Animal models used to test the interactions between infectious agents and products of cigarette smoked implicated in sudden infant death syndrome

Nicola M. Sayers a, David B. Drucker a,b,*

a School of Biological Sciences, University of Manchester, Manchester M13 9PT, UK
b Department of Dental Medicine and Surgery, Turner Dental School, University of Manchester, Manchester M15 6FH, UK.

Received 22 October 1998; revised 24 February 1999; accepted 24 February 1999

Abstract

Animal test systems are reviewed that have relevance to sudden infant death syndrome (SIDS) are reviewed. These test interactions between infectious agents (or their toxins) and products of cigarette smoke. Infectious agents implicated in SIDS include members of the enterobacteria and clostridia, Staphylococcus aureus and Streptococcus pyogenes. Smoking is thought to be the single most preventable cause of SIDS. Tobacco smoke contains many extremely toxic products including cyanide and nicotine. Many animal test systems are available to examine the potency of bacterial toxins and smoke-derived components. These include mice, hamsters, rats and chick embryos. Such systems reveal synergy between bacterial toxins, especially endotoxin and superantigens. They have also demonstrated potentiation of low levels of bacterial toxin by low levels of both nicotine and its primary metabolite, cotinine. These findings suggest a possible causal explanation for the fact that passive exposure to cigarette smoke is a risk factor in sudden infant death syndrome. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Sudden infant death syndrome; Microbiology; Smoking; Animal model

1. Introduction

Sudden infant death syndrome (SIDS) is unique in that its diagnosis excludes any identifiable cause of death [1]. This elusive aspect of SIDS means that an animal model to simulate a SIDS-like death is extremely desirable. A number of animal models exist in which death is quick, without outward signs of distress or illness and with similar post mortem findings to SIDS. Togari et al. [2] used a new born pig model in which a reduction in the cerebral blood flow during episodes of hypercarbia resulted in a cessation of respiratory movements which was reversible upon restoration of the cerebral blood flow. Tong et al. [3] performed site-specific cardiac denervation in a pig model to simulate abnormal cardiac innervation, 11.5% of the pigs tested died spontane-
ously with no obvious cause. Kemp et al. [4] used a rabbit model to illustrate the lethal potential of re-breathing in the prone position so that death occurred quietly in rabbits forced to rebreathe air for prolonged periods of time. A gnotobiotic rat model showed rapid death following the simultaneous, subcutaneous injection of *Staphylococcus aureus* and *Escherichia coli* cultures [5]. The rats died without any signs of terminal illness and with very few inflammatory changes in the lungs, heart and liver detected by histological examination. Jakeman et al. [6] developed a neonatal ferret model to investigate the influenza virus-enhanced lethality of staphylococcal α- and τ-toxins, endotoxin and diphtheria toxin which resulted in sudden death without prior clinical symptoms or extensive post mortem pathology.

The underlying causes of SIDS have been attributed to a number of environmental, sociological and biological factors including upper respiratory tract infections, the carriage of toxigenic bacteria and passive exposure to cigarette smoke. In this review, the authors attempt to summarise how animal models have been used to investigate the interaction between components of cigarette smoke and microbial species associated with SIDS.

## 2. Evidence that infectious agents are involved in SIDS

Microbiological examination has shown the presence of bacterial toxins or toxigenic bacteria in post mortem tissues of many SIDS victims [7]. SIDS is associated with a mild viral infection of the upper respiratory tract and occurs most commonly between 2–4 months of age when the systemic maternal IgG and infant IgG production are low. SIDS victims harbour a different range of bacterial species in the upper respiratory tract compared to controls [8], usually, one of which will be a toxigenic species [9]. Toxigenic bacteria isolated from SIDS victims include *Clostridium perfringens*, *Clostridium botulinum*, *Clostridium difficile*, enterobacteria including *E. coli*, *S. aureus* and *Streptococcus pyogenes* [8,10–13]. *C. botulinum* toxins A, B, C, F and G [14], *C. perfringens* enterotoxin and α-toxin [13], *E. coli* Vero-cell cytotoxin and heat labile enterotoxin [15,16], staphylococcal toxic shock syndrome toxin-1 TSST-1 [17] and enterotoxins [13] have also been isolated from SIDS victims. The combined effects of low IgG, a mild viral infection and exposure to common toxigenic bacteria, is suggested to lead to toxemia and rapid death [18]. Increased expression of Lewisα antigen on respiratory epithelia of SIDS victims enhances the binding of a range of bacteria and yeasts including *S. aureus*, group A streptococci and *Candida* spp. [10].

The known features of SIDS, viz. seasonality, predominantly nocturnal occurrence, sleeping position, twinning and sibling associations, might be explained with respect to increased exposure to bacteria and their toxins. Respiratory infections are more common in the winter months predisposing to the risk of secondary bacterial infections. Respiratory infections also compromise the clearance of mucosal secretions from the upper respiratory tract, resulting, during sleep, in pooling of bacteria and toxins which is exacerbated by sleeping in the prone position [19]. Bottle-feeding, common in SIDS victims, has been claimed to lead to colonisation of the gastrointestinal tract by toxigenic bacteria such as *E. coli* [15], *C. difficile* [20], *C. perfringens* and *S. aureus* [13].

Some bacterial toxins can cause rapid death. Well known examples are infant botulism, toxic shock syndrome and Gram-negative bacterial endotoxin which adds credence to the theory that bacterial toxins can trigger infant death suddenly and without extensive post mortem pathology [21]. Some bacteria isolated from SIDS victims produce superantigens. Examples include staphylococcal enterotoxins A, B, C1, C2, C3, D, E, TSST-1, exfoliating toxin A, exfoliating toxin B and streptococcal exotoxins A, B, C, F and M protein [22–24]. Superantigens do not require processing by antigen presenting cells such as B-cells, monocytes and dendritic cells [22]. They can activate between 5 and 20% of the remaining T-cell population [22] releasing large levels of interleukins, TNF-α and IFN-γ [24]. This may represent an important link between SIDS and superantigens. SIDS victims may consist of a sub-population of infants that have a large population of T-cells with a complementary receptor so that activation of T-cells would result in a massive generation of cytokines.
3. Evidence that exposure to cigarette smoke is involved in SIDS

3.1. Epidemiology

Environmental tobacco smoke is recognised as a health hazard to non-smokers, especially neonates and young children [25], and should be avoided, especially when pregnant [26]. A number of epidemiological studies have identified exposure to maternal smoking both in utero and post partum as an independent risk factor for SIDS [27]. The odds ratios quoted for maternal smoking and SIDS may range anywhere from 2.0 to 5.0 [28–31]. In addition, a dose-response relationship between (a) the extent of exposure to tobacco smoke and (b) an increased risk of SIDS has been found [32,33]. Many researchers think that smoking is the single most preventable cause of SIDS: up to two thirds of all cases should be preventable [32]. Aligne and Stoddard [34] estimated that in the USA, between 1980 and 1996, approximately 2000 SIDS cases could be attributed to tobacco smoke. Difranza and Lew [35] estimated that figure between 1200 and 2200 deaths from SIDS annually. Despite this, very little is known of the mechanism(s) by which tobacco by-products might bring about SIDS.

3.2. Effects of smoke components on physiology in relation to SIDS

Smoking has been associated with increased respiratory illnesses [36,37], an altered hypoxic awakening response [38] and a low birth weight [39], all of which have been associated with SIDS. Tobacco smoke contains a large number of carcinogenic, teratogenic and immunosuppressive compounds such as acrolein and cyanide, the concentrations of which found in one inhalation of smoke can inhibit the oral leukocyte function [40].

Nicotine is part of the particulate/vapour phase of cigarette smoke along with tar. A 70-kg adult can absorb as much as 1 mg of nicotine from one cigarette which is the equivalent of 14.2 μg kg⁻¹ body weight [41]. Nicotine is a toxic central nervous system stimulant which in large doses can cause convulsions, depression of respiration and water retention via the stimulated production of anti-diuretic hormone. Nicotine can also stimulate tachycardia, increase blood pressure, increase vascular peripheral resistance with an accompanying decreased skin temperature, increased bowel motility and also depress salivary and respiratory secretions after an initial stimulation [42,43]. Nicotine is readily absorbed through the skin, respiratory tract and buccal membranes. It is metabolised by the liver, kidneys and lungs and excreted via the kidneys [42]. Nicotine is also an immunosuppressor which can transiently depress chemotaxis of systemic polymorphonuclear leukocytes (PMNs), the viability and phagocytic activity of oral PMNs and levels of systemic IgG, IgM and oral IgA [43]. Its other activities include inhibition of neutrophil and monocyte superoxide and IL-1β production [44] as well as upregulation of lipopolysaccharide (LPS)-mediated monocyte secretion of prostaglandin E₂ [45]. Nicotine also affects the levels of certain cytokines and their receptors [46]. In addition, nicotine can also enhance increased expression of the endotoxin binding receptor CD14 on target cells [45]. In an oral context, nicotine can also inhibit the growth, collagen and fibronectin production of gingival fibroblasts [47], which may ultimately lead to a diminished wound healing response in oral tissues [48]. Nicotine can enhance the growth of some oral bacteria. This is achieved by reducing either the oxidation-reduction potential (Eₚₒ) or mucosal blood flow which leads to a reduction of immunoglobulins and leukocytes at the site of infection [43,49–51].

3.3. Dilution with exposure time

Estimation of exposure to cigarette smoke suffered by SIDS victims prior to death is calculated from the levels of tobacco by-products and metabolites post mortem in hair, nails, saliva, urine and pericardial fluid. These substances include nicotine and its immediate metabolites which include cotinine [52,53], serum thiocyanate [54], N²-nitrosornicotinone (NNN) [55], 3-vinylpyridine [56], benzo(alpha)pyrine [57] and nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [58]. Alternatively, markers of hypoxia such as 2,3-diphosphoglycerate have also been used to estimate nicotine exposure [59]. In a study of nicotine and cotinine levels in the pericardial fluid, Rajs et al. [60] found that 25% of all SIDS victims had cotinine levels exceeding 30 ng ml⁻¹ which in-
icates prolonged exposure to smoke prior to death. Milerad et al. [61] also showed that using cotinine levels in the pericardial fluid, SIDS victims were, significantly more likely than controls, more frequently and heavily exposed to nicotine.

4. Animal models to investigate SIDS

Animal models used in SIDS research are a combination of both those used to mimic SIDS and those used to investigate specific aspects of the syndrome. This distinction is particularly important when interpreting the results from such animal models/assays.

4.1. Smoking and SIDS

Investigations into the effects of whole smoke, specific chemicals and their metabolites have been performed on a limited number of animal models. In addition, in vivo assays to investigate the effect of whole smoke, specific chemicals in tobacco and their metabolites on the responses of animals in utero and post partum to environmental stress such as hyperoxia have been used.

Nicotine has been shown to depress both the primary and secondary immune response of the lungs, lymph nodes and spleen of mice which may account for increased pulmonary infections in smokers [43]. Similarly, Ortega et al. [62] showed that after a 15-min exposure to whole cigarette smoke, containing approximately 1.1 mg of nicotine, mouse alveolar macrophages displayed a significant reduction in their attachment to and phagocytosis of Candida albicans. The most relevant in vivo models for SIDS are those where nicotine and its metabolites are placed in contact with the oral or respiratory mucosa. Chen et al. [63] painted the cheek pouch epithelium of hamsters with nicotine and NNN, alone or in combination. They observed an increase in histological changes, in cheek and gastric mucosa, with nicotine and NNN alone. When applied in combination, these effects were enhanced. Wang et al. [57] also studied the buccal epithelium of Syrian hamsters after exposure to nicotine and benzo (a) pyrene (BP), alone or in combination. They showed that nicotine and BP significantly decreased the production of epidermal growth factor (EGF) and increased the number of EGF receptors. When applied in combination, an enhanced response was observed.

Both rabbits [64] and rats [65] have been used to assess the effect of whole smoke and nicotine, respectively, on lipid metabolism. Milerad et al. [66] used the lamb as a model to show the effect of nicotine on the sensitivity of peripheral chemoreceptors to oxygen tensions. Nicotine infused at 0.5 μg kg⁻¹ min⁻¹ significantly depressed the ventilatory response to hyperoxia and increased the ventilatory response to hypoxia.

Rat models are the most popular for investigating the effects of smoking on the foetus and neonate. Anand and Anand [67] found that for pregnant rats exposed to whole cigarette smoke, their pups' lungs displayed a reduced anti-oxidant status. When challenged in utero with 2–6 mg kg⁻¹ day⁻¹ nicotine, rat pups displayed a decreased response to hypoxic challenges after birth and the mortality was higher with higher doses of nicotine [68,69]. The underlying cause of this altered response was a deficiency in adrenomedullary catecholamines required to maintain the cardiac rhythm during hypoxia leading to a rapid decline in heart rate. This might be important in relation to the prevalence of SIDS deaths between midnight and 8.00 a.m. Tolson et al. [70] attributed nicotine/hypoxia-induced damage of cardiac tissue in a rat in utero/post partum model for this altered response. Holgert et al. [71] found that 3-day old rat pups exposed to nicotine and hypoxia showed a markedly increased peripheral-arterial chemoreceptor sensitivity which manifested as an increase in the release of dopamine and production of the enzyme tyrosine hydroxylase. In contrast, Bamford et al. [72] and Schuen et al. [73] did not find any alteration in the response of rat pups to hypoxia after exposure to nicotine in utero.

At the very least, animal models have shown that tobacco and in particular nicotine are carcinogenic, cytotoxic, neurotoxic and genotoxic [74]. These observations are particularly pertinent to SIDS as they illustrate four points. (1) Tobacco products can affect sites of the body away from the initial site of application. (2) The metabolites of tobacco products are biologically active increasing the toxic effect of inhalation of smoke after the primary products have been cleared from the system. (3) Tobacco can pro-
foundly affect how the neonate copes with fluctuations in oxygen tensions. (4) Tobacco smoke can alter the way in which a neonate deals with an infection. This may be due to an alteration in the intensity of the immune response, alteration in the levels of cytokines and their receptors or enhancement of the potency of a particular infectious agent or toxin.

5. Infectious agents, synergy and SIDS

5.1. Examples of animal models used in SIDS research

Siarakas et al. [75,76] used a non-anaesthetised rabbit model for SIDS to test the effects of bacterial toxins sometimes isolated from SIDS victims. The toxins they used were *C. perfringens* enterotoxin and α-toxin, staphylococcal enterotoxin B, *E. coli* heat stable toxin, Gram-negative endotoxin and *C. difficile* enterotoxin A and cytotoxin B. Siarakas et al. showed that injection of these toxins intravenously had a profound effect on the cardio-respiratory system. Levels of adrenaline and nor-adrenaline increased accompanied by a slow depression of their metabolism. Death would result without visible signs of agitation distress or spasm. Similarly, Lindgren et al. [77] showed that lambs exhibited an altered laryngeal chemostimulation reflex when infected with respiratory syncytial virus. Infected lambs displayed an increased inhibition of minute ventilation and a delayed recovery to normal breathing, resuscitation was required for 40% of the lambs.

5.2. Synergy between bacterial toxins

Animal models often illustrate the impact of synergy between bacteria and/or their toxins which have implications for SIDS research. The mouse peritoneal model used by a number of groups illustrates the lethality of mixed populations of bacteria. The inoculation of a mixed population of *Pseudomonas aeruginosa*, *S. aureus* and *Bacteriodes* spp. was shown to result in death of the animal [78,79]. Ushijima et al. [80] showed that there is increased abscess formation and lethality in the model if both *E. coli* and *B. fragilis* were present. The latter anaerobe relies upon haemolysin production by *E. coli*. TSST-1-producing *S. aureus* and endotoxin also interact synergistically to produce mortality in the mouse [81]. Similar synergistic enhancement of lethality was observed between viable cultures of *E. coli* and *S. aureus* in the gnotobiotic rat SIDS model [5]. The two bacterial species were non-lethal when tested individually, but produced rapid and silent death when combined with no distinct post mortem pathology.

Drucker et al. [82] and Sayers et al. [83] showed that toxins from Gram-positive and Gram-negative bacteria could act synergistically to enhance lethality when injected into the chorio-allantoic vein of 11-day old chick embryos. Using the same in vivo assay, the lethality of toxins from staphylococcal and/or enterobacterial species can be enhanced by the addition of endotoxin [84], nicotine [41,85] cotinine [86]. Gram-negative bacterial endotoxin and TSST-1 interact synergistically when tested concomitantly in rabbits [51,87], rats [81] and chick embryos [88]. The timing of administration of both toxins is important for synergy to occur. Fujikawa et al. [89] showed the greatest effect in rabbits when TSST-1 was administered 4 h before endotoxin. Endotoxin can originate from endogenous Gram-negative flora. In a rabbit model, endogenous endotoxin enhanced mortality when TSST-1 was administered, which was abolished by pre-treatment with polymyxin B [51]. TSST-1 enhances the actions of endotoxin by inhibiting its clearance from the blood by the reticuloendothelial system [51,89]. Other bacterial toxins which have been shown to interact synergistically with endotoxin include cholera toxin [90], staphylococcal enterotoxins B and C1, streptococcal pyrogenic exotoxin C [50] and erythrogenic toxins [91].

5.3. Synergy between virus infection and bacterial toxins

The in vitro effects of a range of toxins have shown to be enhanced by a wide range of viruses including the Sendai virus, adenovirus, influenza virus and picornavirus [6]. The same effect has also been shown in a mouse model between Gram-negative bacterial endotoxin and frog virus 3, mouse hepatitis virus and lymphocytic choriomeningitis virus [6]. Using a neonatal ferret model, Jakeman et al. [6] showed that an existing influenza virus in-
fection enhanced the lethality of staphylococcal α- and τ-toxins, endotoxin and diptheria toxin. They also found that viral titres were unaffected, indicating that the activities of the toxins were enhanced by viral infection and not vice versa, which resulted in sudden death without clinical symptoms or extensive post mortem pathology. The underlying mechanism of this viral-mediated synergy probably occurs through an increased release of cytokines and reactive molecules from cells exacerbated by viral infection [92].

The role of synergy in SIDS may take many forms. Animal assays have shown that bacteria, bacterial toxins or environmental toxins such as nicotine may interact synergistically at concentrations which may be considered clinically unimportant [41,82–86,93]. Viruses, which are associated with SIDS cases prior to death, may enhance secondary bacterial infections of the upper respiratory tract as well as synergise the lethal actions of the toxins they produce [6,18]. In SIDS victims, the actions of viruses, bacterial toxins such as endotoxin and environmental toxins such as nicotine may, acting alone or in concert, stimulate an excessive inflammatory response leading to either systemic shock or apnoea [83].

6. Interactions between infectious agents, exposure to smoke and SIDS

6.1. Nicotine, cotinine and bacterial toxins

The chick embryo assay has been successfully used by a number of research groups to assess the toxic effects of a wide range of bacteria, bacterial toxins and environmental toxins. Amongst others, Schmidt et al. [94] tested purified Salmonella enteritidis endotoxin, De Azavedo et al. [88] examined E. coli endotoxin and TSST-1 and McKendrick [91] compared the extracellular products of a wide range of both Gram-positive and Gram-negative bacteria.

Using the 11-day old chick embryo assay for toxicity, Sayers et al. [83] tested extracellular toxins from pairs of Gram-positive and -negative bacteria isolated from the nasopharynges of SIDS victims \((n = 2)\) and nicotine, either alone or in combination. The first bacterial species pair was \(S. \) aureus plus Klebsiella pneumoniae and the second was \(S. \) aureus and \(E. \) coli. The combination of nicotine at 40 ng with non-lethal individual bacterial toxins showed a synergistic effect. The combination of nicotine and toxins from both of the bacterial species in a pair gave a highly significant increase in lethality to the chick embryos \((P < 0.00001)\). An adult absorbs approximately 1 mg of nicotine from one cigarette \((14.2 \mu g \text{ kg}^{-1})\). A chick embryo is approximately 1000 times smaller than the average man and would therefore receive approximately 1 \(\mu g\) per cigarette \((1.428 \times 10^{-2} \mu g \text{ nicotine kg}^{-1})\). In these experiments, eggs injected with dilute nicotine alone received 40 ng embryo\(^{-1}\) which is approximately 0.57 \(\mu g \text{ kg}^{-1}\) body weight, assuming a mean embryo weight of 70 g. This value is equivalent to a nicotine concentration in the embryo of the same order as that produced in man by 0.05 cigarettes. When diluted a further 32-fold, the nicotine solution was able to transform a non-lethal mixture of bacterial toxins into those lethal to 42 and 76% of the chick embryos tested.

The effect of cotinine, the primary metabolite of nicotine in man, on the lethality of bacterial toxins in the chick embryo assay has also been investigated [86]. Cotinine is effective at levels observed in smokers.

7. Conclusions

When evaluating the results of animal models and assays, it is important to be cautious when extrapolating from animals to humans. The results obtained may possibly be conflicting and the routes of administration of the test substance to the animal may never occur in SIDS victims. As experimentation on neonates is ethically impossible, it is important to use all available resources at our disposal in the search for the underlying mechanism(s) of SIDS.

References


