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.

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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 hypoimmune 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 hypoimmune 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. 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 pneumonie</td>
<td>2k</td>
</tr>
<tr>
<td>3(s)</td>
<td>S aureus</td>
<td>3a</td>
<td>Enterobacter cloacae</td>
<td>3k</td>
</tr>
<tr>
<td>4(s)</td>
<td>S aureus</td>
<td>4a</td>
<td>KI pneumonie</td>
<td>4k</td>
</tr>
<tr>
<td>5(s)</td>
<td>S aureus</td>
<td>5a</td>
<td>Proteus mirabilis</td>
<td>5pm</td>
</tr>
<tr>
<td>6(s)</td>
<td>S aureus</td>
<td>6a</td>
<td>KI oxytoca</td>
<td>6ko</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>KI oxytoca</td>
<td>8ko</td>
</tr>
</tbody>
</table>

* Two enterobacterial species isolated from PNS; S = SIDS victim; N = normal health 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  100  50  25</td>
</tr>
<tr>
<td>1</td>
<td>S epidermidis</td>
<td>0   0   0   0</td>
</tr>
<tr>
<td>2</td>
<td>S aureus</td>
<td>100 46 36  18</td>
</tr>
<tr>
<td>3</td>
<td>S epidermidis</td>
<td>18  9   0   0</td>
</tr>
<tr>
<td>4</td>
<td>S aureus</td>
<td>100 82 29  10</td>
</tr>
<tr>
<td>5</td>
<td>S aureus</td>
<td>0   0   0   0</td>
</tr>
<tr>
<td>6</td>
<td>S epidermidis</td>
<td>0   0   0   0</td>
</tr>
<tr>
<td>7</td>
<td>S aureus</td>
<td>18  18  0   0</td>
</tr>
<tr>
<td>8</td>
<td>S epidermidis</td>
<td>0   0   0   0</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) and enterobacterial species 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.

All E coli strains were tested for the production of verotoxin I-L. Enzyme linked immunosorbent assay plates were coated with crude hydat cyst material. Supernatant and cell lysates were added and bound toxin was detected with rabbit polyclonal sera to each toxin. Goat anti-rabbit phosphate 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.

Toxigenicity testing

The bacterial isolates chosen were screened for lethal toxigenicity using a chick embryo assay system originally described by Eichhorn. 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), d-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 at 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. Briefly, the absorbance (λ285 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. 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.
Toxin synergy

Table 3  Percentage lethal toxicity of entrobacterial toxins over a range of concentrations

<table>
<thead>
<tr>
<th>Toxin concentration (%)</th>
<th>Enterobacterial code</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200</td>
</tr>
<tr>
<td>kp</td>
<td>100</td>
</tr>
<tr>
<td>2kp</td>
<td>91</td>
</tr>
<tr>
<td>3kc</td>
<td>100</td>
</tr>
<tr>
<td>4kc</td>
<td>91</td>
</tr>
<tr>
<td>4cc</td>
<td>100</td>
</tr>
<tr>
<td>5pm</td>
<td>100</td>
</tr>
<tr>
<td>6ko</td>
<td>100</td>
</tr>
<tr>
<td>7ec</td>
<td>73</td>
</tr>
<tr>
<td>8ko</td>
<td>91</td>
</tr>
<tr>
<td>8ec</td>
<td>100</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>Toxin concentration (%)</th>
<th>Staphylococcal code</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200</td>
</tr>
<tr>
<td>1a</td>
<td>27</td>
</tr>
<tr>
<td>2a</td>
<td>100</td>
</tr>
<tr>
<td>3a</td>
<td>82</td>
</tr>
<tr>
<td>4a</td>
<td>36</td>
</tr>
<tr>
<td>5a</td>
<td>55</td>
</tr>
<tr>
<td>6a</td>
<td>64</td>
</tr>
<tr>
<td>7a</td>
<td>55</td>
</tr>
<tr>
<td>8a</td>
<td>82</td>
</tr>
</tbody>
</table>

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

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 enterotoxin.

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 lethalitys, in all cases, were between 177 and 1011% greater than expected lethalitys 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). Entrobacteria 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. SIDS babies are
more likely than normal healthy infants to har-
bour lethally toxigenic bacteria,4, 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 entero-
bacteria 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 ($chi^2$ test, p = 0.05).

There is substantial evidence showing that
tSST-1 potentiates the activity of endogenous
endo- 
toxin from the blood by the reticuloendothelial
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-
gastically to stimulate the synthesis of inter-
leukin-1 from rat macrophages,27 and tumour
necrosis factor $\alpha$ 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 $\alpha$ 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, the 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 Wienneke, Central Public
Health Laboratory, London, for determining staphylococcal
enterotoxin and tSST-1 production, and Dr Derek Law, Hope
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1 Benetelle KH, Albani M. Are there tests predictive for
prolonged apnoea and SIDS? A review of epidemiological
2 Brooks JG. Apnea of infancy and sudden infant death syndrom.
3 Anonymous. Dramatic drop in SIDS after reduce the risk
campaign. FSID Newsletter 1993;43:1.
4 Williams AL. Sudden infant death syndrome. Aust NZ J
5 Beckwith JB. The sudden infant death syndrome. Curr Prob
6 Breslow NE, Day NE. Statistical methods in cancer research.
7 Valdes-Dapena MA. Sudden and unexpected death in in-
fancy, a review of the world literature 1954-66. Pediatrics
8 Williams AL, Wren EC, Bretherton L. Respiratory viruses
9 Miller AD. Recent theories on the cause of cot death. BMJ
10 Li DK, Darling JR. Maternal smoking, low birth weight,
and ethnicity in relation to sudden infant death syndrome.
11 Kandall SR, Gaines J, Habel L, Davidson G, Jessop D.
Relationship of maternal substance abuse to subsequent
sudden infant death syndrome in offspring. J Pediatr
12 Anonymous. Nicotine addiction: a report of the Surgeon
General. Washington DC: US Department of Health and
13 Morris JA, Haran D, Smith A. Hypothesis: common bac-
terial toxins are a possible cause of sudden infant death
14 Jakeman K, Rushon DI, Smith H, Sweet C. Exacerbation of
bacterial toxicity to infant ferrets by influenza virus: possible
role in sudden infant death syndrome. J Infect Dis
1991;163:35:40.
15 Telford DR, Morris JA, Hughes P, Conway AR, Lee S,
Barson AJ, et al. The nasopharyngeal bacterial flora in
16 McKendrick N, Drucker DB, Morris JA, Telford DR, Bar-
son AJ, Oppenheim BA. Bacterial toxins: a possible cause
17 Lee S, Barson AJ, Drucker DB, Morris JA, Telford DR.
Lethal challenge of nontuberculous weaning rats with
bacterial isolates from cases of sudden infant death syndrome.
18 Drucker DB, Aluyi HA, Morris JA, Telford DR, Gibbs A.
Lethal synergistic action of toxins of bacteria isolated from
sudden infant death syndrome. J Clin Pathol 1992;45:
799-801.
19 Drucker DB, Aluyi HA, Morris JA, Telford DR, Oppenheim
BA, Crawley BA. Possibility of separating toxins from
bacteria associated with sudden infant death syndrome
using animal exchange chromatography. J Clin Pathol;
1992;45:802-5.
20 Wienneke AA. The detection of enterotoxin and toxic shock
syndrome toxin-1 production by strains of Staphylococcus
aureus with commercial RPLA kits. Int J Food Microbiol
1987;8:25-30.
21 Law D, Ganguli Leela A, Donohue-Rolfe A, Acheson DWK.
Detection by ELISA of low numbers of Shiga-like toxins
producing Escherichia coli in mixed cultures after growth
in the presence of mitomycin C. J Med Microbiol;
22 Eichhorn EA. A technique for the intravenous inoculation
23 Marrak P, Kapper J. The Staphylococcal enterotoxins and
24 Holland JB, Kenny B, Blight M. Haemolysin secretion from
25 Bettelheim KA, Goldwater PN, Dwyer BW, Bourne AJ,
Smith DL. Toxicogenic Escherichia coli associated with
22:467-74.
26 Schaffert PN. Alteration of immune function by sta-
phylococcal pyrogenic exotoxin type C: possible role in
27 Beezhold DH, Best GK, Bonventre PF, Thompson M.
Synergistic induction of interleukin-1 by endotoxin and
toxic shock syndrome toxin-1 using rat macrophages. Infect
28 Lundemose JB, Smith H, Sweet C. Cytokine release from
human peripheral blood leucocytes incubated with endo-
toxin with and without prior infection with influenza virus:
relevance to the sudden infant death syndrome. Int J Exp
29 Hindler F, Schmidt A, Gong J-H, Bender A, Spenger H,
Niau M, et al. Influenza A virus infects macrophages
and stimulates release of tumor necrosis factor-alpha.
30 Clements P, Jensen CJ, Hannou C, Seborg M, Norn
S. Influenza A virus potentiates baclophen histamine release
causing a bronchoconstriction-induced complement activation.
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