Lethal synergy between toxins of staphylococci and enterobacteria: implications for sudden infant death syndrome

N M Sayers, D B Drucker, J A Morris, D R Telford

Abstract

Aim—To test the hypothesis that lethal synergy occurs between toxin preparations of nasopharyngeal staphylococci and enterobacteria from sudden infant death syndrome (SIDS) victims and matched healthy infants.

Methods—SIDS and matched healthy babies were studied if both staphylococcal and enterobacterial strains were isolated from the nasopharynx. The lethality of toxin preparations from each bacterial isolate (separately and combined) was assessed over a range of dilutions using the chick embryo assay system.

Results—Staphylococci and enterobacteria were isolated together from the nasopharynx of seven SIDS babies but from only one normal healthy infant. Enterobacterial toxins were lethal at high dilutions. Staphylococcal toxins were less toxic. Simultaneous testing in the chick assay of staphylococcal and enterobacterial toxins, from each baby, at non-lethal concentrations enhanced lethality levels by 177 to 1011% compared with lethality expected by an additive effect alone.

Conclusions—Synergy occurs between the toxins of nasopharyngeal staphylococci and enterobacteria. This combination of strains is more likely to occur in the nasopharynx of SIDS victims than that of healthy infants.

Keywords: Sudden infant death syndrome, toxins, synergy.

Sudden infant death syndrome (SIDS) is still the major cause of postneonatal infant mortality during the first 12 months of life in the UK. This is despite a noticeable fall in the number of cases in recent years which has been associated with the national campaign by the Foundation for the Study of Infant Deaths (FSID). SIDS is characterised by a number of features: (a) the number of deaths attributed to SIDS peaks at two to three months of age, becoming rare after one year of age; (b) SIDS cases peak during the winter months and between midnight and 6.00 am while the infant is sleeping; (c) viral infection of the upper respiratory tract has been suggested to be a predisposing factor of SIDS. A number of risk factors have been strongly associated with SIDS: these include sleeping position, maternal drug abuse and maternal smoking.

One hypothesis put forward which explains the age distribution of SIDS as well as the other characteristic features is the “common bacterial toxin hypothesis”. This hypothesis states that an infant is protected by maternal antibodies from toxins produced by common commensal bacteria during the first one to two months of life. However, between two to three months of age maternal antibody protection wanes and infant immunoglobulin production ins. Therefore, the infant is effectively hypo-immune between two to three months of age. Viral infections of the upper respiratory tract can lead to a supra-infection of the nasopharyngeal microflora. If a virally induced super-infection occurs while an infant is hypoinnune then that infant is at risk of SIDS.

Subsequent studies have shown that SIDS victims are significantly more likely to harbour staphylococci, streptococci and enterobacteria in their nasopharynx than matched healthy controls. Moreover, it has also been shown that the nasopharyngeal flora of SIDS victims is significantly more likely than that of normal healthy infants to harbour a lethally toxigenic strain.

Preliminary published data suggest that SIDS associated bacteria and/or their toxins may act synergistically: initial studies have shown that staphylococci and enterobacteria and their toxins may be lethally synergistic. In addition, when toxin preparations from Escherichia coli are fractionated by anionic chromatography, the fractions produced are less lethal individually, than when combined.

The aim of this study was to investigate a series of babies to test the hypothesis that SIDS victims are more likely than matched healthy infants to have lethal toxin synergy associated with the nasopharyngeal flora.

Methods

The bacteria used in this study had been previously isolated from pernasal swabs (PNS) taken from SIDS victims and from normal healthy controls. The method of sampling has been described elsewhere.

SUBJECT SELECTION

Matched pairs of babies (48 SIDS victims and 48 normal healthy controls) were previously selected based on the following criteria: SIDS victims and normal healthy controls were matched for sex, age at time of death (SIDS
Table 1 Bacteria isolated from PNS swabs of SIDS victims and normal healthy infants

<table>
<thead>
<tr>
<th>Baby</th>
<th>Staphylococcal species</th>
<th>Study code</th>
<th>Enterobacterial species</th>
<th>Study code</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(s)</td>
<td>S epidermidis</td>
<td>1e</td>
<td>Klebsiella oxytoca</td>
<td>ko</td>
</tr>
<tr>
<td>2(s)</td>
<td>S aureus</td>
<td>2a</td>
<td>KI pneuniaae</td>
<td>2k</td>
</tr>
<tr>
<td>3(s)</td>
<td>S aureus</td>
<td>3a</td>
<td>Enterbacter cloacae</td>
<td>3ec</td>
</tr>
<tr>
<td>4* (s)</td>
<td>S aureus</td>
<td>4a</td>
<td>KI pneuniaae</td>
<td>4k</td>
</tr>
<tr>
<td>5(s)</td>
<td>S aureus</td>
<td>5a</td>
<td>E coli</td>
<td>4ec</td>
</tr>
<tr>
<td>6(s)</td>
<td>S aureus</td>
<td>6a</td>
<td>Proteus mirabilis</td>
<td>5pm</td>
</tr>
<tr>
<td>7(s)</td>
<td>S aureus</td>
<td>7a</td>
<td>E coli</td>
<td>7ec</td>
</tr>
<tr>
<td>8(s)</td>
<td>S epidermidis</td>
<td>8a</td>
<td>Kl oxytoca</td>
<td>8k</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E coli</td>
<td>8ec</td>
</tr>
</tbody>
</table>

* Two enterobacterial species isolated from PNS; S = SIDS victim; N = normal healthy infant.

Table 2 Bacteria isolated from PNS of matched babies. Percentage lethality of toxins from bacteria in chick embryo assay system over a range of dilutions

<table>
<thead>
<tr>
<th>Baby</th>
<th>Species isolated from PNS</th>
<th>Toxic concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>200</td>
</tr>
<tr>
<td>1</td>
<td>S epidermidis</td>
<td>0 0</td>
</tr>
<tr>
<td>2</td>
<td>S aureus</td>
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</tr>
<tr>
<td>3</td>
<td>S epidermidis</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>S aureus</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>S aureus</td>
<td>0 0</td>
</tr>
<tr>
<td>6</td>
<td>S epidermidis</td>
<td>9 0</td>
</tr>
<tr>
<td>7</td>
<td>S aureus</td>
<td>0 0</td>
</tr>
<tr>
<td>8</td>
<td>S aureus</td>
<td>18</td>
</tr>
</tbody>
</table>

100% = standard toxin preparation (see text for details).

BACTERIA ISOLATED

Eight babies conformed to the above criteria and details are presented in table 1, along with the staphylococcal and enterobacterial species isolated. Where appropriate, table 2 outlines the bacterial species tested from the PNS of matched babies.

Bacterial identification was confirmed using commercially available kits (BioMereux (UK) Ltd, Basingstoke, UK). Enterobacterial species were identified using Rapid 20 E and staphylococcus species were identified using API STAPH. All commercial identification kits were used strictly in accordance with the manufacturer's instructions. All isolates were preserved at -20°C.

The strains of staphylococcus were tested for the production of enterotoxins A to D using the SET-RPLA Kit (TD900, Unipath, Basingstoke, UK). The manufacturer's instructions were followed except that the dilutions of the culture filtrates used were 1 in 2, 1 in 10 and 1 in 100. Enterotoxin analyses were carried out as described previously.20

All E coli strains were tested for the production of verotoxin-1 as described previously.21 Briefly, bacteria were grown overnight in tryptone soya broth containing mitomycin C (200 ng/ml). Cultures were then centrifuged and the supernatant tested for the presence of verotoxin-2, the cell pellet was lysed with polymyxin, centrifuged and the supernatant tested for verotoxin-1. Enzyme linked immunosorbent assay plates were coated with crude hya1id cyst material. Supernatant and cell lysates were added and bound toxin was detected with rabbit polyclonal sera to each toxin. Goat anti-rabbit phosphatase conjugate followed by substrate was used to detect bound polyclonal antibody.

As can be seen from table 1, two staphylococcal species were isolated: Staphylococcus aureus and Staphylococcus epidermidis. S aureus predominated being isolated from seven babies, whereas S epidermidis was isolated from only one baby.

TOXICENICITY TESTING

The bacterial isolates chosen were screened for lethal toxiicinity16,17 using a chick embryo approach originally described by Eichhorn.22

The isolates were grown on a defined medium onto which a sterile dialysis membrane was overlain. The latter medium was composed of D-glucose (2·0 g/l), yeast extract (2·0 g/l), potassium dihydrogen phosphate (3·0 g/l), di-potassium hydrogen phosphate (7·0 g/l), sodium citrate (0·5 g/l), magnesium sulphate (0·1 g/l), ammonium sulphate (1·0 g/l), and agar (15·0 g/l). The bacteria were grown overnight on this medium at 37°C after which time bacteria and super-membranous cell products were harvested. Harvesting was achieved by washing with Hank's balanced salt solution (HBSS) without phenol red but with sodium bicarbonate (Sigma, Poole, Dorset, UK). The concentration of the resulting bacterial suspension was assessed following a method previously described.11 Briefly, the absorbance (λ595 nm) of the bacterial suspension diluted in phosphate buffered saline (PBS) was ascertained using a double-beam spectrophotometer (Pye-Unicam, Cambridge, UK). To facilitate comparison between different bacterial suspensions, a standard absorbance (A = 5·0, following dilution corrections) was considered notionally to be an undiluted solution. The bacteria were then removed from suspension by centrifugation followed by filter sterilisation through a 0·2 μm pore membrane filter (Gelman Sciences, Michigan, USA). The crude toxin preparation derived from each bacterial isolate was considered to be at the same concentration as the original bacterial suspension from which it was derived. Crude toxin preparations were stored at -20°C and diluted as required with HBSS.

The lethality of each crude toxin preparation was assessed by intravenous injection into 11 day old chick embryos.16-19,22 Each egg was injected with 50 μl of each putative toxin preparation at the desired concentration, the embryo was replaced if injection resulted in haemorrhage. Each egg was re-candled after a minimum of 18 hours to determine the survival of the embryo.

PRE-SYNERGY TESTING

Putative toxins from staphylococci were tested at twice the standard concentration, undiluted and diluted (1 in 2 and 1 in 4) with HBSS.
Putative toxins from enterobacteria were tested at twice the standard concentration, undiluted and diluted (1 in 2, 1 in 4, 1 in 8, 1 in 16, 1 in 32, 1 in 64, and 1 in 128) with HBSS. Two separate toxin preparations from each individual bacterium were tested in a minimum of 11 eggs. Embryos were also challenged with HBSS alone as negative controls. The dilution factor for both staphylococcal and enterobacterial toxins was noted at which lethal toxicity in the chick embryo was less than or equal to 20% of eggs challenged. At this point the toxicity was considered minimal.

SYNERGY EXPERIMENTS
Staphylococcal and enterobacterial toxins from individual babies were combined at non-lethal concentrations determined in the previous experiments. Fifteen chick embryos were used to test each combination.

Results
Analysis of enterotoxin production by the staphylococci used showed that 2sa (table 1) produced enterotoxin A, 3sa (table 1) produced enterotoxins C and D, 8sa (table 1) produced enterotoxin D, while the remaining staphylococci did not produce any detectable enterotoxin or toxic shock syndrome toxin 1 (TSST-1). No E coli strain produced detectable verotoxin.

The pre-synergy experiments showed that toxin preparations derived from enterobacteria (table 3) were considerably more lethal than those from staphylococci (table 4). The dilution of toxin preparations from staphylococci used in the synergy experiments ranged from 25 to 50% whereas toxins from enterobacteria were only used at 0-8% concentrated (1 in 128 dilution) in the synergy experiments.

In the synergy experiments (figure) the combination of toxin preparations from staphylococci and enterobacteria from any given baby produces a lethality which exceeds that expected by addition of the percentage lethality of individual toxins. Statistical analysis using the Wilcoxon signed rank test showed that the excess lethality in toxin combination experiments was highly significant (p = 0.004). The observed lethality, in all cases, were between 177 and 1011% greater than expected lethalities assuming an additive effect.

Discussion
The possibility that toxin preparations from enterobacteria and staphylococci of SIDS victims might act synergistically in the chick embryo toxin assay has been confirmed in this study.

Staphylococci produce a wide range of extracellular toxins, such as enterotoxins (A, B, C1, C2, C3, D, and E), TSST-1, exfoliating toxins (A and B), and pyrogenic exotoxins (A, B and C).23 Enterobacteria also produce a wide range of both cellular and extracellular toxins; these include endotoxin, haemolysins (A and B), heat labile/stable toxins, vero-cell cytotoxin, Shiga-like toxin, and proteases.24 25 SIDS babies are

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**Table 3** Percentage lethal toxicity of enterobacterial toxins over a range of concentrations

<table>
<thead>
<tr>
<th>Enterobacterial code</th>
<th>200</th>
<th>100</th>
<th>50</th>
<th>25</th>
<th>12-25</th>
<th>6-25</th>
<th>3-15</th>
<th>1-6</th>
<th>0-8</th>
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<tr>
<td>1ko</td>
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<td>91</td>
<td>91</td>
<td>73</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>27</td>
<td>9*</td>
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<tr>
<td>2kp</td>
<td>91</td>
<td>82</td>
<td>82</td>
<td>82</td>
<td>82</td>
<td>64</td>
<td>64</td>
<td>27</td>
<td>18</td>
</tr>
<tr>
<td>3ec</td>
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<td>100</td>
<td>73</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>59</td>
<td>18</td>
</tr>
<tr>
<td>4ec</td>
<td>91</td>
<td>82</td>
<td>73</td>
<td>55</td>
<td>46</td>
<td>46</td>
<td>27</td>
<td>9</td>
<td>0*</td>
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<tr>
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<td>73</td>
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<td>64</td>
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<td>55</td>
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<td>27*</td>
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<tr>
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<td>82</td>
<td>64</td>
<td>73</td>
<td>55</td>
<td>36</td>
<td>36*</td>
</tr>
</tbody>
</table>

* Dilutions used in synergy experiments; 100% = standard toxin preparation (see text for details).

**Table 4** Percentage lethal toxicity of staphylococcal toxins over a range of concentrations

<table>
<thead>
<tr>
<th>Staphylococcal code</th>
<th>200</th>
<th>100</th>
<th>50</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>1se</td>
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<td>0</td>
<td>0*</td>
<td>0</td>
</tr>
<tr>
<td>2sa</td>
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<td>0</td>
<td>0*</td>
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<tr>
<td>3sa</td>
<td>82</td>
<td>18</td>
<td>0</td>
<td>0*</td>
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<tr>
<td>4sa</td>
<td>36</td>
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<td>0*</td>
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<tr>
<td>5sa</td>
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<td>0*</td>
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<td>6sa</td>
<td>64</td>
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<td>8sa</td>
<td>82</td>
<td>9</td>
<td>0</td>
<td>0*</td>
</tr>
</tbody>
</table>

* Dilutions used in synergy experiments; 100% = standard toxin preparation (see text for details).
more likely than normal healthy infants to har- 
bour lethally toxigenic bacteria, and in par-
ticular enterobacteria and staphylococci.1 The 
results of this study show that it may be possible 
for staphylococcal strains normally associated 
with low toxicity, such as S epidermidis, to 
contribute to lethal synergistic effects. More-
over, it has been shown that of the sta-
phylococcal strains tested only three produced 
enterotoxins, whereas all staphylococcal strains 
stimulated a lethal synergistic effect with enter-
obacterial toxins.

The ratio of both staphylococci and enterob-
aeria being simultaneously isolated from the 
nasopharynx of SIDS babies compared with 
normal healthy infants, in this study, was 7:1, 
respectively. As synergy can only occur if toxins 
from both staphylococci and enterobacteria are 
present, it is statistically more probable that 
synergy will occur in SIDS babies than in nor-
mal healthy infants (χ² test, p = 0.05).

There is substantial evidence showing that 
TSST-1 potentiates the activity of endogenous 
endotoxin by inhibiting the clearance of endo-
toxin from the blood by the reticuloendothelial 
cell system.26 This mode of action may also be 
true for other toxins, including enterotoxins A, 
B, C, and streptococcal pyrogenic exotoxin 
type C. TSST-1 and endotoxin also act syner-
gistically to stimulate the synthesis of inter-
leukin-1 from rat macrophages,27 and tumour 
necrosis factor α and interleukin-1 from human 
peripheral blood leucocytes.28

It has also been shown that bacterial toxins 
can act synergistically with influenza virus A. 
Using a ferret model, the lethality of influenza 
A is increased by bacteria,29 as well as endotoxin 
alone. Endotoxin and influenza A also increase 
the release of tumour necrosis factor α and 
histamine.30

Although not all of the staphylococci tested 
produced enterotoxins, it is plausible that other 
toxins are produced which act synergistically 
with enterobacterial toxins, particularly endo-
toxin.

To avoid lethal synergy in infants between 
toxins from Gram positive and Gram negative 
bacteria, key toxins must be neutralised. 
This might be achieved by immunisation. 
Pro-
phylactic antibiotics could not be administered 
sufficiently early because of the sudden onset 
of SIDS.

The authors wish to thank Antonnette Wieneke, Central Public Health Laboratory, London, for determining staphylococcal enterotoxin and TSST-1 production, and Dr Derek Law, Hope Hospital, Salford, for determining E. coli verotoxin production. We would also like to thank the Foundation for the Study of Infant Deaths for their financial support.

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