Pyrogenic toxins of *Staphylococcus aureus* in sudden unexpected nocturnal deaths in adults and older children: factors influencing the control of inflammatory responses to toxic shock syndrome toxins

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Abstract

Sudden unexpected nocturnal deaths (SUND) occur in young immigrant workers, mainly from south-east Asia, who are 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 the UK and it has been suggested that these or other toxins with superantigen properties might induce strong inflammatory responses leading to sudden unexpected nocturnal deaths. 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 by 50% in a model system, (3) to assess in a model system the effects on inflammatory responses to toxic shock syndrome toxin-1 (TSST) of cortisol levels present at night, during the day and under conditions of physiological stress. Enzyme linked immunosorbent assays were used to assess levels of IgG to TSST, staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin C (SEC). Human buffy coats were used to examine the effect of IgG to the toxins for neutralising activity and the effect of cortisol on induction of inflammatory mediators. Tumour necrosis factor α (TNF-α) was detected by a bioassay with L929 cells, interleukin-6 (IL-6) and interleukin-10 (IL-10) were measured by an enzyme linked immunosorbent assay. IL-6 and TNF-α levels elicited by the toxins were not reduced by night time levels 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 responses. Stress levels of cortisol (40–80 μg dl⁻¹) significantly reduced all three cytokines earlier than the normal day time levels. The majority of the population tested had sufficient antibodies to reduce TNF-α and IL-6 responses elicited by TSST and SEC in the model system. In the age range in which most sudden unexpected nocturnal death cases occur (20–39 years), males had significantly lower levels of IgG to TSST compared with females. If these toxins play a role in precipitating the series of events leading to sudden unexpected nocturnal death, the higher levels of IgG to the toxins observed in females might explain partly the much higher prevalence of these deaths among men in this age range. If inflammatory responses play a role in sudden unexpected nocturnal death, the inability of the night time levels of cortisol to control IL-6 and TNF-α in the model system might reflect these interactions in vivo. The methods developed for detection of the toxins in tissue samples and the quantitative IgG assays for anti-toxins can be applied...
to investigation of SUND victims to test the hypothesis that some of these deaths are precipitated by pyrogenic staphylococcal toxins. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Sudden unexpected nocturnal death; Pyrogenic staphylococcal toxin; Cortisol; Anti-toxin

1. Introduction

Sudden unexpected nocturnal deaths (SUND) occur in young immigrant workers, mainly from southeast Asia, who are employed in wealthier countries such as Singapore and Saudi Arabia. These deaths usually occur at night and cannot be explained following thorough autopsy investigations including toxicology and microbiology. SUND has several epidemiological features in common with sudden infant death syndrome (SIDS): the night time occurrence, a recent history of minor infection, smoking or exposure to cigarette smoke, a higher incidence among males [1,2].

Bacterial toxins with superantigen activities, in particular the pyrogenic toxins of Staphylococcus aureus such as the toxic shock syndrome toxin-1 (TSST-1), have been implicated in unexpected deaths in older children [3] and adults [4,5]. It has been suggested that inflammatory responses induced by the toxins contribute to the series of events leading to both SIDS and SUND. There are two ways in which inflammatory responses induced by the toxins could be controlled: (1) neutralisation of toxin activity by antibodies; and (2) reduction of inflammatory responses by cortisol.

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 [6]. In vivo studies of human volunteers have also demonstrated that the highest TNF responses to endotoxin were obtained when the toxin was administered at night [7]. 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 in a model system the effects on inflammatory responses to TSST-1 of cortisol levels present at night (5–10 μg dl⁻¹), during the day (10–20 μg dl⁻¹) and under conditions of physiological stress (40–80 μg dl⁻¹) [8].

2. Materials and methods

2.1. Levels of IgG to staphylococcal pyrogenic toxins in adults

Serum samples (n = 100) from autopsies were obtained from the Forensic Medicine Unit. Samples from 10 males and 10 females in each of the following age ranges were assessed: 20–29, 30–39, 40–49, 50–59, 60–69. Each sample was examined by an enzyme linked immunosorbent assay (ELISA) for IgG antibodies to TSST, staphylococcal enterotoxin C (SEC) and staphylococcal enterotoxin A (SEA). The plates were coated with 100 μl per well of TSST, SEC or SEA (Toxin Technology) (0.1 μg ml⁻¹) in coating buffer which consisted of 15 mM Na₂CO₃, 35 mM NaH₂CO₃ and 3 mM sodium azide (pH 9.6). The plates were incubated overnight at 4°C and washed three times with washing buffer containing 0.05% (v/v) Tween 20 and 0.1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Blocking buffer containing 1% (w/v) BSA in PBS was added to each well and the plates were incubated for 30 min. The blocking buffer was removed and serum samples diluted one in 50 in blocking buffer were added to duplicate wells. After washing 3 times, horseradish peroxidase (HRP)-conjugated sheep anti-human IgG from the Scottish Antibody Production Unit diluted one in 100 in blocking buffer was added to the wells and incubated for 60 min at 37°C with continuous shaking, then washed three times with washing buffer. The substrate (100 μl) was added to the wells. It contained 40 μg O-phenylenediamine in 100 ml of 0.1 M phosphate citrate buffer (0.1 M Na₂HPO₄ and 0.1 M citric acid, pH 5) activated by 40 ml 30% (v/v) H₂O₂ immediately before use. The colour change

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was stopped after 10–20 min by adding 100 µl 12.5% (v/v) H₂SO₄. The absorbance at 490 nm (A₄₉₀) was determined by an ELISA plate reader (Dynatech) and corrected by subtracting the A₄₉₀ of the corresponding blank well containing each of the components except the serum sample. The results were expressed in µg ml⁻¹ derived from the IgG standard curve in each experiment [9].

2.1.1. Collection of 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 Dulbecco’s modified essential medium (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 x 10⁶ cells ml⁻¹ in DMEM with 10% (v/v) human serum (each buffy coat with its respective serum), 1% (w/v) glutamine (Gibco), penicillin (100 IU ml⁻¹) and streptomycin (200 µg ml⁻¹) (Gibco).

2.1.2. The effect of cortisol on leukocyte responses to TSST-1

Cells obtained in Section 2.1.1 (500 µl per well) were placed in 24-well tissue culture plates and 250 µl of TSST-1 was added to the cells at a final concentration of 0.1 µg ml⁻¹. These were incubated at 37°C 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 ml⁻¹. The cells were incubated at 37°C in a humidified incubator with 5% CO₂. At different times (0, 4, 8, 16, 24, 36 and 72 h), 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 tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10). The results for the different concentrations of cortisol were expressed as a percentage of the control containing toxin alone (100%).

2.1.3. Neutralisation of toxin activity by pooled human sera

The experimental procedure in Section 2.1.2 was used to assess the neutralising effect on TSST-1 of a pool of sera prepared from the samples in Section 2.1. The levels of TNF-α and IL-6 induced by toxin treated with dilutions of the pooled sera were compared with the 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 in Section 2.1.2 but the effects of antibodies to the toxin rather than cortisol levels was examined. The toxins plus medium, pooled antisera plus medium and toxins plus dilutions of the pool were incubated for 16 h at 37°C before addition to the cells.

2.2. Cytokine assays

2.2.1. ELISA for IL-6 and IL-10

Flat-bottomed microtitre plates (96 wells) were incubated overnight at 4°C with 100 µl per well of mouse monoclonal antibody specific for IL-6 (R and D Systems) (1 µg ml⁻¹) diluted in coating buffer. The plates were washed three times with washing buffer and 100 µl blocking buffer were added to each well for 30 min at room temperature. The blocking buffer was removed, the plates were washed three times and supernatant samples (100 µl) added to duplicate wells. Dilutions of recombinant human IL-6 standard (R and D Systems) (1 µg ml⁻¹) diluted in coating buffer. The plates were washed three times with washing buffer and 100 µl blocking buffer were added to each well for 30 min at room temperature. The blocking buffer was removed, the plates were washed three times and supernatant samples (100 µl) added to duplicate wells. Dilutions of recombinant human IL-6 standard (R and D Systems) ranging from 0.1 to 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 three times and 100 µl of polyclonal goat anti-human IL-6 (R and D Systems) diluted one in 100 in blocking buffer was added to detect IL-6 bound to the wells. After incubation for 2 h at 37°C with continuous shaking, the plates were washed three times and 100 µl of HRP-conjugated donkey anti-sheep/goat IgG (Scottish Antibody Production Unit) diluted one in 100 in.
Fig. 1. IgG bound 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).
Fig. 2. The effect of dilutions of the pooled serum samples on induction of IL-6 by TSST-1 (x-axis = dilutions of the pooled samples).

Fig. 3. The effect of dilutions of the pooled serum samples on induction of IL-6 by SEC (x-axis = dilutions of the pooled samples).
blocking buffer were added to the wells. The plates were incubated for 1 h at 37°C with continuous shaking and washed three times. The substrate OPD (100 μl) was added and the colour change allowed to develop. The $A_{490}$ was determined as in Section 2.1 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 (R and D Systems) and results were expressed in ng ml$^{-1}$.

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

2.2.2. Bioassay for TNF-α

The L929 cells were used for the bioassay for TNF-α by the method described by Delahooke et al. [10].

3. Results

3.1. Levels of IgG bound 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 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 bound to SEA between males and females in any of the groups (Fig. 1 and Table 1).

3.2. Neutralisation of staphylococcal toxins by pooled antisera

In the absence of antibody, TNF-α and IL-6 from cells treated with toxin were detected at 4 h and the levels rose steadily until 16–20 h. The levels of IgG

Fig. 4. The effect of dilutions of the pooled serum samples on induction of TNF-α by TSST-1 (x-axis = dilutions of the pooled samples).
(μg ml⁻¹) binding to TSST-1, SEC and SEA were determined for the pooled sera and dilutions of the pool were tested for their ability to reduce the cytokine production by the toxins.

Figs. 2-5 summarise the effects of incubating the toxins with dilutions of the pooled sera on induction by inflammatory mediators. For 0.1 μg TSST, 0.4 μg ml⁻¹ IgG was needed for approximately a 50% reduction of both TNF-α and IL-6. For 0.1 μg ml⁻¹ SEC, 1.25 μg ml⁻¹ IgG was needed for a 50% reduction of IL-6 and 0.5 μg ml⁻¹ for a 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 the SEA-induced production of either TNF-α or IL-6 by 50%.

### Table 1

<table>
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<th>Age Group</th>
<th>TSST-1</th>
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<td>Mean</td>
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<td>84</td>
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<tr>
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<td>P value</td>
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<td>30-39</td>
<td>Mean</td>
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<td>57</td>
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<tr>
<td>40-49</td>
<td>Mean</td>
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<td>90</td>
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<tr>
<td></td>
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<td>0.49</td>
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<td>Mean</td>
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<td>96</td>
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<td>60-69</td>
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### 3.3. Protective levels of anti-toxins in the population examined

Among the samples tested, 14% of males and 8% of females had less than 0.4 μg ml⁻¹ IgG needed for a 50% reduction of TNF-α and IL-6 in response to...
Fig. 6. 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 seven donors).
Fig. 7. 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 seven donors).
Fig. 8. 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 seven donors).
TSST-1. The opposite pattern was observed for SEC, 8% of males and 12% of females had less than 1.25 W g ml⁻³ needed for neutralisation of IL-6. The percentage of males (4%) and females (6%) with less than 0.5 W gm l⁻³ needed for a 50% reduction of TNF-α was similar.

3.4. The effect of cortisol levels on induction of TNF-α, IL-6 and IL-10 from buffy coats by TSST-1

None of the cortisol levels examined significantly reduced or enhanced the production of TNF-α or IL-6 in the absence of toxin.

3.4.1. The effect of night time levels of cortisol

Night time levels of cortisol in adults range from 5 to 10 µg dl⁻¹. Compared with the positive control containing toxin alone, there was no significant reduction in TNF-α or IL-6 by either 5 µg dl⁻¹ or 10 µg dl⁻¹ of cortisol over 36 h. At 16 h, there was a significant enhancement of production of TNF-α (P = 0.008) and IL-6 (P = 0.01). The IL-10 production was significantly decreased at 16 (P = 0.01), 24 (P = 0.008) and 36 h (P = 0.02) (Fig. 6).

3.4.2. The effect of day time levels of cortisol

Day time levels of cortisol in adults range from 10 to 20 µg dl⁻¹. Compared with the positive 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). The IL-10 production was significantly enhanced at 8 h (P = 0.048) and decreased at 16–36 h but not significantly (Fig. 7).

3.4.3. The effect of stress levels of cortisol

Stress levels of cortisol in adults range from 40 to 80 µg dl⁻¹. Compared with the positive control containing toxin alone, from 4 to 24 h, there was a significant reduction of TNF-α production and from 4 to 36 h, a significant reduction of IL-6. IL-10 was significantly decreased by high levels of cortisol from 16 to 36 h (Fig. 8).

4. Discussion

The results obtained are significant in relation to some of the epidemiological risk factors identified for SUND.

4.1. Night time occurrence of SUND

Most SUND cases occur at night when cortisol levels are the lowest. The night time level observed in normal subjects (5–10 µg dl⁻¹) did not reduce the production of pro-inflammatory cytokines in the model system. Similar results were observed in parallel studies on day time and night time levels of cortisol found in infants with a modification of the protocol used in these studies [11]. At several time points, they 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 a circadian rhythm and exhibit an increased production during the night when cortisol levels are the lowest [6,7]. Day time levels had an inhibitory effect on the IL-6 production but not TNF-α in response to the toxins. Stress levels of cortisol significantly reduced all three cytokines.

4.2. 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-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 compared with females. TSST-1 has been identified in tissues of a 6-years old child [3] and two adults who died suddenly [4,5]. 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 in this age range.

4.3. Neutralisation of inflammatory responses to pyrogenic toxins

Results indicate that there might be a small proportion of the population with antibody levels lower than that needed to neutralise the individual toxins.
Studies by our group [12,13] 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 [1].

4.4. Genetic control of inflammatory responses to bacterial toxins

Studies indicate that there are significant genetic factors associated with the control of both pro- and anti-inflammatory responses to endotoxin [14,15]. Fatal outcome of meningococcal infection was related to low levels of TNF responses and/or high levels of IL-10 responses obtained with in vitro studies of first degree relatives of the patients [15]. There is evidence of individual differences in response to TSST-1. Studies by Raza et al. [16] found that a small proportion of the population exhibited TNF responses to TSST-1 that were twice the level observed with their respective unstimulated cells. 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. This is an area for further investigation in relation to SUND as well as SIDS.

4.5. Conclusions

The methods developed for detection of toxins [4,5] and quantitative IgG assays for anti-toxins can be applied to investigation of SUND victims to test the hypothesis that some of these deaths are precipitated by pyrogenic staphylococcal toxins.

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References


