Epidemiol. Infect. (1993). **110**, 507–517

Factors enhancing adherence of toxigenic *Staphylococcus aureus* to epithelial cells and their possible role in sudden infant death syndrome

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(*Accepted 13 January 1993*)

SUMMARY

Toxigenic strains of *Staphylococcus aureus* have been suggested to play a role in sudden infant death syndrome (SIDS). In this study we examined two factors that might enhance binding of toxigenic staphylococci to epithelial cells of infants in the age range in which cot deaths are prevalent: expression of the Lewis^a antigen and infection with respiratory syncytial virus (RSV). By flow cytometry we demonstrated that binding of three toxigenic strains of *S. aureus* to cells from non-secretors was significantly greater than to cells of secretors. Pre-treatment of epithelial cells with monoclonal anti-Lewis^a or anti-type-1 precursor significantly reduced bacterial binding ($P < 0{\cdot}01$); however, attachment of the bacteria correlated only with the amount of Lewis^a antigen detected on the cells ($P < 0{\cdot}01$). HEp-2 cells infected with RSV bound significantly more bacteria than uninfected cells. These findings are discussed in context of factors previously associated with SIDS (mother's smoking, bottle feeding and the prone sleeping position) and a hypothesis proposed to explain some cases of SIDS.

INTRODUCTION

The suggestion that microorganisms are involved in the aetiology of some cases of sudden infant death syndrome (SIDS) is based on evidence from a variety of epidemiological and pathological studies. SIDS occurs during the period when maternal antibodies have declined and the infant immune system is immature. There is a marked seasonal variation in SIDS; the risk increases in autumn and winter when respiratory infections are more common [1, 2]. There is often a history of upper respiratory tract infection in these infants [3]. Mother's smoking was identified as a factor for SIDS in the New Zealand studies [4]. Smoking and passive exposure to cigarette smoke have been associated with increased risk of respiratory tract infections [5] and with carriage of potentially pathogenic microorganisms [6, 7]. At post-mortem examination, there is often evidence of

minor inflammation and infection of the respiratory tract in many SIDS infants [8].

There are conflicting results on the role of viruses [9, 10] and toxigenic intestinal bacteria in SIDS [11–15]. Recently, it has been suggested that nasopharyngeal colonization by toxigenic strains of *Staphylococcus aureus*, particularly those producing the toxic shock syndrome toxin 1 (TSST-1), might be involved in some of these infant deaths [16]. Pyrogenic toxins are produced by many strains of *S. aureus*, and similar ones are found among some strains of group A β-haemolytic *Streptococcus pyogenes*. These are 'superantigens' that have significant physiological effects such as induction of fever (> 38 °C), possibly by direct action on the hypothalamus or by their induction of tumor necrosis factor (TNF) and interleukin-1 by monocytes [17]. *S. aureus* was isolated from a higher proportion of SIDS infants (41·3%), compared with a control population (28·3%) [18]. In this study we examined two factors that might enhance density of colonization by toxigenic staphylococci in infants during the period when they are at risk of SIDS: expression of the Lewis^a blood group antigen, and infection with respiratory syncytial virus (RSV).

Non-secretion, the genetically controlled inability of an individual to secrete the glycoprotein form of his/her ABO blood group antigens, is associated with susceptibility to a number of bacterial diseases and with asymptomatic carriage of some potentially pathogenic microorganisms [19], including group A streptococci [20]. The secretor gene also influences expression of the Lewis blood group antigens; non-secretors produce only Lewis^a; secretors produce Lewis^b predominantly but also variable amounts of Lewis^a. The amount of Lewis^a present in secretors depends on the relative activities of the fucosyl transferases coded for by the secretor gene and the Lewis gene. If the secretor fucosyl transferase acts first on the precursor chain, the Lewis enzyme can add fucose to the subterminal sugar of the chain to produce Lewis^b. If the Lewis fucosyl transferase acts on the precursor first, the Lewis^a molecule cannot act as a substrate for the secretor enzyme.

One of the hypotheses proposed to explain the increased proportion of non-secretors among carriers of some potentially pathogenic bacteria and yeasts is that the Lewis^a antigen is one of the host cell receptors for some microorganisms [19, 21, 22]. In this context, the reported high proportion of infants expressing Lewis antigens during the first year of life was of particular interest [23]. Among infants, the enzyme coded for by the secretor gene appears to be less efficient than that coded for by the Lewis gene. Although the majority of infants are secretors (75–80%), during the first year of life they will express easily detectable amounts of Lewis^a. The peak in the proportion of infants expressing Lewis^a is 2–4 months, similar to the highest incidence of cot deaths.

It has been reported that both natural and experimental viral infections enhance colonization by *S. aureus* [24]. RSV infected cells bind greater numbers of *Neisseria meningitidis* and type b *Haemophilus influenzae* compared with uninfected cells [25]. As RSV infects almost half of all infants by the age of 12 months and is most prevalent in the winter months [26], it is an obvious candidate for investigation of the role viruses might play in enhanced colonization by toxigenic bacteria.

These observations prompted the following questions:

1. Is Lewis[a] detected in SIDS infants?
2. Do strains of toxigenic *S. aureus* bind in greater numbers to non-secretor cells expressing larger amounts of Lewis[a]?
3. As some viral infections increase carriage of staphylococci, do tissue culture cells infected with RSV bind greater numbers of staphylococci than uninfected cells?

The results of these investigations are discussed in the context of epidemiological data on the role of microorganisms in SIDS.

SUBJECTS AND METHODS

Respiratory tract secretions (89) obtained from SIDS infants during autopsy were provided by Dr J. N. Inglis and Dr P. Molyneaux (Regional Virus Laboratory, City Hospital, Edinburgh) and by Dr A. Gibson (Royal Hospital for Sick Children, Glasgow). These were examined for presence of H [27] and Lewis antigens [28] by enzyme-linked immunoassays (ELISA) described previously.

S. aureus strains NCTC 10652, NCTC 10654, NCTC 10655, NCTC 10656, NCTC 10657, NCTC 11965, and NCTC 8532 were obtained from Dr A. Wieneke Central Public Health Laboratory, Colindale. Strains 40654 and 41206 were kindly provided by Dr J. Medcraft, Public Health Laboratory Service, Department of Microbiology, Reading, Berkshire. The strains and the toxins they produced are listed in Table 1. The bacteria were grown on nutrient agar or for some experiments on blood agar to examine the effect of medium on binding to epithelial cells.

Buccal epithelial cells (BEC) were obtained from pairs of healthy secretor and non-secretor donors matched as closely as possible for ABO blood group, age and sex. ABO groups of the donors were determined by slide agglutination of erythrocytes with monoclonal anti-A and anti-B antibodies (Scottish National Blood Transfusion Service). Secretor status was determined from saliva by haemagglutination inhibition assays [29], initially confirmed by tube agglutination with monoclonal anti-Lewis[a] or anti-Lewis[b] antibodies (Scottish National Blood Transfusion Service) and later by ELISAs for H and Lewis antigens.

BEC were collected by rubbing the inside of the cheeks with cotton swabs. To remove the cells, the swabs were agitated in 10 ml phosphate-buffered saline (PBS) (pH 7·2). They were washed twice in PBS in a Sorvall RT 6000 centrifuge (300 g for 10 min) and the concentration adjusted to 2×10^5 ml^{-1} after determination of the number of cells microscopically in an improved Neubauer haemocytometer.

Bacteria were labelled with fluorescein isothiocynate (FITC) by a modification of the method of Wright and Jong [30]. A heavy suspension of bacteria in PBS was prepared and washed twice by centrifugation at 1000 g for 20 min. The bacterial pellet was resuspended in 4 ml of a freshly prepared 0·04% solution of FITC in sodium carbonate (0·05 M) and sodium chloride (0·1 M) (pH 9·2). The mixture was incubated at 37 °C for 20 min and washed twice with PBS. The pellet was

resuspended in PBS and filtered through a Millipore membrane filter (5 μm pore size) to remove clumps of bacteria.

The bacterial concentration was determined by measuring the optical density of the suspension at 540 nm. The linear relationship between optical density and total count was determined for each strain.

Binding of bacteria to BEC of secretors and non-secretors

BEC (200 μl) were mixed with 200 μl of the FITC-labelled bacteria at the following ratios of bacteria per cell: 80:1, 160:1 and 320:1. The mixtures were incubated at 37 °C for 30 min with gentle shaking in an orbital incubator (Gallenkamp). The cells were washed twice with PBS by centrifugation at 300 g for 10 min to remove unattached bacteria. The samples were resuspended in 300 μl 1% buffered paraformaldehyde (Sigma) and stored in the dark at 4 °C until analysed.

Analysis of binding assays

Each sample was analysed by flow cytometry with an EPICS-C (Coulter Electronics, Luton, UK) equipped with a 5 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 bitmap. The bitmap included the main population of the cells and excluded debris and clumps from further analysis. The percentage of cells with fluorescence greater than background level was recorded on a one-parameter histogram which measured fluorescence on a logarithmic scale. The mean fluorescence channel values for positive cells were obtained from a one-parameter histogram measuring fluorescence on a linear scale. The results were analysed by Immunoanalysis (Coulter). a computer programme that subtracts the values of the control population from the test population at each channel of the two histograms. The binding index (BI) of each sample was calculated by multiplying the percentage of fluorescent cells by the mean fluorescence channel value.

Inhibition of bacterial binding

BEC obtained from secretors or non-secretors were treated with monoclonal anti-Lewis^a (LM112/161.8, kindly supplied by Dr R. H. Fraser, Law Hospital. Carluke) or monoclonal antibody to the type 1 precursor chain (anti-precursor type 1) (clone no 619/102, Russel Fine Chemicals, Chester) for 60 min at 37 °C. After washing twice by centrifugation at 300 g for 10 min, the attachment assay was performed and analysed as described above.

Binding of bacteria to HEp-2 cells

Methods for assessment of binding of *S. aureus* strains NCTC 8532 and NCTC 10655 to uninfected and RSV-infected HEp-2 tissue culture cells and analysis of results were as described for *H. influenzae* [25].

Detection of Lewis^a and type 1 precursor antigens on BEC of secretor and non-secretor donors

Buccal epithelial cells (200 μl, 2×10^5 ml^{-1}) from 7 secretors, 7 non-secretors and 3 secretor donors whose erythrocytes were not agglutinated by either anti-Lewis^a

or anti-Lewis^b monoclonal antibodies (Scottish National Blood Transfusion Service) (Lewis-negative individuals) were incubated with 200 μl of monoclonal antibodies to Lewis^a (1/5) or precursor type 1 (1/10) for 60 min at 37 °C. The cells were washed twice with PBS at 300 g for 10 min and incubated with 200 μl rabbit anti-mouse IgM conjugated with FITC (1/200) (Sigma). The FITC-labelled antibody was also added to 200 μl of cells which had not been treated with the first antibody as a control. After 30 min incubation in an orbital shaker at 37 °C, the cells were washed twice with PBS. They were resuspended in 300 μl 1% buffered paraformaldehyde and stored in the dark at 4 °C until analysed. The cells were analysed on an EPICS-C flow cytometer as described above and the binding indices calculated as before.

Statistical methods

All analyses were carried out on the logarithms of the binding indices which conformed more closely to a normal distribution than the raw values. Differences between groups were tested by paired or unpaired *t* tests as appropriate, and confidence limits for the mean values in one group were expressed as a percentage of those in the other by taking antilogarithms. Analysis of covariance was used to test whether binding levels were associated with the amount of anti-Lewis^a in secretors and non-secretors tested on different days, while the association in a similar experiment with anti-precursor on a single day was tested by Pearson correlation. Three-factor analysis of variance was used to test whether RSV infection was related to binding of HEP2 cells at two different concentrations of bacteria per cell.

RESULTS

Detection of Lewis^a antigen in body fluids of SIDS infants

Lewis^a antigen was detected in 63/89 (71%) of the specimens from SIDS infants.

Binding of S. aureus *to epithelial cells of secretors and non-secretors*

The results of the binding assays are presented in Table 1 as the 95% confidence limits for binding of the bacteria to non-secretor cells expressed as a percentage of binding to cells from matched secretors. Confidence intervals for which both values are above 100 indicated significant evidence that cells from non-secretors bound more staphylococci than cells from secretors.

There was no significant difference in binding to cells of non-secretors compared with binding to cells of secretors observed for the non-toxigenic strain NCTC 8532 or five of the toxin producing strains: NCTC 41206, NCTC 40654, NCTC 10654, NCTC 10656 and NCTC 10652. Three of the toxigenic isolates NCTC 10655, NCTC 11965 and NCTC 10657, showed higher binding to cells from non-secretors compared with binding to cells from secretors (Table 1). There was significantly higher binding of NCTC 10655 and NCTC 10657 to non-secretor cells at all ratios of bacteria : cells tested, while for strain NCTC 11965 there was significantly higher binding to cells of non-secretors only with the lowest ratio of bacteria.

Table 1. *95% confidence limits for binding of* Staphylococcus aureus *to non-secretor cells expressed as a percentage of binding to cells from matched secretors*

Strain no.	Toxin produced	Bacteria per cell 80	160	320
NCTC 8532	—	66–145	69–146	31–182
NCTC 10652	A	63–198	63–227	50–301
NCTC 10654	B	67–153	75–185	75–206
NCTC 10655	C	138–228†	107–249*	107–237*
NCTC 10656	D	61–136	79–175	67–178
NCTC 10657	A, B	110–197*	101–161*	117–165†
NCTC 11965	A, TSST-1	102–201*	92–192	81–203
40654	A	82–182	54–189	91–222
41206	B	74–147	75–172	74–158

* $P < 0{\cdot}05$; † $P < 0{\cdot}01$.

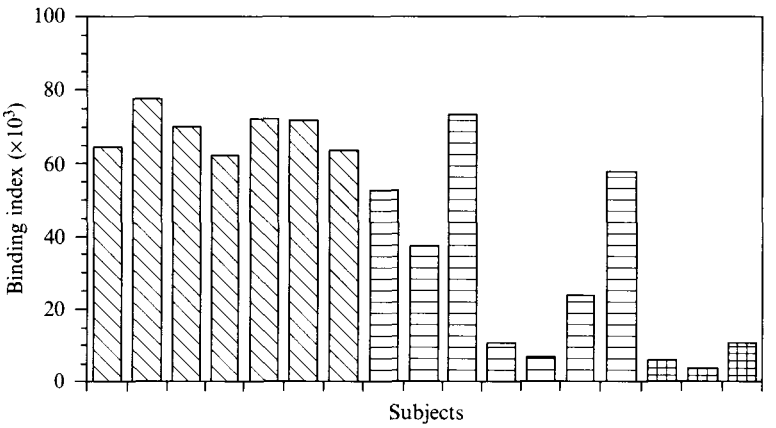

Fig. 1. Binding of monoclonal anti-Lewis^a antibody to epithelial cells of non-secretors (▨), secretors (▤) and Lewis^{a-b} (▦) secretors.

Binding of anti-Lewis^a or anti-precursor antibody to epithelial cells of secretors and non-secretors

The epithelial cells from the three individuals whose erythrocytes were not agglutinated by either anti-Lewis^a or anti-Lewis^b (Lewis-negative) bound consistantly low levels of anti-Lewis^a. Cells from non-secretors bound high levels of the antibody. While the mean binding index for secretors (36707) was approximately half that for non-secretors (79148), there was considerable variation in the amount of antibody bound by individual secretors (Fig. 1). There was no consistent pattern of binding of the anti-precursor monoclonal to cells of secretors, non-secretors or Lewis-negative individuals.

Inhibition of bacterial binding

Binding of *S. aureus* NCTC 10655 to BEC obtained from 7 secretors of 7 non-secretors treated with anti-Lewis^a antibody was significantly lower compared with binding of the bacteria to untreated cells from the same donors ($t = -4{\cdot}46$.

D.F. = 13, $P < 0{\cdot}001$, 95% CI 55–81%). A similar pattern was observed for binding of NCTC 10655 to cells of 7 non-secretors and 10 secretors treated with monoclonal anti-precursor type 1 compared with untreated cells ($t = 8{\cdot}19$, D.F. = 16, $P < 0{\cdot}001$, 95% CI 58–72%).

Binding of NCTC 8532 to the cells from the same donors treated with anti-Lewis^a antibody was significantly lower compared with binding of the bacteria to untreated cells ($t = -4{\cdot}97$, D.F. = 11, $P < 0{\cdot}001$, 95% CI 43–72%). Inhibition of NCTC 8532 with anti-precursor was not examined because of the limited amount of antibody.

Binding of bacteria with reference to detection of Lewis^a *antigen or precursor antigen*

Binding of *S. aureus* NCTC 10655 was correlated with the amount of monoclonal anti-Lewis^a antibody detected on the BEC of secretors and non-secretors (D.F. = 12, $t = 5{\cdot}03$, $P < 0{\cdot}001$). A similar pattern was found with NCTC 8532 ($t = 4{\cdot}24$, D.F. = 12, $P < 0{\cdot}001$). There was no significant correlation between the amount of anti-precursor detected on epithelial cells and binding of NCTC 10655 or NCTC 8532.

Binding of S. aureus *to RSV infected cells*

Strains NCTC 8532 and NCTC 10655 were assessed in seven experiments to compare binding of bacteria to HEp-2 cells and RSV-infected HEp-2 cells. For both isolates the binding indices were greater for the virus infected cells: NCTC 8532 ($P < 0{\cdot}001$, 95% CI 117–155%), NCTC 10655 ($P < 0{\cdot}05$, 95% CI 109–155%).

DISCUSSION

The results provided information for the three questions to be investigated in this study. Lewis^a antigen was detected in secretions of 71% of SIDS infants examined. There was increased binding of some toxigenic strains of *S. aureus* to epithelial cells from non-secretor donors; and RSV-infected cells bound more staphylococci than uninfected cells.

The lack of relevant control populations is a major criticism of epidemiological studies of cot deaths. There is, however, evidence from several surveys that *S. aureus* is isolated from the nasopharynx of approximately 35–40% of SIDS infants [16, 18, 31] compared with 28% from healthy infants [18]. TSST-1 has been demonstrated in the renal tubular cells of some SIDS infants but not in a comparison group of infants who were not cot death victims [32]. The pyrogenic toxins of *S. aureus* and group A streptococci are powerful 'superantigens' that can induce release of cytokines that might trigger a cascade of events leading to shock or damage to the respiratory or cardiac systems [17].

Because density of colonization might be an important consideration in the hypothesis that these toxins play a role in some cot deaths, factors suggested to enhance colonization were assessed in the study reported here. Non-secretors of ABO blood group antigens are over-represented among carriers of group A streptococci [20]. We tested the hypothesis that epithelial cells from non-secretor donors might bind greater numbers of toxigenic staphylococci than cells from

secretors. Three of the eight toxigenic strains, including one producing TSST-1. did show significantly higher binding to non-secretor cells.

Attachment of strain NCTC 10655 which bound in greater numbers to non-secretor cells was significantly inhibited by pretreatment of the host cells with either monoclonal anti-Lewis^a antibody or monoclonal anti-precursor type-1 antibody. Anti-precursor type 1 antibody was examined because it has been suggested that bacteria might bind to the precursor portion of the ABO or Lewis blood group antigens; and glycosylation of the precursor to H or Lewis antigens decreased the accessibility of the binding site [33]. There was a significant correlation between binding of NCTC 10655 and binding of anti-Lewis^a antibody to the host cell, but there was no correlation between binding of this strain with the amount of anti-precursor antibody detected on the cells. As the anti-precursor monoclonal was prepared by immunization with Lewis^a antigen, the inhibition of bacterial binding observed following pretreatment of cells with anti-precursor might be due to cross-reactivity with Lewis^a although none was indicated by the manufacturer.

Although NCTC 8532 did not bind in greater numbers to non-secretor cells compared with secretor cells, its binding was inhibited by treatment of the epithelial cells with anti-Lewis^a and there was a significant correlation between its binding to the donor cells and the amount of anti-Lewis^a antibody bound by the cells. From Fig. 1, it is apparent that some secretors bind as much anti-Lewis^a as most non-secretors. For the experiments comparing the binding of staphylococci to cells from secretors and non-secretors, the donors were matched only according to sex, age and ABO blood group; and the same pairs of secretor/non-secretor donors were not available for each experiment. At that time there was no information on the amount of anti-Lewis^a bound by the cells of individual donors.

Early experimental work suggested that there are multiple receptors for staphylococci on human epithelial cells [34]. If Lewis^a is one of the receptors for some strains of staphylococci or group A streptococci capable of producing pyrogenic toxins, the expression of this antigen among young infants might enhance their colonization by these bacteria. Viral infections have been shown to enhance carriage of staphylococci [24]; and disease due to TSST-1 has been reported to follow influenza or ''flu-like' illnesses [35]. Binding of both meningococci and type b *H. influenzae* to tissue culture cells derived from human epithelium (HEp-2) was substantially increased if the cells were infected with RSV [25]; and, in this study, both the toxigenic and non-toxigenic strain tested bound in greater numbers to the RSV infected cells. If there are similar interactions *in vivo*, infection with RSV common during the first year of life might also enhance colonization by these bacteria.

These studies suggest two factors that might enhance staphylococcal colonization of young infants; however, all infants who become colonized do not become SIDS victims. The following hypothesis is an attempt to correlate our laboratory findings with factors identified in epidemiological studies of cot deaths in New Zealand: mother's smoking, prone sleeping position and bottle feeding [4].

Mother's smoking which was associated with carriage of meningococci among children [36] might increase the risk of initial exposure to potentially pathogenic bacteria in two ways. First, epithelial cells from smokers have been shown to bind

greater numbers of staphylococci than cells from non-smokers [37]. Smoking also enhances susceptibility to respiratory viral infections; and epithelial cells from individuals with natural or experimental viral infections bound more staphylococci compared with those from individuals who were not infected with a virus [37].

In addition to the two factors examined in the present study, others that might enhance density of colonization of infants by staphylococci include passive exposure to cigarette smoke which decreases mucocilary clearance. Infants in the age range in which the peak of SIDS occurs have little or no mucosal or systemic immunity to staphylococci or to the pyrogenic toxins. The effect of breast feeding on carriage of staphylococci or susceptibility to these toxins is unknown; however, in studies on the possible role of toxigenic clostridia in cot deaths, *Clostridium difficile* was isolated from significantly fewer breast fed infants compared with formula fed infants, and *Cl. difficile* toxin was detected only in the faeces of formula-fed infants [38].

The pyrogenic toxins are produced between 37–40 °C; and they are produced in greater quantities at the higher temperatures [17]. Three factors associated with SIDS might increase the infant's temperature and thereby enhance toxin production: respiratory infection; overwrapping with clothing or bedding; and the prone sleeping position. In the prone sleeping position, infants lose less heat than in the supine position. [39–41]. The synergistic effect between increased temperature and increased toxin production might account for the high temperatures recorded for some of these infants at autopsy. Among 24 infants who died suddenly and whose rectal temperatures were measured immediately before refrigeration, 10 had temperatures > 38 °C and 5 were > 40 °C [42]. In addition to the heat-shock hypothesis of cot death, the increased release of IL-1 induced by the toxins might contribute to prolonged sleep apnoea as suggested by Guntheroth [43].

Studies are underway to assess levels of Lewis[a] in saliva of infants, exposure to cigarette smoke and presence of RSV on carriage of toxigenic staphylococci and streptococci. Results of these studies should provide evidence to refute or confirm the scheme proposed.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Scottish Cot Death Trust. M.W.R. is a recipient of a postgraduate studentship from the government of Pakistan and an Overseas Research Scholarship (ORS) award.

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