toxins can also amplify

the effects of endotoxic shock over 100,000 fold (Bohach *et al.*, 1990). Both IL-1 and TNF-α are thought to induce fever by the production of prostaglandin E_2 in the preoptic area of hypothalamus (Dinarello *et al.*, 1986; Dinarello, 1996). IL-1 has been suggested to cause deep sleep associated with prolonged periods of apnea in infants (Guntheroth, 1989).

Pyrogenic toxins of *S. aureus* are produced between 37-40° C and in greater quantities at the higher temperatures (Bohach *et al.*, 1990). Under most circumstances, the temperature of the nasopharyngeal mucosa is below 37°C due to the cooling effect of the passage of air during breathing. If there are environmental factors that increase the temperature of the mucosal surfaces, *e.g.* low grade fever and blocked nasal passages due to viral infection, this might result in a "microenvironment" in which the temperature is sufficiently raised to induce toxin production (Blackwell *et al.*, 1995).

1.3.4 Synergy or additive effects of toxins and other factors.

A single infectious agent alone is probably not responsible for SUND or SIDS. Synergistic effects between infectious agents or their toxins, and environmental factors might be involved in these sudden deaths. In experimental models, synergy occurs between toxins of the same micro-organism or between toxins of two different micro-organisms, as in the case of an enhanced lethality of staphylococcal toxin in the presence of preparations from *E. coli* in the chick embryo model (Drucker *et al.*,

1992). Similarly, *S. aureus* TSST-1 and endotoxin showed enhanced toxicity on chicken embryos when both toxins were combined (de Azavedo *et al.*, 1985).

Nicotine potentiates the lethal action of synergistic combination of bacterial toxins (Sayers *et al.*, 1995). Viral infections have been demonstrated to enhance the lethality of bacterial toxins in an animal model (Jakeman *et al.*, 1991) and can also enhance TNF-α production from human cells in response to bacterial toxins (Lundemose *et al.*, 1993). Studies by (Sarawar *et al.*, 1994) on staphylococcal toxins and a symptomatic viral infection in mice indicated that the inflammatory response to viral infections could promote the induction of TNF-α and other cytokines. (Stiles *et al.*, 1999) demonstrated in mice that there is a synergistic effect between staphylococcal enterotoxins or TSST-1 and lipopolysaccharide (LPS) measured by increased production of proinflammatory cytokines and decreased temperature. The lethal synergistic effect between bacterial exotoxins and LPS from different species has been demonstrated in the chick embryo model (Sayers *et al.*, 1995).

1.3.5 Factors enhancing colonisation by toxigenic bacteria

There are 3 factors that might enhance frequency or density of colonisation by staphylococci or *B. pertussis*: expression of the Lewis^a antigen; infection by respiratory virus; and exposure to cigarette smoke.

1.3.5.1 Expression of Lewis^a antigen

The peak incidence of SIDS occurs in the 2-4 month age range. During this period, 80-90% of infants express the Lewis^a antigen and *S. aureus* is isolated from approximately 40% infants in this age range (Aniansson *et al.*, 1992; Blackwell *et al.*, 1999; Harrison *et al.*, 1999). It has been demonstrated that the Lewis^a antigen is one of the receptors for 6 strains of staphylococci producing pyrogenic toxins A, B C. and TSST-1 and 2 non-toxigenic strains of staphylococci (Saadi *et al.*, 1993). Both staphylococci and *B. pertussis* bind to the Lewis^a antigen, and the closely related Lewis^x antigen on epithelial cells (Blackwell *et al.*, 1995), and a protein adhesin on toxigenic staphylococci that binds to Lewis^a has been identified (Saadi *et al.*, 1994).

There are, however, no data on the expression of this antigen on epithelial cells of the groups affected by SUND.

1.3.5.2 Virus infection and bacterial toxin

Both natural virus infections and live influenza vaccines have been demonstrated to enhance colonisation by potentially pathogenic bacteria (Ramirez-Ronda *et al.*, 1981). Laboratory studies have demonstrated enhanced binding of staphylococci, *B. pertussis,* pneumococci and several Gram-negative species identified in SIDS infants to HEp-2 cells infected with respiratory syncytial virus (RSV), (El Ahmer *et al.*, 1996; Saadi *et al.*, 1996). *In vitro*, RSV-infected cells expressed increased levels of

CD14 and CD18 to which endotoxin binds (El Ahmer *et al.*, 1996; Raza *et al.*, 1999). A similar pattern has been observed in this model system with influenza virus (El Ahmer *et al.*, 1999).

1.3.5.3 Effect of cigarette smoke

Smokers are more frequently and heavily colonised by potentially pathogenic bacteria (Blackwell *et al.*, 1990; Blackwell *et al.*, 1992). Staphylococci, *B. pertussis*, pneumococci and several Gram-negative species bound in greater numbers to buccal epithelial cells from smokers compared with those from non-smokers (Saadi *et al.*, 1996; El Ahmer *et al.*, 1999). Smokers have more viral upper respiratory tract infections, which have been found to enhance colonisation by staphylococci (Ramirez-Ronda *et al.*, 1981). Stress is associated with increased susceptibility to viral infection (Cohen *et al.*, 1991) and with increased cigarette consumption. Passive coating of epithelial cells with cigarette smoke extract showed enhanced bacterial binding but no change in expression of antigens receptors Lewis ^{a} , Lewis ^{b} Lewis ^{x}, fibrinogen and fibronectin (El Ahmer *et al.*, 1999).

1.4 Inflammatory responses in SIDS and SUND

SIDS and SUND are suggested to be due to pathophysiological responses elicited by a combination of microbial products and / or cigarette smoke when the host's endocrine responses are less able to "damp down" inflammatory mediators. It is

likely that the major damage is due to overproduction of inflammatory mediators by autoamplification due to synergistic effects of the body's responses to viral infection, bacterial toxins and possibly components of cigarette smoke.

Over the past 15 years our understanding of the pathophysiology of inflammation and septic shock has progressed considerably. Microbial constituents such as lipopolysaccharide (LPS, endotoxin) of Gram-negative bacteria, and the exotoxins or cell-wall fragments of Gram-positive bacteria have been shown to be potent activators of a wide range of cells, including monocytes, neutrophils and endothelial cells. Upon stimulation, these cells release pro-inflammatory mediators which play a critical role in the host's response to invasion by micro-organisms, and serve to coordinate the cellular and humoral components that contribute to the development of inflammatory and immune reactions (Glauser *et al.*, 1994). The cytokine mediators (TNF-α, interleukins, chemokines and colony-stimulating factors) exert chemotactic effects for immune cells, enhance the expression of the MHC class 1 and II antigens, and participate in the activation and proliferation of B and T lymphocytes.

While inflammation is an essential feature of the host defences, it needs to be regulated to insure that excessive or unnecessary tissue damage is not produced. A lack of inflammatory response as seen in immunosuppressed patients is detrimental and promotes infection. On the other hand, excessive inflammatory reactions associated with septic shock are harmful. Indeed, severe sepsis and septic shock are a manifestation of an overwhelming, dysregulated inflammatory host response

characterized by massive activation of several systems leading to multiple organ dysfunction and death in a high proportion of cases (Heumann *et al.*, 1998).

1.4.1 Role of cytokines in pathogenesis of septic shock

The presence of bacterial infection is associated with the production of many pro-inflammatory cytokines including TNF-α, IL-1 and IL6 (Dinarello, 1996). The cytokines mediate the range of symptoms associated with infection such as fever, anorexia, and fatigue. Purified TNF-α and IL-1 induce changes similar to those induced by endotoxin which include haemorrhagic and ischaemic necrosis of the gastrointestinal tract, haemorrhages and leukocyte infiltration in the lungs, acute tubular necrosis of the kidneys, metabolic acidosis, stress hormone elaboration, hypotension, circulatory collapse and death. When these changes are induced by Gram-negative bacteria shown to evoke the cytokines cascade, they can be completely inhibited by specific inhibition of TNF-α or IL-6 (Ohlsson *et al.*, 1990).

1.4.2 Control of cytokine production in response to endotoxin

Interleukin (IL-10), interleukin (IL-4), and interleukin (IL-3) decrease or may even completely suppress the synthesis of pro-inflammatory cytokines (Bogdan & Nathan, 1993). IL-10, a pluripotent molecule, exerts its anti-inflammatory effects by inhibiting cytokine synthesis in macrophages and Th1 T cells (Fiorentino *et al.*, 1991). These anti-inflammatory cytokines act primarily by inhibiting transcription of

pro-inflammatory cytokine genes (Wang *et al.*, 1994). Circulating anti-inflammatory cytokines exert profound inhibitory effects. IL-10 was reported to protect mice against staphylococcal enterotoxin B-induced lethal shock when administered before or concurrently with staphylococcal enterotoxin B (Bean *et al.*, 1993).

1.4.3 Cytokines and sleep.

Several cytokines have been demonstrated to be somnogenic. For example, in experimental systems, IL-1 enhances slow-wave sleep (Krueger *et al.*, 1984). IL-1 and TNFα have been shown to exhibit a high degree of temporal regulation in humans, and it is very likely that they are either regulated by sleep or are themselves important regulators of normal sleep (Krueger & Majde, 1995). Other cytokines have no sleep-enhancing potency, *e.g.* IL-2 and IL-6 (Krueger & Majde, 1995). Patients with sleep apnea syndrome experience disturbed sleep patterns; they have less sleep at night and spells of sleep during the day. Night time TNFα, IL-6, interferon γ and IL-1 peaks observed in normal individuals are observed during the day in these patients (Entzian *et al.*, 1996).

Circadian variation in cytokine release is influenced by the synthesis and release of endogenous hormones such as melatonin and cortisol (Utiger, 1992). Melatonin modulates the effect of antibody response and exhibits antagonist effects on corticosterone-induced immunosuppression effects, which have been demonstrated in experimental animals (Maestroni *et al.*, 1986).

1.4.4 Diurnal variation in levels of cortisol and responsiveness of cortisol to inflammatory response.

Cortisol and other glucocorticoids suppress virtually all components of the immune response including release of cytokines and inflammatory mediators such as eicosanoids, bradykinin, serotonin and histamine (Reichlin, 1993). The hypothesis that anti-inflammatory actions of glucocorticoids are physiological, (Munck *et al.*, 1984) implies that in the absence of glucocorticoids, inflammatory responses should be exacerbated. Laue *et al.* (1988) found that the inflammatory response to subcutaneous carrageenin in normal rats is enhanced by administration of the glucocorticoids receptor antagonist RU 486 in the resting state. Glucocorticoids are probably the prime immuno-regulators secreted during stress. (Reichlin, 1993). This became evident in experiments in which stressing of adrenalectomized or hypophysectomized rats caused inhibition of mitogen-stimulated lymphocyte proliferation (Keller S.E. *et al.*, 1991).

Cytokine-induced glucocorticoid secretion and glucocorticoid inhibition of cytokine synthesis act as important safeguards in preventing cytokine overreaction (Costas *et al.*, 1996). Measurement of diurnal variations in plasma levels of TNFα or IL-6 in relation to level of adrenocorticotropic hormone (ACTH) and cortisol in response to endotoxin showed that during the day there was no effect on the endotoxin-induced increase in plasma TNFα or IL-6. Subjects who received endotoxin in the evening

when endogenous glucocorticoid levels were low showed about twice the increase in plasma ACTH and cortisol as those who received endotoxin in the morning when endogenous glucocorticoid levels were high. These results demonstrate diurnal variations in susceptibility to endotoxin that may be due to a suppression of the biologic effects of TNFα and IL-6 by endogenous glucocorticoids (Pollmacher *et al.*, 1996).

In adults, circadian variations in the concentration of cortisol have been noted with minimal secretion rates of the hormone occurring in the early hours of the morning, and the adrenal gland is more responsive to a standard dose of ACTH during the day than after midnight (Van Cauter *et al.*, 1996) when many SUND deaths occur (Goh *et al.*, 1993). Circadian variations in the toxicity of TNF-α may be involved. It was reported that TNF-α induced lethality for mice varied up to 9-fold across the day, being lowest in the second half of the active period and highest at the end of the resting period (Hrushesky *et al.*, 1994). Diurnal variations on the effects of TNF-α and IL-6 on the rectal temperature and the hypothalamic-pituitary-adrenal (HPA) system may be related to the circadian rhythm in plasma cortisol levels (Fantuzzi *et al.*, 1993). For example, both adrenalectomy (Bertini *et al.*, 1988) and glucocorticoid receptor type II blockade (Brouckaert *et al.*, 1992) sensitise animals to the lethal effects of TNF-α, suggesting that endogenous glucocorticoids play a protective role against the toxic effects of cytokines.

1.4.5 Enhancement of cytokine levels by glucocorticoids

In contrast, glucocorticoids have been shown to promote the production and release of several cytokines. Liao *et al.* (1995) have shown that corticosterone induces the cytokines IL-6 and TNF-α when administered at either basal (35 ng ml^{-1}) or stress related (350 ng ml^{-1}) levels in an *in situ* liver perfusion system in the absence of other stimuli. When infused together with endotoxin, the basal dose potentiates cytokines production, whereas the stress-related dose acted suppressively.

1.4.6 Stress, hormone levels and cytokines

Stimulation of the HPA axis and glucocorticoids by stress induce a negative feedback loop which exists between peripheral inflammatory cytokines and the HPA axis, in which cytokines promote hypothalamic corticotropin-releasing hormone (CRH) release and subsequent activation of the pituitary-adrenal axis (Sternberg *et al.*, 1992). The effects of basal non-stress concentrations of endogenous corticosteroids on specific aspects of immune function are largely unknown. The effects of physiological fluctuations in corticosteroid levels have not been systematically examined in humans. Although studies of the adrenal gland responses indicate an important regulatory role of the HPA axis on susceptibility to inflammation, definitive evidence for the precise regulation of immune functions by the HPA axis in humans does not exist.

Stress and non-stress levels of glucocorticoids are regulated through two different types of glucocorticoid receptors. The type 1 high affinity, (mineralocorticoid receptor) mediates non-stress circadian fluctuations in glucocorticoids. The type 2 low affinity (glucocorticoid receptor) mediates stress levels of glucocorticoids (Sutanto & de Kloet, 1991).

Administration of a pharmacological dose of hydrocortisone suppressed the production of IL-β, IL-6, and TNF-α whereas administration of a physiological dose of hydrocortisone suppressed only TNFα production. Stress-induced levels of glucocorticoids achieved during exercise suppressed IL-β and TNF-α production (De Rijk *et al.*, 1997). Extensive exercise cause impaired neutrophil bactericidal function, which increased the susceptibility to infection. This was suggested to be due to the induced increases in cortisol level which suppressed not only neutrophils but also B and T cell function (Fukatsu *et al.*, 1996).

Mental stress and microbial infection are both associated with a variety of endocrine changes including activation of the HPA system (Solomon, 1987). The inflammatory response to infection with Gram-negative bacteria represents a different form of stress induced by endotoxins which stimulate the synthesis and release of cytokines including IL-1, IL-6, and TNF-α *in vitro* (Flad *et al.*, 1989), and IL-6, and TNF-α in vivo (Mackensen *et al.*, 1991).

During septic shock, cortisol values range from normal to 20 times the high normal value (Schein *et al.*, 1990). This discrepancy could have several explanations such as disruption of the circadian rhythm of cortisol in severely stressed patients or impairment of the HPA (Perrot *et al.*, 1993). All these factors could lead to the wide variation in measured adrenocortical response to severe septic shock. Finlay and McKee reported increased mortality when morning cortisol levels showed a progressive fall to less than 12 $\mu g\ dl^{-1}$ (Finlay & McKee, 1982).

1.4.7 Role of Prostaglandins on inflammatory responses

Prostaglandins have been suggested to play a role in the generation of the circadian changes in body temperature because prostaglandin synthesis inhibitors suppress the physiological nocturnal temperature rise in rats (Scales & Kluger, 1987). In addition, prostaglandins are involved in both the pyrogenic and HPA system responses to endotoxin in humans; ibuprofen (an anti prostaglandin) blocks both temperature and cortisol increases (Martich *et al.*, 1992). Studies in rats suggest that HPA-system activation by TNF-α is mediated by prostaglandins in a manner independent of endogenous glucocorticoid feedback, because even in adrenalectomixed animals, TNF-α-induced ACTH release can be blocked by indomethacin (Sharp & Matta, 1993). Diurnal variations in cytokine-induced prostaglandin synthesis might represent a corticosteroid-independent mechanism modulating the human host response to endotoxin in a 24-h period (Pollmacher *et al.*, 1996).

1.5 Aims of the study

The major aim of the study was to develop methods to test the hypothesis that pyrogenic toxins of *S. aureus* are involved in some cases of SUND in Saudi Arabia.

The specific objectives were:

1. to develop an adequate method for tissue sampling that does not result in disfiguring of the body;
2. to develop a method for detecting bacterial toxins in tissue and body fluids;
3. to examine the effect of night time, day time and stress levels of cortisol on inflammatory response to pyrogenic staphylococcal toxins;
4. to examine the levels of antibodies to pyrogenic toxins in relation to age and sex;
5. to examine the effect of different level of antibodies to pyrogenic toxins on their induction of inflammatory responses.

Chapter 2

General materials and methods

2.1 The toxins, antibodies, horseradish peroxidase (HRP) or flourescein isothiocyanate (FITC) labelled antibodies to toxins and other antigens are listed in tables 2.1and 2.2

Table 2.1 Toxins and suppliers

Toxin	Supplier
Staphylococcal enterotoxin A	Toxin Technology
Staphylococcal enterotoxin B	Toxin Technology
Staphylococcal enterotoxin C	Toxin Technology
Toxic shock syndrome toxin-1	Toxin Technology

Table 2.2 Antibodies and suppliers

Antibody	Source	Supplier
Anti-SEA	Rabbit	Toxin Technology
Anti-SEA	Sheep	Toxin Technology
Anti-AEC	Rabbit	Toxin Technology
Anti-AEC	Sheep	Toxin Technology
Anti-TSST-1	Rabbit	Toxin Technology
Anti-TSST-1	Sheep	Toxin Technology
HRP anti-SEA	Rabbit	Toxin Technology
HRP anti-SEC	Rabbit	Toxin Technology
HRP anti-TSST-1	Rabbit	Toxin Technology
HRP anti-human IgG	Sheep	SAPU [1]
HRP anti-sheep/goat	Donkey	SAPU
Monoclonal anti-IL-6	mouse	R&D Systems [2]
Polyclonal anti-human IL-6	goat	R&D Systems
Monoclonal anti-IL-10	mouse	R&D Systems
Polyclonal anti-human IL-10	goat	R&D Systems
FITC anti-rabbit IgG	goat	Sigma [3]

1. Scottish Antibody Production Unit, Carluke, Lanarkshire
2. R&D Systems, Abingdon, UK
3. Sigma, Poole, Dorset.

2.2 Phosphate buffered saline

Phosphate buffered saline (PBS) contained $Na_2 HPO_4$ (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).

2.3 Enzyme linked immunosorbent assays (ELISA)

2.3.1 ELISA reagents

2.3.1.1Coating buffer.

The coating buffer consisted of 15mM Na_2CO_3, 35 mM NaH_2CO_3 and 3mM sodium azide (pH 9.6).

2.3.1.2 Washing buffer

The washing buffer contained 0.05% (v/v) Tween 20 and 0.1% (w/v) bovine serum albumin (BSA) in PBS.

2.3.1.3 Blocking buffer

Blocking buffer contained 1% (w/v) BSA in PBS

2.3.1.4 Substrate.

The substrate contained 40 μg O-phenylenediamine in 100 mi of 0.1M phosphate citrate buffer (0.1 M Na_2HPO_4 and 0.1 M citric acid, pH 5) activated by 40 μl 30% (v/v) H_2O_2 immediately before use.

2.3.1.5 Stopping solution

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

2.4 Tissue culture: culture media, supplements and cells.

2.4.1 Dulbecco's modified essential medium

Dulbecco's modified essential medium (DMEM) (Sigma) was used as the basis of the culture media for both human peripheral blood leukocytes and L-929 cells. Growth medium (GM) for L-929 consisted of DMEM with 1% (w/v) glutamine (Gibco), 100 IU ml^{-1} penicillin (Gibco), 200 μg ml^{-1} streptomycin (Gibco), and 10%

(v/v) foetal calf serum (FCS). For human peripheral blood leukocytes the FCS was substituted with 10% (v/v) human serum each buffy coat with its respective serum.

2.4.2 Human peripheral blood leukocytes

One day old buffy coats (50 ml) from donors of blood group O were obtained from the Scottish National Blood Transfusion Service, Royal Infirmary of Edinburgh. The samples were collected between 9:00-16:30 h. They were diluted 1 in 4 in sterile PBS under aseptic conditions. The diluted blood (40 ml) was layered carefully on Histopaque (12 ml) (Sigma) in (50 ml) sterile plastic centrifuge tubes and centrifuged for 30 min at 300 x g. Leukocytes (monocytes and lymphocytes) were collected from the interface and washed twice in DMEM at 250 x g for 10 min. Viable cells were counted by the trypan blue exclusion method with a Neubauer haemocytometer and diluted to 2×10^{6} cells ml^{-1} in DMEM with 10% (v/v) human serum (2.4.1).

2.4.3 L929 cells

The L929 mouse fibroblast cell line was obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). Cells were cultured in GM (2.4.1) at 37°C in a 5% CO_2 atmosphere until they reached a semi-confluent state.

Chapter 3

Needle biopsy for collection of tissue samples

3.1 INTRODUCTION

The explosion of new technology and imaging techniques have to a large extent resulted in a decline in the autopsy as a form of medical investigation (Huston *et al.*, 1996; Lipsky, 1993). Several studies have shown a 10% to 41% incidence of major discrepancies between clinical diagnoses, including those listed on death certificates, and the diagnoses found at autopsy (Harris & Blundell, 1991). This is still happening despite the technological advances.

Without an autopsy, there can be no certainty that the clinical diagnosis is entirely correct; furthermore, the extent and varieties of pathological and mutual interactive processes cannot be fully assessed, nor the efficacy of treatment fully evaluated. Without autopsy there cannot be any guarantee of the quality of medical care, indeed most of modern medicine and pathology is based on knowledge gleaned from the autopsy (Davis & Peterson, 1996).

3.1.1 What constitutes an adequate autopsy?

An autopsy is only part of an investigation of a sudden and unexpected death, and has to be associated with a full scene investigation and an investigation of the past medical, social and family history of the deceased. Ideally an adequate autopsy would also involve the participation of numerous individuals other than the pathologist; these include the medical examiner/coroner/ procurator fiscal, public health officials, the patient's physician, and the police or other agency which is officially tasked with the investigation of deaths. Collaboration between such agencies enhances the ability of the pathologist to determine accurately the cause and circumstances of death. A review of the medical and family history, and of all other information collated from the relevant agencies, such as social welfare and health care services, is essential.

A complete autopsy consists of a full systematic external and internal examination of the body, routine microscopic examination of representative blocks from different organs, and toxicological, microbiological, and biochemical investigations on material collected at necropsy. More sophisticated tests aimed at looking for evidence of stimulation of the inflammatory responses would be a useful adjunct, though these are not yet widely available. Similarly, culture of cells taken at autopsy to determine the presence of genetically-determined and hereditary disorders should also follow when appropriate in the interest of the remaining offspring and siblings of the deceased.

3.1.2 Decline in autopsy rate.

Over the last two decades, the rate of autopsies has declined worldwide (Lipsky, 1993; Huston *et al.*, 1996; McManus & Wood, 1996; Setlow, 1996). Among the reasons for this decline are the vast improvements and the resolution available in modern diagnostic techniques, financial constraints of hospitals which divert the resources away from the autopsy room to clinical needs, fear of litigation and a variety of personal, cultural and religious motives of both physicians (who fail to seek consent for the autopsy) and of the family members of the deceased (who prefer not to permit the autopsy taking place) (Huston *et al.*, 1996).

The issue of religious objection to an autopsy is another emotionally-laden matter. It becomes particularly manifest in the post-mortem investigations on orthodox Jews, which are deemed essential by law and are initiated by the Medical Examiner in the United States (Mittleman *et al.*, 1992) and by the relevant authorities in Israel (Avrahami *et al.*, 1995). Similar objections are raised by Muslims, Hindus and some Roman Catholics.

3.1.3 Variation in autopsies in countries in which SUND is a problem

SUND is a considerable problem in Singapore and Saudi Arabia, and it is useful to consider the methods of investigations of such deaths in these two countries.

3.1.3.1 Singapore

Singapore follows a system similar to that in Britain. SUNDs are reported to the Coroner as are other violent, unnatural and suspicious deaths. About 3000 unexpected deaths are reported every year and there is a 70% autopsy rate (Goh *et al.*, 1993). The autopsies are carried out to very high standards and are backed further by full laboratory investigations.

3.1.3.2 Saudi Arabia

In Saudi Arabia, autopsies are infrequent and indeed are not performed routinely on bodies of persons dying suddenly and unexpectedly unless the circumstances indicate the death was non-accidental or non-natural. It is indeed difficult to persuade the appropriate authorities to instruct a post-mortem examination in Saudi Arabia because of cultural objections and the extensive bureaucracy that is required to allow such examinations to proceed. Many Saudis believe that dissection of the human body is a form of desecration, and although this is not strictly forbidden on religious

grounds, they insist for such reasons that an autopsy is not performed on their relatives. The only authority on which an autopsy can be instructed is that of the Governor of the Region in which the death has occurred. This is a cumbersome procedure and permission is usually given only to exclude criminal activity. This permission is still subject to the full consent being given by the next of kin of the deceased which, for the reasons outlined above, is not usually forthcoming.

3.1.4 Alternative autopsy methods

One method of sampling individual organs after death is to use a needle. Understandably this method will only enable major pathology to be excluded and is not as conclusive and as complete as a conventional autopsy. This method has been attempted particularly in those situations where autopsy consent would not have been available. This procedure can provide tissues or fluid samples.

Foroudi *et al.*, (1995) in their study from a large city mortuary in New South Wales involving 21 non-traumatic, non-suspicious, coronial deaths found that post-mortem needle sampling was inferior to the ordinary autopsy both in terms of the yield in terms of pathological diagnoses and in terms of erroneous diagnosis. Post-mortem needle sampling should never replace a complete autopsy, but might be useful in circumstances in which conventional autopsy is not possible for a variety of reasons. It should only be offered as last possible alternative to families who will not consent to a complete autopsy.

Post mortem laparoscopic autopsy is another less invasive technique which does not disfigure the body and is more readily accepted by families of the deceased who have refused permission for a full post-mortem examination (Avrahami *et al.*, 1995). This system was applied in a series of examination which were carried out in Tel Aviv on twenty cadavers and comparison of the findings of the procedure in the abdominal area showed a 100 % correlation in all cases of intra-peritoneal haemorrhages, hepatic and spleenic injury, diaphragmatic tear, gunshot wounding and small bowel perforation. Retroperitoneal and rectal injuries and tumours were not discovered. This technique, therefore, has its limitations in terms of completeness and is only a poor substitute for a complete autopsy.

Since the result of this study would be applied to deaths in countries where objections to autopsy would be frequent, the first objective was to develop a quick, relatively non-invasive and reliable method to collect representative and adequate samples of those internal organs which would be most relevant to the study under circumstances in which a complete autopsy would not be possible. Needle biopsy techniques were, therefore, examined to determine if samples from the brain, liver, and spleen could be obtained without any obvious or identifiable disfiguring of the body.

3.2 Materials and methods

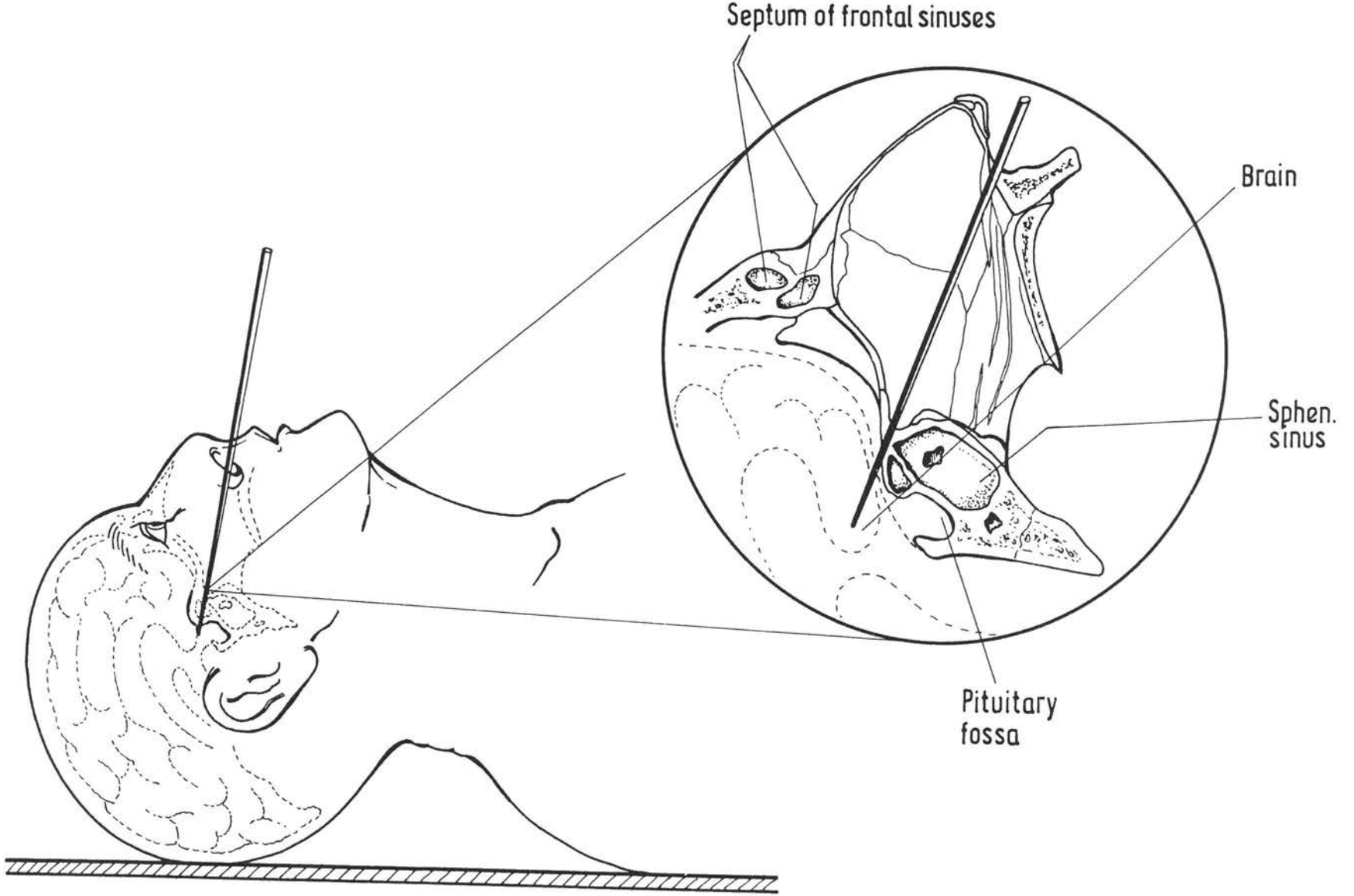
3.2.1 Collection of tissues by biopsy needle

A Trucut biopsy needle (15.2 cm long cannula with a 2.0 mm diameter core) (Baxter Health Care Corporation, USA). More than one core of each of the organs was taken by separate piercings with the needle to obtain representative samples of the organ. The tissue samples not used for histology were stored at -20°C. The core obtained was a cylinder of tissue up to 3.0 cm in length.

3.2.1.1 Brain

A Trucut biopsy needle was introduced through the nose and inserted through the cribriform plate of the ethmoid bone in the roof of the nose to reach the adjacent part of the brain which is usually the mid-brain (Figure 3.1).

fig 3·1


Septum of frontal sinuses
Brain
Sphen.
sinus
Pituitary
fossa

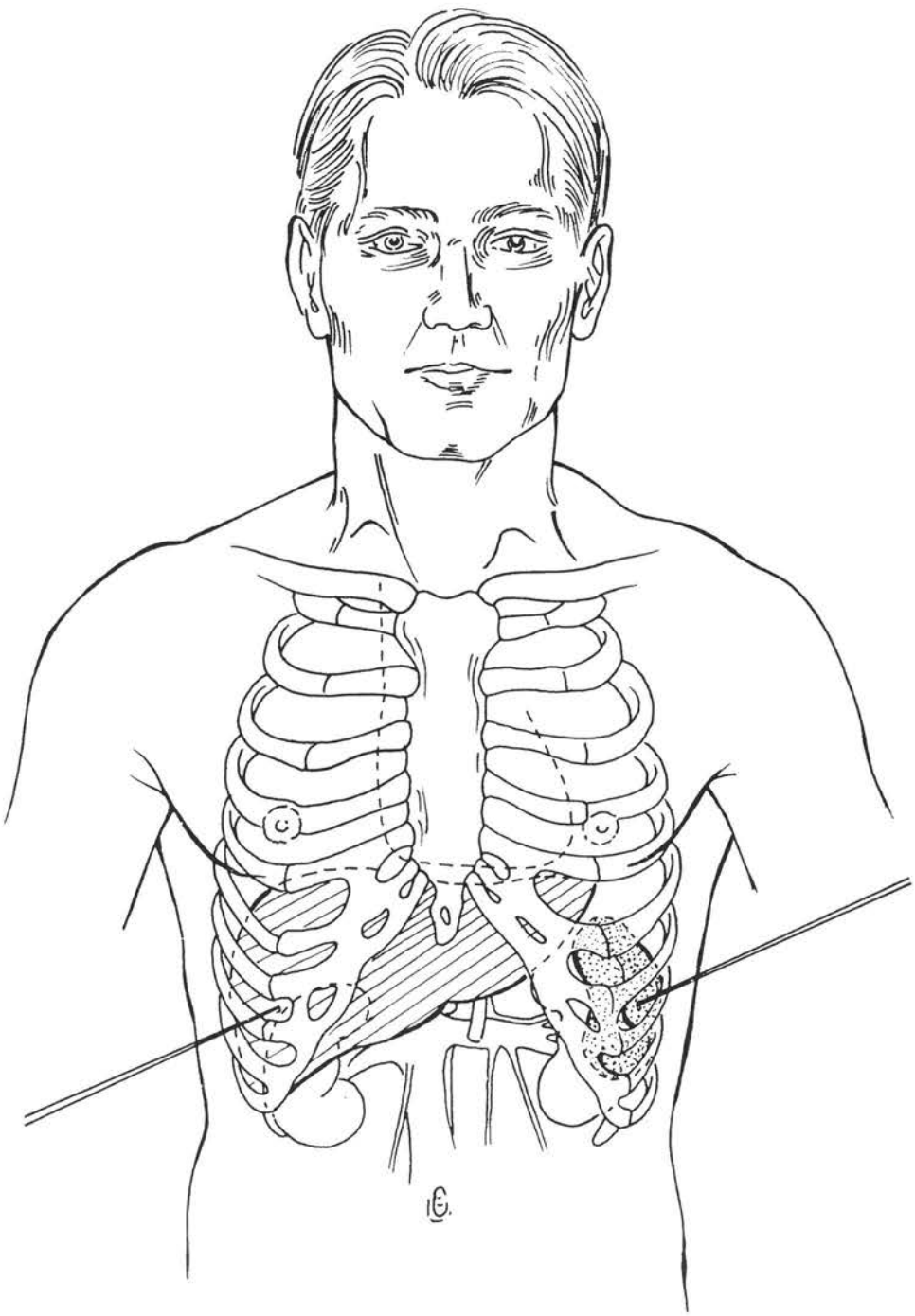
3.2.1.2 Liver

The liver was sampled by a similar needle through the intercostal space in the mid-axillary line between the 9th and the 10th ribs or the 10th and the 11th ribs (figure 3.2) on the right.

3.2.1.3 Spleen

The spleen was sampled through the 10th intercostal space in the mid-axillary line on the left (figure 3.2).

fig 3·2



3.2.2. Processing and staining

Part of the tissue samples obtained were fixed in buffered formalin and prepared for histopathological examination using standard techniques to produce Haematoxylin and Eosin (H and E) stained sections.

The tissue cores obtained were fixed in buffered formalin and processed on a Tissue Tec machine and embedded in paraffin wax. Slides were automatically machine cut at 6 micron levels and stained with (H and E) in a standard histopathological laboratory.

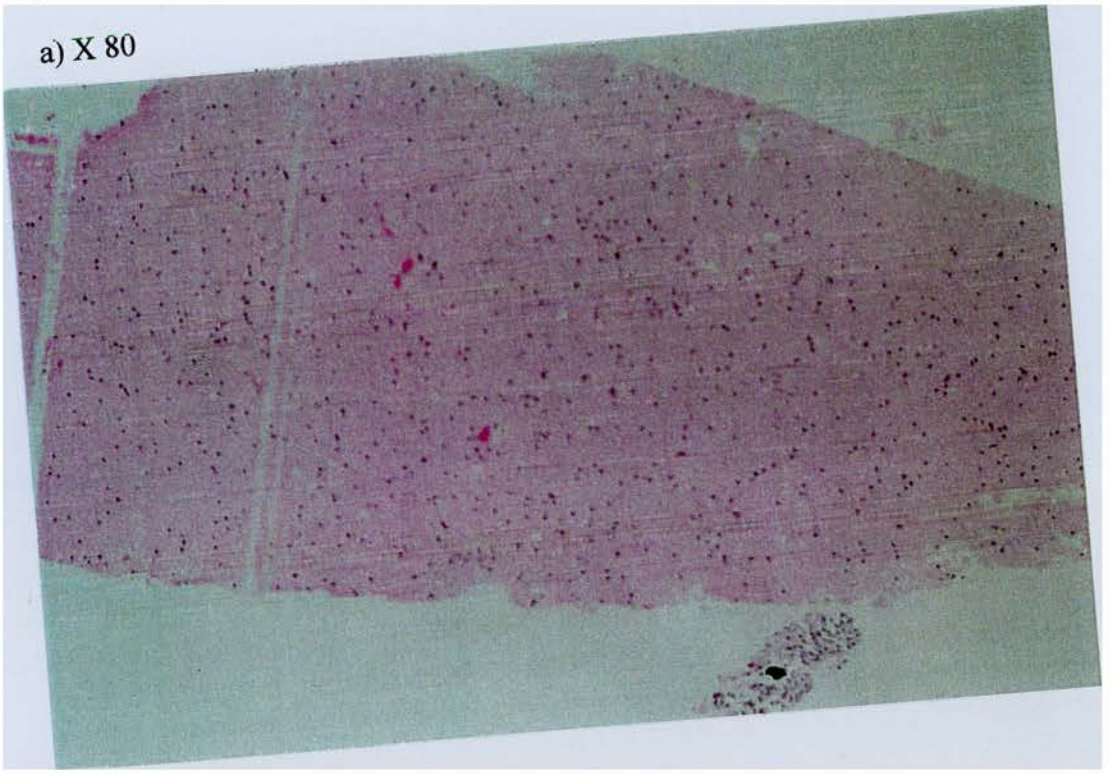
3.3 Results

3.3.1 Histological staining of collected tissues by biopsy needle

All the specimens of tissue obtained (N=3) were identified by microscopy as brain (Figure 3.4), spleen (Figure 3.5) and liver origin (Figure 3.6).

Figure 3.4 Micrograph of brain section stained wi

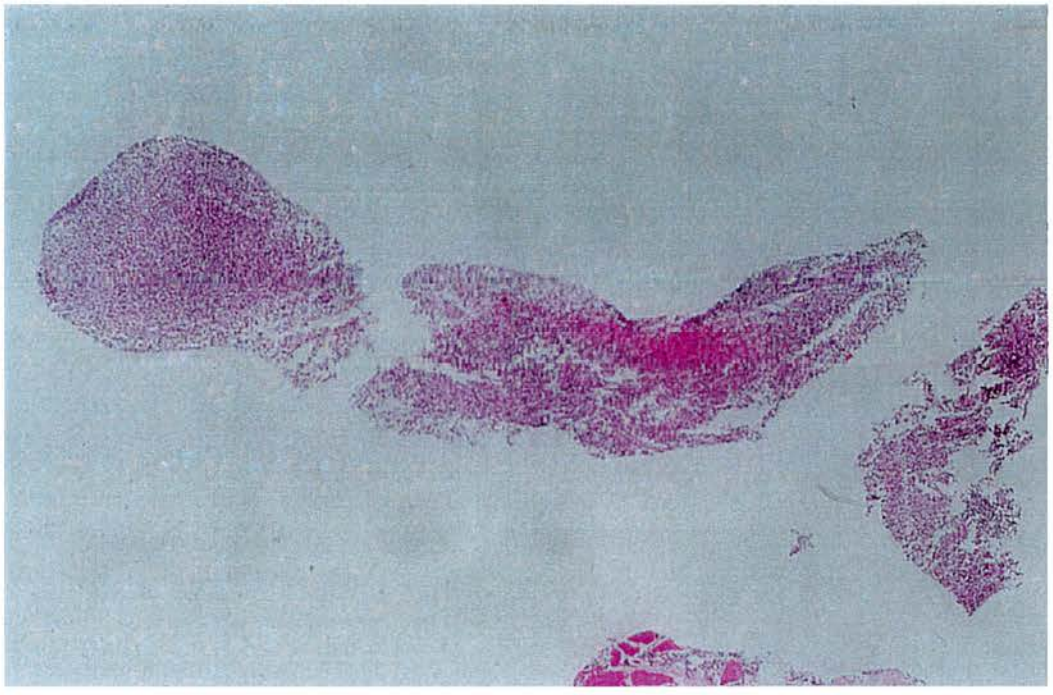
a) X 80



b) X 200

Figure 3.5 Micrograph of spleen section stained with H and E.

a) X 80



b) X 200

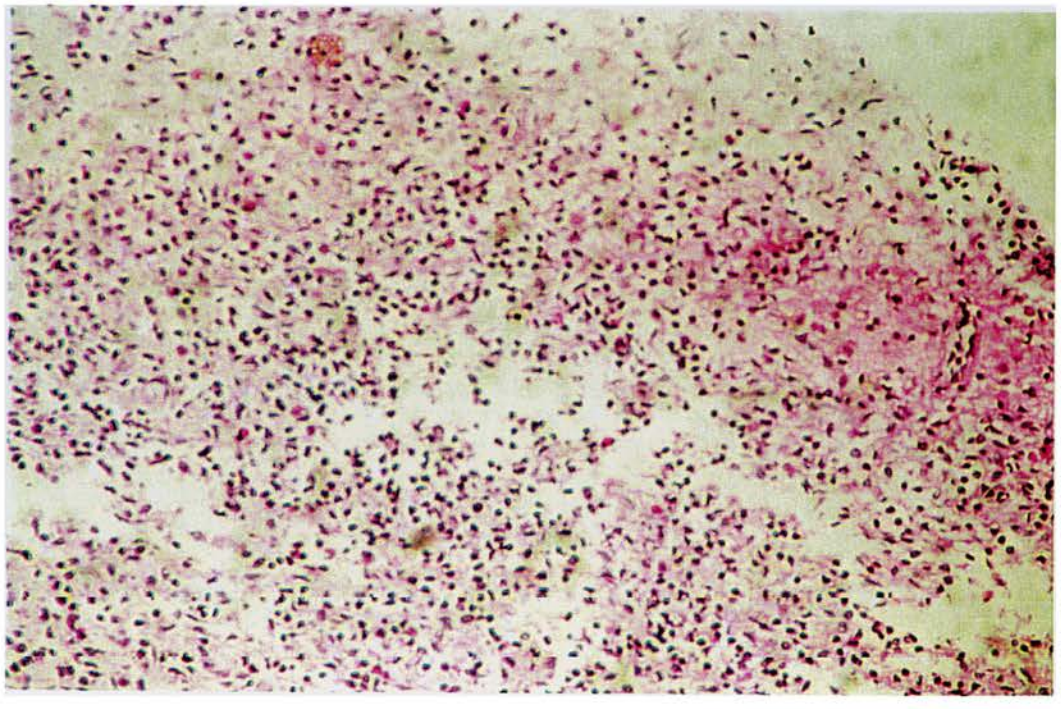
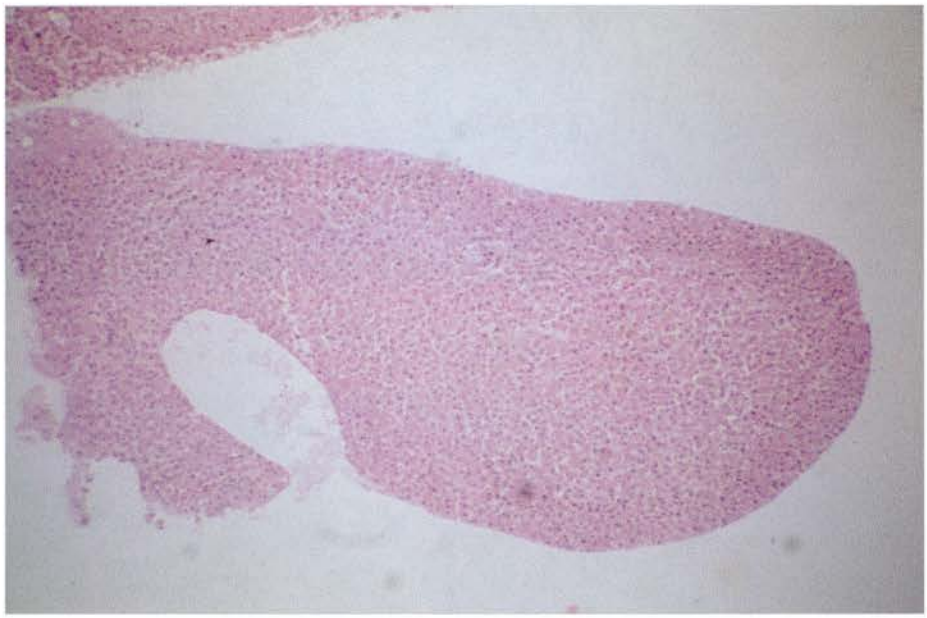
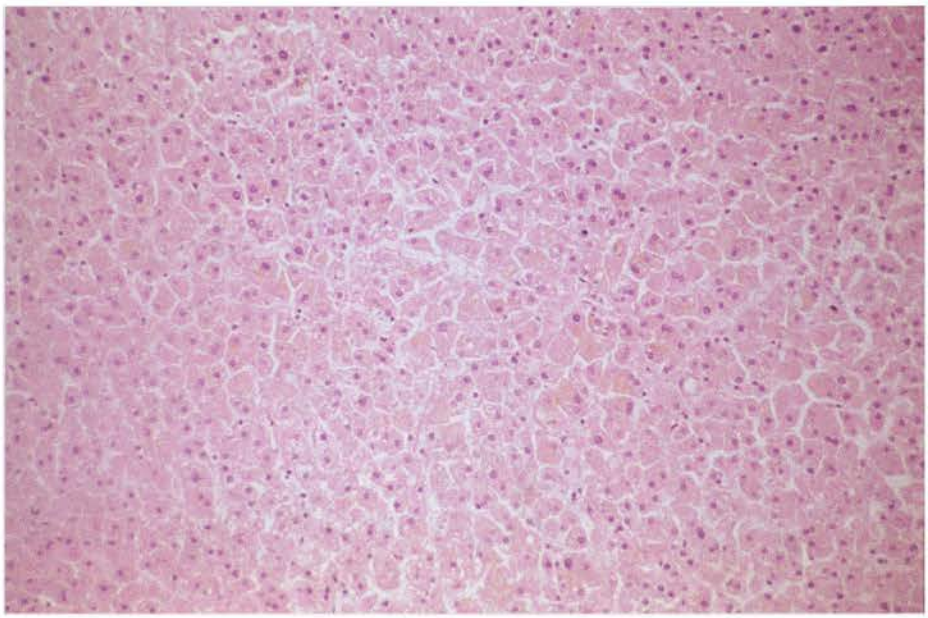


Figure 3.6 Micrograph of liver section stained with H and E.

a) X 80



b) X 200



3.4 DISCUSSION

The needle biopsy technique, although yielding a small quantity of tissue for examination, is a useful substitute when a full autopsy cannot be performed. This technique is swift, non-disfiguring, and as such, more easily agreed by the bereaved who have 'possession of the body'. It need not be carried out in a mortuary but can be carried out in the ward or side room. It could be carried out rapidly following the pronouncement of the fact of death. This would ensure that decomposition and autolysis are kept to a minimum.

The samples of the organs thus obtained, particularly if several cores are obtained, can be subjected to a wide range of investigations including histological examination. If the cores are preserved adequately, immunological, microbiological and other tests can be carried out. The tissue can be utilised for histochemical and similar studies.

This method might be more readily acceptable to the bereaved when post-mortem material is required and when permission for a complete conventional autopsy can not be obtained. It is not a substitute for the full autopsy, but it is a measure that salvages some of the information which would otherwise be unavailable if no post mortem examination were carried out.

In relation to the cultural restraints under which I will be collecting material to investigate these deaths, it is expected to provide appropriate tissues to screen for bacterial toxins.

Chapter 4

Detection of pyrogenic toxins of *Staphylococcus aureus* in human tissues

4.1 Introduction

S. aureus strains that produce pyrogenic toxins, including that associated with toxic shock syndrome (TSS), have been isolated from SIDS infants (Telford *et al.*, 1989). It has been suggested that the series of events by which toxigenic bacteria might precipitate SIDS (Blackwell *et al.*, 1995) is also involved in some cases of SUND among immigrant workers as some of the risk factors are similar to those of SIDS (Blackwell *et al.*, 1994).

The pyrogenic toxins of *S. aureus* and *Streptococcus pyogenes* are superantigens that have profound effects on the inflammatory responses of the host. They exert a powerful effect on the inflammatory system, stimulating T-cells, which subsequently induce the formation of large amounts of cytokines. Generation of an overwhelming inflammatory response may lead to shock and death (Lindsay *et al.*, 1994).

Previous studies by Malam *et al* (1992) and Newbould *et al* (1989 used immunohistochemical method to identify staphylococcal toxins. There were problems with non-specific background binding of the antibodies to tissues. There were also problems with detecting the toxin after the tissues had been fixed for sometime. The objective of this part of study was to develop ELISA and flow cytometry methods for detecting toxins in autopsy tissues.

4.2 Materials and Methods

4.2.1 Collection of samples

Tissue specimens were obtained from the following sources: 1) unfixed tissues (brain, spleen, kidney) from SUND cases in Saudi Arabia, (n = 4) collected by needle biopsy; 2) 49 formalin fixed brain tissue samples from SIDS (n =30) and non-SIDS infants (n = 19) prepared by Dr. J. Hilton, Institute of Forensic Medicine, NSW, Australia; 3) formalin fixed samples from the Forensic Medicine Unit from two adults who died unexpectedly following episodes of vomiting and two sex and age matched accident victims. The positive control for these studies was a fixed sample of brain tissue obtained from a 6 year old child who died suddenly and unexpectedly following infection with parainfluenza virus and from whom TSST-1 producing *S. aureus* was isolated from his respiratory tract and the corresponding toxin identified in his tissues (Bentley *et al.*, 1997). Samples of brain, spleen and kidney were taken from the 6 year old child and the 4 adult cases from the Forensic Medicine Unit.

4.2.2 Preparation of tissues for analysis

The extracts of brain, kidney or spleen were prepared by grinding small pieces of the frozen or fixed tissue in a homogeniser with 2 ml of PBS per 0.05 g of tissue. When the extract was very viscous, it was filtered through a syringe packed with nylon wool. The extract was collected and centrifuged for 10 min at 300 x *g* and the supernatants were stored at –20° C until used in the ELISA. The cells from each sample were resuspended in 3% (v/v) acetic acid, washed twice with PBS and tested in the flow cytometry assay.

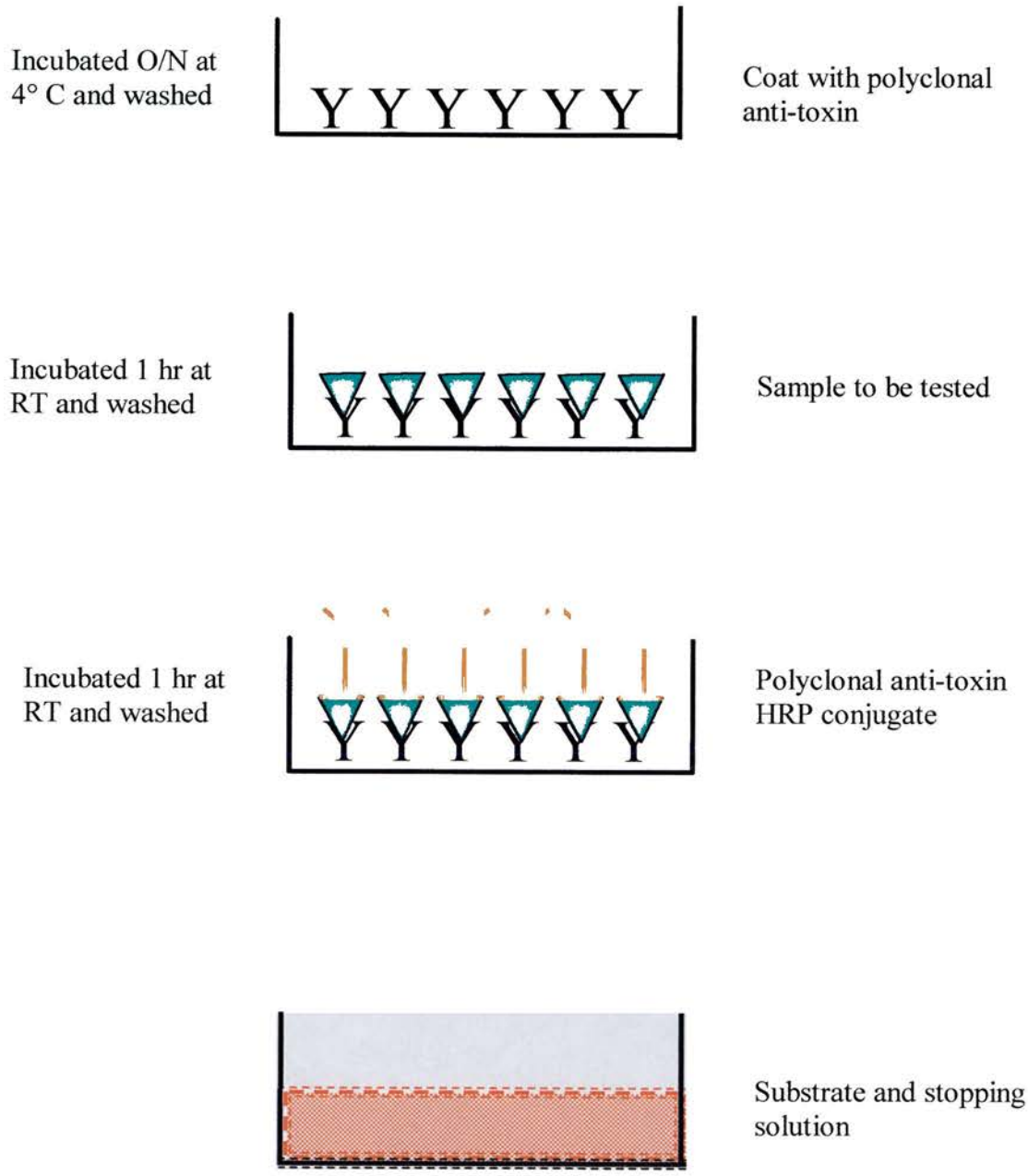
4.2.3. Detection of toxin by ELISA

The method was adapted from that of Morissette *et al* (1991) for detection of staphylococcal toxins in cheese. TSST-1, SEA, SEB and SEC1 were detected by a capture ELISA illustrated in (Figure 4.1). PBS containing 4% (v/v) polyethylene glycol 6000 (PEG) was used as the general diluent. Individual ELISA plates were coated overnight at 4°C with 100 µl of polyclonal sheep antibodies to the individual toxins diluted 1 in 100 in coating buffer. The coated plates were washed three times with washing buffer (2.3.1.2) and blocked with blocking buffer which contained PBS supplemented with gelatine 1% (w/v) for 1 h. After washing 3 times, 100 µl of dilutions of the homologous toxin or 100 µl of the supernatant from the tissue preparations were added to duplicate wells and incubated for 2 h at room

temperature. After washing 3 times, 100 µl HRP sheep anti-TSST-1, anti-SEA, anti-SEB or anti-SEC1 diluted 1 in 100 in blocking buffer were added to the appropriate well for 2 h. Finally, the plates were washed 3 times and 100 µl of the OPD substrate was added. The colour was allowed to develop in the dark and the reaction was stopped within 20 min by adding the stopping solution (2.3.1.6).

Absorbance at 490 nm (A_{490}) was determined by an ELISA plate reader (Dynatech). The spectrophotometer was blanked on the control to which neither supernatant nor toxin was added. Samples were tested in duplicate and the readings averaged. The purified toxins were tested in the ELISA to determine the lower limit of detection and to assess cross reactivity assay. In each experiment, the extract from the 6 years old child in whose tissues TSST-1 has been identified previously by the ELISA methods (Bentley *et al.*, 1997) was used as the positive control. The negative control was the extract from a road accident victim in whose tissues staphylococcal toxins were not detected in the flow cytometry assay. OD values greater than the negative control were classed as positive.

Figure 4.1 Detection of toxin by ELISA



O/N = over night RT = room temperature

4.2.4. Detection of toxins by flow cytometry

Cells obtained from the homogenates of brain, kidney or spleen were washed twice by centrifugation in PBS at 300 x *g* for 10 min, resuspended and counted in a haemocytometer. The cells were adjusted to $2.5x10^{5}$ ml^{-1} and 200 µl of the cell suspension were added to 200 µl PBS (control) or 200 µl of polyclonal rabbit antiserum (test) to TSST-1. After incubation at 37°C for 60 min with gentle shaking (100 rpm) in an orbital incubator, the samples were washed twice in PBS by centrifugation at 300 x *g* for 10 min. Goat anti-rabbit IgG conjugated with FITC (200 µl diluted 1 in 100 in PBS) was added to each sample and incubated at 37oC for 20 min in the orbital incubator. The FITC-labelled antibody was also added to 200 µl of cells treated with PBS as a negative control. The cells were washed by centrifugation, resuspended in 150 µl PBS and fixed with 100 µl of 1% (v/v) buffered paraformaldehyde. The samples were stored in the dark at 4oC until analysed by flow cytometry.

Each sample was analysed with a Coulter EPICS "XL" flow cytometer (Coulter Electronics, Luton, UK) equipped with a 55 W laser with a power output of 200 mW at 488 nm. The cells were selected from a display of forward angle light scatter versus 90^{o} light scatter (granularity) by means of a bit map. The bitmap included the main population of cells. A minimum of 1000 cells was analysed for each sample. Fluorescence greater than the background levels was recorded on a one-parameter histogram measuring fluorescence on a logarithmic scale. The percentage of

fluorescent cells and the mean channel value results were analysed by Immunoanalysis (Coulter), a computer programme that subtracts the values of the control population from the test population at each channel of the two histograms. The results obtained with the control sample to which only FITC-labelled anti-rabbit IgG had been added were used as the background control for the test sample. Test samples were defined as positive if 10% or more of the cells showed increased immunofluorescence compared with the control. The percentage of cells in each sample showing fluorescence greater than the negative control (cells treated only with the FITC anti-sheep IgG) was recorded on a one parameter histogram measuring fluorescence on a logarithmic scale.

4.2.5. Statistical methods

The data from the flow cytometry analysis for detection of TSST-1 on cells from cases and controls were subjected to chi-square test.

4.3 Results

4.3.1 Detection of toxins by ELISA

4.3.1.1 Sensitivity of ELISA for Staphylococcus toxins:

The capture ELISA was used to determine the sensitivity of the assay with purified toxins. A calibration curve was produced for the different concentrations of the toxins ranging from 1.0 to 500 ng. An A_{490} value of 0.1 was equivalent to 1.8 ng TSST-1, 1 ng SEA, 8 ng SEB and 6 ng SEC. The results are illustrated in Figures 4.2, 4.3 ,4.4 and 4.5.

Figure 4.2 Standard Curve - TSST -1

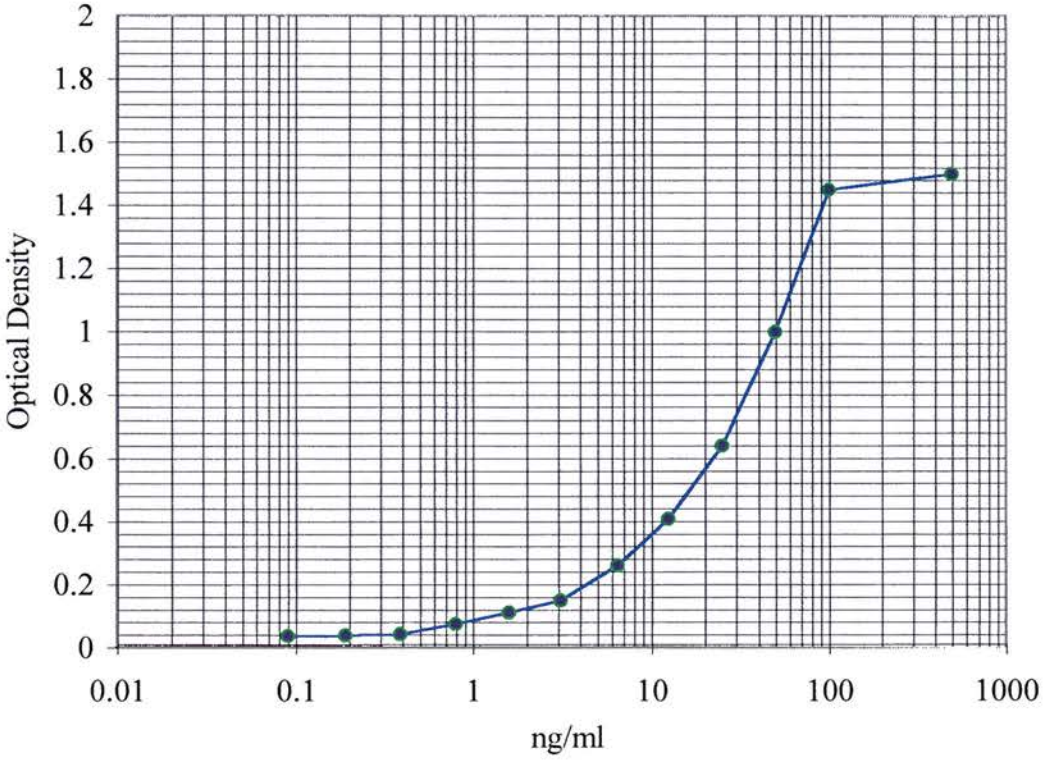


Figure 4.3 Standard Curve - SEA

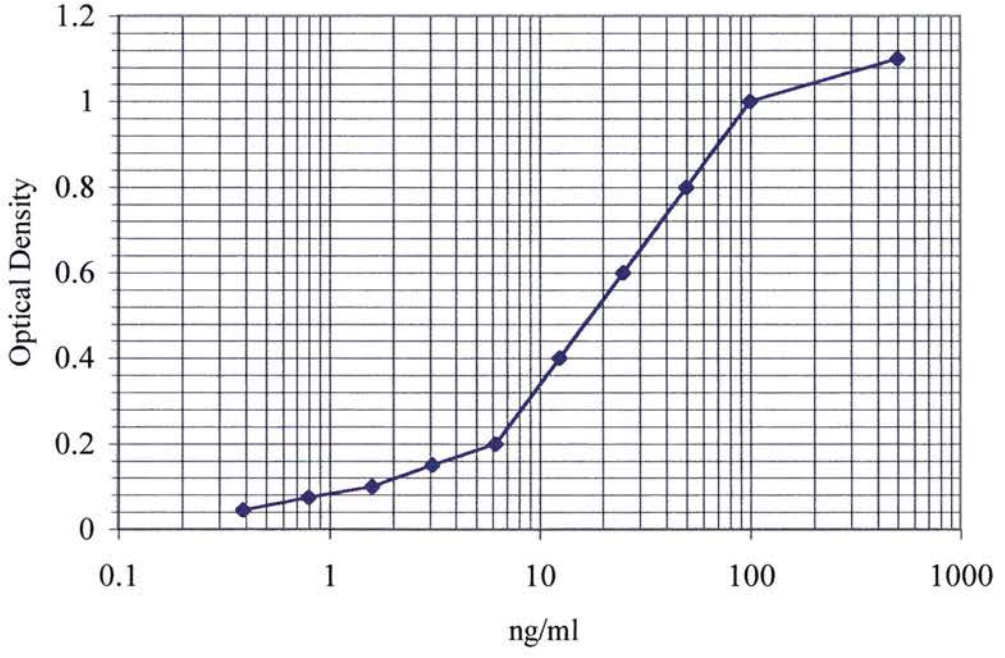


Figure 4.4. Standard Curve - SEB

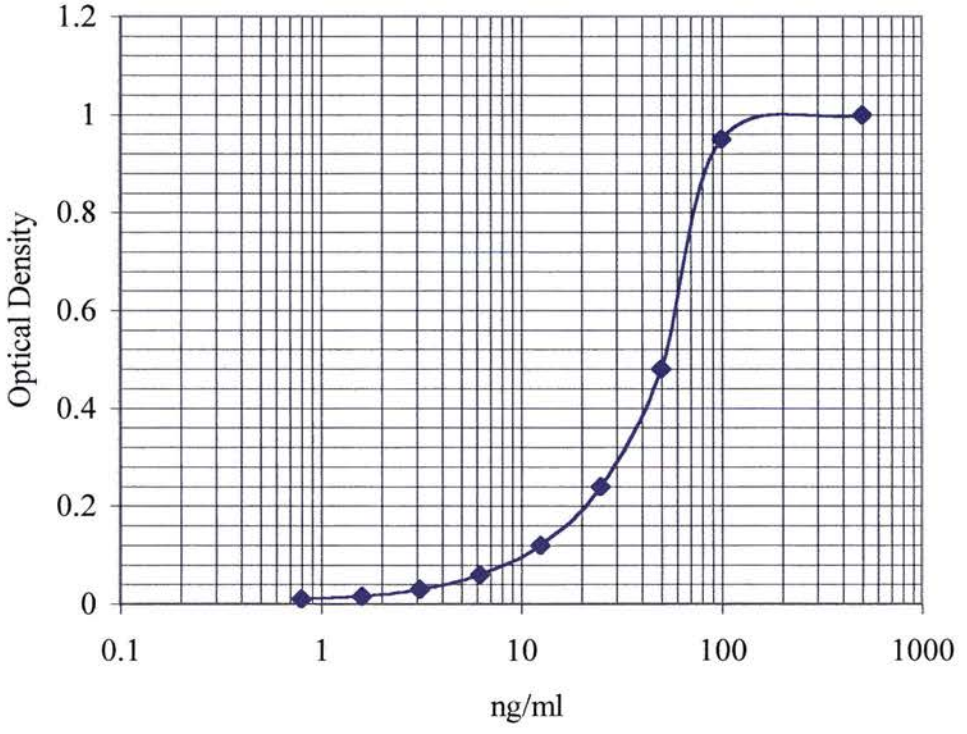
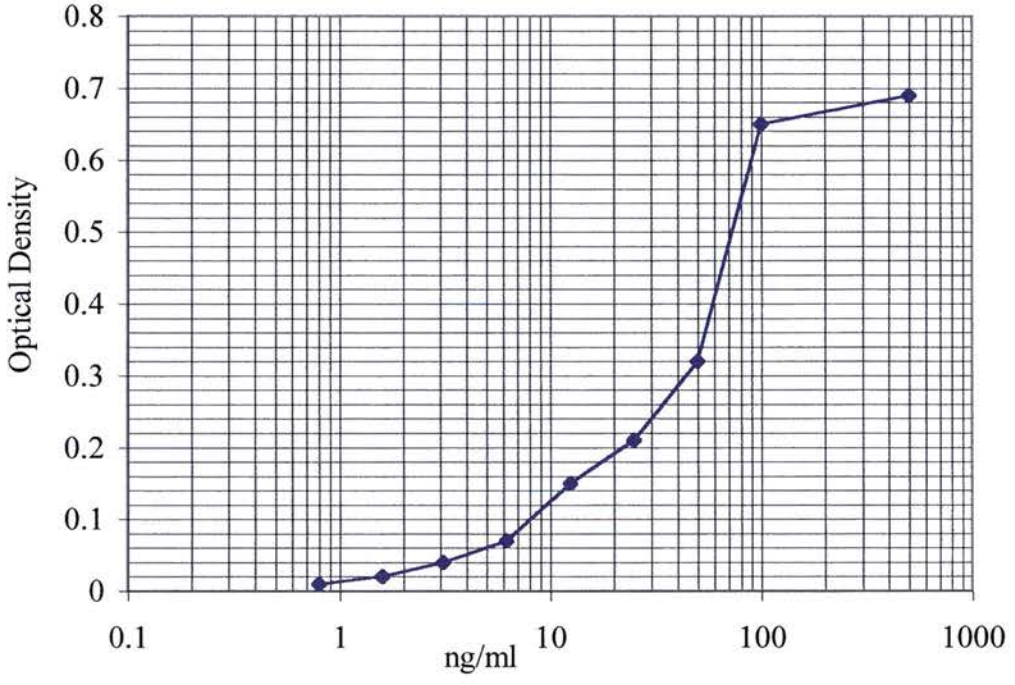


Figure 4.5 Standard curve - SEC



4.3.1.2 Specificity of ELISA for Staphylococcus toxins

The specificity of the antibodies for the toxins in the capture ELISA was assessed. The mean of the A_{490} value for the homologous toxins / anti-toxin combination was taken as 100% and mean A_{490} values obtained with the heterologous toxins expressed as percent obtained with the homologous toxin. In this assay, each toxin was tested at 100 ng ml^{-1}. Table 4.1 represents the mean of 3 assays

The anti-TSST-1 appeared to be most specific of the 4 antitoxins tested with A_{490} values for the heterologous toxins < 1% that of the homologous toxins. With anti-SEA, A_{490} values > 10% compared with the homologous toxin were observed for TSST-1, SEB and SEC. A_{490} values with anti-SEB were >10% for TSST-1 but <10% for SEA and SEC. With anti-SEC, OD values obtained with each of heterologous toxin were <10% that with SEC.

Table 4.1. Specificity of capture ELISA for staphylococcal toxins

Antibody	Antigen			
	TSST-1	SEA	SEB	SEC
	A_{490} (%)	A_{490} (%)	A_{490} (%)	A_{490} (%)
TSST-1	1.43 (100)	0.004 (0.3)	0.001 (0.07)	0.003 (0.2)
SEA	0.82 (75)	1.10 (100)	0.14 (12.7)	0.25 (22.7)
SEB	0.12 (17.6)	0.094 (10)	0.95 (100)	0.06 (6.3)
SEC	0.045 (7)	0.038 (6)	0.025 (4)	0.65 (100)

4.3.3 Detection of toxins by flow cytometry

4.3.2.1 Detection of toxins in different tissues

The highest readings were obtained with cells from the brain from a 6 year old child (positive control) which were greater than those observed with cells from the spleen and the lowest readings were found with cells from the kidney (Figure 4.7)

Brain tissues from the two adults who died following episodes of vomiting were positive. No toxins were detected in brain, spleen or kidney of the adult road traffic victims (Figure 4.8).

Figure 4.7 Detection of toxin in a) brain, b) spleen and c) kidney tissues from 6 year old child (positive control)

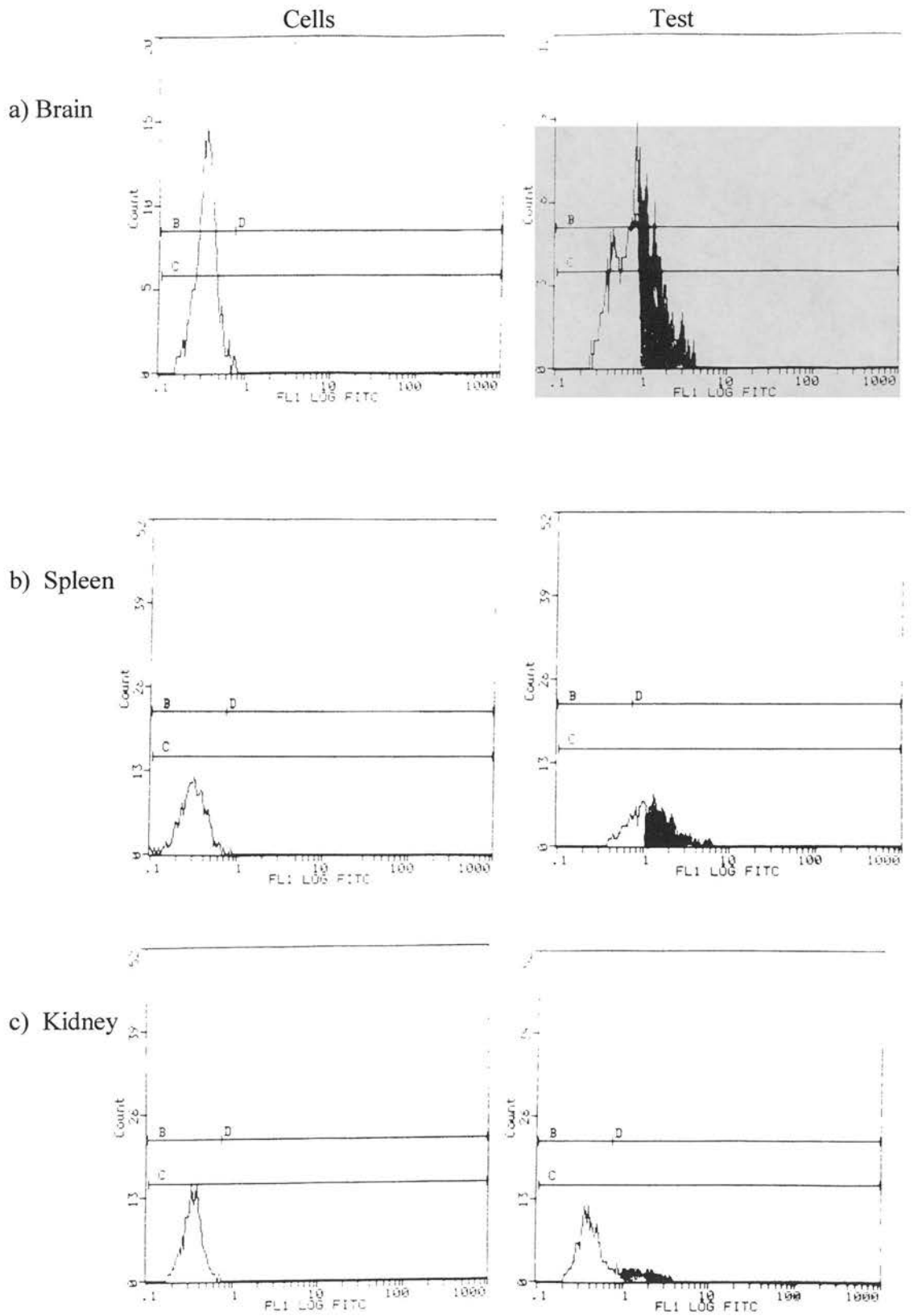
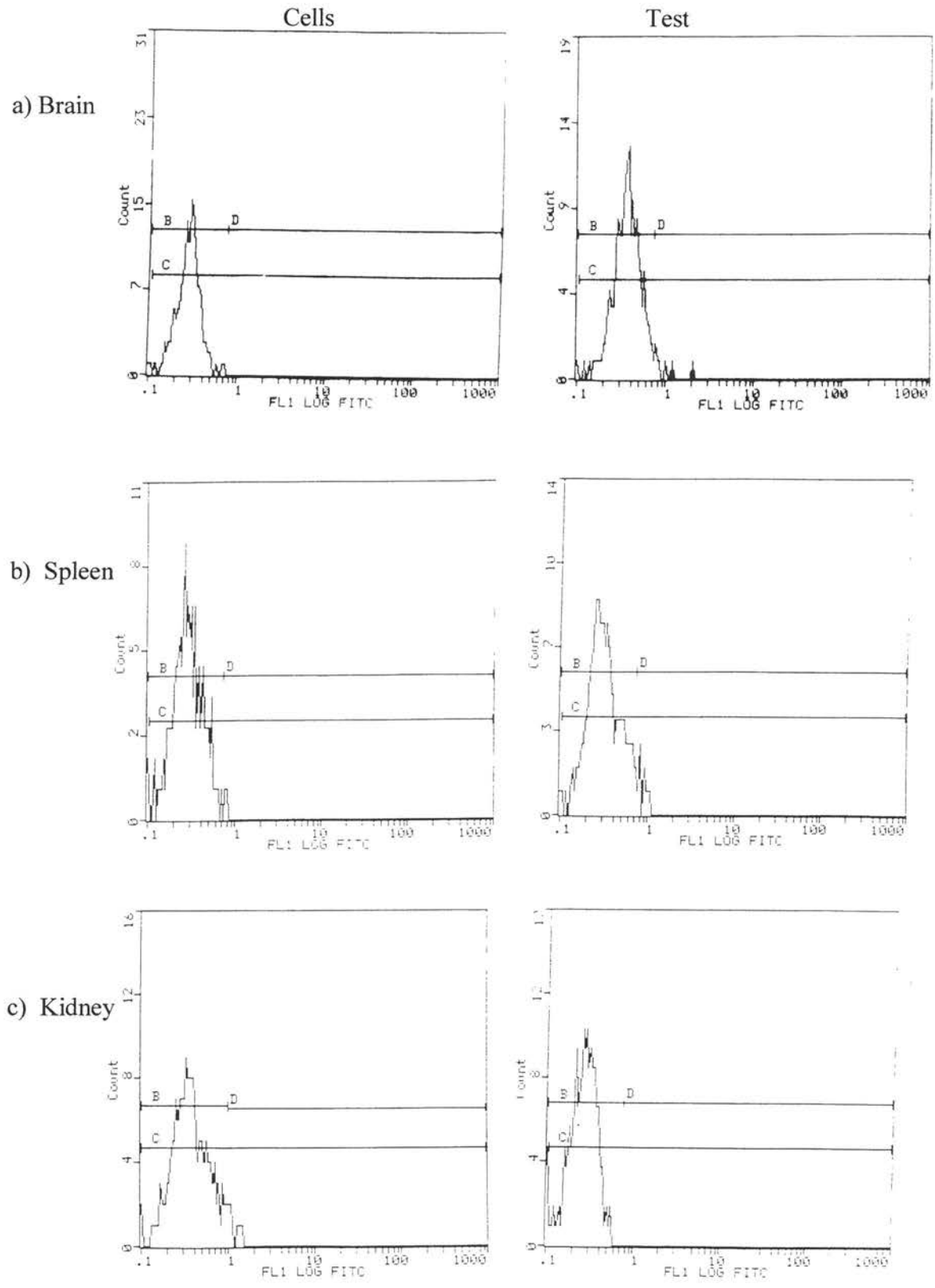


Figure 4.8 Detection of toxin in a) brain, b) spleen and c) kidney tissues from road traffic victims



4.3.4 Detection of toxins by ELISA in tissues of SIDS and SUND

4.3.4.1 Detection of toxin by ELISA

Because the results obtained with anti-TSST-1 were the most specific in the ELISA, it was used to screen the specimens from the SIDS and non-SIDS cases.

The concentration of TSST-1 in the fixed brain of the positive control (6 year old child) detected by ELISA was calculated to be 8.1 ng ml^{-1} by these assays. The Australian samples were tested more than 18 months after fixation. Only 1 of the 49 samples showed a detectable value by ELISA and this was 3.4 ng ml^{-1}. None of the 4 samples collected from SUND cases in Saudi Arabia were positive for TSST-1.

4.3.5. Detection of TSST-1 by flow cytometry

4.3.5.1 Detection of TSST-1 in the Australian samples

A specimen was considered to be positive if the percentage of cells with fluorescence above the negative control treated with FITC-labelled rabbit-anti IgG was ≥ 10%. All assays were carried out with no knowledge of the diagnosis. The value for the positive control was 23%

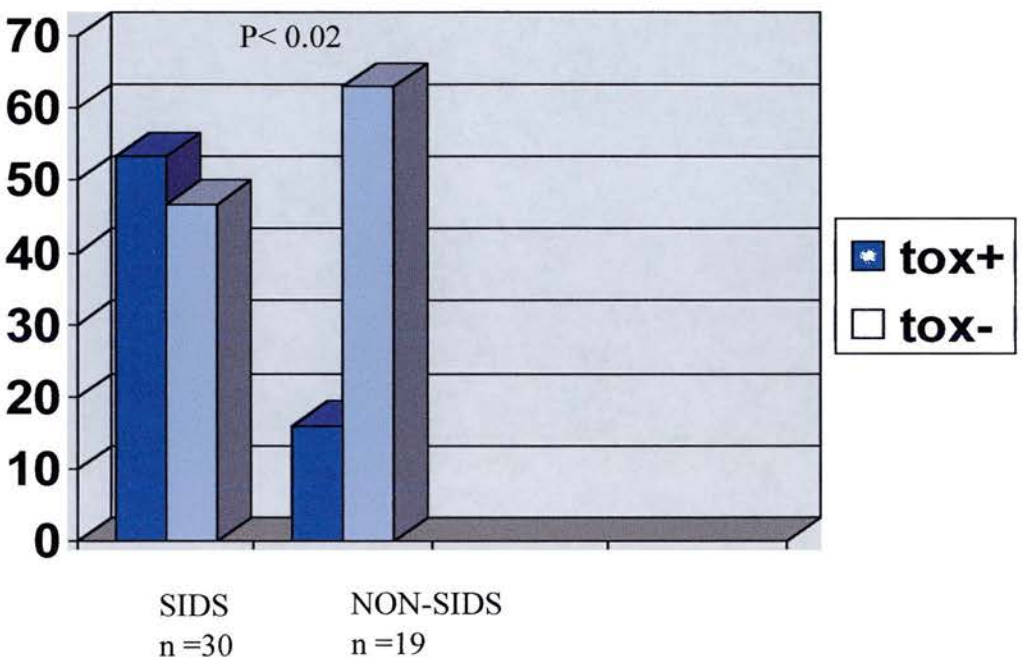
TSST-1 was detected on cells in 16 of the 30 cases of SIDS (53%) and 3 of 19 non-SIDS cases (16%) (p =0.02, 95% CI 1.28, 37.91, OR= 6.07). All the other samples

tested were negative. Two of the 3 non-SIDS cases died of pneumonia and one of complications related to cystic fibrosis (Table 4.2) (Figure 4.6).

Table 4.2 Detection of TSST-1 by flow cytometry in tissues of Australian samples from SIDS (n = 30) and Non-SIDS (n = 19)

Sample	Sex	Age / week	% positive cells	Diagnosis
1	Female (F)	16	26.4	SIDS (S)
2	F	36	17.5	S
3	Male (M)	3	3.2	S
4	F	36	0.2	S
5	F	12	46.8	S
6	M	16	15.9	S
7	F	44	11	S
8	F	16	0.2	S
9	F	12	73	S
10	M	4	5	S
11	F	10	21.2	S
12	M	4	37.5	S
13	M	7	13.3	S
14	M	48	9.7	S
15	M	12	0.5	S
16	M	16	1.3	S
17	F	16	13	S
18	F	8	66.3	S
19	M	11	3.3	S
20	M	5	4	S
21	M	2	9	S
22	M	12	10	S
23	F	24	1.7	S
24	F	40	3	S
25	F	68	2.7	S
26	F	12	40	S
27	F	20	0.9	S
28	F	12	62.2	S
29	M	12	2.5	S
30	F	12	0.2	S
31	F	20	24.9	NON-SIDS
32	M	20	3.2	NS
33	M	72	0.4	NS
34	M	20	4	NS
35	M	52	2	NS
36	F	28	1.5	NS
37	F	20	0.9	NS
38	M	24	42.2	NS
39	M	8	44.5	NS
40	F	20	3.5	NS
41	F	12	5.2	NS
42	F	12	1	NS
43	F	156	0.5	NS
44	F	156	0.3	NS
45	M	48	1.6	NS
46	F	12	0.8	NS
47	M	5	8.3	NS
48	M	24	3.5	NS
49	F	12	0.3	NS

Figure 4.6 Identification of TSST-1 in brain tissue of SIDS and non-SIDS (Australian infants)



4.3.5.2 Detection of TSST-1 in the samples from SUND cases

Three of the 4 of SUND cases were collected after more than 4 weeks after death. Case no. 3 collected within 2 days by needle biopsy is the only one which showed a border line value (8.5 %).

4.4 Discussion

In a pilot study, carried out by colleagues in the laboratory, one or more staphylococcal toxins were identified in frozen tissues or serum in 10/19 (53%) local SIDS infants. *S. aureus* was isolated from 7 of the 10 infants in whose tissues toxins were found. Of the 9 infants whose samples were toxin negative, *S. aureus* was not isolated from 8 (Fisher's exact test, $P < 0.02$). The one staphylococcal isolated obtained from the toxin-negative infant did not produce any of the 4 pyrogenic toxins examined in this study. Among the 7 staphylococcal isolates, 4 produced the corresponding to those toxins found in the tissues. The results obtained by ELISA agreed with those obtained by flow cytometry in each case. Formalin-fixed samples from 13 French infants were tested within 12 months of collection and TSST-1 or SEC were identified in 7/13 (55%).

Detection of pyrogenic toxins in the frozen samples corresponded with isolation of *S. aureus* from local SIDS infants. For the frozen samples, the flow cytometry method

gave positive results for those samples that were positive by ELISA and negative results for those in which no toxins were detected by ELISA.

Samples from age matched infants who died of other causes were not available in the pilot studies. The Australian samples (30 SIDS and 19 non-SIDS) were the best collection of cases and controls that could be obtained.

4.4.1 Method

4.4.1.1 Detection of toxin in different tissues

Immunohistochemical methods for detection of toxins in host tissues were only partly successful. The toxins are difficult to detect in fixed tissues if kept for long time. Cross-reactivity between toxin and human tissues with some polyclonal antibodies was noted (unpublished observation, Dr. A A. Zorgani)

Immunohistochemical methods were developed in a rat model to determine in which tissues toxins might be located. The kidney appeared to be the target organ in this model and TSST-1 and SEC were subsequently identified in kidney tissues of SIDS infants (Malam *et al.*, 1992; Newbould *et al.*, 1989). With tissues from the adults who died suddenly and unexpectedly, the highest readings observed with the flow cytometry method were in brain tissue compared with spleen or kidney from the same patient.

The results with ELISA indicate that as with the immunohistochemical method, fixation alters the toxin and limits the use of both these methods for tissues fixed for more than 12 months.

4.4.1.2 Comparison of flow cytometry and ELISA method.

Results indicate that the ELISA has some limitations in relation to detection of the toxins in fixed tissues. Formalin fixed samples from French infants were tested within a year of collection; for these specimens, both ELISA and the flow cytometry methods gave similar results (Zorgani *et al.*, 1999). The Australian samples were collected in 1995 and tested in 1997; for these, only the flow cytometry method gave positive results for the majority of fixed samples. With the immunohistochemical method, the toxins were identified in approximately three times as many SIDS infants as controls : TSST-1 in 18% compared with 6% among infants who died of other causes; SEC in 36% of SIDS infants and 12% among the comparison group (Malam *et al.*, 1992; Newbould *et al.*, 1989). Similar ratios were observed in the present study with the flow cytometry method for the collection of tissues from the Australian SIDS infants (53%) and their respective comparison group (16%).

For frozen and recently fixed samples, either ELISA or flow cytometry can be used; however, for tissues fixed for 18 months or longer, based on the results obtained with the Australian samples, flow cytometry is recommended. Based on the experiments to assess specificity of the anti-toxin, the studies will be limited to assays for TSST-1 and possibly SEC1.

4.4.2 Comparison of results for SIDS infants from different countries

Detection of staphylococcal toxins in tissues of SIDS infants was not restricted from samples from local isolates. There were positive samples among 62 specimens from SIDS infants from 3 different countries. The flow cytometry method was applicable to all three sets of samples. The percentages of positive samples from each country were similar, Scotland 53%, France 55% Australia 53%.

4.5 Conclusion

The method developed for detection of toxins in tissues of SIDS cases can be applied to the study of SUND. The size of the samples obtained with the needle biopsy might be a limiting factor. Samples from countries such as Singapore in which thorough autopsies and sufficient quantities of tissues have been collected could be screened by these methods. Cross reactivity of polyclonal antisera to SEC1, SEA and SEB

limited the study to the use of anti-TSST-1. Monoclonal antibodies to the staphylococcal toxins might improve the specificity of the immunohistochemical, ELISA and flow cytometry methods, but at present they are not commercially available.

Chapter 5

The effect of cortisol on inflammatory response induced by pyrogenic toxins.

5.1 Introduction

SUND deaths usually occur at night. If these deaths are triggered by pyrogenic toxins alone or in conjunction with other inflammatory agents, what are the factors that could control these inflammatory responses? One would be cortisol levels and another would be presence of antibodies to the toxins.

It has been demonstrated *in vitro* that inflammatory responses to endotoxins are greater with leukocytes obtained at night than those obtained from the same individual during the day. These were correlated with lower levels of cortisol detected at night compared with higher day time levels (Entzian *et al.*, 1996). *In vivo* studies of human volunteers have also demonstrated highest TNF responses to endotoxin were obtained when the toxin was administered at night (Pollmacher *et al.*, 1996). The objectives of this part of the study were to assess in a model system the effects on inflammatory responses induced by TSST-1 in the presence of human

night time levels (5-10μg dl^{-1}), day time levels (10-20μg dl^{-1}) and physiological stress levels (40-80μg dl^{-1}) of cortisol (Guyton & Hall, 1996).

5.2 Materials and Methods

5.2.1 Collection of human peripheral blood leukocytes

Human peripheral blood leukocytes were collected, counted and cultured as described in 2.4.2.

5.2.2 Effect of TSST-1 or cortisol on induction of inflammatory mediators

Cells 2 x 10^{6} ml^{-1} (500 μl/well) were placed in 24 well tissue culture plates and 250 μl of TSST-1 at various concentration (0.05, 0.1,0.5,or 1μg ml^{-1}) added to the wells. The cells were incubated at 37°C in a humidified incubator with 5% CO_2. At different times (0, 4, 8, 16, 24, 36, and 72h), the cell supernatants were collected in sterile tubes and centrifuged at 250 x g for 10 min. The supernatants were stored at -20°C until assayed for TNFα, IL-6 and IL-10. The results for the mean of the different concentrations of toxins were used to determined the amount to be used to test the effects of cortisol on induction inflammatory mediators by TSST-1. Cortisol dilutions (5-80 μg dl^{-1}) were incubated with the cells over the same time periods and supernatants tested for TNFα, IL-6 and IL-10.

5.2.3 Effect of cortisol on leukocyte responses to TSST-1

Cells 2 x 10^6 ml^{-1} (500 µl/well) were placed in 24 well tissue culture plates and 250 µl of TSST-1 added to the cells at a final concentration of 0.1µg ml^{-1}. These were incubated at 37oC for 30 min and 250 µl of different concentrations of water soluble hydrocortisone (Sigma) diluted in DMEM were added to give a final concentration of 5, 10, 20, 40, 60 or 80 µg dl^{-1}. The cells were incubated at 37°C in a humidified incubator with 5% CO_2. At different times (0, 4, 8, 16, 24, 36, and 72h), the cell supernatants were collected in sterile tubes and centrifuged at 250 x g for 10 min. The supernatants were stored at -20°C until assayed for TNFα, IL-6 and IL-10. The results for the mean of the different concentrations of cortisol were compared to the mean of the control containing toxin alone.

5.2.4 Cytokine assays

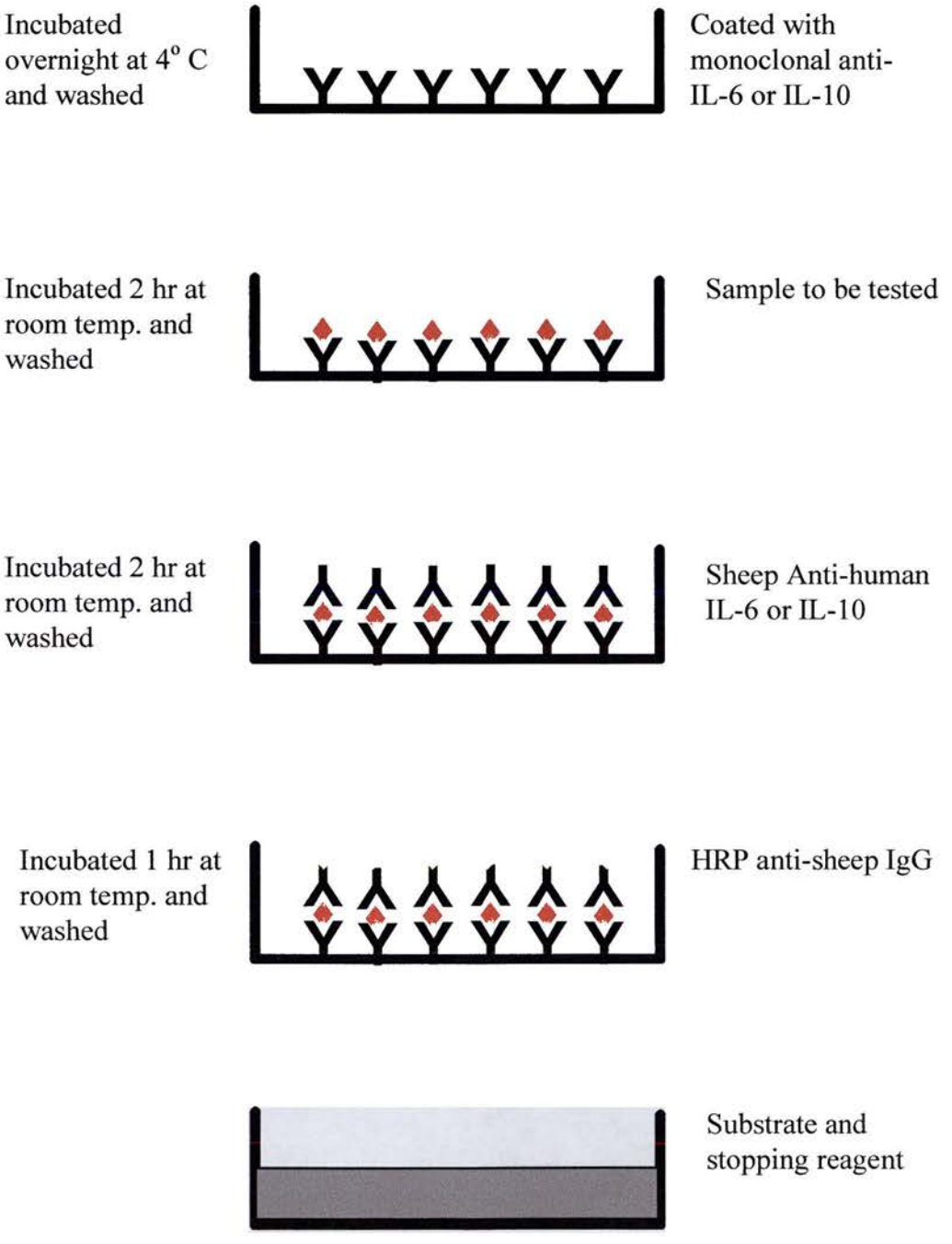
5.2.4.1 ELISA for IL-6 and IL-10 (Figure 5.1)

Flat-bottomed microtiter plates (96 wells) were incubated over night at 4°C with 100 µl per well of mouse monoclonal antibody specific for IL-6 (1 µg ml^{-1}) diluted in coating buffer. The plates were washed 3 times with washing buffer and 100 µl of blocking buffer were added to each well for 30 min at room temperature. The blocking buffer was removed, the plates were washed 3 times and supernatant samples (100 µl) added to duplicate wells. Dilutions of recombinant human IL-6

standard (R&D Systems) ranging from 0.1 ng –100 ng ml^{-1} were made in blocking buffer and added to duplicate wells. The plates were incubated for 2 h at 37 °C with continuous shaking in an orbital incubator. The plates were washed 3 times and 100 µl of polyclonal goat anti-human IL-6 diluted 1 in 100 in blocking buffer were added to detect IL-6 bound to the wells. After incubation for 2 h at 37°C with continuous shaking, the plates were washed 3 times and 100 µl of HRP-conjugated donkey anti-sheep/goat IgG diluted 1 in 100 in blocking buffer were added to the wells. The plates were incubated for 1 h at 37°C with continuous shaking and washed 3 times. The substrate OPD (100µl) (2.3) was added and the colour change allowed to develop. The A_{490} was determined and corrected by subtracting the absorbance of the corresponding blank well containing each of the components except the cell supernatant. The amount of IL-6 in each sample was determined relative to the recombinant human IL-6 standard and results were expressed in ng ml^{-1}.

The same protocol employing mouse monoclonal anti-IL-10 and polyclonal goat anti-IL-10 serum was used to detect IL-10. The amount of IL-10 in each sample was determined relative to the recombinant human IL-10 standard and results were expressed in ng ml^{-1}.

Figure 5.1 ELISA for detection of IL-6 & IL-10



5.2.4.2 Bioassay for TNF-α

The method described by Delahooke *et al* 1995 was used (Figure 5.2). L929 cells were dislodged by 0.1% trypsin (w/v) (Gibco, Paisley, UK) to avoid cell clumping and washed by centrifugation at 300 x g. Cells were resuspended in growth medium and adjusted to 3.0×10^5 ml^{-1}. Cells (100µl) were placed in flat-bottomed wells of 96 well tissue culture plates and incubated at 37°C in a humidified incubator containing 5% CO_2 for 24 h.

The growth medium was discarded and replaced with 100 µl per well of test supernatant diluted 1 in 1 in assay medium composed of DMEM containing 5% (v/v) FCS, 1 mM L-glutamine and 2 mg ml^{-1} actinomycin D (Sigma). The supernatants were tested in duplicate. Dilutions of a standard human TNF-α obtained from the National Institute for Biological Standards and Controls (NIBSC) (Hertfordshire, UK) were prepared in the assay medium. Duplicates of each dilution of the standard ranging from 0 to 100 IU ml^{-1} were added to wells. The TNF standard was used in every plate; 6 wells with 100 µl of assay medium were included as controls and another 6 wells without L929 cells were included in each plate as blanks. All were treated identically to the test samples.

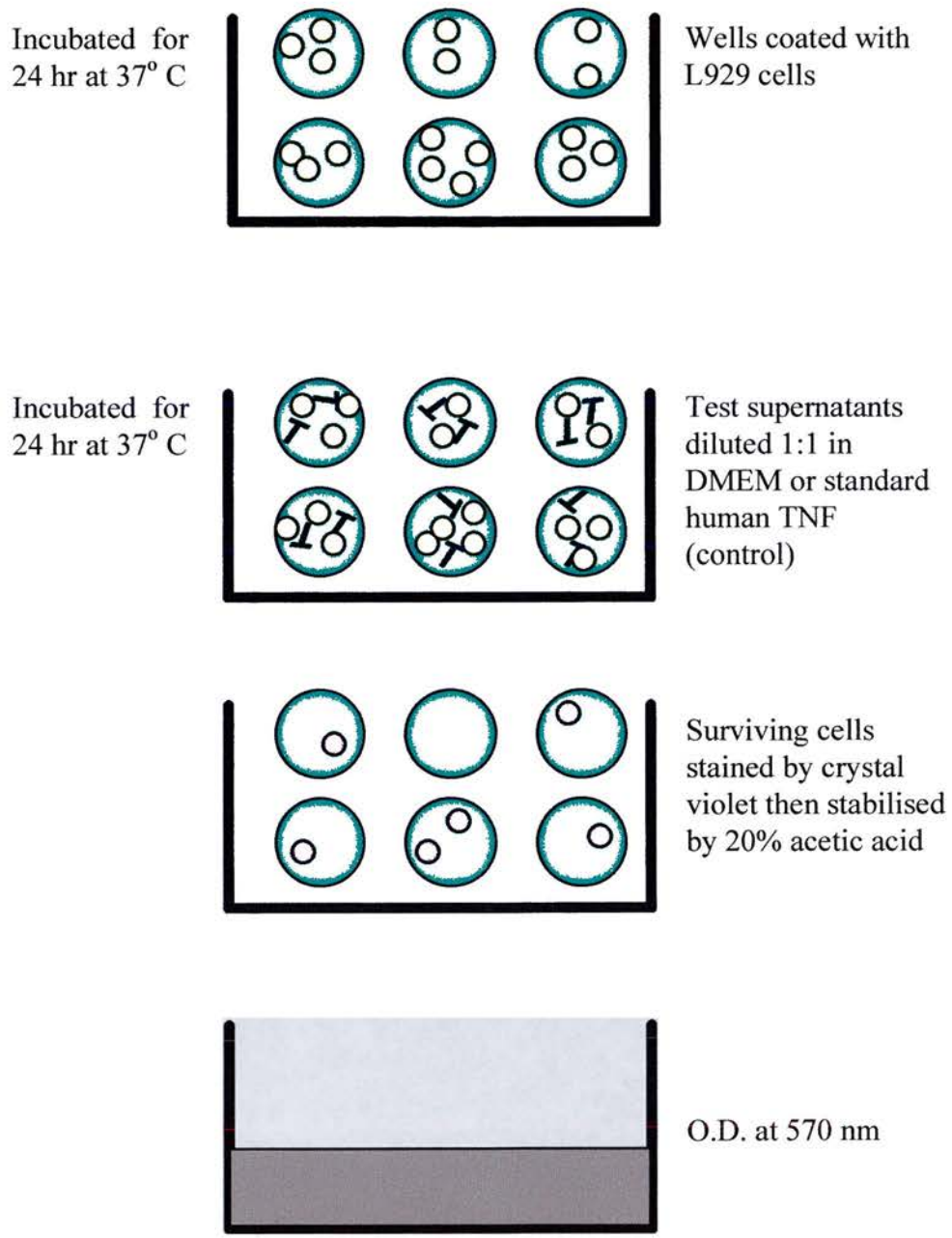
Plates were incubated at 37°C in a humidified incubator containing 5% CO_2 for another 24 h. The supernatant in each well was removed and replaced with a solution containing 0.5% (w/v) crystal violet and 20% (v/v) methanol in distilled water which had been filtered through Whatman No.1 filter paper. After 2 min the plates were

washed vigorously with tap water and allowed to dry. Each well received 50 µl of 20% (v/v) acetic acid to solubilise the dye in the stained cells. Any air bubbles were removed and absorbance was measured at 570 nm with a plate reader (MR 700 Dynatech Laboratories). The amount of TNF in each sample was determined relative to the TNF standard curve and results were expressed in IU ml^{-1}.

5.2.5 Statistical analysis

Student t-test for paired sample was used for statistical analysis.

Figure 5.2 TNF-α bioassay



5.3 Results

5.3.1 Effect of time and TSST-1 concentration on induction of inflammatory mediators

TSST-1 at various concentration (0.05, 0.1,0.5,or 1μg ml^{-1}) increased production of (TNF-α IL-6 and IL-10). Maximum production was observed with 0.1 μg ml^{-1} at of 16 hours for TNF-α and IL-6 and 24 hr for IL-10 (Figure 5.3).

5.3.2 Effect of cortisol levels on induction of TNF-α, IL-6 and IL-10 from buffy coats

Incubation of cells with cortisol at concentration ranging between (5-80 μg dl^{-1}) did not affect the production of TNF-α, IL-6 or IL-10 compared with the control containing medium alone. Results for the various concentration of cortisol are compared with those for the positive control containing TSST-1 (Figure5.4).

Figure 5.3 The effect of time and TSST-1 concentration on induction of (a) TNF-α, (b) Il-6 (c) IL-10

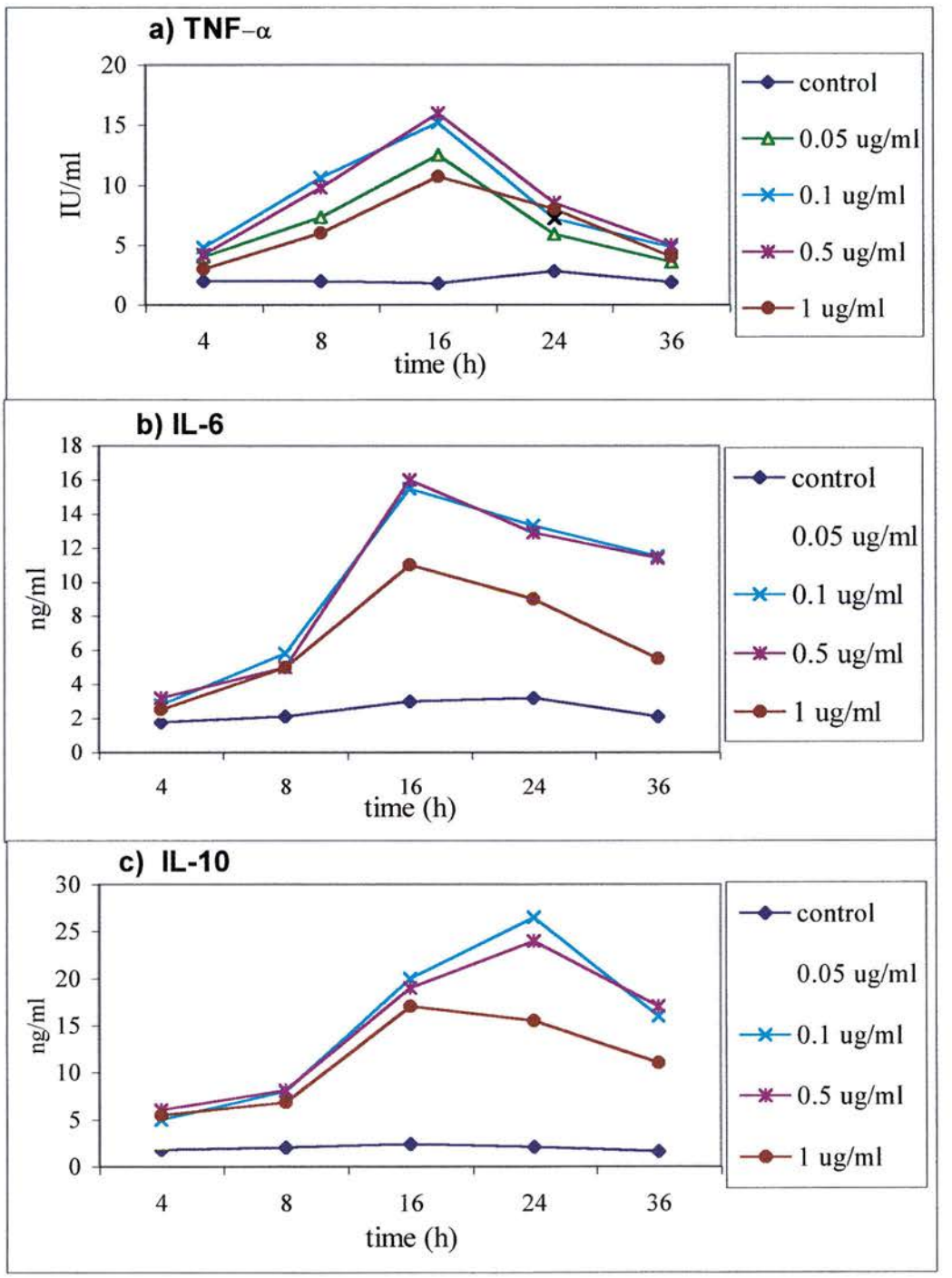
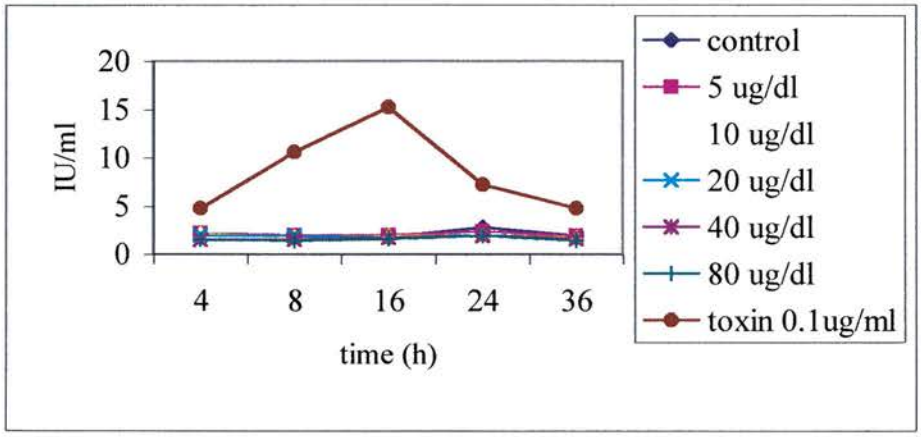
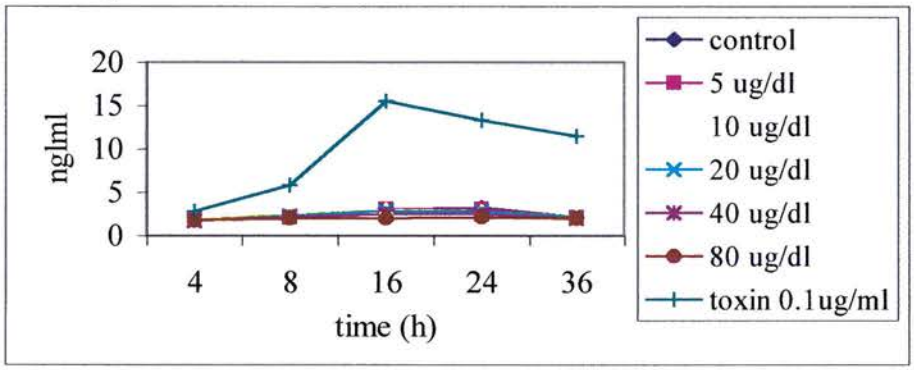


Figure 5.4 The effect of cortisol levels on induction of (a) TNF-a, (b) Il-6 (c) IL-10

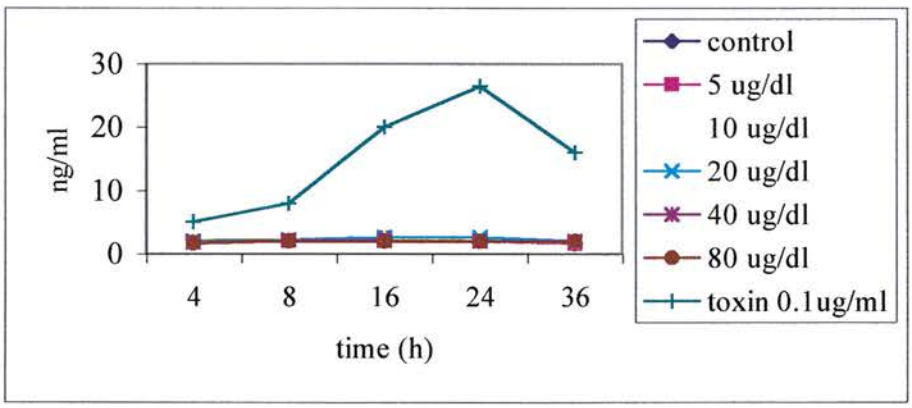
a) TNF-α



b) IL-6



c) IL-10



5.3.3 Effect of cortisol levels on induction of inflammatory mediators by TSST-1.

5.3.3.1 Effect of night time levels of cortisol on induction of inflammatory mediators by TSST-1.

Night time levels of cortisol in human adults range from 5-10 μg dl^{-1}. Compared with the control containing toxin alone, there was no significant reduction in TNF-α or IL-6 by either 5μg dl^{-1} or 10 μg dl^{-1} of cortisol over 36 h. At 16 hours there was significant enhancement of production TNF-α (P=0.008) and IL-6 (P=0.01). IL-10 production was significantly decreased at 16 (P=0.01), 24 (P=0.008) and 36 h (P=0.02) (Figure 5.5 and Table 5.1).

5.3.3.2 Effect of day time levels of cortisol on induction of inflammatory mediators by TSST-1.

Day time levels of cortisol in adults range from 10-20 μg dl^{-1}. Compared with the control containing toxin alone, there was no significant effect of cortisol on TNF-α production, but IL-6 was significantly reduced at 8 (P=0.007), 16 (P=0.006) and 24 h (P=0.034). IL-10 production was significantly enhanced at 8 h (P=0.048) and decreased at 16-36 h but not significantly (Figure 5.6 and Table 5.2).

5.3.3.3 Effect of stress levels of cortisol on induction of inflammatory mediators by TSST-1.

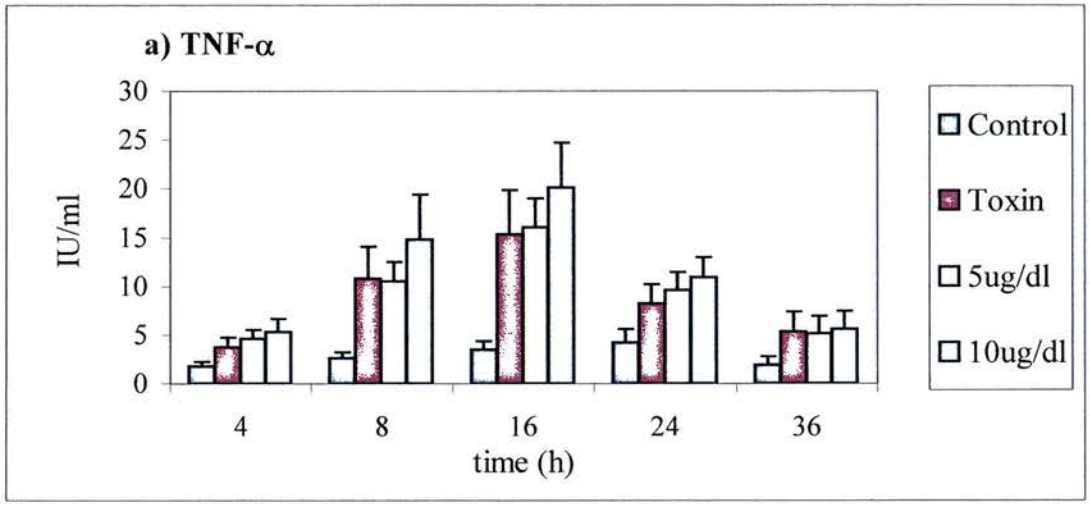
Stress levels of cortisol in adults range from 40-80 μg dl^{-1}. Compared with the control containing toxin alone, from 4 to 24 h there was significant reduction of TNF-α production and from 4-36 h significant reduction of IL-6. IL-10 was significantly decreased by high levels of cortisol from 16-36 h (Figure 5.7 and Table 5.3).

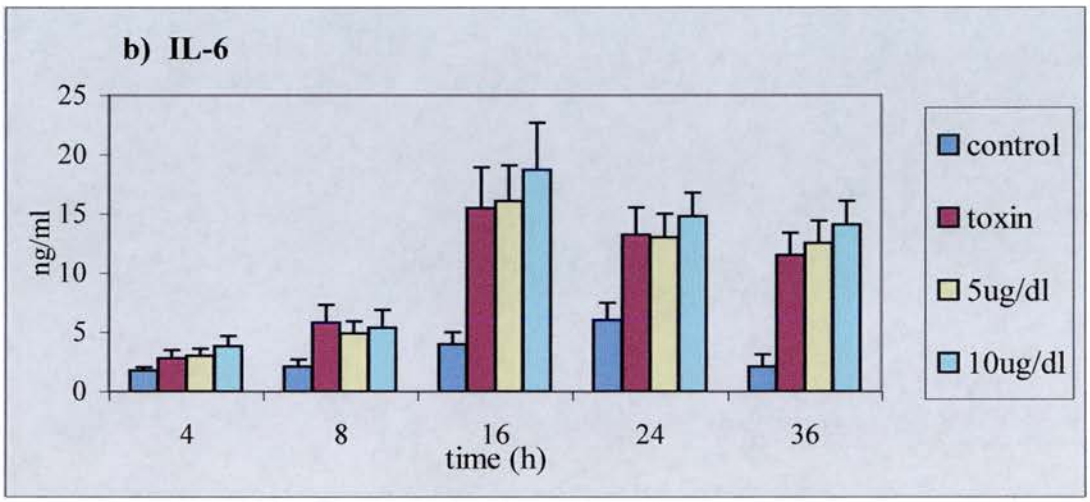
Table 5.1. Effect of night time level of cortisol (μg dl $^{-1}$) on induction of TNF-α, IL-6 & IL-10 from buffy coats by TSST-1. (7 donors)

		4hrs			8 hrs			16 hrs			24 hrs			36 hr		
		mean	**95% CI**	***p* value**	***mean***	**95% CI**	***p* value**	***mean***	**95% CI**	***p* value**	***mean***	**95% CI**	***p* value**	***mean***	**95% CI**	***p* value**
TNF-α IU ml $^{-1}$	Cells	1.7			2.6			3.5			4.2			1.8		
	Toxin + cells	**3.75**	**0.26, 3.7**	**0.029**	**10.75**	**1.6, 14.6**	**0.021**	**15.3**	**2.8, 20.7**	**0.017**	8.2		NS	**5.35**	**0.17, 6.7**	**0.042**
	Cortisol 5	4.6		NS	10.5		NS	16		NS	**9.6**	**0.21, 2.7**	**0.028**	5.1		NS
	Cortisol 10	5.3		NS	14.7		NS	**20.1**	**1.7, 7.9**	**0.008**	10.9		NS	5.6		NS
IL-6 ng ml^{-1}	Cells	1.75			2.1			4			4.5			2		
	Toxin + cells	2.8		NS	**5.8**	**0.97, 6.3**	**0.015**	**15.5**	**5.3, 17.6**	**0.003**	**13.25**	**3.6, 10.8**	**0.002**	**11.5**	**5, 13.7**	**0.001**
	Cortisol 5	3		NS	4.9		NS	16.1		NS	13		NS	12.5		NS
	Cortisol 10	3.8		NS	5.37		NS	**18.7**	**1, 5.4**	**0.01**	14.8		NS	**14.1**	**1.5, 3.7**	**0.001**
IL-10 ng ml^{-1}	Cells	4			3			3.6			3.25			1.37		
	Toxin + cells	5		NS	**7.88**	**2.6, 7.1**	**0.001**	**20.6**	**6.9, 27**	**0.005**	**25.75**	**8.3, 36.7**	**0.007**	**16.12**	**5.1, 24.4**	**0.008**
	Cortisol 5	4.25		NS	10.2		NS	12		NS	16.8		NS	12.8		NS
	Cortisol 10	**3.6**	**-2.4, -0.28**	**.02**	6.12		NS	**6.63**	**-23.8, - 4.2**	**0.01**	**9.8**	**-26.2, -5.6**	**0.008**	**6.5**	**-17.8, - 1.3**	**0.02**

* 95% CI of mean difference

Figure 5.5 The effect of night time levels of cortisol (5-10 μg dl^{-1}) on induction of (a) TNF-α, (b) IL-6, (c) IL-10 in response to TSST-1 (means of 7 donors)





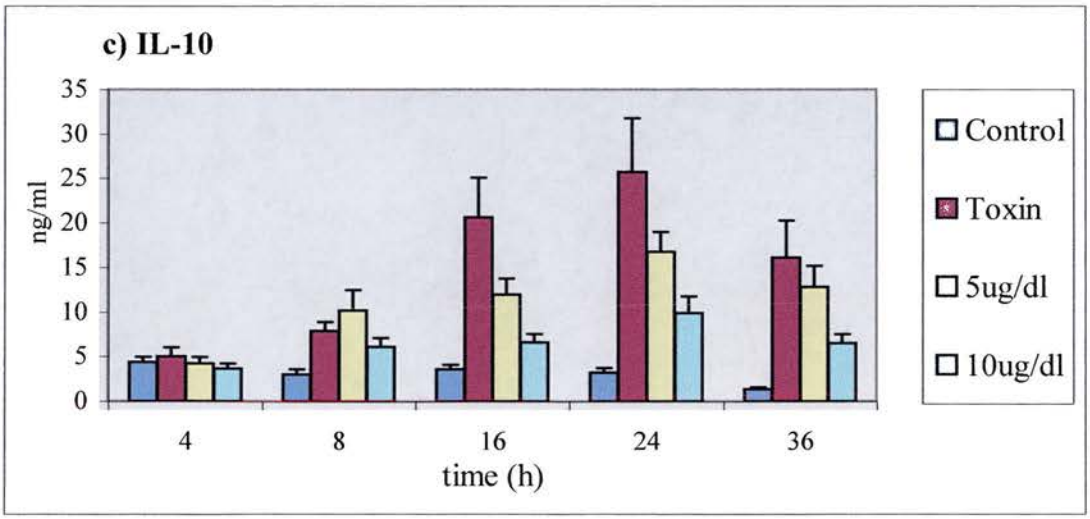
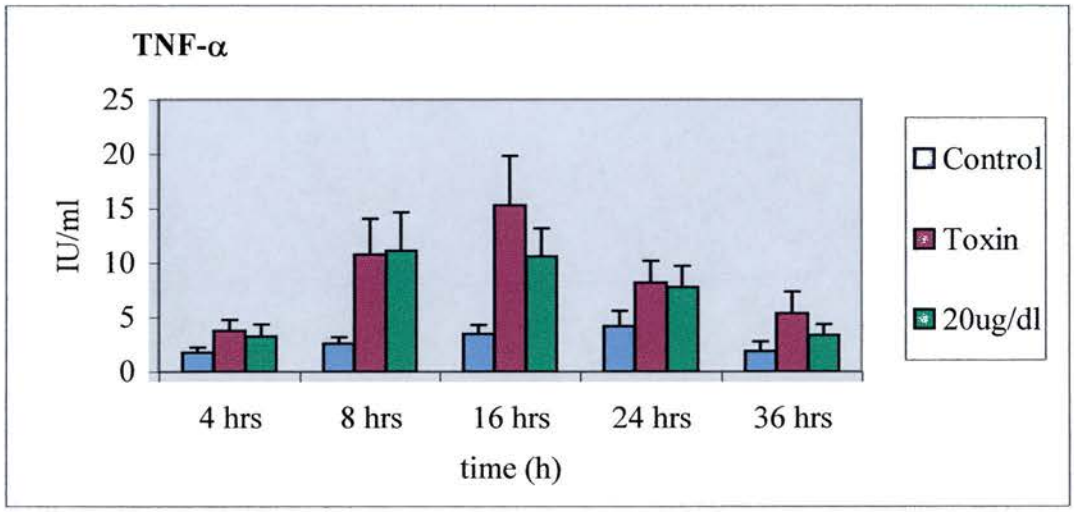


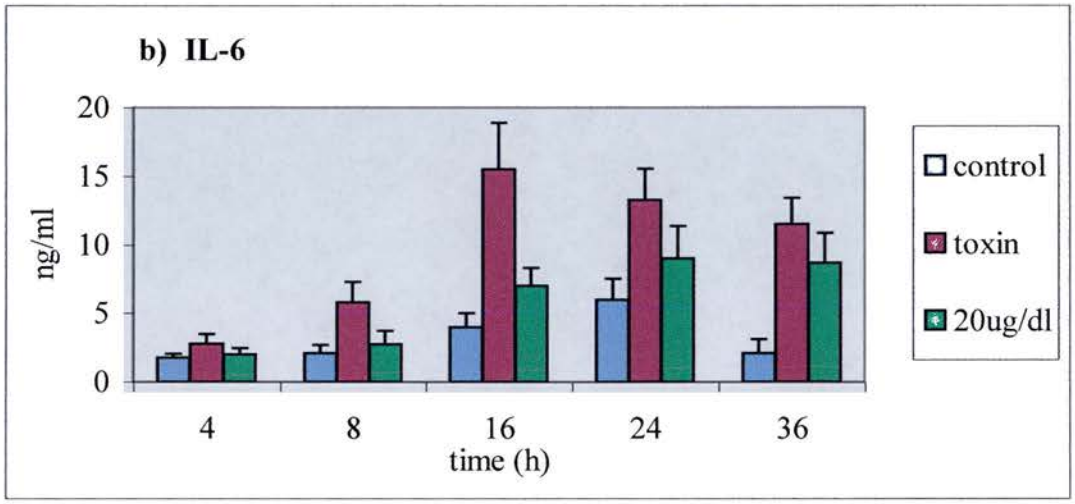
Table 5.2. Effect of daytime level of cortisol (μg dl $^{-1}$) on induction of TNF-α, IL-6 & IL-10 from buffy coats by TSST-1 (7 donors)

		4hrs			8 hrs			16 hrs			24 hrs			36 hr		
		mean	**95% CI**	***p* value**	***mean***	**95% CI**	***p* value**	***mean***	**95% CI**	***p* value**	***mean***	**95% CI**	***p* value**	***mean***	**95% CI**	***p* value**
TNF-α IU ml $^{-1}$	Cells	1.7			2.6			3.5			4.2			1.8		
	Toxin +Cells	**3.75**	**0.26, 3.7**	**0.029**	**10.75**	**1.6, 14.6**	**0.021**	**15.3**	**2.8, 20.7**	**0.017**	8.2		NS	**5.35**	**0.17, 6.7**	**0.042**
	Cortisol 20	3.25		NS			NS	10.6		NS	7.76		NS	3.38		NS
IL-6 ng ml^{-1}	**Toxin +cells**	2.8		NS	**5.8**	**0.97, 6.3**	**0.015**	**15.5**	**5.3, 17.6**	**0.003**	**13.25**	**3.6, 10.8**	**0.002**	**11.5**	**5, 13.7**	**0.001**
	Cortisol 20	2		NS	**2.75**	**- 4.9, -1.1**	**0.007**	7	**-13.5, -3.4**	**0.006**	**9**	**-7.1, -.3**	**0.034**	8.7		NS
IL-10 ng ml^{-1}	**Toxin +cells**	5		NS	**7.88**	**2.6, 7.1**	**0.001**	**20.63**	**6.9, 27**	**0.005**	**25.75**	**8.3, 36.7**	**0.007**	**16.12**	**5.1, 24.4**	**0.008**
	Cortisol 20	5.7		NS	**16.12**	**0.06, 16.4**	**0.048**	14.3		NS	22.3		NS	15.75		NS

* 95% CI of mean difference

Figure 5.6 The effect of day time levels of cortisol (20 μg dl^{-1}) on induction of (a) TNF-α, (b) IL-6, (c) IL-10 in response to TSST-1 (means of 7 donors)





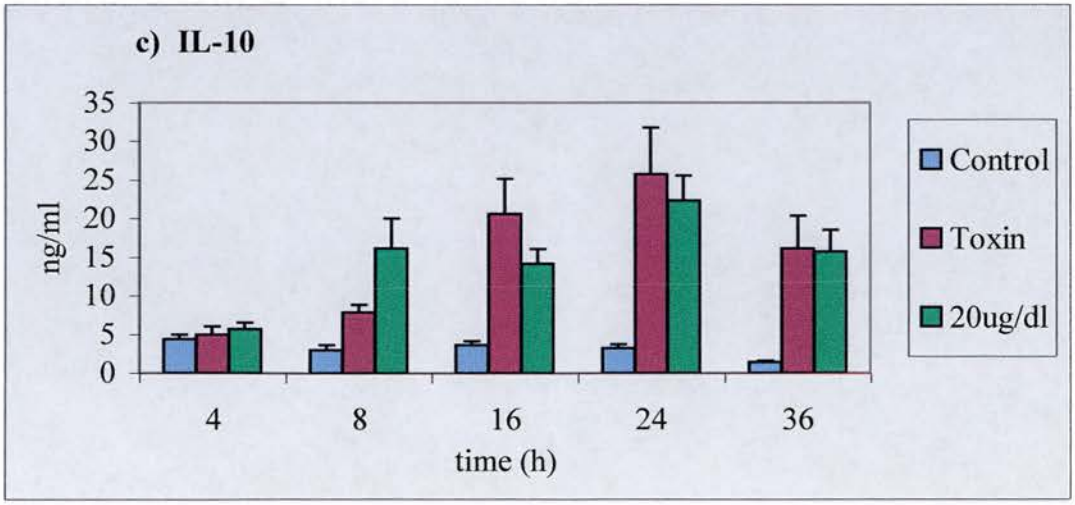
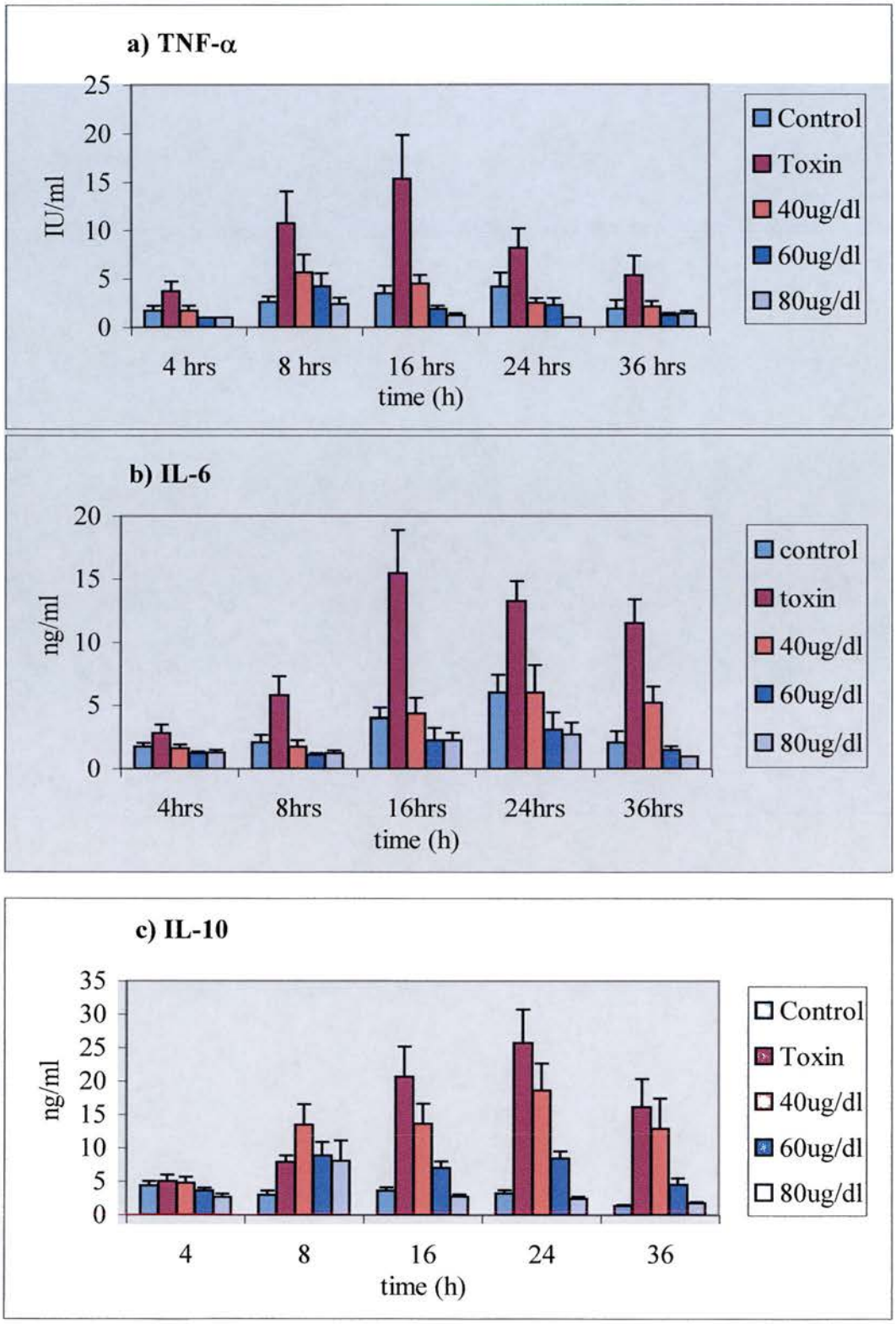


Table 5.3. Effect of stress levels of cortisol (μg dl $^{-1}$) on induction of TNF-α, IL-6 & IL-10 from buffy coats by TSST-1. (7 donors)

		4hrs			8 hrs			16 hrs			24 hrs			36 hr		
		mean	95% CI	*p* value	*mean*	95% CI	*p* value	*mean*	95% CI	*p* value	*mean*	95% CI	*p* value	*mean*	95% CI	*p* value
TNF-α IU ml^{-1}	Cells	1.7			2.6			3.5			4.2			1.8		
	Toxin +cells	**3.75**	**0.26, 3.7**	**.029**	**10.75**	**1.6, 14.6**	**0.021**	**15.3**	**2.8, 20.7**	**0.017**	8.2		NS	**5.35**	**0.17, 6.7**	**0.042**
	Cortisol 40	**1.75**	**-3.8, - 0.1**	**0.04**	**5.7**	**-9.9, - 6.4**	**.048**	**4.5**	**-21, - 0.5**	**0.042**	**2.5**	**-11.2, -2**	**0.049**	2.1		NS
	Cortisol 60	**1**	**-5, - 0.4**	**0.026**	**4.25**	**-12, - 0.1**	**0.046**	**1.87**	**-24.5, - 2.2**	**0.025**	2.3		NS	1.25		NS
	Cortisol 80	**1**	**-5, - 0.4**	**0.026**	**2.37**	**-14.6, -2**	**0.017**	**1.25**	**-24.8, - 3.2**	**0.018**	**1**	**-12.1, -2.1**	**0.012**	1.37		NS
IL-6 ng ml^{-1}	**Toxin +cells**	2.8		NS	**5.8**	**0.97, 6.3**	**0.015**	**15.5**	**5.3, 17.6**	**0.003**	**13.25**	**3.6, 10.8**	**0.002**	**11.5**	**5, 13.7**	**0.001**
	Cortisol 40	**1.6**	**- 4.9, -2.3**	**0.043**	**1.75**	**-7, - 1**	**0.014**	**4.37**	**-17.3, - 4.9**	**0.004**	**6**	**-10, -4.4**	**.00**	**5.2**	**-9.6, -2.8**	**0.003**
	Cortisol 60	1.25		NS	**1.12**	**-8.7, - 0.5**	**0.030**	**2.25**	**-20.5, - 5.9**	**0.003**	**3.12**	**-13.6, -6.6**	**.00**	**1.5**	**-14.2, -5.7**	**0.001**
	Cortisol 80	**1.3**	**-3.4, -2.8**	**0.046**	**1.25**	**-8.7, - 0.4**	**0.036**	**2.25**	**-21.5, - 4.9**	**0.007**	**2.75**	**-16.3, -4.6**	**0.004**	**1**	**-14.9, -6**	**0.001**
IL-10 ng ml^{-1}	**Toxin +cells**	5		NS	**7.88**	**2.6, 7.1**	**0.001**	**20.63**	**6.9, 27**	**0.005**	**25.75**	**8.3, 36.7**	**0.007**	**16.12**	**5.1, 24.4**	**0.008**
	Cortisol 40	4.7		NS	13.5		NS	13.6		NS	**18.6**	**-13.9, - 3.2**	**0.042**	12.8		NS
	Cortisol 60	3.6		NS	8.8		NS	**7**	**-25.4, - 1.86**	**0.028**	**8.5**	**-30.1, - 4.3**	**0.016**	**4.5**	**-19.8, -3.4**	**0.01**
	Cortisol 80	**2.6**	**-4.6, -0.05**	**0.045**	8.1		NS	**2.75**	**-28.5, -7.3**	**0.005**	**2.38**	**-37.1, -9.56**	**0.005**	**1.75**	**-24.1, -4.6**	**0.01**

* 95% CI of mean difference

Figure 5.7 The effect of stress levels of cortisol (40-80 μg dl^{-1}) on induction of (a) TNF-α, (b) IL-6, (c) IL-10 in response to TSST-1 (means of 7 donors)



5.5 Discussion

The objective of these experiments was to assess the production of three inflammatory mediators (TNF-α, IL-6 and IL-10) in response to TSST-1 and to show how their production was affected by hydrocortisone levels found in adults at night, during the day and under condition of physiological stress. The results obtained were assessed in relation to some of the epidemiological risk factors identified for SUND.

Most SUND cases occur at night when cortisol levels are lowest. The night time level observed in normal subjects (5-10 μg dl^{-1}) did not reduce production of pro-inflammatory cytokines in the model system. Similar results were observed in parallel studies on daytime and night time levels of cortisol found in infants with a modification of the protocol used in these studies (Gordon,1999). At several time points, low levels of cortisol significantly enhanced both TNF-α and IL-6, and significantly reduced IL-10, production in the model system.

Inflammatory responses to endotoxin (IL-6 and TNF-α) are significantly affected by hormonal levels which are associated with circadian rhythm and exhibit increased production during the night when cortisol levels are lowest (Entzian *et al.*, 1996; Pollmacher *et al.*, 1996). Day time levels had an inhibitory effect on IL-6 production

but not TNF-α in response to the toxins. Stress levels of cortisol significantly reduced all three cytokines.

Extrapolation of these *in vitro* studies to the human situation is difficult. The dynamic situation found in the living organism cannot be reproduced, particularly the effects of feed-back mechanisms which have a bearing on both interleukin production and hydrocortisone levels. The only reasonable deduction that can however be made is that at night time, there could be a decreased control of inflammatory mediators induced by infectious agents.

Only one stimulant (TSST-1) was tested in this system, but combinations of microbial products (bacterial toxins and virus infection) and products of cigarette smoke might result in higher levels of inflammatory mediators (Raza *et al.*, 1999).

Chapter 6

The effect of antibodies on inflammatory responses induced by pyrogenic toxins

6.1 Introduction

Antibodies can reduce the severity of toxic shock syndrome as demonstrated by protection of rabbits from a lethal toxaemia with TSST-1 by anti TSST-1 antibodies (Bonventre *et al.*, 1988). Clinical infections with *S. aureus* result in higher concentration of antibodies to the staphylococcal-toxins (Jozefczyk, 1974). Normal pooled IgG contains antibodies against a major group of superantigens and staphylococcal toxins, which modulates a possible immunoregulatory role for the antibodies *in vivo* (Takei *et al.*, 1993). A monoclonal antibody to TSST-1 reacted with TSST-1 in ELISA and blocked induction of interleukin 1 by TSST-1 (Beezhold *et al.*, 1987). In normal individuals, the prevalence of serum antibodies to staphylococcal toxins increases with age (Vergeront *et al.*, 1983; Notermans *et al.*, 1983). In contrast to controls of the same age group, antibodies directed against TSST-1 are usually absent or minimal in sera of patients with toxic shock syndrome

(Bonventre *et al.*, 1984; Notermans *et al.*, 1983). The lack of specific IgG antibodies to TSST-1 in patients could be of pathogenic significance and help to explain the susceptibility to TSST in certain individuals (Christensson *et al.*, 1986).

Over 90% of *S. aureus* isolated from women with toxic shock syndrome associated with menstruation produce the toxin (Bonventre *et al.*, 1988). The incidence of menstrual TSST-1 decreased in the last few years, and this can be explained by the changes in the absorbency and composition of tampons available (Schuchat & Broome, 1991). In recent years, it became clear that non-menstrual TSS may result from focal infections with *S. aureus* strains which do not produce TSST-1 and staphylococcal toxins other than TSST-1 may precipitate the illness by similar or identical pathways (Garbe *et al.*, 1985; Schlievert, 1986; Schlievert *et al.*, 1995). There are some cases of TSS associated with viral respiratory infections such as influenza (MacDonald *et al.*, 1987; Newbould *et al.*, 1989; Schlievert *et al.*, 1995).

The objectives of the present study were: 1) to assess the levels of antibodies to pyrogenic staphylococcal toxins in the general population; 2) to assess the levels of IgG to the toxins needed to reduce the production of inflammatory mediators in a model system by 50%; 3) to assess the results in relation to the age range affected by SUND and the prevalence of their deaths among men.

6.2. Materials and Methods

6.2.1 Levels of antitoxin in adults.

6.2.1.1 Subjects.

Serum samples (n=100) from autopsies were obtained from the Forensic Medicine Unit collected during autopsies carried out routinely. Samples from 10 males and 10 females in each of the following age ranges was assessed: 20-29; 30-39; 40-49; 50-59; 60-69.

6.2.1.2 ELISA for detection of antitoxin

Each sample was examined by ELISA for IgG antibodies to TSST, SEC, SEA. The plates were coated with 100 µl/well of 0.1 µg ml^{-1} TSST, SEC or SEA in coating buffer and incubated overnight at 4 °C. The plates were washed 3 times with washing buffer, 100 µl of blocking buffer were added to each well and the plates were incubated for 30 min. The blocking buffer was removed, serum samples diluted 1:50 (100 µl) added to duplicate wells and the plates incubated for 60 min at 37°C with continuous shaking. The wells were washed 3 times with washing buffer, HRP-conjugated sheep anti-human IgG (100 µl) diluted 1 in 100 in blocking buffer was added to the wells and the plates incubated for 60 min at 37^0C with continuous shaking. The wells were washed 3 times with washing buffer, the OPD substrate (100 µl) was added to the wells and the colour change was stopped after 10 - 20 min by

adding 100 µl of H_2SO_4. The A_{490} was determined by an ELISA plate reader (Dynatech) and corrected by subtracting the OD of the corresponding blank well containing each of the components except the serum sample. The results were expressed in ng ml^{-1} derived from the IgG standard curve from 1-100 ng in each experiment (Zorgani *et al.*, 1996).

6.2.2.1 Collection of human peripheral blood monocytes

Collection of human peripheral blood monocytes was discussed in 5.2.1.

6.2.2 .2 Neutralisation of toxin activity by pooled human sera

The experimental procedure in 5.2.3 was used to assess the neutralising effect on TSST-1 of a pool of sera prepared from the samples in 5.2.1. The levels of TNF-α and IL-6 induced from cells by toxin treated with dilutions of the pooled sera were compared with results of cells incubated with toxin alone or with serum dilutions with no toxin. The results obtained with the serum treated toxin were expressed as percentage of the positive control, the toxin alone (100%). The assays were carried out under the same conditions as 5.2.3. The toxins plus medium, pooled antiserum plus medium, and toxins plus dilutions of the pool were incubated for 16 h at 37°C before addition to the cells.

6.2.3 Cytokine assays

6.2.3.1 The ELISA for IL-6 was described in 5.2.4.1 (Figure 5.1)

6.2.3.2 Bioassay for TNF-α was described in 5.2.4.2 (Figure 5.2)

6.2.4 Statistical analysis

Student t-test for paired sample was used for data analysis.

6.3 Results

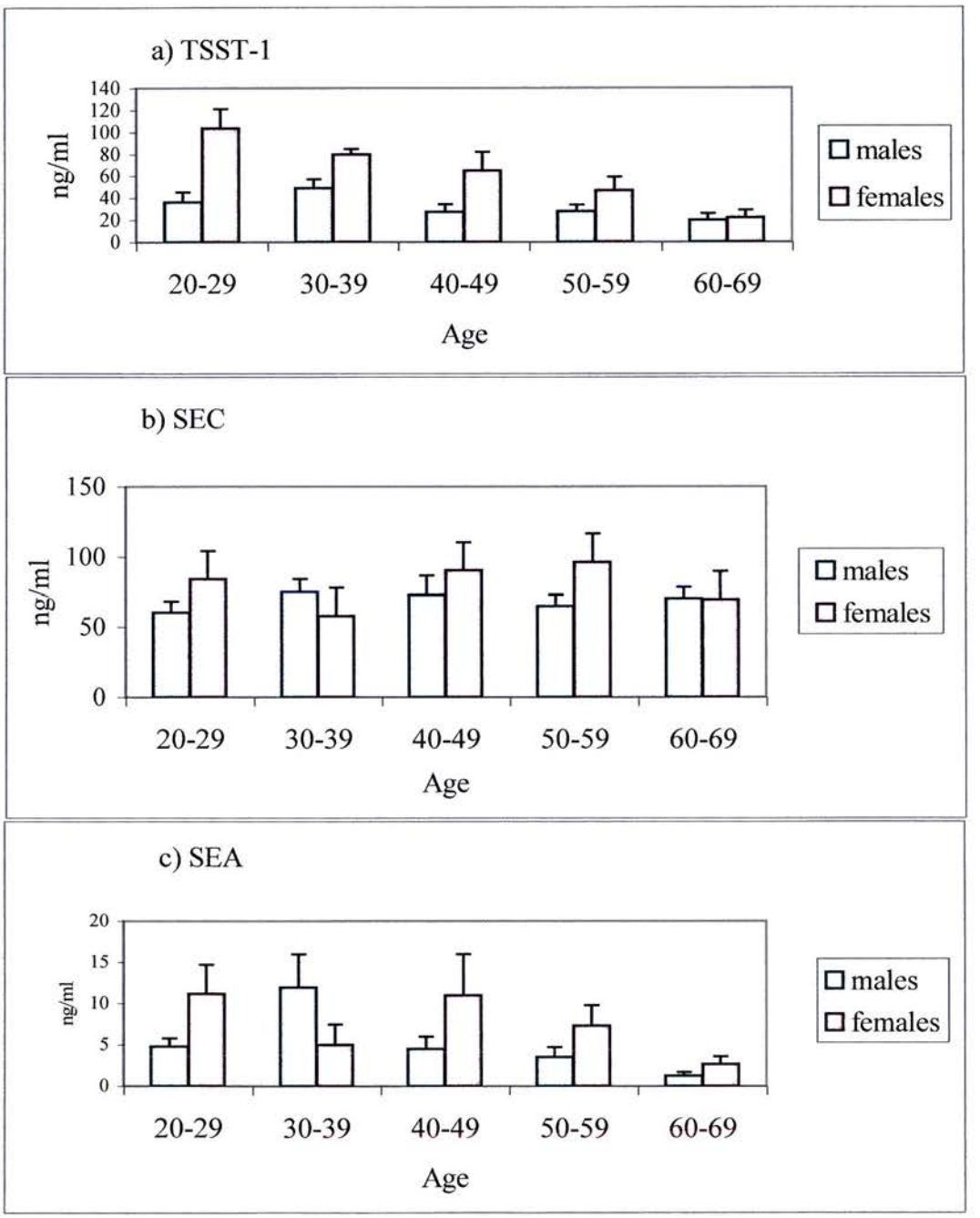
6.3.1 Levels of IgG bindings to toxins in samples from males and females

In general, females had higher levels of IgG which bound to the toxins in the ELISA. Compared with the results for males, IgG levels to TSST-1 were significantly higher in females in the 20-29 (P= 0.007) and the 30-39 (P=0.006) age groups in which the majority of SUND cases occurs. In most age groups, there were no significant differences for IgG that bound to SEC, but in the 50-59 age groups females had higher levels than males (P=0.014). There was no significant difference in levels of IgG bindings to SEA between males and females in any of the groups (Figure 6.1 and Table 6.1)

Table 6.1. Levels of IgG (ng ml^{-1}) that bound to staphyloccocal toxins in sera from males compared with females (10 pairs of males and females per age range)

	20-29			**30-39**			**40-49**			**50-59**			**60-69**		
	Mean		***p* value**	***Mean***		***p* value**	***Mean***		***p* value**	***Mean***		***p* value**	***Mean***		***p* value**
	F	***M***		***F***	***M***		***F***	***M***		***F***	***M***		***F***	***M***	
TSST-1 F/ M	**103**	**36**	**0.007**	**81**	**49**	**0.006**	64	27	0.06	47	27	0.15	22	20	0.81
SEC F/M	84	60	0.08	57	75	0.3	90	72	0.49	**96**	**65**	**0.014**	69	70	0.92
SEA F/M	11	4	0.17	5	12	0.27	11	4	0.26	7	3	0.2	2.7	1.3	0.173

Figure 6.1. IgG binding to (a) TSST-1, (b) SEC, (c) SEA in sera from 50 males (n=10 per age group) and 50 females (n=10 per age group).

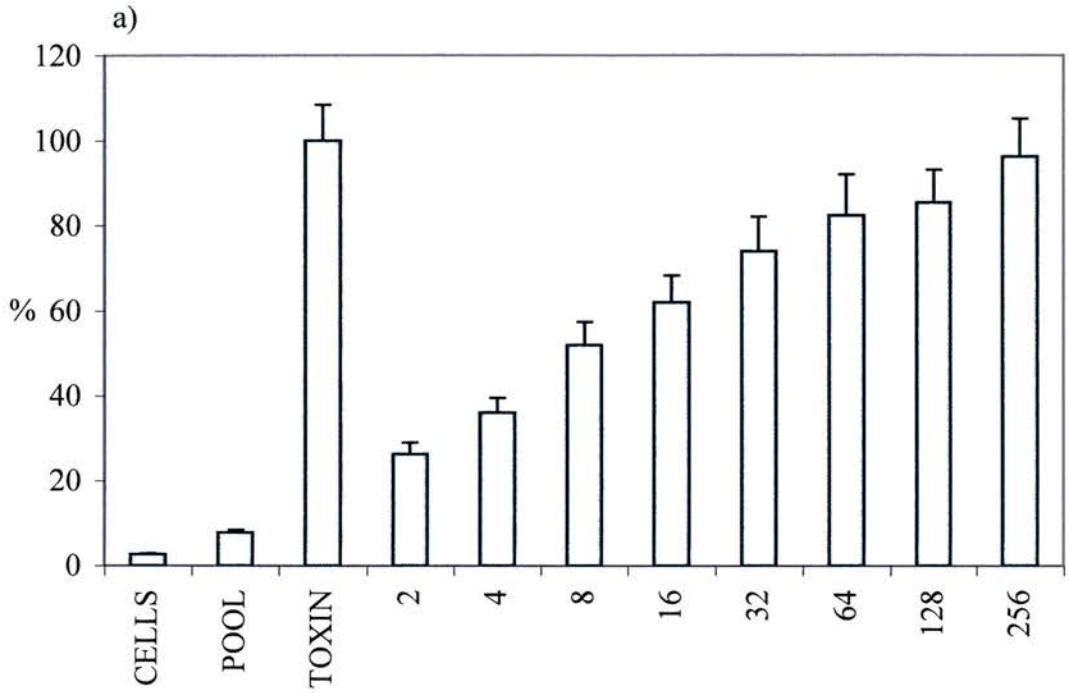


6.3.2 Neutralisation of staphylococcal toxins by pooled antisera

In the absence of antibody, TNF-α and IL-6 from cells treated with toxin was detected at 4 h and the levels rose steadily until 16-20 h (Figure 5.5-5.6). The IgG (ng ml $^{-1}$) binding to TSST-1, SEC and SEA were determined for the pooled sera by the quantitative ELISA and dilutions of the pool tested for their ability to reduce cytokine production by the toxins. The pool contained 2.7 μg ml^{-1} IgG that bound TSST-1, 4 μg ml^{-1} that bound to SEC and 0.4 μg ml^{-1} that bound to SEA.

Figures 6.2-6.3 summarise the results of 7 experiments with the toxins and toxins treated with dilutions of the pooled sera. For 0.1 μg TSST, 0.4 μg ml^{-1} IgG was needed for approximately 50% reduction of both cytokines. For 0.1 μg ml^{-1} SEC, 1.25 μg ml^{-1} IgG was needed for 50% reduction of IL-6 and 0.5 μg ml^{-1} for 50% reduction of TNF-α production. Compared with levels of IgG that bound to TSST-1 or SEC, the levels of IgG bound to SEA were negligible. None of the dilutions of the pooled sera contained enough antibodies to reduce SEA induced production of either TNF-α or IL-6 by 50% (Figure 6.4).

Figure 6.2. Effect of dilutions of the pooled serum samples on induction of IL-6 by (a) TSST-1 (b) SEC



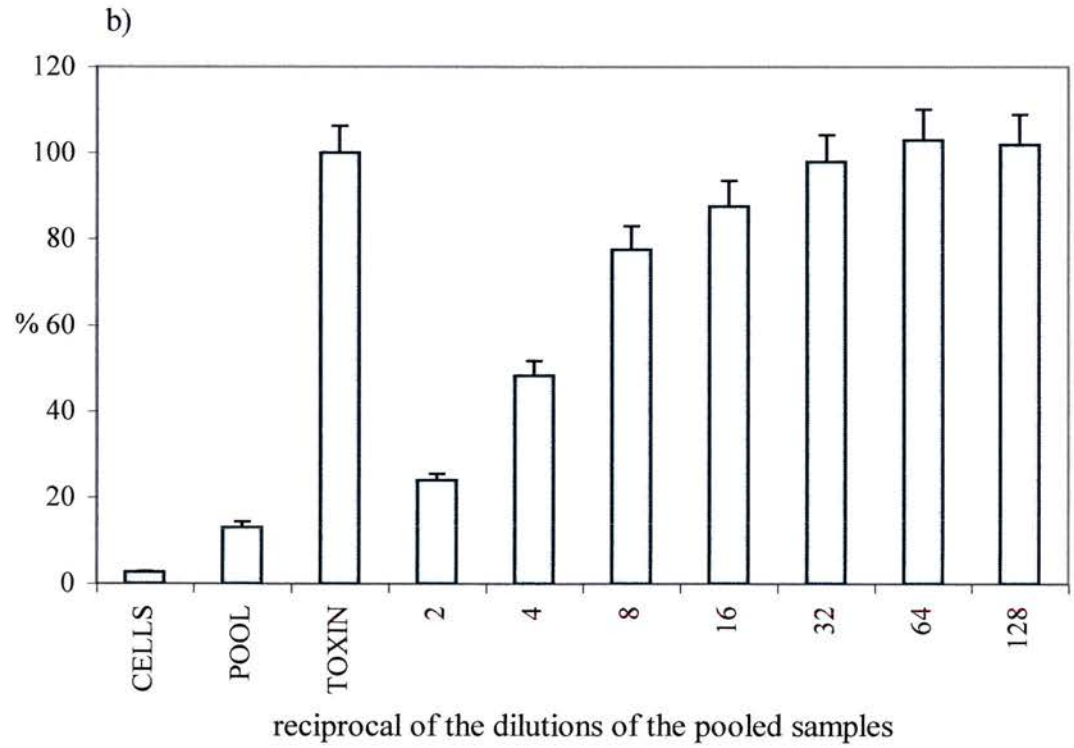
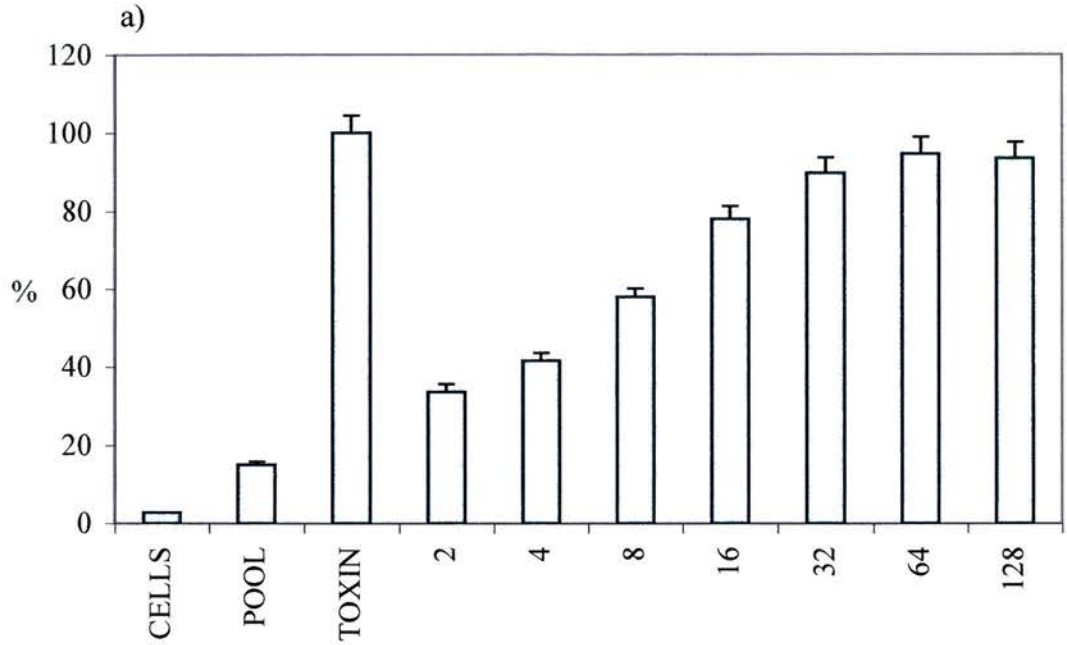


Figure 6.3. Effect of dilutions of the pooled serum samples on induction of TNF-α by a) TSST-1 and b) SEC



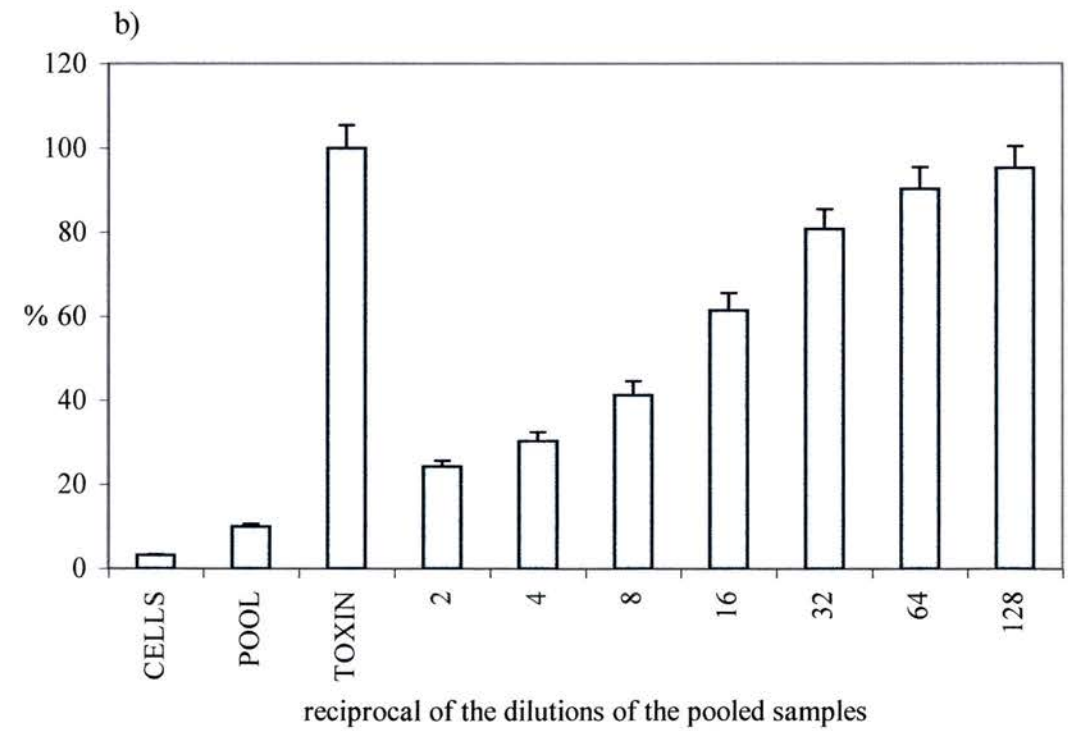
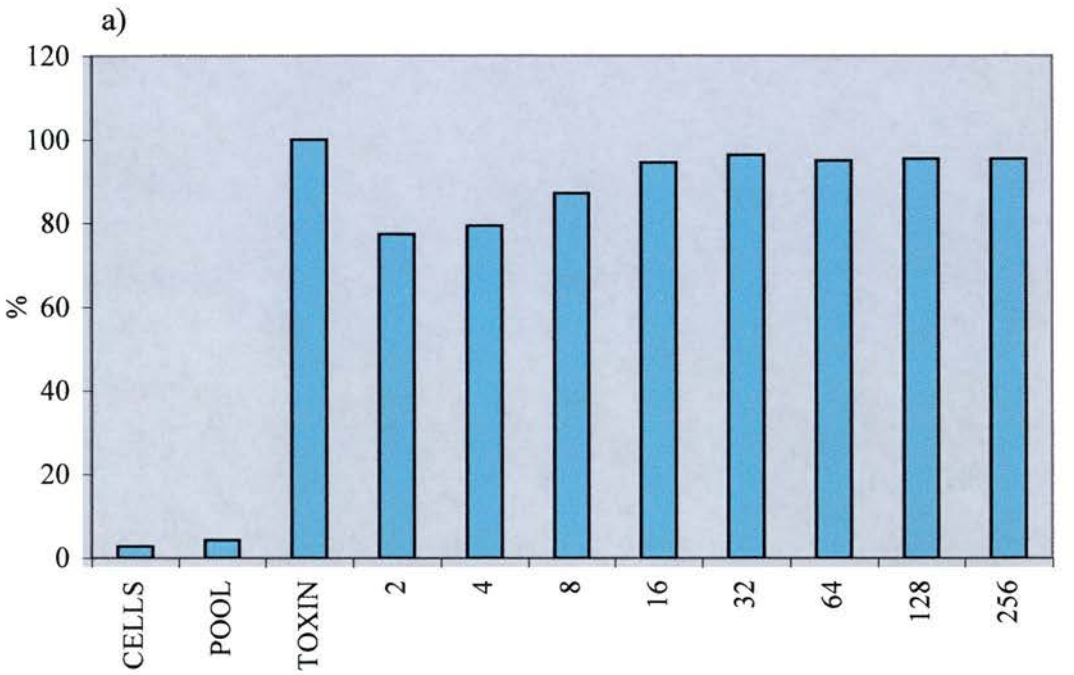
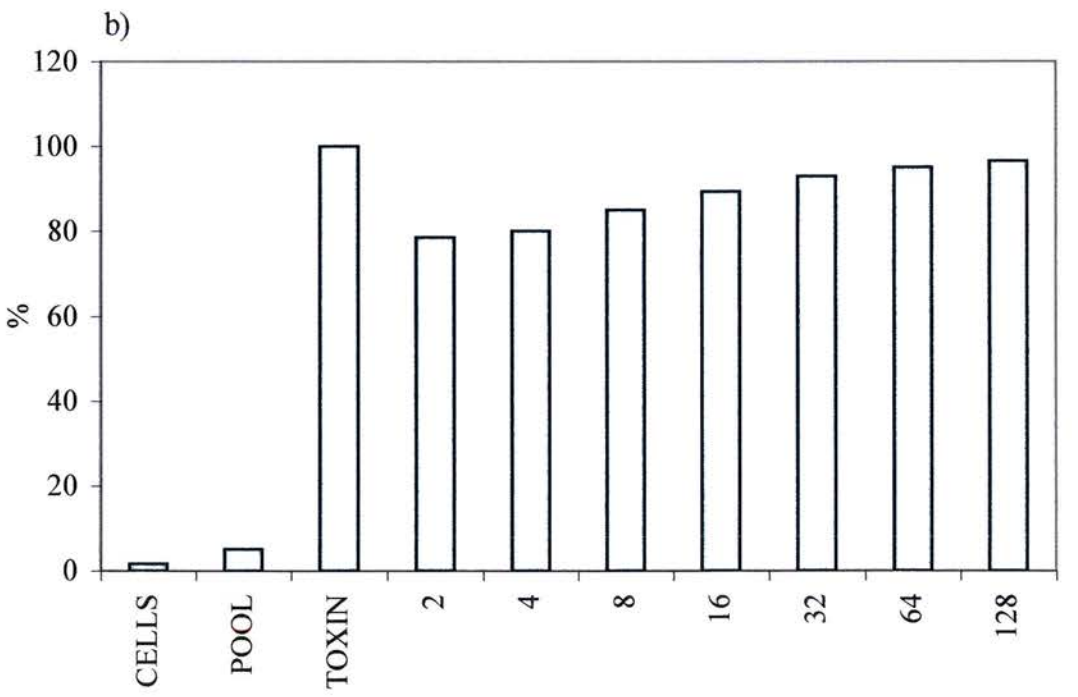


Figure 6.4. Effect of dilutions of the pooled serum samples on
a) induction of IL-6 by SEA and b) induction of TNF-α by SEA





6.3.3 Protective levels of antitoxins in the population examined

Among the samples tested (14%) of males and (8%) of females had less than 0.4 μg ml^{-1} of IgG that bound to TSST-1 in the ELISA. The opposite pattern was observed for SEC, (8%) of males and (12%) of females had less than 1.25 μg ml^{-1} needed for neutralisation of IL-6; however, the percentage of males (4%) and females (6%) with less than 0.5 μg ml^{-1} needed for neutralisation of TNF-α was similar.

6.4 Discussion

The results obtained with the quantitative ELISA for IgG levels to the toxins and the effect of the IgG antitoxin levels on neutralisation of the toxin activity were assessed in relation to the some of the epidemiological risk factors of SUND

6.4.1 Levels of IgG in the population tested in relation to age and prevalence of SUND in men

The majority of the population tested had sufficient antibodies to reduce TNF and IL-6 responses elicited by TSST-1 and SEC in the model system. In the age range in which most SUND cases occur (20-39 years), males had significantly lower levels of IgG to TSST compared with females. TSST-1 has been identified in tissues of a 6 year old child (Bentley *et al.*, 1997) and two adults who died suddenly and

unexpectedly (chapter 4). If these toxins play a role in precipitating the series of events leading to SUND, the higher levels of IgG to the toxins observed in females might partly explain the much higher prevalence of these deaths among men.

6.4.2 Neutralisation of inflammatory responses to pyrogenic toxins

Results indicate there might be a small proportion of the population with antibody levels lower than that needed to neutralise the individual toxins. Studies by Essery *et al* (1999) indicate that immunisation against diphtheria pertussis and tetanus (DPT) induces IgG antibodies cross reactive with staphylococcal pyrogenic toxins. The majority of Thai immigrants affected by SUND were in the age group that would not have been immunised against childhood diseases (Blackwell *et al.*, 1994). Antibody levels in sera of women with TSS or history of TSS were lower than normal women with no history of TSS (Bonventre *et al.*, 1984). Levels of antibodies to these toxins have not been assessed in SUND victims.

6.4.3 Genetic control of inflammatory responses to bacterial toxins

Studies indicate that there are significant genetic factors associated with control of both pro- and anti-inflammatory responses to endotoxin (Westendorp *et al.*, 1995). Fatal outcome of meningococcal infection was related to low levels of TNF responses and/or high levels of IL-10 responses obtained *in vitro* studies of first degree relatives of the patients (Westendorp *et al.*, 1997). There is evidence of

individual differences in response to TSST-1. A small proportion of the individuals expressed TNF-α responses to TSST-1 twice the level observed with their respective unstimulated cells (Raza *et al.*, 1999). There have been no studies on inflammatory responses in the immigrant populations affected by SUND in comparison with the local populations in the host country.

6.4.4 Conclusion

In conclusion, the methods developed for quantitative IgG assays of antitoxins can be applied to investigation of SUND victims to test the hypothesis that some of these deaths are precipitated by pyrogenic staphylococcal toxins in the absence of or low levels of antibodies to the pyrogenic staphylococcal toxins.

Chapter 7

The effect of IL-10 on inflammatory response induced by pyrogenic toxins

7.1 Introduction

Interleukin–10 (IL-10) was originally identified as an inhibitory factor for cytokine synthesis. It exerts its anti-inflammatory effects by inhibiting cytokine synthesis in macrophages and T helper 1 (Th1) cells (Fiorentino *et al.*, 1991; Seder & Paul, 1994). In mice Th1 cells secrete IL-2, IFN-γ and TNF and are responsible for the development of the cell-mediated response which is important for removal of intracellular bacterial pathogens and viruses. T helper 2 (Th2) cells secrete IL-4, IL-5, IL-10 and IL-13 and are responsible for the stimulation of specific imunoglobulin production and control of macrophage function (Morel & Oriss, 1998).

Circulating anti-inflammatory cytokines exert profound inhibitory effects. When normal monocytes and plasma from patients with sepsis were mixed with LPS, plasma IL-10 was found to suppress the response of normal monocytes to LPS

(Brandtzaeg *et al.*, 1996). IL-10 was reported to protect mice against staphylococcal enterotoxin B-induced lethal shock when administered before or concurrently with staphylococcal enterotoxin B (Bean *et al.*, 1993). Following a challenge injection in mice of staphylococcus enterotoxin-A (SEA) introduced four days after the priming injection of SEA, IL-10 production from Th2 cells is significantly enhanced while that of Th1 cells is suppressed (Sundstedt *et al.*, 1997).

IL-10 stimulates DNA replication of tonsillar B cells activated either via their antigen receptor or via cross-linking of surface CD40 antigen, it also enhances their viability (Rousset *et al.*, 1992). IL-10 has been shown to induce anti-CD40 activated human tonsillar B cells to secrete large amounts of IgG, IgA, and IgM (Defrance *et al.*, 1992). IL-10 enhanced immunoglobulin production by *S. aureus* activated B cells (Itoh *et al.*, 1994; Itoh & Hirohata, 1995). It has been shown that peripheral blood mononuclear cells and B cells from healthy IgA deficient individuals produced significantly higher levels of IgG when stimulated with IL-10 compared with infection prone IgA deficient patients (Friman *et al.*, 1996). IL-10 is synthesized late by activated human monocytes and has been shown to inhibit its own production in an autocrine fashion (de Waal *et al.*, 1991; de Waal *et al.*, 1992). This negative feed back loop may afford an important mechanism to prevent prolonged suppression of immune or inflammatory responses.

IL-10 production was found to be defective in patients with adult respiratory distress syndrome which is mediated by inflammatory responses (Chollet-Martin *et al.*,

1994). In the studies in chapter 5, some of the subjects appeared to have lower IL-10 responses at 16hr and 24hr (Table 7.1and 7.2)

The objective of the present study were: 1) to determine if recombinant IL-10 was effective as cortisol in reducing pro-inflammatory responses in the model system; 2) to determine if the levels of recombinant IL-10 needed for reduction of pro-inflammatory responses were similar to that elicited from leukocyte in response to staphylococcal toxins.

7.2 Materials and Methods

7.2.1. Collection of human peripheral blood leukocytes

Buffy coats cells were prepared as described in 2.4.2

7.2.2. Effect of IL-10 on leukocyte responses to TSST-1, SEA, and SEC.

Human leukocytes 2 x 10^6 obtained as in 2.4.2 (500 µl/well) were placed in 24 well tissue culture plates and 250 µl of TSST-1, SEA or SEC were added to the cells at a final concentration of 0.1µg ml^{-1}. These were incubated at 37°C for 30 min and 250 µl of different concentrations of IL-10 diluted in DMEM were added to give a final

concentration of 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3 or 1.5 ng/ml^{-1}. The cells were incubated at 37°C for 16 h in a humidified incubator with 5% CO_2. The cell supernatants were collected in sterile tubes and centrifuged at 250 x g for 10 min. The supernatants were stored at -20°C until assayed for TNFα (5.2.4.2) and IL-6 (5.2.4.1). The results for the different concentrations of IL-10 were expressed as a percentage of the control containing toxin alone.

7.2.3 Statistical analysis

Student t-test for paired samples was used for data analysis.

7.3 Results

7.3.1 Proinflammatory cytokine levels in relation to IL-10 levels of individual donors

Tables 7.1 and 7.2 summarise the cytokine levels at 16 hr and 24 hr in relation to the unstimulated cells, IL-10 levels for donors 1- 4 increased approximately (6-10) fold by 16 hr, but for donors 5 – 8, (2-4) fold increase were observed. Pro-inflammatory responses for donors 1 – 4 were lower in response to the toxin. IL-6 increased (2.5-5) folds that of the unstimulated cells and TNF-α increased (4-6) fold. For donors 5 – 8, IL-6 levels increased from 2.6 to 10 times that of the control and TNF-a 1.25 to 13 times of the control. Even at 24 hr only one donor in the low responder group had IL-10 levels above 20 ng ml $^{-1}$ (Table 7.2). For donor 8, there was no difference between levels of IL-10 at 16 and 24 hr.

Table 7.1 Cytokines levels induced with TSST-1 by 16 hr

	TNF-α			IL-6			IL-10		
donor	No toxin IU/ml^{-1}	Toxin IU/ml^{-1}	X increase ✳	No toxin ng/ml^{-1}	Toxin ng/ ml^{-1}	X increase ✳	No toxin ng/ ml^{-1}	Toxin ng/ml^{-1}	X increase ✳
1	2	9.5	X 4.7	2	8	X 4	4	30	X 7
2	2	9	X 4.5	1	5	X 5	2	20	X 10
3	1	6	X 6	4	10	X 2.5	6	40	X 7
4	2	9	X 4	5	15	X 3	3	20	X 6
5	4	30	X 7	1	8	X 8	5	12	X 2
6	3	40	X 13	2	20	X 10	2	8	X 4
7	4	5	X 1.25	10	26	X 2.6	4	15	X 4
8	2	14	X 7	7	32	X 4	3	10	X 3

✳ X fold increase compared with non-stimulator control.

Table 7.2 Cytokines levels induced with TSST-1 by 24 hr

	TNF-α			IL-6			IL-10		
donor	No toxin IU/ml^{-1}	Toxin IU/ml^{-1}	X increase ✳	No toxin ng/ml^{-1}	Toxin ng/ml^{-1}	X increase ✳	No toxin ng/ml^{-1}	Toxin ng/ml^{-1}	X increase ✳
1	2	9.5	X 4.7	4	10	X 2.5	4	30	X 7
2	3	5	X 1.6	2	8	X 4	2	24	X 12
3	2	7	X 3.5	5	9	X 2	5	50	X 10
4	2	5	X 2.5	7	14	X 2	3	34	X 11
5	8	10	X 1.25	3	6	X 2	4	20	X 5
6	5	10	X 2	5	18	X 3	1	10	X 10
7	4	6	X 1.5	10	18	X 1.8	3	28	X 9
8	6	12	X 2	12	25	X 2	3	10	X 3

✳ X fold increase compared with non-stimulator control.

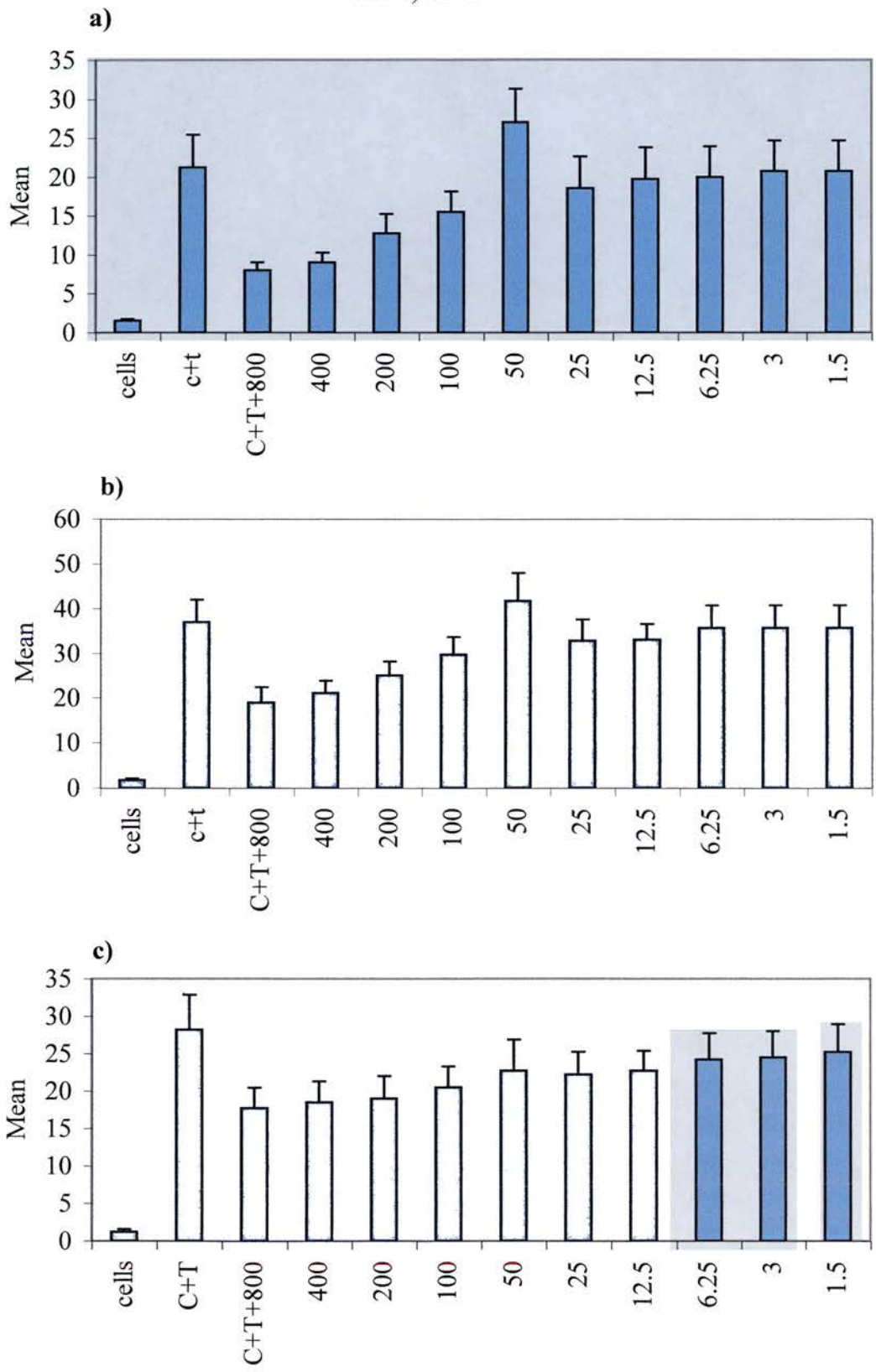
7.3.2 Effect of IL-10 levels on induction of TNF-α from buffy coats by TSST-1, SEC or SEA

IL-10 levels ranging from 800-1.5 ng ml^{-1} were tested for their effect on induction of TNF-α by the three toxins Compared with the positive control containing toxin alone, there was a significant reduction in TNF-α induced by TSST-1 with concentrations of IL-10 between 800-100 ng ml^{-1}. In response to SEC, IL-10 significantly reduced induction of TNF-α at concentrations between 800-100 ng ml^{-1}. At 50 ng ml^{-1} of IL-10, there was a significant enhancement of production TNF-α induced by both TSST-1 (P=0.005) and SEC (P=0.032). There was a significant reduction in TNF-α induced by SEA at a concentration of IL-10 between 800-50 ng ml^{-1}. In contrast to the results with TSST-1 and SEC, there was no significant enhancement with 50 ng ml^{-1} (Figure 7.1 and table 7.3).

Table 7.3. Effect of IL-10 (ng ml^{-1}) on TNF-α induced by TSST-1, SEC and SEA (n=5)

	TNF-α								
	TSST-1			SEC			SEA		
	mean	95% CI	*p* value	*mean*	95% CI	*p* value	*mean*	95% CI	*p* value
Cells	1.5			1.75			1.25		
Cell + Toxin	**21.25**	**6.6, 32.8**	**0.017**	**37**	**19, 51.4**	**0.006**	**28.25**	**41.2, 12.7**	**0.009**
C.T IL-10 800	**8**	**-23.4, -3.07,**	**0.026**	**19**	**-30.9, -5**	**0.022**	**17.75**	**-17.9, -3**	**0.02**
IL-10 400	**9**	**-21.8, -2.66**	**0.027**	**21.5**	**-30.9, -0.04,**	**0.05**	**18.5**	**-15.8, -3.6**	**0.015**
IL-10 200	**12.7**	**-13.9, -3.06**	**0.016**	**25**	**-22.7, -1.2,**	**0.038**	**19**	**-15.2, -3.2**	**0.016**
IL-10 100	**15.5**	**- 11.3, -0.18**	**0.046**	**29**	**-12.3, -2.1,**	**0.020**	**20.5**	**-13.8, -1.6**	**0.028**
IL-10 50	**27**	**3.3, 8.1**	**0.005**	**41**	**0.7, 8.7**	**0.032**	**22.75**	**-10.77, -0.22**	**0.045**
IL-10 25	**18.5**	**-5.13, -0.3**	**0.035**	**32.75**	**-5.7, -2.7,**	**0.003**	22.25	-0.75, 12.7	0.066
IL-10 12.5	19.75	-.091, 3.1	0.058	33	-2.3, 10.3	0.139	22.75	-1.1, 12.18	0.079
IL-10 6.25	20	-0.75, 3.2	0.141	35.75	-0.2, 2.7	0.08	24.25	-1.5, 9.5	0.10
IL-10 3	20.75	-1.09, 2.09	0.39	35.75	-0.27, 2.7	0.08	24.5	-0.4, 7.9	0.065
IL-10 1.5	20.75	-1.09, 2.09	0.39	35.75	-0.27, 2.7	0.08	25.2	-0.6, 6.6	0.081

Figure 7.1. The effect of IL-10 (ng ml^{-1}) on TNF-α induced by a) TSST, b) SEC and c) SEA



7.3.3 Effect of IL-10 levels on induction of IL-6 from buffy coats by TSST-1, SEC or SEA

IL-10 levels ranging from 800-1.5 ng ml^{-1} were tested for their effect on induction of IL-6 by the three toxins. Compared with the positive control containing toxin alone, there was a significant reduction in IL-6 induced by TSST-1, SEC and SEA at concentrations between 800-25 ng ml^{-1} (Figure 7.2 and table 7.4).

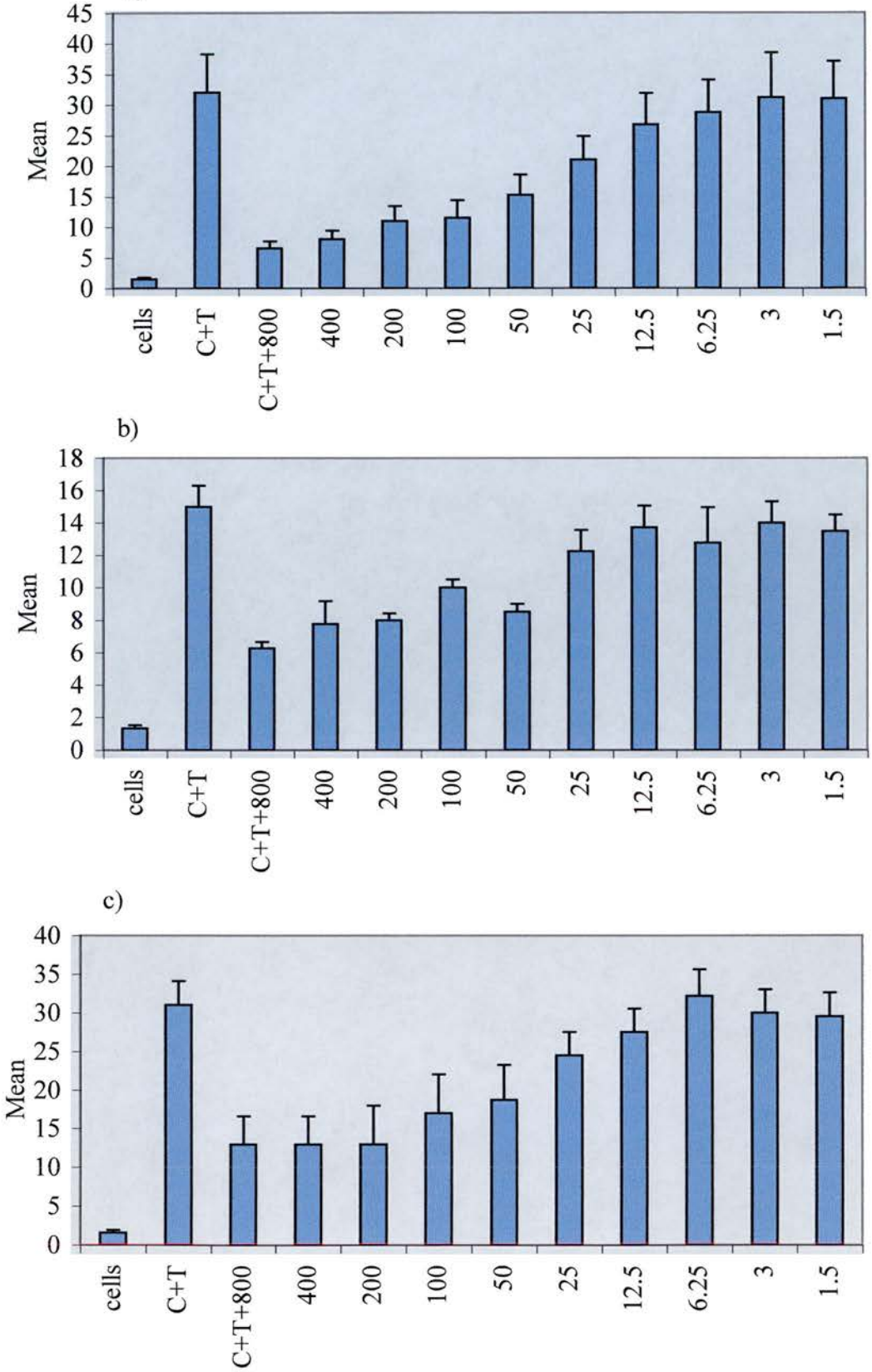
For 100 ng ml^{-1} TSST, 200 ng ml^{-1} of IL-10 reduced induction of TNF-α by approximately 50%. For 100 ng ml^{-1} SEC, 800 ng ml^{-1} were needed for the same effect. For 100 ng ml^{-1} SEA, none of the concentrations of IL-10 were able to reduce TNF-α by 50%.

For 100 ng ml^{-1} TSST, 50 ng ml^{-1} of IL-10 reduced induction of IL-6 by approximately 50%. For 100 ng ml^{-1} SEC, between 800 and 50 ng ml^{-1} were needed to reduce the level of IL-6 by approximately 50%. For 100 ng ml^{-1} SEA, between 200 and 100 ng ml^{-1} were needed for the same effect. (Figures 7.1-7.2).

Table 7.4. Effect of IL-10 (ng ml^{-1}) on IL-6 induced by TSST-1, SEC and SEA (n=5)

	IL-6								
	TSST-1			SEC			SEA		
	mean	95% CI	*p* value	*mean*	95% CI	*p* value	*mean*	95% CI	*p* value
Cells	1.5			1.38			1.6		
Cell + Toxin	**32**	**10.8, 50.1**	**0.016**	**15**	**9.9, 17.2**	**0.001**	**31**	**20.2, 38.5**	**0.002**
C.T IL-10 800	**6.5**	**- 42.6, - 8.3**	**0.018**	**6.25**	**-13.3, -4.17**	**0.009**	**13**	**-28.3, -7.6**	**0.012**
IL-10 400	**8**	**- 40, -7.9**	**0.018**	**7.75**	**-10.7, -3.72**	**0.007**	**13**	**-28.3, -7.6**	**0.012**
IL-10 200	**11**	**-35.29, -6.7**	**0.018**	**8**	**-11.6, -2.3**	**0.018**	**15**	**-28.5, -3.47**	**0.027**
IL-10 100	**11.5**	**-33.5, -7.4**	**0.015**	**10**	**-7.5, -2.4**	**0.009**	**17**	**-25.1, -2.82**	**0.028**
IL-10 50	**15.25**	**-29.3, -4.19**	**0.024**	**7.5**	**-12.9, -2.06**	**0.022**	**18.75**	**-22.2, -2.23**	**0.030**
IL-10 25	**21**	**-19-, 2.99**	**0.022**	**12.25**	**-5.1, -0.36**	**0.035**	**24.5**	**-11.09, -1.9**	**0.020**
IL-10 12.5	**26.7**	**-10.5, -0.007**	**0.050**	13.75	-3.2, 0.7	0.141	**27.5**	**-6.54, -0.45**	**0.035**
IL-10 6.25	28.75	-0.9, 7.4	0.09	11	-3.6, 11.6	0.19	32.25	-2.7, 0.27	0.080
IL-10 3	31.25	-2.7, 4.2	0.5	14	-0.29, 2.29	0.092	30	-0.29, 2.29	0.092
IL-10 1.5	31	-1.9, 3.9	0.3	13.5	-0.55, 3.5	0.1	29.5	-0.5, 3.5	0.1

Figure 7.2. The effect of IL-10 (ng ml^{-1}) on IL-6 induced by a) TSST, b) SEC and c) SEA



7.4 Discussion

The results obtained in this study indicate that IL-10 elicited from leukocytes in response to toxins and recombinant IL-10 exerted suppressive influences on the production of proinflammatory cytokines (TNF-α and IL-6). This confirms previous observations that IL-10 potently inhibits the production of Th1 cytokines (Fiorentino *et al.*, 1991; Seder & Paul, 1994). IL-10 production is controlled in part by genetic factors. In families in which there was a severe or fatal case of meningococcal disease, there was evidence of low TNF and/or high IL-10 production among the first-degree relatives of the principal case. The presence of both carried a very high risk of the fatal outcome (Westendorp *et al.*, 1997), among the 8 donors tested in response to TSST-1, 4 had levels of IL-10 below 20 ng ml^{-1} at 16 hr, and only 1 of the 4 had levels > 25 ng ml^{-1} at 24 hr. The levels for donor 8 remained unchanged from 16-24 hr.

With the model used in this study, the lowest concentration of IL-10 recombinant needed to reduce significantly IL-6 production at 16 h was 25 ng ml^{-1}. In response to TSST-1, at 16 h more than half of the donors produced IL-10 levels in or near the range needed (20-40 ng ml^{-1}) to reduce IL-6 and TNF-α production. Donor 6 had particularly low levels of IL-10 (8 ng ml^{-1}) induced by TSST-1 were not related to levels present in the controls without toxin. None of the donors treated produced levels of IL-10 needed in the assay system for reduction of TNF-α (minimum of 100 ng ml^{-1}) in response to TSST-1.

IL-10 was reported to protect mice against staphylococcal toxins mostly through its ability to inhibit TNF-α production by T cells (Bean *et al.*, 1993). It was also demonstrated that IL-10 protects mice from LPS induced lethal shock through its ability to prevent TNF-α production as well as that of other pro-inflammatory cytokines (Gerard *et al.*, 1993; Howard *et al.*, 1993). IL-10 has been the subject of various studies on control of inflammation *in vivo* (Opal *et al.*, 1998; Selzman *et al.*, 1998). IL-10 would be predicted to have a protective effect in humans in relation to inflammatory responses induced by staphylococcus toxins.

If poor IL-10 production is associated with adult respiratory distress syndrome (Chollet-Martin *et al.*, 1994), it might be of value to test the hypothesis that SUND victims had a genetically determined low level of IL-10 responses.

Chapter 8

General discussion

8.1 results of the study in relation to the original objectives

SUND is a significant problem in Saudi Arabia. The failure of an investigation which includes an autopsy to produce an adequate cause of death in a sudden and unexpected fatality is always viewed as a problem to be kept at an absolute minimum. In Saudi Arabia there is high immigrant population of over two million manual workers and about 60 deaths of the SUND category occur among these expatriate workers every year. This is a source of significant difficulty and some embarrassment to govermental agencies who have to deal with consular and embassy officials, and with the bereaved families. If at least some of these deaths could be explained in a more direct and concrete fashion, the task of these officials would be made easier and the families of the deceased would more readily accept a scientific explanation of the mode of death.

The general aim of the project was to develop methods to investigate the hypothesis that some of these deaths are triggered by staphylococcal toxins. A similar hypothesis has been proposed for SIDS and some of the methods were developed using material

from SIDS infants and infants who died of other causes. The basis of this hypothesis is that the common pathogen, *Staphylococcus aureus,* which frequently colonises the upper respiratory passages, under suitable conditions namely viral infection, an elevated local temperature and cigarette smoking may be induced to proliferate and to produce pyrogenic toxins in significant quantities which in turn act as superantigens and induce a generalised acute inflammatory and immunological response in the host. Unless these responses are naturally damped down or curtailed then these toxins may be present in sufficient quantities as to damage vital organs. During sleep there is a decreased level of corticosteroids in comparison with daytime levels. Under conditions of stress such as homesickness in workers in a foreign country with a long working week, the immunological responses may also be altered and decreased. The presence of unneutralised toxins in large enough quantities under such conditions may induce intrinsic biochemical internal changes before any pathological changes of any significance can be identified at autopsy or the histological examination of tissues taken at the time of the post-mortem examination.

The specific objectives were:

1. to develop an adequate method for tissue sampling that does not result in disfiguring of the body;
2. to develop a method for detecting toxins in tissue and body fluids;
3. to examine the effect of night time, day time and stress levels of cortisol on inflammatory responses to pyrogenic staphylococcal toxins;

4. to examine the levels of antibodies to pyrogenic toxins in relation to age and sex;
5. to examine the effect of different levels of antibodies to pyrogenic toxins on induction of inflammatory responses.

8.1.1 Collection of tissues samples

In Saudi Arabia autopsies are not performed routinely on bodies of persons dying suddenly and unexpectedly unless the circumstances indicate the death was non-accidental or non-natural. It is difficult to carry out autopsies in Saudi Arabia because of cultural objections and other legal implications. The first objective of this study was to develop a quick, non-invasive and reliable method to collect samples of internal organs without any obvious or identifiable disfiguring of the body. A needle biopsy technique produced a sufficient yield of tissue from brain, liver, and spleen, this is minimally invasive and much more acceptable method and allows the securing of sufficient tissue for histological examination and screening for toxins.

I will be able to use this limited collection of material when I return home as there should be no objection based on religious and cultural considerations to its use.

8.1.2 Detection of staphylococcal toxins in tissues

For this hypothesis to be plausible, it is essential that one is able to detect staphylococcal toxins in human tissues derived at autopsy in the appropriate cases and that these test are specific and sensitive.

Pyrogenic staphylococcal toxins cause life-threatening disease that is characterised by the rapid onset of high fever, shock, capillary leakage, and multi-organ dysfunction (Bohach *et al.*, 1990). These superantigens can cause sudden death in adults (Schlievert, 1993) and have been implicated in SIDS (Malam *et al.*, 1992; Newbould *et al.*, 1989; Murrell *et al.*, 1993).

ELISA and flow cytometry methods were developed to screen fresh and fixed tissues for TSST-1, SEA, SEB, and SEC. The methods can compensate for non-specific background binding noted in immunohistochemical method used by (Malam *et al.*, 1992; Newbould *et al.*, 1989). The flow cytometry method detected toxin in both frozen and fixed tissues, but ELISA was suitable only for frozen tissues or those fixed for less than 12 months. Assay for TSST-1 are the most specific as the cross reactivity detected with other toxins was less than 1% in the ELISA. Among a set of tissues from 30 SIDS infants and 19 infants who died of other causes the proportion of SIDS in whom TSST-1 was detected was 53% compared with 16% in the comparative group.

Owing to a lack of availability of tissues from SUND cases, these studies could not be replicated as has been done for SIDS cases.

8.1.3 Cortisol levels and the night time prevalence of SUND

It has been suggested that pyrogenic toxins with superantigenic properties might induce strong inflammatory responses leading to SUND. Inflammatory responses are less well controlled at night when most of these deaths occur. Leukocytes from human buffy coats were used to examine the effect of different concentrations of cortisol on the inflammatory mediators induced by TSST-1.

Night time levels of cortisol (5-10 $\mu g\ dl^{-1}$) significantly enhanced both TNF-α and IL-6 and significantly reduced IL-10 production in the model system at 16 hr following exposure to the toxin. Day time levels of cortisol (20 $\mu g\ dl^{-1}$) had an inhibitory effect on IL-6 production but not on TNF-α in response to the toxins. Stress levels of cortisol (40-80 $\mu g\ dl^{-1}$) significantly reduced the levels of all the three cytokines. These studies indicate that diurnal variation of cortisol may play an important role in the regulation of specific cytokine production and night time levels were not sufficient to control IL-6 and TNF-α in the model system used. In a similar model system, the same responses were observed for levels of cortisol ($\leq 5\ \mu g\ dl^{-1}$) present at night in infants who had developed their adult-type circadian rhythm (Gordon, 1999).

8.1.4 IgG levels specific to staphylococcal toxins in the general population in relation to age and sex of SUND victims

ELISA was used to assess naturally occurring levels of IgG to TSST-1, SEA and SEC. The majority of the population tested had sufficient antibodies to bind with and neutralise TNF-α and IL-6 responses elicited by TSST-1 and SEC in the model system. In the age range in which most of SUND deaths occur (20-39 years), males had significantly lower levels of IgG to TSST-1 compared with females. This might be related to tampon usage by women and their subclinical exposure to toxigenic staphylococci. The presence of these antibodies also indicates that normal individuals are naturally exposed to the staphylococcal toxins. The absence of antibodies to TSST-1 in serum of toxic shock syndrome patients (Bonventre *et al.*, 1984) and the presence of anti-TSST-1 antibodies in a large proportion of healthy subjects (Notermans *et al.*, 1983) indicates that absence of antibodies to the toxins is a major risk factor for susceptibility to TSST-1 (Notermans *et al.*, 1983; Christensson *et al.*, 1986).

If a similar pattern of anti-staphyococcal toxins can be found in populations at risk of SUND, the lower levels of IgG to the toxins observed in men might partly explain the much higher prevalence of these deaths among males.

8.1.5 Effect of antibody levels on induction of inflammatory responses.

The results obtained indicate there might be a small proportion of the population tested with antibody levels lower than those needed to neutralise the individual toxins. Studies by Essery *et al.*, (1999) indicate that immunisation against diphtheria, pertussis and tetanus (DPT) induces IgG antibodies that cross-react with staphylococcal pyrogenic toxins. The majority of Thai immigrants affected by SUND were in the age group that would not have been immunised against childhood diseases (Blackwell *et al.*, 1994).

It would therefore be important to follow up this aspect of the hypothesis by access to the medical records of these individuals and by looking at variation in antibody levels in relation to geographical area, ethnic group or changes in the working environment in specially designed epidemiological studies.

8.2 Results in relation to other postulated hypotheses suggested for SUND.

If staphylococcal toxins are the cause of some SUND cases, then males at night time would be more vulnerable especially if they had not been vaccinated in childhood or later and have not been exposed to staphylococcal antigens in the past or have intrinsically low production of such antitoxins for any other reasons.

Among the hypotheses suggested for SUND, the findings of this study could be relevant to cardiac abnormalities, stress and infections. Diet suggested as a possible cause in relation to the low thiamine content of food and the changes in potassium levels as a consequence of certain foods could not account for the predominance of males among the victims as both men and women usually shared the same diet. As hypokalaemia tends to be more prevalent in females than in males (Nilwarangkur *et al.*, 1990), it is difficult to attribute chronic potassium deficiency as a cause of SUND which mainly involves males.

8.2.1 Cardiac abnormalities

Ventricular fibrillation has been postulated as an ultimate cause of SUND deaths (Kirschner *et al.*, 1986). Prolonged QT intervals were observed within groups at high risk of SUND (Munger *et al.*, 1991). The long QT syndrome is characterised by a tendency to ventricular fibrillation and, sometimes, fatal syncopal attacks (Park & Guntheroth, 1978). Arrhythmia has been reported as a side-effect of treatment of patients with cancer with TNF-α, IL-2 and IFN-γ (Muc *et al.*, 1996; Eskander *et al.*, 1997). Negative ionotropic and arrhythmogenic effects were observed in cultured myocytes exposed to IL-1, IL-2, IL-3 or TNF-α (Weisensee *et al.*, 1993).

In the hypothesis postulated, high levels of cytokines could be induced overnight without any counterbalancing and neutralising responses by the body. In such

situations the elevated levels of cytokines such as TNF-α and IL-6 induced by infectious agents and / or superantigens, such as the staphylococcal toxins, might therefore induce arrhythmia in susceptible individuals.

8.2.2 Stress

Mental and emotional problems such as home sickness are thought to contribute to a significant proportion of SUND cases. Mental stress is known to induce cardiac arrhythmia and is associated with increased susceptibility to viral infections of the upper respiratory tract (Lown, 1982). Stress was associated in a dose-response manner with an increased risk of acute infectious respiratory illness, and this risk was attributable to increased rates of infection rather than to an increased frequency of symptoms after infection (Cohen *et al.*, 1991). Stress is thought to influence immune function through autonomic nerves innervating lymphoid tissue `(Felten & Olschowka, 1987) or hormone-mediated alteration of immune cells (Rabin *et al.*, 1989). Stimulation of the HPA axis and glucocorticoids by stress induces a negative feedback loop that exists between peripheral inflammatory cytokines and the HPA axis, in which cytokines promote hypothalamic corticotropin-releasing hormone (CRH) release and subsequent activation of the pituitary-adrenal axis (Sternberg *et al.*, 1992). Mental stress and microbial infection are both associated with a variety of endocrine changes including activation of the HPA system (Solomon, 1987).

Stress leads to increased cigarette smoking in smokers in which could result in increased risk of viral upper respiratory tract infection, both smoking and viral infection increase colonization with staphylococci (Ramirez-Ronda *et al.*, 1981). In addition, cells from the mouth of smokers bind more pathogenic bacteria, including *S. aureus* (Saadi *et al.*, 1996; El Ahmer *et al.*, 1999).

The consequences of all these changes, even in the presence of increased concentrations of corticosteroids, is to put an excessive toxin burden on susceptible individual which in some individuals may be sufficient as to cause death.

8.2.3 Infection

The results of this study indicate two areas that need to be considered in relation to the role of infection and SUND: 1) interactions between micro-organisms and factors such as cigarette smoke implicated in these deaths; 2) control of inflammatory responses elicited by the infectious agents.

8.2.3.1 Interactions between micro-organisms

In experimental models, synergy occurs between toxins of the same micro-organism or between toxins of two different micro-organisms, as in the case of an enhanced lethality of staphylococcal toxin in the presence of preparations from *E. coli* in the

chick embryo model (Drucker *et al.*, 1992). Stiles *et al* (1999) found that in mice there is a synergistic effect between staphylococcal enterotoxins or TSST-1 and lipopolysaccharide (LPS) measured by increased production of proinflammatory cytokines.

Virus infections also enhance susceptibility to bacterial toxins. Both natural virus infections and live influenza vaccines have been demonstrated to enhance colonisation by potentially pathogenic bacteria (Ramirez-Ronda *et al.*, 1981). Laboratory studies have demonstrated enhanced binding of staphylococci, *B. pertussis*, pneumococci and several Gram-negative species to HEp-2 cells infected with respiratory syncytial virus (RSV) (El Ahmer *et al.*, 1996; Saadi *et al.*, 1996), or influenza virus (El Ahmer *et al.*, 1999 a). Viral infections have also been demonstrated to enhance the lethality of bacterial toxins in animal models (Jakeman *et al.*, 1991) and can also enhance TNF-α production from human cells in response to bacterial toxins (Lundemose *et al.*, 1993). Studies by Sarawar *et al* (1994) on staphylococcal toxins and asymptomatic viral infection in mice indicated that the inflammatory response to viral infections could enhance the induction of TNF-α and nitric oxide.

Smokers are more frequently and heavily colonised by pathogenic bacteria (El Ahmer *et al.*, 1999). Nicotine potentiates the lethal action of synergistic combination of bacterial toxins in the chick embryo model (Sayers *et al.*, 1995) and a water soluble

cigarette smoke extract has been shown to enhance release of TNF-α from monocytes infected with RSV (Raza *et al.*, 1999).

Mild respiratory or gastrointestinal infections were often present in many SUND victims prior to death. Many of them had been taking either conventional or herbal remedies (Goh *et al.*, 1993). A single infectious agent alone is probably not responsible for SUND or SIDS. Synergistic effects between infectious agents or toxins and environmental factors are more likely to be involved in these sudden deaths.

8.2.3.2 Genetic control of inflammatory responses to infectious agents.

There is evidence that different individuals show a gentically-determined variation in their ability to produce certain cytokines, and this predisposition may result in an unusual and perhaps damaging reaction on exposure to certain toxins.

IL-10, IL-4, and IL-3 decrease or may even completely suppress the synthesis of pro-inflammatory cytokines (Bogdan & Nathan, 1993). Circulating anti-inflammatory cytokines exert profound inhibitory effects. IL-10 was reported to protect mice against staphylococcal enterotoxin B-induced lethal shock when administered before or concurrently with staphylococcal enterotoxin B (Bean *et al.*, 1993).

The results obtained in this study indicate that IL-10 exerted suppressive influences on the production of proinflammatory cytokines (TNF-α and IL-6). This confirms previous observations that IL-10 potently inhibits the production of Th1 cytokines (Fiorentino *et al.*, 1991; Seder & Paul, 1994).

IL-10 production is controlled in part by genetic factors. In families in which there was a severe or fatal case of meningococcal disease, there was evidence of low TNF and /or high IL-10 production among the first-degree relatives of the primary case. The presence of both carried a very high risk of the fatal outcome (Westendorp *et al.*, 1997).

Some of the donors in the study produced low levels of IL-10, and the increase in TNF-α or IL-6 compared with the levels in the absence of TSST-1 were higher for these individuals. The study found that the lowest concentration of recombinant IL-10 needed to reduce significantly IL-6 production at 16 h was 25 ng ml^{-1}. In the model used in this study more than half of the donors produced IL-10 levels in or near the range needed (20-40 ng ml^{-1}) to reduce IL-6 production. None of the donors treated produced levels of IL-10 needed in the assay system for reduction of TNF-α (minimum of 100 ng ml^{-1}) in response to TSST-1. However, donors with high levels of IL-10 showed generally lower levels of TNF-α or IL-6 compared with donors who produced low levels of IL-10.

These studies confirm an inter-individual variation in cytokine production and responses. This would need to be looked at further and systematically in families of SUND victims.

8.2.3.3 Circadian rhythm and control of inflammatory responses

Circadian variation in cytokine release is influenced by the synthesis and release of endogenous hormones such as melatonin and cortisol (Utiger, 1992) my own studies were confined to cortisol.

In adults, circadian variations in the concentration of cortisol have been noted with minimal secretion rates of the hormone occurring in the early hours of the morning. The adrenal gland is more responsive to a standard dose of ACTH during the day than after midnight (Van Cauter *et al.*, 1996) when many SUND deaths occur (Goh *et al.*, 1993). Cortisol and melatonin have been suggested to affect diurnal variations in the levels of IFN-γ and IL-10 observed *in vitro* studies in which blood samples taken from donors at different time of the day were challenged with bacterial endotoxin. IFN-γ was highest and IL-10 was lowest during the early morning hours and correlated negatively with plasma cortisol levels and positively with melatonin levels (Petrovsky *et al.*, 1998). Cytokine-induced glucocorticoid secretion and glucocorticoid inhibition of cytokine synthesis act as important safeguards in preventing cytokine overreaction (Costas *et al.*, 1996).

Most SUND cases occur at night when cortisol levels are lowest. The night time level observed in normal subjects (5-10 μg dl^{-1}) did not reduce production of pro-inflammatory cytokines in the model system. At several time points, low levels of cortisol significantly enhanced both TNF-α and IL-6 and significantly reduced IL-10 production in the model system. Inflammatory responses to endotoxin (IL-6 and TNF-α) are significantly affected by hormonal levels which are associated with circadian rhythm and exhibit increased production during the night when cortisol levels are lowest (Entzian *et al.*, 1996; Pollmacher *et al.*, 1996). Day time levels had an inhibitory effect on IL-6 production but not on TNF-α in response to the toxins. Stress levels of cortisol significantly reduced all three cytokines.

8.2.3.4 Control of inflammatory responses by antibodies to infectious agents

Antibodies to toxins can reduce the severity of toxic shock syndrome as demonstrated by protection of rabbits from a lethal toxaemia with TSST-1 with an exogenous adminsistration of pre-prepared antibodies (Bonventre *et al.*, 1988). Clinical infections with *S. aureus* result in higher concentrations of antibodies to the staphylococcal toxins (Jozefczyk, 1974). Normal pooled IgG contains antibodies against a major group of superantigens and staphylococcal toxins which play an immunoregulatory role *in vivo* (Takei *et al.*, 1993). A monoclonal antibody to TSST-1 reacted with TSST-1 in ELISA and blocked induction of interleukin 1 by TSST-1

(Beezhold *et al.*, 1987). In normal individuals, the prevalence of serum antibodies to staphylococcal toxins increases with age (Vergeront *et al.*, 1983; Notermans *et al.*, 1983). In contrast to controls of the same age group, antibodies directed against TSST-1 are usually absent or minimal in sera of patients with toxic shock syndrome (Bonventre *et al.*, 1984; Notermans *et al.*, 1983). The lack of specific IgG antibodies to TSST-1 in patients could be of pathogenic significance and help to explain the susceptibility to TSS in certain individuals (Christensson *et al.*, 1986).

Over 90% of *S. aureus* isolated from women with toxic shock syndrome associated with menstruation produce the toxin (Bonventre *et al.*, 1988). In recent years, it became clear that non-menstrual TSS may result from focal infections with *S. aureus* strains in which staphylococcal toxins other than TSST-1 precipitate the illness by similar or identical pathways (Garbe *et al.*, 1985; Schlievert, 1986; Schlievert *et al.*, 1995). There are some cases of TSS associated with viral respiratory infections such as influenza (MacDonald *et al.*, 1987; Newbould *et al.*, 1989; Schlievert *et al.*, 1995).

There have been no studies published to indicate whether SUND victims have normal, decreased or elevated levels of antitoxin antibodies. This study, which I hope to be able to carry out in my own country, may assist in linking more directly the *in vitro* phenomena with these deaths.

8.3 Activity of cytokines in relation to SUND.

Most SUND deaths are associated with sleep. Sleep is a common manifestation of infection, and several cytokines have been demonstrated to be somnogenic. For example, in experimental systems, IL-1 enhances slow-wave sleep (Krueger *et al.*, 1984). IL-1 and TNF-α have been shown to exhibit a high degree of temporal regulation in humans, and it is very likely that they are either regulated by sleep or are themselves important regulators of normal sleep (Krueger & Majde, 1995). Patients with sleep obstructive apnea syndrome experience disturbed sleep patterns; they have less sleep at night and spells of sleep during the day. Night time TNFα, IL-6, INF γ and IL-1 peaks observed in normal individuals are found during the day in these patients (Entzian *et al.*, 1996). Most SIDS deaths occur during sleep between midnight and 8.00 a.m. when many somnogenic cytokines (IL-1, IL-2, IL-6 and TNF-α) are at a peak. Human recombinant TNF-α and IL-1 were shown to cause prolonged slow wave sleep and to suppress the rapid eye movement stage of sleep (Shoham *et al.*, 1987; Krueger *et al.*, 1987; Krueger & Majde, 1995)

Circadian variations in the toxicity of TNF-α may be involved. It was reported that TNF-α induced lethality for mice varied up to 9-fold across the day, being lowest in the second half of the active period and highest at the end of the resting period (Hrushesky *et al.*, 1994). Diurnal variations on the effects of TNF-α and IL-6 on the rectal temperature and the hypothalamic-pituitary-adrenal (HPA) system may be related to the circadian rhythm in plasma cortisol levels (Fantuzzi *et al.*, 1993).

8.4 Future studies

8.4.1 Methods

The aim of the study was to develop methods to examine the possible role of pyrogenic toxins in SUND. Each year approximately 60-80 SUND are referred to the Forensic Medicine Department in the Eastern province of Saudi Arabia where I will have access to the bodies. The methods described will be applied to specimens from these victims, and I will try to set-up comparative studies with the Forensic Medicine Institute in Singapore.

8.4.2 Populations at risk

No studies on antibody levels to the staphylococcal toxins or differences in inflammatory responses of populations at high risk of SUND have been carried out.

8.4.2.1 Antibodies to the toxins in immigrant workers

In Saudi Arabia, blood is obtained from immigrant workers as part of the health screening procedures. Specimens obtained will be screened for levels of antibodies to the staphylococcal toxins and compared with levels of antibodies to these toxins in serum from local populations.

8.4.2.2 Genetic control of cytokine responses

Leukocytes extracted from blood samples from the immigrant workers will be screened for induction of proinflammatory and anti-inflammatory cytokines in response to TSST-1 and the results compared with those obtained with samples from the local Saudi population.

If blood samples were obtain soon after death, studies similar to those in chapter 5 could be carried out to examine proinflammatory and anti-inflammatory responses to toxins in SUND victims. Samples from victims of accidental deaths matched for age and sex from the same ethnic group would be appropriate controls. If these samples proved difficult to obtain, studies of inflammatory responses of first-degree relatives of SUND victims, and in families in which there were no cases of SUND, would be an alternative approach. This was the method used by Westendorp et al., (1997) to evaluate differences in responses to endotoxin in relation to fatal meningococcal diseases; however, access to families of SUND victims would require the cooperation of local authorities in countries from which the immigrant workers come. These studies would be the basis to test the hypotheses that SUND victims have high responses of pro-inflammatory cytokines (TNF-α and IL-6) and low levels of anti-inflammatory cytokines (IL-10) in response to TSST-1 or other staphylococcal toxins.

8.5 Conclusion

The work carried out in this study did not directly answer the questions, "Are the pyrogenic staphylococcal toxins involved in triggering the events leading to SUND?" It has however given fresh insight to the association between the production of toxins by *Staphylococci* and the variation that occurs naturally due to circadian changes in steroid levels. It has also demonstrated a potential inter-individual variability in the amounts and varieties of cytokines produced in relation to exposure to toxins. This could at least in part be genetically determined and may be ethnically related. It has, also led to development of methods that can be applied to future studies of SUND victims in Saudi Arabia within the constraints of the local legal, cultural and religious practices.

References

ADLER, S.R. (1995). Refugee stress and folk belief: Hmong sudden deaths. *Social Science & Medicine* **40**, 1623-1629.

ANDERSON, R.E., HILL, R.B., BROUDY, D.W., KEY, C.R. & PATHAK, D. (1994). A population-based autopsy study of sudden, unexpected deaths from natural causes among persons 5 to 39 years old during a 12-year period. *Human Pathology.* **25**, 1332-1340.

ANIANSSON, G., ALM, B., ANDERSSON, B., LARSSON, P., NYLEN, O., PETERSON, H., RIGNER, P., SVANBORG, M. & SVANBORG, C. (1992). Nasopharyngeal colonization during the first year of life. *Journal of Infectious Diseases.* **165**, Suppl-42

APONTE, G. (1960). The enigma of 'Bangungut'. *Annals of Internal Medicine* **52**, 1258-1260.

AVRAHAMI, R., WATEMBERG, S., DANIELS-PHILIPS, E., KAHANA, T. & HISS, J. (1995). Endoscopic autopsy. *American Journal of Forensic Medicine and Pathology.* **16**, 147-150.

BARON, R.C., THACKER, S.B., GORELKIN, L., VERNON, A.A., TAYLOR, W.R. & CHOI, K. (1983). Sudden death among Southeast Asian refugees. An unexplained nocturnal phenomenon. *Journal of American Medical Association.* **250**, 2947-2951.

BEAN, A.G., FREIBERG, R.A., ANDRADE, S., MENON, S. & ZLOTNIK, A. (1993). Interleukin 10 protects mice against staphylococcal enterotoxin B-induced lethal shock. *Infection & Immunity.* **61**, 4937-4939.

BEEZHOLD, D.H., BEST, G.K., BONVENTRE, P.F. & THOMPSON, M. (1987). Synergistic induction of interleukin-1 by endotoxin and toxic shock syndrome toxin-1 using rat macrophages. *Infection & Immunity.* **55**, 2865-2869.

BENTLEY, A.J., ZORGANI, A.A., BLACKWELL, C.C., WEIR, D.M. & BUSUTTIL, A. (1997). Bacterial toxins and sudden unexpected death in a young child. *Forensic Science International.* **88**, 141-146.

BERTINI, R., BIANCHI, M. & GHEZZI, P. (1988). Adrenalectomy sensitizes mice to the lethal effects of interleukin 1 and tumor necrosis factor. *Journal of Experimental Medicine* **167**, 1708-1712.

BETTELHEIM, K.A., CHANG, B.J., ELLIOTT, S.J., GUNZBURG, S.T. & PEARCE, J.L. (1995). Virulence factors associated with strains of Escherichia coli from cases of sudden infant death syndrome (SIDS). *Comparative Immunology, Microbiology & Infectious Diseases* **18**, 179-188.

BETTELHEIM, K.A., GOLDWATER, P.N., DWYER, B.W., BOURNE, A.J. & SMITH, D.L. (1990). Toxigenic Escherichia coli associated with sudden infant death syndrome. *Scandinavian Journal of Infectious Diseases.* **22**, 467-476.

BLACKWELL, C.C., BUSUTTIL, A., WEIR, D.M., SAADI, A.T. & ESSERY, S.D. (1994). Sudden unexpected nocturnal deaths among Thai immigrant workers in Singapore. The possible role of toxigenic bacteria. *International Journal of Legal Medicine* **106**, 205-208.

BLACKWELL, C.C., MACKENZIE, D.A., JAMES, V.S., ELTON, R.A., ZORGANI, A.A., WEIR, D.M. & BUSUTTIL, A. (1999). Toxigenic bacteria and Sudden Death Syndrome (SIDS): nasopharyngeal flora during the first year of life. *FEMS Immunology & Medical Microbiology (in press).*

BLACKWELL, C.C., TZANAKAKI, G., KREMASTINOU, J., WEIR, D.M., VAKALIS, N., ELTON, R.A., MENTIS, A. & FATOUROS, N. (1992). Factors affecting carriage of Neisseria meningitidis among Greek military recruits. *Epidemiology & Infection* **108**, 441-448.

BLACKWELL, C.C., WEIR, D.M. & BUSUTTIL, A. (1995). Infectious agents, the inflammatory responses of infants and sudden infants death syndrome (SIDS). *Molecular Medicine Today* **1**, 72-78.

BLACKWELL, C.C., WEIR, D.M., JAMES, V.S., TODD, W.T., BANATVALA, N., CHAUDHURI, A.K., GRAY, H.G., THOMSON, E.J. & FALLON, R.J. (1990). Secretor status, smoking and carriage of *Neisseria meningitidis*. *Epidemiology & Infection* **104**, 203-209.

BOGDAN, C. & NATHAN, C. (1993). Modulation of macrophage function by transforming growth factor beta, interleukin-4, and interleukin-10. *Annals of the New York Academy of Sciences.* **685**, 713-739.

BOHACH, G.A., FAST, D.J., NELSON, R.D. & SCHLIEVERT, P.M. (1990). Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. *Critical Reviews in Microbiology.* **17**, 251-272.

BONE, R.C. (1994). Gram-positive organisms and sepsis. *Archives of Internal Medicine.* **154**, 26-34.

BONVENTRE, P.F., LINNEMANN, C., WECKBACH, L.S., STANECK, J.L., BUNCHER, C.R., VIGDORTH, E., RITZ, H., ARCHER, D. & SMITH, B. (1984). Antibody responses to toxic-shock-syndrome (TSS) toxin by patients with TSS and by healthy staphylococcal carriers. *Journal of Infectious Diseases.* **150**, 662-666.

BONVENTRE, P.F., THOMPSON, M.R., ADINOLFI, L.E., GILLIS, Z.A. & PARSONNET, J. (1988). Neutralization of toxic shock syndrome toxin-1 by monoclonal antibodies *in vitro* and *in vivo*. *Infection & Immunity.* **56**, 135-141.

BRANDTZAEG, P., OSNES, L., OVSTEBO, R., JOO, G.B., WESTVIK, A.B. & KIERULF, P. (1996). Net inflammatory capacity of human septic shock plasma evaluated by a monocyte-based target cell assay: identification of interleukin-10 as a major functional deactivator of human monocytes. *Journal of Experimental Medicine.* **184**, 51-60.

BROUCKAERT, P., EVERAERDT, B. & FIERS, W. (1992). The glucocorticoid antagonist RU38486 mimics interleukin-1 in its sensitization to the lethal and interleukin-6-inducing properties of tumor necrosis factor. *European Journal of Immunology.* **22**, 981-986.

BROWNE, K.F., PRYSTOWSKY, E., HEGER, J.J., CHILSON, D.A. & ZIPES, D.P. (1983). Prolongation of the Q-T interval in man during sleep. *American Journal of Cardiology* **52**, 55-59.

CHESNEY, P.J., DAVIS, J.P., PURDY, W.K., WAND, P.J. & CHESNEY, R.W. (1981). Clinical manifestations of toxic shock syndrome. *Journal of American Medical Association* **246**, 741-748.

CHOLLET-MARTIN, S., ROUSSET, F., CHASTRE, J., GIBERT, C., BANCHEREAU, J. & GOUGEROT-POCIDALO, M.A. (1994). Cytokines in adult respiratory distress syndrome. *Lancet* **344**, 1440

CHRISTENSSON, B., JOHANSSON, P.J. & OXELIUS, V.A. (1986). Imbalanced serum IgG subclass pattern in toxic shock syndrome patients: deficiency of specific IgG1 and IgG4 subclass antibodies to toxic shock syndrome toxin 1. *Clinical & Experimental Immunology* **66**, 443-449.

COHEN, S., TYRRELL, D.A. & SMITH, A.P. (1991). Psychological stress and susceptibility to the common cold. *New England Journal of Medicine* **325**, 606-612.

COSTAS, M., TRAPP, T., PEREDA, M.P., SAUER, J., RUPPRECHT, R., NAHMOD, V.E., REUL, J.M., HOLSBOER, F. & ARZT, E. (1996). Molecular and functional evidence for in vitro cytokine enhancement of human and murine target cell sensitivity to glucocorticoids. TNF-alpha priming increases glucocorticoid inhibition of TNF-alpha-induced cytotoxicity/apoptosis. *Journal of Clinical Investigation.* **98**, 1409-1416.

DAVIS, G.J. & PETERSON, B.R. (1996). Dilemmas and solutions for the pathologist and clinician encountering religious views of the autopsy. *Southern Medical Journal.* **89**, 1041-1044.

DE AZAVEDO, J.C., LUCKEN, R.N. & ARBUTHNOTT, J.P. (1985). Effect of toxic shock syndrome toxin 1 on chicken embryos. *Infection & Immunity.* **47**, 710-712.

DE WAAL, M., ABRAMS, J., BENNETT, B., FIGDOR, C.G. & DE VRIES, J.E. (1991). Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *Journal of Experimental Medicine* **174**, 1209-1220.

DE WAAL, M., YSSEL, H., RONCAROLO, M.G., SPITS, H. & DE VRIES, J.E. (1992). Interleukin-10. *Current Opinion in Immunology* **4**, 314-320.

DEFRANCE, T., VANBERVLIET, B., BRIERE, F., DURAND, I., ROUSSET, F. & BANCHEREAU, J. (1992). Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *Journal of Experimental Medicine* **175**, 671-682.

DELAHOOKE, D.M., BARCLAY, G.R. & POXTON, I.R. (1995). Tumor necrosis factor induction by an aqueous phenol-extracted lipopolysaccharide complex from Bacteroides species. *Infection & Immunity.* **63**, 840-846.

DERIJK, R., MICHELSON, D., KARP, B., PETRIDES, J., GALLIVEN, E., DEUSTER, P., PACIOTTI, G., GOLD, P.W. & STERNBERG, E.M. (1997). Exercise and circadian rhythm-induced variations in plasma cortisol differentially regulate interleukin-1 beta (IL-1 beta), IL-6, and tumor necrosis factor-alpha (TNF alpha) production in humans: high sensitivity of TNF alpha and resistance of IL-6. *Journal of Clinical Endocrinology & Metabolism* **82**, 2182-2191.

DINARELLO, C.A. (1996). Biologic basis for interleukin-1 in disease. *Blood* **87**, 2095-2147.

DINARELLO, C.A., CANNON, J.G., WOLFF, S.M., BERNHEIM, H.A., BEUTLER, B., CERAMI, A., FIGARI, I.S., PALLADINO, M.A.J. & O'CONNOR, J.V. (1986). Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. *Journal of Experimental Medicine* **163**, 1433-1450.

DRUCKER, D.B., ALUYI, H.A., MORRIS, J.A., TELFORD, D.R., OPPENHEIM, B.A. & CRAWLEY, B.A. (1992). Possibility of separating toxins from bacteria associated with sudden infant death syndrome using anion exchange chromatography. *Journal of Clinical Pathology.* **45**, 802-805.

EL AHMER, O.R., ESSERY, S.D., SAADI, A.T., RAZA, M.W., OGILVIE, M.M., WEIR, D.M. & BLACKWELL, C.C. (1999). The effect of cigarette smoke on adherence of respiratory pathogens to buccal epithelial cells. *FEMS Immunology & Medical Microbiology.* **23**, 27-36.

EL AHMER, O.R., RAZA, M.W., OGILVIE, M.M., BLACKWELL, C.C., WEIR, D.M. & ELTON, R.A. (1996). The effect of respiratory virus infection on expression of cell surface antigens associated with binding of potentially pathogenic bacteria. *Advances in Experimental Medicine & Biology.* **408**, 169-177.

EL AHMER, O.R., RAZA, M.W., OGILVIE, M.M., ELTON, R.A., WEIR, D.M. & BLACKWELL, C.C. (1999). Binding of bacteria to HEp-2 cells infected with influenza A virus. *FEMS Immunology & Medical Microbiology (in press).*

ENTZIAN, P., LINNEMANN, K., SCHLAAK, M. & ZABEL, P. (1996). Obstructive sleep apnea syndrome and circadian rhythms of hormones and cytokines. *American Journal of Respiratory & Critical Care Medicine* **153**, 1080-1086.

ESKANDER, E.D., HARVEY, H.A., GIVANT, E. & LIPTON, A. (1997). Phase I study combining tumor necrosis factor with interferon-alpha and interleukin-2. *American Journal of Clinical Oncology* **20**, 511-514.

ESSERY, S.D., RAZA, M.W., SAADI, A.T., WEIR, D.M., BUSUTTIL, A. & BLACKWELL, C.C. (1999). The protective effect of immunisation in relation to Sudden Infant Death Syndrome. *FEMS Immunology & Medical Microbiology.(in press)*

FANTUZZI, G., CANTONI, L., SIRONI, M. & GHEZZI, P. (1993). Inhibitors of cytochrome P450 suppress tumor necrosis factor production. *Cellular Immunology* **150**, 417-424.

FARIS, E.A. & FATHALLA, E. (1992). Bangungut syndrome sudden unexpected nocturnal death among Philippino and Thai construction workers in the eastern province. Saudi Arabia. *Saudi Medical Journal.* **13**, 531-533.

FELTEN, S.Y. & OLSCHOWKA, J. (1987). Noradrenergic sympathetic innervation of the spleen: II. Tyrosine hydroxylase (TH)-positive nerve terminals form synapticlike contacts on lymphocytes in the splenic white pulp. *Journal of Neuroscience Research.* **18**, 37-48.

FINLAY, W.E. & MCKEE, J.I. (1982). Serum cortisol levels in severely stressed patients. *Lancet* **1**, 1414-1415.

FIORENTINO, D.F., ZLOTNIK, A., VIEIRA, P., MOSMANN, T.R., HOWARD, M., MOORE, K.W. & O'GARRA, A. (1991). IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *Journal of Immunology* **146**, 3444-3451.

FLAD, H.D., LOPPNOW, H., FEIST, W., WANG, M.H., BRADE, H., KUSUMOTO, S., RIETSCHEL, E.T. & ULMER, A.J. (1989). Interleukin 1 and tumor necrosis factor: studies on the induction by lipopolysaccharide partial structures. *Lymphokine Research.* **8**, 235-238.

FOROUDI, F., CHEUNG, K. & DUFLOU, J. (1995). A comparison of the needle biopsy post mortem with the conventional autopsy. *Pathology.* **27**, 79-82.

FRIMAN, V., HANSON, L.A., BRIDON, J.M., TARKOWSKI, A., BANCHEREAU, J. & BRIERE, F. (1996). IL-10-driven immunoglobulin production by B lymphocytes from IgA-deficient individuals correlates to infection proneness. *Clinical & Experimental Immunology* **104**, 432-438.

FUKATSU, A., SATO, N. & SHIMIZU, H. (1996). 50-mile walking race suppresses neutrophil bactericidal function by inducing increases in cortisol and ketone bodies. *Life Sciences.* **58**, 2337-2343.

GARBE, P.L., ARKO, R.J., REINGOLD, A.L., GRAVES, L.M., HAYES, P.S., HIGHTOWER, A.W., CHANDLER, F.W. & BROOME, C.V. (1985). Staphylococcus aureus isolates from patients with non-menstrual toxic shock syndrome. Evidence for additional toxins. *Journal of American Medical Association* **253**, 2538-2542.

GERARD, C., BRUYNS, C., MARCHANT, A., ABRAMOWICZ, D., VANDENABEELE, P., DELVAUX, A., FIERS, W., GOLDMAN, M. & VELU, T. (1993). Interleukin 10 reduces the release of tumour necrosis factor and prevents lethality in experimental endotoxemia. *Journal of Experimental Medicine* **177**, 547-550.

GLAUSER, M.P., HEUMANN, D., BAUMGARTNER, J.D. & COHEN, J. (1994). Pathogenesis and potential strategies for prevention and treatment of septic shock: an update. *Clinical Infectious Diseases.* **18**, Suppl-16

GOH, K.T., CHAO, T.C. & CHEW, C.H. (1990). Sudden nocturnal deaths among Thai construction workers in Singapore. *Lancet* **335**, 1154

GOH, K.T., CHAO, T.C., HENG, B.H., KOO, C.C. & POH, S.C. (1993). Epidemiology of sudden unexpected death syndrome among Thai migrant workers in Singapore. *International Journal of Epidemiology.* **22**, 88-95.

GORDON, A.E. (1999) The protective effective of breast feeding in relation to sudden infant death syndrome. *PhD Thesis, University of Edinburgh,* 1999.

GUNTHEROTH, W.G. (1989). Interleukin-1 as intermediary causing prolonged sleep apnea and SIDS during respiratory infections. *Medical Hypotheses.* **28**, 121-123.

GUYTON, A.C. & HALL, J. (1996). The Adrenocortical Hormones. In *Textbook of Medical Physiology*. 963-967. New York: W. B Saunders Company.

HARRIS, M.D. & BLUNDELL, J.W. (1991). Audit of necropsies in a British district general hospital. *Journal of Clinical Pathology.* **44**, 862-865.

HARRISON, L.M., MORRIS, J.A., TELFORD, D.R. & BROWN, S.M. (1999). The nasopharyngeal bacterial flora in infancy: effects of age, gender, season, viral upper respiratory tract infection and sleeping position. *FEMS Immunology & Medical Microbiology in (press).*

HELFANT, R.H. (1986). Hypokalemia and arrhythmias. *American Journal of Medicine* **80**, 13-22.

HEUMANN, D., GLAUSER, M.P. & CALANDRA (1998). Monocyte deactivation in septic shock. *Current Opinion in Infectious Diseases* **11**, 279-283.

HOWARD, M., MUCHAMUEL, T., ANDRADE, S. & MENON, S. (1993). Interleukin 10 protects mice from lethal endotoxemia. *Journal of Experimental Medicine* **177**, 1205-1208.

HRUSHESKY, W.J., LANGEVIN, T., KIM, Y.J. & WOOD, P.A. (1994). Circadian dynamics of tumor necrosis factor alpha (cachectin) lethality. *Journal of Experimental Medicine* **180**, 1059-1065.

HUSTON, B.M., MALOUF, N.N. & AZAR, H.A. (1996). Percutaneous needle autopsy sampling *Modern Pathology.* **9**, 1101-1107.

ITOH, K. & HIROHATA, S. (1995). The role of IL-10 in human B cell activation, proliferation, and differentiation. *Journal of Immunology* **154**, 4341-4350.

ITOH, K., INOUE, T., ITO, K. & HIROHATA, S. (1994). The interplay of interleukin-10 (IL-10) and interleukin-2 (IL-2) in humoral immune responses: IL-10 synergizes with IL-2 to enhance responses of human B lymphocytes in a mechanism which is different from upregulation of CD25 expression. *Cellular Immunology* **157**, 478-488.

JAKEMAN, K.J., RUSHTON, D.I., SMITH, H. & SWEET, C. (1991). Exacerbation of bacterial toxicity to infant ferrets by influenza virus: possible role in sudden infant death syndrome. *Journal of Infectious Diseases.* **163**, 35-40.

JOZEFCZYK, Z. (1974). Specific human antibodies to enterotoxins A, B, and C1 of Staphylococcus: their increased synthesis in staphylococcal infection. *Journal of Infectious Diseases* **130**, 1-7.

KANAI, K. & DEJSIRILERT, S. (1988). Pseudomonas pseudomallei and melioidosis, with special reference to the status in Thailand. *Japanese Journal of Medical Science & Biology.* **41**, 123-157.

KELLER S.E., SCHLEIFER S.J. & DEMETRIKOPOULOS M.K. (1991). Stress-induced changes in immune function in animals: hypothalamo-pituitary-adrenal influences. In *Psychoneuroimmunology*, eds. ADER R., FELTEN D.L. & COHEN N., pp. 771-787. New York: Academic Press, Inc.

KIECOLT-GLASER, J.K. & GLASER, R. (1988). Methodological issues in behavioral immunology research with humans. *Brain, Behavior & Immunity.* **2**, 67-78.

KIRSCHNER, R.H., ECKNER, F.A. & BARON, R.C. (1986). The cardiac pathology of sudden, unexplained nocturnal death in Southeast Asian refugees. *Journal of American Medical Association* **256**, 2700-2705.

KRUEGER, J.M., DINARELLO, C.A., SHOHAM, S., DAVENNE, D., WALTER, J. & KUBILLUS, S. (1987). Interferon alpha-2 enhances slow-wave sleep in rabbits. *International Journal of Immunopharmacology* **9**, 23-30.

KRUEGER, J.M. & MAJDE, J.A. (1995). Cytokines and sleep. *International Archives of Allergy & Immunology* **106**, 97-100.

KRUEGER, J.M., WALTER, J., DINARELLO, C.A., WOLFF, S.M. & CHEDID, L. (1984). Sleep-promoting effects of endogenous pyrogen (interleukin-1). *American Journal of Physiology.* **246**, t-9

LAUE, L., KAWAI, S., BRANDON, D.D., BRIGHTWELL, D., BARNES, K., KNAZEK, R.A., LORIAUX, D.L. & CHROUSOS, G.P. (1988). Receptor-mediated effects of glucocorticoids on inflammation: enhancement of the inflammatory response with a glucocorticoid antagonist. *Journal of Steroid Biochemistry* **29**, 591-598.

LIAO, J., KEISER, J.A., SCALES, W.E., KUNKEL, S.L. & KLUGER, M.J. (1995). Role of corticosterone in TNF and IL-6 production in isolated perfused rat liver. *American Journal of Physiology.* **268**, t-706

LINDGREN, C., MILERAD, J. & LAGERCRANTZ, H. (1997). Sudden infant death and prevalence of whooping cough in the Swedish and Norwegian communities. *European Journal of Pediatrics* **156**, 405-409.

LINDSAY, J.A., JOHNSON, H.M., WALLACE, F.M. & SOOS, J.M. (1994). Can superantigens trigger sudden infant death?. *Medical Hypotheses.* **43**, 81-85.

LIPSKY, M.S. (1993). Autopsy: role of the family physician. *American Family Physician.* **47**, 1605-1610.

LOWN, B. (1982). Mental stress, arrhythmias and sudden death. *American Journal of Medicine* **72**, 177-180.

LUNDEMOSE, J.B., SMITH, H. & SWEET, C. (1993). Cytokine release from human peripheral blood leucocytes incubated with endotoxin with and without prior infection with influenza virus: relevance to the sudden infant death syndrome. *International Journal of Experimental Pathology.* **74**, 291-297.

MACDONALD, K.L., OSTERHOLM, M.T., HEDBERG, C.W., SCHROCK, C.G., PETERSON, G.F., JENTZEN, J.M., LEONARD, S.A. & SCHLIEVERT, P.M. (1987). Toxic shock syndrome. A newly recognized complication of influenza and influenzalike illness. *Journal of American Medical Association* **257**, 1053-1058.

MACKENSEN, A., GALANOS, C. & ENGELHARDT, R. (1991). Treatment of cancer patients with endotoxin induces release of endogenous cytokines. *Pathobiology* **59**, 264-267.

MAESTRONI, G.J., CONTI, A. & PIERPAOLI, W. (1986). Role of the pineal gland in immunity. Circadian synthesis and release of melatonin modulates the antibody response and antagonizes the immunosuppressive effect of corticosterone. *Journal of Neuroimmunology.* **13**, 19-30.

MALAM, J.E., CARRICK, G.F., TELFORD, D.R. & MORRIS, J.A. (1992). Staphylococcal toxins and sudden infant death syndrome. *Journal of Clinical Pathology.* **45**, 716-721.

MARRACK, P. & KAPPLER, J. (1990). The staphylococcal enterotoxins and their relatives. *Science* **248**, 705-711.

MARTICH, G.D., PARKER, M.M., CUNNION, R.E. & SUFFREDINI, A.F. (1992). Effects of ibuprofen and pentoxifylline on the cardiovascular response of normal humans to endotoxin. *Journal of Applied Physiology.* **73**, 925-931.

MCMANUS, B.M. & WOOD, S.M. (1996). The autopsy. Simple thoughts about the public needs and how to address them. *American Journal of Clinical Pathology.* **106**, Suppl-4

MITTLEMAN, R.E., DAVIS, J.H., KASZTL, W. & GRAVES, W.M.J. (1992). Practical approach to investigative ethics and religious objections to the autopsy. *Journal of Forensic Sciences.* **37**, 824-829.

MOREL, P.A. & ORISS, T.B. (1998). Crossregulation Between Th1 and Th2 Cells. *Critical Reviews in Immunology* **18**, 275-303.

MORISSETTE, C., GOULET, J. & LAMOUREUX, G. (1991). Rapid and sensitive sandwich enzyme-linked immunosorbent assay for detection of staphylococcal enterotoxin B in cheese. *Applied & Environmental Microbiology* **57**, 836-842.

MORRIS, J.A. (1999). The common bacterial toxins hypothesis of sudden infant death syndrome. *FEMS Immunology & Medical Microbiology.(in press)*

MORRIS, J.A., HARAN, D. & SMITH, A. (1987). Hypothesis: common bacterial toxins are a possible cause of the sudden infant death syndrome. *Medical Hypotheses.* **22**, 211-222.

MUC, M., BARANOWSKI, M., BRACZKOWSKI, R., ZUBELEWICZ, B. & KOZOWICZ, A. (1996). Cardiotoxic effect of the TNF alpha preparation given intravenously to patients with advanced neoplasms. *Przeglad Lekarski.* **53**, 78-82.

MUNCK, A., GUYRE, P.M. & HOLBROOK, N.J. (1984). Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine Reviews.* **5**, 25-44.

MUNGER, R.G. (1987). Sudden death in sleep of Laotian-Hmong refugees in Thailand: a case-control study. *American Journal of Public Health* **77**, 1187-1190.

MUNGER, R.G., PRINEAS, R.J., CROW, R.S., CHANGBUMRUNG, S., KEANE, V., WANGSUPHACHART, V. & JONES, M.P. (1991). Prolonged QT interval and risk of sudden death in South-East Asian men. *Lancet* **338**, 280-281.

MURRELL, W.G., STEWART, B.J., O'NEILL, C., SIARAKAS, S. & KARIKS, S. (1993). Enterotoxigenic bacteria in the sudden infant death syndrome. *Journal of Medical Microbiology.* **39**, 114-127.

NEWBOULD, M.J., MALAM, J., MCILLMURRAY, J.M., MORRIS, J.A., TELFORD, D.R. & BARSON, A.J. (1989). Immunohistological localisation of staphylococcal toxic shock syndrome toxin (TSST-1) antigen in sudden infant death syndrome. *Journal of Clinical Pathology.* **42**, 935-939.

NICOLL, A. & GARDNER, A. (1988). Whooping cough and unrecognised postperinatal mortality. *Archives of Disease in Childhood.* **63**, 41-47.

NILWARANGKUR, S., NIMMANNIT, S., CHAOVAKUL, V., SUSAENGRAT, W., ONG, a.Y., VASUVATTAKUL, S., PIDETCHA, P. & MALASIT, P. (1990). Endemic primary distal renal tubular acidosis in Thailand. *Quarterly Journal of Medicine* **74**, 289-301.

NIMMANNIT, S., MALASIT, P., CHAOVAKUL, V., SUSAENGRAT, W., VASUVATTAKUL, S. & NILWARANGKUR, S. (1991). Pathogenesis of sudden unexplained nocturnal death (lai tai) and endemic distal renal tubular acidosis. *Lancet* **338**, 930-932.

NOTERMANS, S., VAN LEEUWEN, W.J., DUFRENNE, J. & TIPS, P.D. (1983). Serum antibodies to enterotoxins produced by Staphylococcus aureus with special reference to enterotoxin F and toxic shock syndrome. *Journal of Clinical Microbiology.* **18**, 1055-1060.

OHLSSON, K., BJORK, P., BERGENFELDT, M., HAGEMAN, R. & THOMPSON, R.C. (1990). Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. *Nature* **348**, 550-552.

OKADA, R. & KAWAI, S. (1983). Histopathology of the conduction system in sudden cardiac death. *Japanese Circulation Journal.* **47**, 573-580.

OPAL, S.M., WHERRY, J.C. & GRINT, P. (1998). Interleukin-10: potential benefits and possible risks in clinical infectious diseases. *Clinical Infectious Disease.* **27**, 1497-1507.

OPPENHEIM, B.A., BARCLAY, G.R., MORRIS, J., KNOX, F., BARSON, A., DRUCKER, D.B., CRAWLEY, B.A. & MORRIS, J.A. (1994). Antibodies to endotoxin core in sudden infant death syndrome. *Archives of Disease in Childhood.* **70**, 95-98.

PARK, M.K. & GUNTHEROTH, W.G. (1978). Long Q-T syndrome: a preventable form of sudden death. *Journal of Family Practice.* **7**, 945-948.

PERROT, D., BONNETON, A., DECHAUD, H., MOTIN, J. & PUGEAT, M. (1993). Hypercortisolism in septic shock is not suppressible by dexamethasone infusion. *Critical Care Medicine* **21**, 396-401.

PETROVSKY, N., MCNAIR, P. & HARRISON, L.C. (1998). Diurnal rhythms of pro-inflammatory cytokines: regulation by plasma cortisol and therapeutic implications. *Cytokine.* **10**, 307-312.

POLLMACHER, T., MULLINGTON, J., KORTH, C., SCHREIBER, W., HERMANN, D., ORTH, A., GALANOS, C. & HOLSBOER, F. (1996). Diurnal variations in the human host response to endotoxin. *Journal of Infectious Diseases.* **174**, 1040-1045.

RABIN, B.S., COHEN, S., GANGULI, R., LYSLE, D.T. & CUNNICK, J.E. (1989). Bidirectional interaction between the central nervous system and the immune system. *Critical Reviews in Immunology* **9**, 279-312.

RAMIREZ-RONDA, C.H., FUXENCH-LOPEZ, Z. & NEVAREZ, M. (1981). Increased pharyngeal bacterial colonization during viral illness. *Archives of Internal Medicine* **141**, 1599-1603.

RAZA, M.W., EL AHMER, O.R., OGILVIE, M.M., BLACKWELL, C.C., SAADI, A.T., ELTON, R.A. & WEIR, D.M. (1999). Infection with respiratory syncytial virus enhances expression of native receptors for non-pilate Neisseria meningitidis on HEp-2 cells. *FEMS Immunology & Medical Microbiology.* **23**, 115-124.

READ, D.H. & HARRINGTON, D.D. (1981). Experimentally induced thiamine deficiency in beagle dogs: clinical observations. *American Journal Veterinary Research.* **42**, 984-991.

REICHLIN, S. (1993). Neuroendocrine-immune interactions. *New England Journal of Medicine* **329**, 1246-1253.

ROUSSET, F., GARCIA, E., DEFRANCE, T., PERONNE, C., VEZZIO, N., HSU, D.H., KASTELEIN, R., MOORE, K.W. & BANCHEREAU, J. (1992). Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America.* **89**, 1890-1893.

RUMBAUGH, K.P., HAMOOD, A.N. & GRISWOLD, J.A. (1999). Analysis of Pseudomonas aeruginosa clinical isolates for possible variations within the virulence genes exotoxin A and exoenzyme S. *Journal of Surgical Research.* **82**, 95-105.

SAADI, A.T., BLACKWELL, C.C., ESSERY, S.D., RAZA, M.W., EL AHMER, O.R., MACKENZIE, D.A., JAMES, V.S., WEIR, D.M., OGILVIE, M.M., ELTON, R.A., BUSUTTIL, A. & KEELING, J.W. (1996). Development and environmental factors that enhance binding of Bordetella pertussis to human epithelial cells in relation to sudden infant death syndrome (SIDS). *FEMS Immunology & Medical Microbiology.* **16**, 51-59.

SAADI, A.T., BLACKWELL, C.C., RAZA, M.W., JAMES, V.S., STEWART, J., ELTON, R.A. & WEIR, D.M. (1993). Factors enhancing adherence of toxigenic Staphylococcus aureus to epithelial cells and their possible role in sudden infant death syndrome. *Epidemiology & Infection* **110**, 507-517.

SAADI, A.T., WEIR, D.M., POXTON, I.R., STEWART, J., ESSERY, S.D., BLACKWELL, C.C., RAZA, M.W. & BUSUTTIL, A. (1994). Isolation of an adhesin from Staphylococcus aureus that binds Lewis a blood group antigen and its relevance to sudden infant death syndrome. *FEMS Immunology & Medical Microbiology.* **8**, 315-320.

SARAWAR, S.R., BLACKMAN, M.A. & DOHERTY, P.C. (1994). Superantigen shock in mice with an inapparent viral infection. *Journal of Infectious Diseases* **170**, 1189-1194.

SAYERS, N.M., DRUCKER, D.B., MORRIS, J.A. & TELFORD, D.R. (1995). Lethal synergy between toxins of staphylococci and enterobacteria: implications for sudden infant death syndrome. *Journal of Clinical Pathology.* **48**, 929-932.

SAYERS, N.M., DRUCKER, D.B., TELFORD, D.R. & MORRIS, J.A. (1995). Effects of nicotine on bacterial toxins associated with cot death. *Archives of Disease in Childhood.* **73**, 549-551.

SCALES, W.E. & KLUGER, M.J. (1987). Effect of antipyretic drugs on circadian rhythm in body temperature of rats. *American Journal of Physiology.* **253**, t-13

SCHEIN, R.M., SPRUNG, C.L., MARCIAL, E., NAPOLITANO, L. & CHERNOW, B. (1990). Plasma cortisol levels in patients with septic shock *Critical Care Medicine* **18**, 259-263.

SCHLIEVERT, P.M. (1986). Staphylococcal enterotoxin B and toxic-shock syndrome toxin-1 are significantly associated with non-menstrual TSS *Lancet* **1**, 1149-1150.

SCHLIEVERT, P.M. (1993). Role of superantigens in human disease. *Journal of Infectious Diseases.* **167**, 997-1002.

SCHLIEVERT, P.M., BOHACH, G.A., OHLENDORF, D.H., STAUFFACHER, C.V., LEUNG, D.Y., MURRAY, D.L., PRASAD, G.S., EARHART, C.A., JABLONSKI, L.M., HOFFMANN, M.L. & CHI, Y.I. (1995). Molecular structure of staphylococcus and streptococcus superantigens *Journal of Clinical Immunology* **15**, Suppl-10S

SCHUCHAT, A. & BROOME, C.V. (1991). Toxic shock syndrome and tampons. *Epidemiologic Reviews.* **13**, 99-112.

SEDER, R.A. & PAUL, W.E. (1994). Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annual Review of Immunology* **12**, 635-673.

SELZMAN, C.H., SHAMES, B.D., MILLER, S.A., PULIDO, E.J., MENG, X., MCINTYRE, R.C.J. & HARKEN, A.H. (1998). Therapeutic implications of interleukin-10 in surgical disease. *Shock* **10**, 309-318.

SETLOW, V.P. (1996). The need for a national autopsy policy. *Archives of Pathology & Laboratory Medicine* **120**, 773-777.

SHARP, B.M. & MATTA, S.G. (1993). Prostaglandins mediate the adrenocorticotropin response to tumor necrosis factor in rats. *Endocrinology* **132**, 269-274.

SHOHAM, S., DAVENNE, D., CADY, A.B., DINARELLO, C.A. & KRUEGER, J.M. (1987). Recombinant tumor necrosis factor and interleukin 1 enhance slow-wave sleep. *American Journal of Physiology.* **253**, t-9

SOLOMON, G.F. (1987). Psychoneuroimmunology: interactions between central nervous system and immune system. *Journal of Neuroscience Research.* **18**, 1-9.

STERNBERG, E.M., CHROUSOS, G.P., WILDER, R.L. & GOLD, P.W. (1992). The stress response and the regulation of inflammatory disease. *Annals of Internal Medicine* **117** , 854-866.

STILES, B.G., CAMPBELL, Y.G., CASTLE, R.M. & GROVE, S.A. (1999). Correlation of Temperature and Toxicity in Murine Studies of Staphylococcal Enterotoxins and Toxic Shock Syndrome Toxin 1. *Infection & Immunity.* **67**, 1521-1525.

SUGAI, M. (1959). A pathological study on sudden and unexpected death especially on the cardiac autopsied by medical examiners in Tokyo. *Acta Patholigica Japonica.* **9 (suppl)**, 723-725.

SUNDSTEDT, A., HOIDEN, I., ROSENDAHL, A., KALLAND, T., VAN ROOIJEN, N. & DOHLSTEN, M. (1997). Immunoregulatory role of IL-10 during superantigen-induced hyporesponsiveness in vivo. *Journal of Immunology* **158**, 180-186.

SUTANTO, W. & DE KLOET, E.R. (1991). Mineralocorticoid receptor ligands: biochemical, pharmacological, and clinical aspects. *Medicinal Research Reviews.* **11**, 617-639.

TAKEI, S., ARORA, Y.K. & WALKER, S.M. (1993). Intravenous immunoglobulin contains specific antibodies inhibitory to activation of T cells by staphylococcal toxin superantigens *Journal of Clinical Investigation.* **91**, 602-607.

TELFORD, D.R., MORRIS, J.A., HUGHES, P., CONWAY, A.R., LEE, S., BARSON, A.J. & DRUCKER, D.B. (1989). The nasopharyngeal bacterial flora in the sudden infant death syndrome. *Journal of Infection* **18**, 125-130.

US CENTRES FOR DISEASE CONTROL. (1988). Sudden unexplained death syndrome among Southeast Asian refugees-United States. *Journal of American Medical Association* **14, 2033**.

UTIGER, R.D. (1992). Melatonin--the hormone of darkness. *New England Journal of Medicine* **327**, 1377-1379.

VAN CAUTER, E., LEPROULT, R. & KUPFER, D.J. (1996). Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol. *Journal of Clinical Endocrinology & Metabolism* **81**, 2468-2473.

VERGERONT, J.M., STOLZ, S.J., CRASS, B.A., NELSON, D.B., DAVIS, J.P. & BERGDOLL, M.S. (1983). Prevalence of serum antibody to staphylococcal enterotoxin F among Wisconsin residents: implications for toxic-shock syndrome. *Journal of Infectious Diseases* **148**, 692-698.

WANG, P., WU, P., SIEGEL, M.I., EGAN, R.W. & BILLAH, M.M. (1994). IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells. *Journal of Immunology* **153**, 811-816.

WEISENSEE, D., BEREITER-HAHN, J., SCHOEPPE, W. & LOW-FRIEDRICH, I. (1993). Effects of cytokines on the contractility of cultured cardiac myocytes. *International Journal of Immunopharmacology* **15**, 581-587.

WERGELAND, H.I., HAAHEIM, L.R., NATAS, O.B., WESENBERG, F. & OEDING, P. (1989). Antibodies to staphylococcal peptidoglycan and its peptide epitopes, teichoic acid, and lipoteichoic acid in sera from blood donors and patients with staphylococcal infections. *Journal of Clinical Microbiology.* **27**, 1286-1291.

WESTENDORP, R.G., LANGERMANS, J.A., DE BEL, C.E., MEINDERS, A.E., VANDENBROUCKE, J.P., VAN FURTH, R. & VAN DISSEL, J.T. (1995). Release of tumor necrosis factor: an innate host characteristic that may contribute to the outcome of meningococcal disease. *Journal of Infectious Diseases* **171**, 1057-1060.

WESTENDORP, R.G., LANGERMANS, J.A., HUIZINGA, T.W., ELOUALI, A.H., VERWEIJ, C.L., BOOMSMA, D.I. & VANDENBROUCKE, J.P. (1997). Genetic influence on cytokine production and fatal meningococcal disease. *Lancet* **349**, 170-173.

WILLIAMS, M.E., GERVINO, E.V., ROSA, R.M., LANDSBERG, L., YOUNG, J.B., SILVA, P. & EPSTEIN, F.H. (1985). Catecholamine modulation of rapid potassium shifts during exercise. *New England Journal of Medicine* **312**, 823-827.

WONGWANICH, S., CHOTANACHAN, P., KONDO, E. & KANAI, K. (1996). Multifactorial pathogenic mechanisms of Burkholderia pseudomallei as suggested from comparison with Burkholderia cepacia. *Southeast Asian Journal of Tropical Medicine & Public Health* **27**, 111-118.

ZORGANI, A.A., ESSERY, S.D., AL MADANI, O., BENTLEY, A.J., JAMES, V.S., MACKENSEN, A., BLACKWELL, C.C., WEIR, D.M. & BUSUTTIL, A. (1999). Detection of Pyrogenic Toxins of Staphylococcus aureus in Sudden Infant Death Syndrome. *FEMS Immunology & MedicalMicrobiology in (press).*

ZORGANI, A.A., ESSERY, S.D., JAMES, V.S., BLACKWELL, C.C., WEIR, D.M. & BUSUTTIL, A. (1997). Detection of pyrogenic staphylococcal toxins in British and French SIDS infants. *International Conference on Sudden Infant Death Syndrome, Dublin, Ireland.*

ZUGHAIER, S.M., RYLEY, H.C. & JACKSON, S.K. (1999). Lipopolysaccharide (LPS) from Burkholderia cepacia is more active than LPS from Pseudomonas aeruginosa and Stenotrophomonas maltophilia in stimulating tumor necrosis factor alpha from human monocytes. *Infect. Immun.* **67**, 1505-1507.