EXHIBIT BE

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Detection of pyrogenic toxins of *Staphylococcus aureus* in sudden infant death syndrome

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Abstract

It has been suggested that pyrogenic toxins of *Staphylococcus aureus* are involved in the series of events leading to some cases of sudden infant death syndrome (SIDS). The objectives of the study were to screen tissues from SIDS infants for pyrogenic toxins and to compare incidence of identification of these toxins among these infants from different countries. An enzyme-linked immunosorbent assay (ELISA) and a flow cytometry method were used to screen body fluids and frozen or formalin-fixed tissues for pyrogenic toxins of *S. aureus*, toxic shock syndrome toxin 1 (TSST), staphylococcal enterotoxins A (SEA), B (SEB), and C_1 (SEC). Toxins were identified in tissues of 33/62 (53%) SIDS infants from three different countries: Scotland (10/19, 56%); France (7/13, 55%); Australia (16/30, 53%). In the Australian series, toxins were identified in only 3/19 (16%) non-SIDS deaths ($\chi^2 = 5.42$, $P < 0.02$). The flow cytometry method was useful for toxin detection in both frozen and fixed tissues, but ELISA was suitable only for frozen tissues or those fixed for less than 12 months. Identification of pyrogenic toxins in $> 50\%$ of SIDS infants from three different countries indicated further investigation into the role the toxins play in cot deaths might result in development of additional measures to reduce further the incidence of these infant deaths.

Keywords: *Staphylococcus aureus*; Toxic shock syndrome toxin; Enzyme-linked immunosorbent assay; Flow cytometry; Sudden infant death syndrome

1. Introduction

Pyrogenic toxins produced by *Staphylococcus aureus* have been implicated in some cases of sudden infant death syndrome (SIDS) [1–4]. These include the toxic shock syndrome toxin 1 (TSST) and staphylococcal enterotoxin C_1 (SEC) [2,3]. We have proposed a series of events by which pyrogenic toxins,

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alone or in conjunction with other infectious agents or products of cigarette smoke, might precipitate SIDS [5,6].

A recent study by our group used an enzyme-linked immunosorbent assay (ELISA) to detect TSST in tissues of a 6-year-old child who died suddenly and unexpectedly following parainfluenza virus infection and from whom *S. aureus* which produced TSST was isolated [7]. Here we report use of ELISA and flow cytometry methods to screen for staphylococcal toxins in frozen and fixed tissues from SIDS infants from three different countries.

2. Subjects and methods

2.1. Sources of samples

Between 1995 and 1997 unfixed samples of serum, lung and kidney tissues obtained from 19 SIDS infants were supplied by Dr J.W. Keeling and Dr N. Smith, the Royal Hospital for Sick Children, Edinburgh. They were stored at −20°C until tested. Nasopharyngeal swabs and respiratory secretions from these infants were cultured on selective media for pathogenic bacteria including *S. aureus*. Isolates obtained from the infants were stored at −20°C.

Fixed samples of brain, kidney and spleen from 13 SIDS infants were supplied by Dr C. Rambaud, Hospital Antoine Beclèere, Paris. These were tested within 12 months of collection. Prof. J. Hilton, Institute of Forensic Medicine, New South Wales, Australia, supplied samples of fixed brain tissue from 30 SIDS infants and 19 infants who died of other causes. These were tested approximately 18 months after collection.

The positive control for these studies included fixed samples of brain tissue obtained from the child who died suddenly and unexpectedly following parainfluenza infection. *S. aureus* producing TSST-1 was isolated from his respiratory tract and the corresponding toxin identified in his tissues [7]. Other controls included frozen and fixed tissues from four adults. Patient 1 was a 47-year-old male and patient 2 a 67-year-old female, both of whom died suddenly following severe vomiting. Full autopsies on both cases, including toxicology, found no morbid anatomical cause for the vomiting or death. Patients 3 (male aged 35) and 4 (female aged 76) were victims of road traffic accidents who died of multiple injuries [8]. All were tested within 9 months after death.

2.2. Preparation of tissues for analysis

Extracts of frozen samples of midbrain, kidney or spleen were prepared by grinding small pieces of the thawed tissue in a homogeniser with 2 ml of phosphate-buffered saline (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 $300 \times g$ and the supernate tested in the ELISA. The cells were resuspended in 3% (v/v) acetic acid, washed twice with PBS and tested in the flow cytometry assay.

Formalin-fixed, paraffin wax-embedded blocks of midbrain, spleen or kidney tissues were deparaffinised in xylene and rehydrated in descending concentrations of alcohol. The tissues were then homogenised and extracted with the procedures used for frozen specimens.

2.3. Detection of toxins in tissues by flow cytometry

Cells obtained from the homogenates of brain, kidney or spleen were washed twice by centrifugation in PBS at $300 \times g$ for 10 min, resuspended and counted in a haemocytometer. The cells were adjusted to 2.5×10^5 ml^{-1} and 200 μl of the cell suspension was added to 200 μl PBS (control) or 200 μl of polyclonal rabbit antitoxin (test). After incubation at 37°C for 60 min with gentle shaking (100 rpm) in an orbital incubator (Gallenkamp), the samples were washed twice in PBS by centrifugation at $300 \times g$ for 10 min. Goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (200 μl diluted 1 in 100 in PBS) (Sigma) was added to each sample and incubated at 37°C for 20 min in the orbital incubator. 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 4°C 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. Cells were selected from a display of forward angle light scatter (size) versus 90° light scatter (granularity) by means of a bit map. The bit map included the main population of cells. Fluorescence greater than the background level 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 program 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.

2.4. Detection of toxins by ELISA

TSST-1, SEA, SEB and SEC_1 were detected by a capture ELISA method used previously which was adapted from that described by Morisette et al. [7,9]. The purified toxins (Toxin Technology, USA) were tested in the ELISA to determine the lower limit of detection and to assess cross-reactivity of the antisera for heterologous toxins. For the cross-reactivity assays, the optical density (OD) value obtained with 5 μg of the homologous toxin was assigned a value of 100 and the OD value obtained with an equivalent amount of heterologous toxin expressed as a percentage of the homologous.

Readings for test samples were corrected by subtracting the OD value of the corresponding blank to which all the components of the assay except the tissue extract were added. Samples were tested in duplicate and the readings averaged. In each experiment, the extract from the 6-year-old in whose tissues TSST-1 had been identified previously by the ELISA methods [7] 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.

2.5. Toxin production by S. aureus isolates

S. aureus isolates were grown in brain heart infusion (BHI) at 40°C for 72 h. The cultures were centrifuged to remove the bacteria and the supernate tested in the ELISA described above. Controls included purified toxins and BHI.

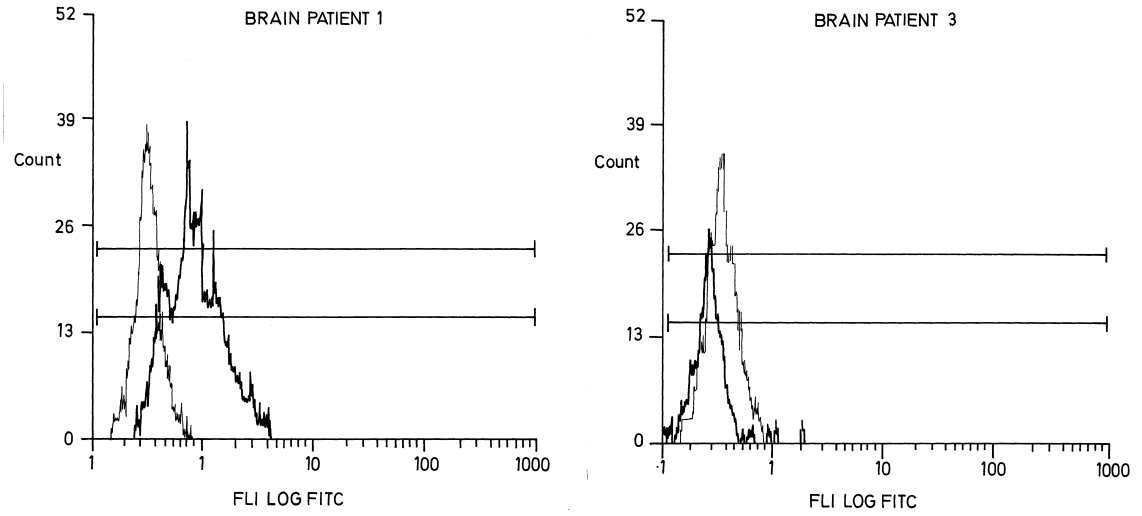

Fig. 1. Detection of TSST-1 in brain tissue from patient 1 and patient 3 (light lines represent results for the control tissues to which only the FITC-labelled anti-rabbit IgG has been added; dark lines represent the test tissues to which anti-TSST-1 has been added).

2.6. Statistical analyses

Statistical analyses were carried out with the Epi-Info package.

3. Results

3.1. Detection of toxins by flow cytometry

Because of the small amounts of tissue available from infants, initial studies were carried out with tissues from the 6-year-old child and the adults. No toxin was detected in brain, spleen or kidney of the adult road traffic victims (patients 3 and 4) by either flow cytometry or ELISA. Fig. 1 illustrates the results obtained with the brain tissue from case 1 and the age- and sex-matched road traffic victim (patient 3). The heavy line is the result obtained with the test sample and the light line the control to which only FITC-labelled anti-rabbit IgG was added. In Fig. 2 the readings obtained with spleen and kidney cells from patient 1 are illustrated. The percentage of cells with fluorescence above the control and the mean fluorescence of the positive cells were greater in each test sample. The highest readings were obtained with cells from the brain which were greater than those observed from the spleen and the lowest readings were found with cells from the kidney. Similar results were obtained with tissues from patient 2 and the control patient 4.

3.2. Detection of toxins by ELISA

Serial dilutions of the purified toxins were tested in the capture ELISA. An optical density of 0.1 corresponded to 1.8 ng TSST, 1 ng SEA, 8 ng SEB and 6 ng SEC. Cross-reactivity between the antisera and heterologous toxins was examined in six separate assays. Compared with the OD for the homologous toxin and antitoxin, there was very little cross-reactivity with the heterologous toxins (Table 1).

3.3. Detection of toxins in frozen samples from SIDS infants

Among the 19 local deaths investigated, there were eight from whom staphylococci were not isolated, and SEA and SEC_1 were identified in kidney tissue of one. There were 10 infants from whom staphylococci were isolated (nine SIDS infants and the 6-year-old boy), and at least one staphylococcal toxin was identified in each of these (Fisher exact test = 0.00025).

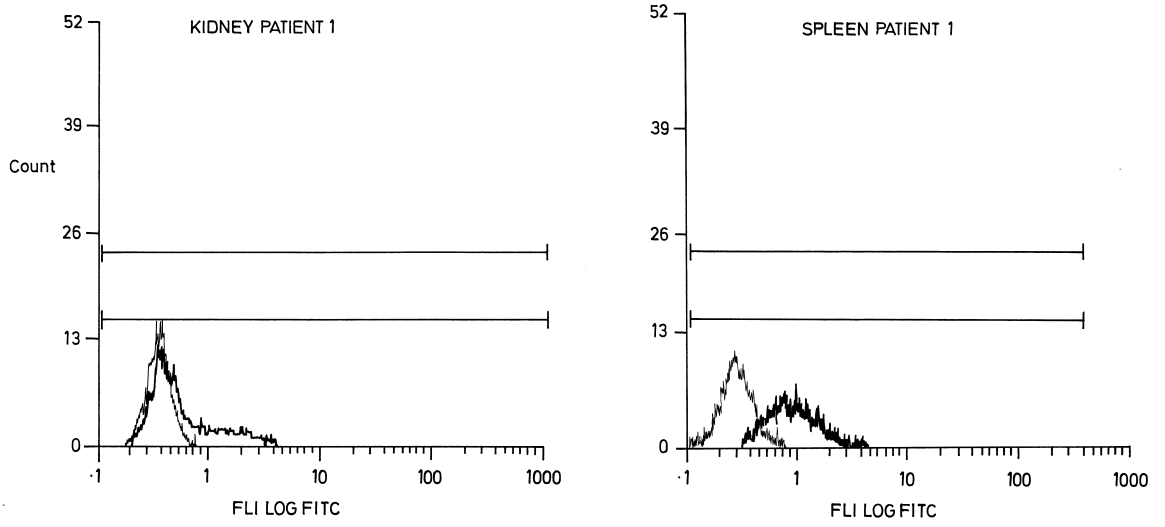

Fig. 2. Detection of TSST-1 in kidney and spleen tissue from patient 1 (light lines represent results for the control tissues to which only the FITC-labelled anti-rabbit IgG has been added; dark lines represent the test tissues to which anti-TSST-1 has been added).

Table 1
Specificity of capture ELISA for staphylococcal toxins

Antibody	TSST-1 A_{490} (%)	SEA A_{490} (%)	SEB A_{490} (%)	SEC 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)

3.4. Detection of toxins in tissues from SIDS cases and controls

We identified one or more staphylococcal toxins in frozen tissues or serum in 10/19 (53%) of local SIDS infants. *S. aureus* was isolated from seven of the 10 infants in whose tissues toxins were found. Of the nine infants whose samples were toxin-negative, *S. aureus* was not isolated from eight (Fisher's exact test, $P < 0.02$). The one staphylococcal isolate obtained from a toxin-negative infant did not produce any of the four pyrogenic toxins examined in this study. Among the seven staphylococcal isolates, four produced the corresponding toxin(s) found in the tissues. The results obtained by ELISA agreed with those obtained by flow cytometry in each case.

3.5. Detection of toxins in formalin-fixed tissues

TSST-1 was identified previously in fixed brain tissue of the 6-year-old and samples from this tissue were included as positive controls for the formalin-fixed samples. The fixed samples from the French infants were tested within 12 months of collection and TSST-1 or SEC were identified in 7/13 (55%). Both ELISA and flow cytometry methods gave complementary results.

The Australian samples were tested more than 18 months after fixation. The flow cytometry method identified TSST-1 in 16/30 (53%) SIDS infants but only 3/19 (16%) in the non-SIDS group ($\chi^2 = 5.42$, $P < 0.02$). Two of the non-SIDS group died of pneumonia and the third died of complications of cystic fibrosis. The ELISA method detected TSST-1 in only one of the specimens positive in the flow cytometry assays.

4. Discussion

Detection of pyrogenic toxins in the frozen samples corresponded closely with isolation of *S. aureus* in our 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.

Another group used a rat model to develop immunohistochemical methods 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 tissue of many SIDS infants [2,3]. With the flow cytometry method, we observed the highest readings in brain tissue compared with spleen or kidney from the same patient. In the rat model, fixation of tissues resulted in decreased detection of toxins with time by the immunohistochemical method (Prof. J.A. Morris, personal communication). Our results indicate that the ELISA has similar limitations. The 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. 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; and SEC in 36% compared with 12% among the comparison group [2,3]. 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%).

It has been suggested that the pyrogenic staphylococcal toxins (which can cause sudden death in previously healthy adults [10] or children [7]) are responsible for precipitating the series of events leading to some cases of SIDS [5,6], and in this study we identified one or more of these toxins in over 50% of local SIDS infants and a similar proportion of French and Australian SIDS cases. *S. aureus* most closely fits the mathematical model of SIDS proposed by Morris et al. [11]. It is the most common isolate from the nose and throat of infants during the first 3 months of life (42–57% of infants) [6,12,13] but it was identified among 86.4% of 37 SIDS infants in this age range [14].

We conclude that the majority of infants will be colonised by *S. aureus*; however, SIDS occurs among a very small proportion. We suggest this reflects the fact that the pyrogenic toxins are induced only between 37 and 40°C, and the temperature of the nasopharynx is usually lower due to the passage of air over the mucosal surfaces [15]. Factors which can increase the temperature of the mucosal surfaces to the permissive temperature for toxin induction could include upper respiratory tract infection and the prone sleeping position [5,6,16]. The current findings indicate that further investigation of the role of these toxins in cot deaths is warranted. Such studies could provide clues to the mechanisms of these deaths, explanations for the protective effect of the supine sleeping position [16] and possibly development of new strategies to reduce further the incidence of SIDS.

Acknowledgements

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