CORRESPONDENCE

Curliated *Escherichia coli*, soluble curlin and the sudden infant death syndrome (SIDS)

Toxigenic bacteria, especially *Escherichia coli* in the gut or nasopharynx, have been associated with babies who have died of SIDS compared with living or dead controls [1–5]. Such toxic bacterial products should be demonstrable in the sera of SIDS babies. Notably, lethal toxicity of serum from SIDS babies (and not appropriate controls) has been demonstrated in chick embryos [6], HeLa cells [7] and mice [8]. The gene encoding the curli structural subunit (curlin, CsgA) is present and transcribed in most natural isolates of *E. coli* but only certain strains are able to assemble the protein subunit into curli [9]. Curli play a role in determining *E. coli* urinary tract pathogenicity [10] and curliated strains have a profound effect on blood pressure and induce significant hypotension when injected into mice intraperitoneally [11], leading us to explore a possible role for curli-producing strains in the causation of SIDS [12]. A random selection of *E. coli* strains, maintained on Columbia agar slopes, which had been collected from the small and large intestines of 92 SIDS babies, 17 dead control babies and 91 healthy baby stools were examined for curlin production by cultural methods on two types of Congo red agar – Congo red-magnesium oxalate agar [13] and YESCA agar [14]. They were incubated at 26°C for 48 h and examined for the typical appearance of colonies taking up the Congo red dye, which represented curli-positivity.

All 92 SIDS *E. coli* isolates produced curli protein on Congo red agar. Significantly fewer control isolates from non-SIDS deaths – 13 (76.5%) of 17 isolates (χ^2 17.79; $p = 0.00002$) – and from healthy babies – 74 (80.4%) of 91 isolates (χ^2 16.3; $p = 0.00005$) – produced curli protein. The strong association between curli production and *E. coli* strains from SIDS cases indicated that sera from SIDS cases might contain soluble curlin antigen (CsgA). Therefore, sera from SIDS cases and age-matched controls were examined immunochemically for the presence of CsgA.

Heart blood samples from SIDS and babies who died of other causes were obtained following approval from the Women's & Children's Hospital Research Ethics Committee. The work complied fully with the South Australian legal requirements relating to the retention of tissues obtained at autopsy.

Dot-immunoblots of SIDS and control sera were prepared by spotting 0.5- μ l volumes of serum diluted in native sample buffer (NSB) on to nitrocellulose membranes (0.45 μ m pore size) (Invitrogen). After fixing in transfer buffer containing methanol 10% and

then blocking with skim-milk 5% in Tris-buffered saline (TBST), the antigens were incubated overnight at room temperature with gentle rocking with a 1 in 2000 dilution (in TBST) of affinity-purified rabbit anti-CsgA (ZB-AIII) kindly provided Dr Zhao Bian, Karolinska Institute, Sweden. Pre-immune rabbit antiserum at the same dilution was used as a negative control.

After washing, the membranes were treated with HRP-conjugated goat anti-rabbit immunoglobulins (Dako A/S, Denmark, code P0448) diluted 1 in 2500 for 2 h and washed in TBS; colour was developed with One StepTM TMB-Blotting (No. 34018; Pierce, Rockford, IL, USA). The titre of CsgA activity in each serum specimen was determined by testing two-fold dilutions in NSB.

Western blots were performed with Tris-glycine 4% and 4–20% precast 10-well 1.5-mm gels (Invitrogen) for native PAGE of SIDS sera and *E. coli*-positive soluble curlin control antigen, prepared by solubilising a loopful of a 48-h CFA agar [14, 15] growth of a known curli-positive *E. coli* K12 strain (DH1) in 100 μ l of formic acid (90%). The solution was micro-centrifuged at 13 000 rpm for 10 min and 10 μ l of supernate were added to 30 μ l of NSB. SIDS sera (20 μ l) were mixed with 20 μ l of NSB and centrifuged similarly; 25 μ l of supernate were loaded on to gel slots with 20 μ l of a mol. wt marker (MultimarkTM Novex, San Diego, CA, USA). Gels were run at 125 V, 20 mA for 1.25 h and transferred on to Hybond-N+ nylon transfer membrane, 0.45 μ m pore size (Amersham Pharmacia Biotech, Little Chalfont, Bucks) at 25 V, 120 mA for 1 h. Membranes were blocked with skim-milk 5% in TBST and incubated overnight at room temperature in a 1 in 5000 dilution of anti-CsgA (ZB-AIII) and then treated as described above for the dot-immunoblots.

All 68 SIDS sera tested in a dot-immunoblot assay with fraction 6 of affinity-purified rabbit-anti-CsgA at a 1 in 2000 dilution gave positive reactions indicating the presence of CsgA curli protein and 45 gave titres up to the last dilution of 1 in 64. Further titration yielded two with titres of 32 768. In six babies, coliforms were grown from heart blood cultures obtained at autopsy. The isolate types and respective titres were: *E. coli* O66:H1, 4; *E. coli* O16:H48, 512; *E. coli* O4:H1, 4096; coliform (unspecified), 64; coliform (unspecified), 512; and coliform (unspecified), 32 768. Forty-one sera, sterile at autopsy, yielded the following titres: 4 (4 sera), 8 (3), 16 (3), 32 (4), 64 (16), 128 (4), 256 (3) and 512 (4). Typical contaminants (coagulase-negative

staphylococci, *Micrococcus* spp., diphtheroids, etc.) were found in 17 heart blood cultures and their respective serum titres and number were as follows: 4 (1), 8 (1), 16 (4), 32 (1), 64 (2), 512 (4), 4096 (2) and 32 786 (2). Heart blood cultures were not performed in four babies. Age-matched control sera from 61 healthy babies and 14 of 21 sera from non-SIDS deaths (including five with sterile heart blood cultures) gave negative results for curli protein. Seven sera from non-SIDS deaths gave positive results (χ^2 5.84; $p = 0.02$). Of the positive babies, one who died in a motor vehicle accident had a coliform (*Enterobacter agglomerans*), and one had a gram-positive coccus isolated from heart blood obtained at autopsy. Both isolates were regarded as contaminants. Three of the seven non-SIDS deaths had sterile heart blood and the remaining two had no blood culture performed. There was no relationship between post-mortem interval and titre of curli. No sera gave positive reactions after membranes had been incubated with pre-immune rabbit serum. Anti-CsgA-treated transfer blots of SIDS sera showed 15-kDa bands clearly identifying CsgA in these sera, confirming the identity of the dot-immunoblot reactions. The sera from which low numbers of typical contaminants and environmental organisms were grown on sterility testing gave negative reactions in colony blot assays.

The debate on the mechanism of death in SIDS has been dominated by physiologists – proponents of respiratory obstruction or sleep apnoea or other theories. Prone sleep position, in which 83% of South Australian SIDS babies are found [16] remains a leading risk factor in SIDS and is claimed to contribute to respiratory obstruction, but the nature of the pathological findings contradict this. Moreover, pathology is helpful in excluding these mechanisms. Many infants succumb in supine and lateral positions and rates of prone SIDS vary considerably geographically and temporally [17].

Evidence supports the idea that SIDS babies are in some way more predisposed to the condition than surviving healthy babies. Indeed, it has been shown that 70% of SIDS babies carry a particular allele of the IL-10 gene, IL-10-592*A allele, which results in a low-producer haplotype [18]. IL-10 is important in immunity against bacterial toxins and this could predispose the baby to these toxins. Furthermore, many pre- and post-natal risk factors for SIDS [10–25] strongly suggest infection, as do poor socio-economic conditions, ethnicity (genetic predisposition or susceptibility), passive smoking and several maternal and environmental factors [26]. Viral infections potentiate bacterial toxins [27] as do nicotine [28] and compounds in cigarette smoke [29]. Furthermore, bacterial toxins synergistically potentiate each other in terms of lethality [30]. Winter seasonality for SIDS also supports a contributory role of viruses [16, 21, 23]. Pathological evidence of acute viral infection is evident in lymph nodes from cases of SIDS [31].

The 'Reducing the Risk' campaign based on epidemiological findings from several studies in relation to prone sleep position [32] perpetuates a biased approach to this enigmatic and important cause of post-neonatal death which accounts for 0.81 deaths/1000 live births in Australia [33], or some 210 SIDS babies annually. Although since 1991 the rate of SIDS seems to have fallen by about 50% in a number of countries, SIDS remains a major contributor to post-neonatal mortality. This apparent fall may only be a reflection of natural variation. An example of this is seen in Swedish figures, showing that the rate in the late 1990s has returned to the level observed in the early 1970s. The decline seen in the early 1990s began before the introduction of the 'back to sleep' campaign [34].

Asphyxia cannot provide the answer because it is not reflected in the pathological evidence; the number and distribution of petechiae are very different in the two conditions whilst heavy fluid-laden lungs is a non-specific finding [35–37]. Other organs (heart, spleen, liver and brain) are heavier than normal in SIDS [37]. Cases of SIDS captured on 24-h computerised memory-monitors (tracing pulse, respiratory rate and blood pressure) also showed that asphyxia was an impossibility [38, 39]. Invoking an asphyxial mechanism for prone position must logically exclude the same mechanism for deaths in supine and lateral positions.

The effect of prone sleep position could be explained hypothetically on the basis of possible differences in rates of delivery to the systemic circulation of gut-derived lethal toxin [40, 41]. A candidate 'toxin' is soluble curli antigen, CsgA, accompanied (or not) by other toxins absorbed through the gut that may reach the circulation *via* the portal system which takes it to the liver. Fatty change (of which toxemia is a cause) is a frequent finding in the livers of SIDS babies [42]. A second route of curli or toxin absorption would be *via* the lymphatic system and the thoracic duct. Curli protein or toxin would be delivered *via* the duct to the innominate vein and thence to the right side of the heart. The first organ exposed to curli protein or toxin would be the lungs followed by the heart and the thymus. These are the organs in which classical pathological findings (petechiae and wet, heavy lungs) are seen in SIDS. Bacterial toxins or other products can perturb basement membranes of small blood vessels leading to the small haemorrhages (petechiae) and fluid-laden organs seen in SIDS. Curli binds to fibronectin [9] and this could precipitate damage to capillary basement membranes and perturb the clotting system. The pattern of distribution of petechiae is distinctive and could be explained by the fact that these would be the first organs exposed (as explained above) or that these organs are replete with toxin receptors [40, 41]. This hypothesis explains why more deaths occur in the prone position in which a lethal amount of toxin is delivered; in other positions, by inference, a usually sublethal dose would be delivered to the

systemic circulation. Furthermore, prone sleep position could assist ingestion of toxigenic organisms contaminating sleeping surfaces. Other pathological findings in SIDS include liquid (unclothed) blood within the chambers of the heart [36, 42], and elevated cross-linked fibrin degradation products [43] (seen in toxæmia and sepsis); in this context, curlin protein represents contact-phase bacterial components which can activate the pro-inflammatory pathway [11, 44] involving reactions with fibrinogen and fibronectin [45] which can lead to depletion of coagulation factors resulting in a hypocoagulability state [43]. The finding of an empty bladder in most SIDS cases [36, 42] suggests decreased renal perfusion (toxæmic shock) during the last sleep. Hypotension could be explained by curlin protein induction of pro-inflammatory cytokines with release of bradykinin or NO, or both [11].

A possible model for SIDS is infant botulism. Arnon *et al.* [46] published a series of cases of infant botulism that had been misdiagnosed as SIDS. Like infant botulism (in which there is ingestion of toxigenic bacteria which colonise the gut, and the demonstration of toxin in the serum), the same model for SIDS is now supported by reasonable evidence [1–4]. Moreover and similarly, we propose that SIDS should be renamed ‘infant curlinism’ in recognition of our findings of curlin protein in all SIDS sera tested and our conclusion that this association is likely to be causal in nature. We await independent confirmation of our findings. A ‘diagnosis’ and rational cause and answer for these deaths have been long awaited. The potential for a vaccine to prevent ‘infant curlinism’ is also exciting. Now there is the challenge of gaining an understanding of the conditions that favour the production, release and absorption of soluble CsgA.

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