RESEARCH ARTICLE

The effect of *Staphylococcus aureus* carriage in late pregnancy on antibody levels to staphylococcal toxins in cord blood and breast milk

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Abstract

We investigated the effect of carriage of *Staphylococcus aureus* in the later stages of pregnancy on levels of antibody specific to the *S. aureus* toxins, staphylococcal enterotoxin B (SEB), staphylococcal enterotoxin C (SEC) and toxic shock syndrome toxin-1 (TSST-1), in cord blood and breast milk and also explored the relationship between levels of antibody in antenatal serum and cord blood. Nasopharyngeal swabs and stool samples were collected on two occasions, from 96 women, during the last 6 weeks of pregnancy. Samples were cultured and *S. aureus* isolates were identified. Antenatal and cord blood samples from the same women and their infants were analysed for IgG antibody to SEB, SEC and TSST-1 by enzyme-linked immunosorbent assay. Breast milk samples were analysed for IgA antibody to the same toxins. We found that *S. aureus* carriage in pregnancy is common and exposure to a toxin-producing isolate boosts immunity. Over 89% of women and infants have some protective antibody to the toxins, and antitoxin IgG levels are higher in cord blood samples compared with antenatal samples. Levels of cord blood IgG and breast milk IgA specific for the staphylococcal toxins vary. Some infants lack protection and could be at risk of toxin-induced disease.

Introduction

It has been suggested that toxins produced by common nasopharyngeal bacteria can act in synergy to cause sudden death in infants with low levels of circulating immunoglobulins (Morris *et al.*, 1987). There is considerable support for this hypothesis from theoretical, experimental and epidemiological studies (Morris, 1999), which show that the hypothesis can explain the key epidemiological features of sudden infant death syndrome (SIDS), including the age distribution, the winter excess of cases and the association with prone sleeping and exposure to tobacco smoke.

A candidate organism for a pathogenic role in SIDS is the bacterium *Staphylococcus aureus*. *Staphylococcus aureus* has been identified as the most common colonizer of the nasopharynx in the early months of life when SIDS incidence reaches a peak (Blackwell *et al.*, 1999; Harrison *et al.*, 1999a). Pyrogenic toxins produced by *S. aureus* have been implicated in SIDS cases (Newbould *et al.*, 1989; Telford *et al.*, 1989; Malam *et al.*, 1992; Murrell *et al.*, 1993; Zorgani *et al.*, 1999), with one study detecting the toxins in the tissues of over 50% of SIDS cases from three different countries (Zorgani *et al.*, 1999).

A study to determine the presence of toxin-specific antibodies in sera found that a significantly smaller proportion of sera from SIDS cases tested positive for IgG to a number of bacterial toxins, including staphylococcal enterotoxin B (SEB), than an age-matched comparison group (Siarakas *et al.*, 1999). Our previous research has revealed that specific maternal IgG to staphylococcal enterotoxin C (SEC) and toxic shock syndrome toxin (TSST-1) is markedly variable in cord blood samples, and in some cases, the samples tested negative (Harrison *et al.*, 2004).

Transplacental transfer of IgG is upregulated in the last trimester of pregnancy (Holt & Jones, 2000) and factors that influence the production of specific maternal IgG during this period should be reflected in cord blood levels. Antibodies contained in breast milk are also a response to a woman's exposure to specific antigens. The aims of this study were to establish whether carriage of toxigenic strains

of *S. aureus* in the later stages of pregnancy influenced the levels of antibody specific to the *S. aureus* toxins (SEB, SEC and TSST-1) in cord blood and breast milk and to explore the relationship between the levels of antibody in antenatal blood and cord blood.

Materials and methods

Population studied

Pregnant women were recruited to the study at antenatal classes. The study had ethical approval from the Preston, Chorley and South Ribble Local Research Ethics Committee and informed consent was obtained from the women.

Sample collection and preparation

Cord blood samples were collected by the midwives. The samples were separated and the serum was stored at −70 °C. Blood samples taken during the second trimester, as part of the normal antenatal procedure, were processed and stored in the same manner as the cord blood samples. Women were visited at home on two occasions during the last 6 weeks of pregnancy. Before each visit, participants collected a stool sample in a sterile container and stored it in a refrigerator. A nasopharyngeal swab was taken at each visit.

Isolation and identification of *S. aureus*

Staphylococcus aureus was isolated and identified from nasopharyngeal swabs using standard clinical laboratory methods. Stool samples were diluted serially in 10-fold steps in peptone water and cultured aerobically on *Staphylococcus* medium no 110 (Oxoid) at 37 °C for 2 days. Colonies of different morphology were enumerated and subcultured on blood agar. Standard methods were used to identify *S. aureus* isolates. All *S. aureus* isolates were stored at −70 °C for further tests.

Detection of IgG to staphylococcal pyrogenic toxins in antenatal and cord blood

Serum samples were examined for IgG antibody to SEB, SEC and TSST-1 by an enzyme-linked immunosorbent assay (ELISA). A modification of the protocol of Al Madani *et al.* (1999) was used. Test and control wells were coated with 100 μL of SEB, SEC or TSST-1 at 1 μg mL^{-1} in carbonate buffer (Sigma). Standard wells were coated with 100 μL of serial dilutions of human IgG (Sigma); concentrations ranged from 5 to 10 000 ng mL^{-1}. Plates were incubated overnight at 4 °C. The wells were then emptied and washed four times with washing buffer [0.05% (v/v) Tween-20 in phosphate-buffered saline (PBST)]. The plates were blocked for 1 h at 37 °C with a buffer containing 5% (w/v) skimmed-milk powder in PBST. Plates were washed four times. Serum samples were diluted in buffer [2.5% (w/v) skimmed-milk powder in PBST] and 100 μL was loaded into each test well. The same buffer (100 μL) was added to standard and control wells. Plates were then incubated for 1 h at 37 °C. After four washes, 100 μL of anti-human IgG, conjugated to horseradish peroxidase (HRP) (Sigma) diluted in buffer [2.5% (w/v) skimmed-milk powder in PBST], was added to the test and standard wells. HRP-labelled sheep anti-SEB (100 μL), HRP-labelled sheep anti-SEC (100 μL) or HRP-labelled sheep anti-TSST-1, diluted 1 in 300 in buffer [2.5% (w/v) skimmed-milk powder in PBST], was added to the appropriate control wells. The plates were incubated for 1 h at 37 °C. After three washes, 100 μL of the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma) was added to each well. The substrate contained 1 mg TMB in 10 mL of 0.05 M phosphate–citrate buffer (pH 5.0) and was activated immediately before use by adding 2 μL of 30% (v/v) H_2O_2. The reaction was stopped after 15 min by the addition of 100 μL 2 M H_2SO_4 and the absorbance was read at 450 nm with a plate reader (Organon Teknika).

Detection of IgA to staphylococcal pyrogenic toxins in breast milk

A modification of the protocol of Gordon *et al.* (1999) was used. Test and control wells of microtitre plates were coated with 100 μL of SEB, SEC or TSST-1 at 1 μg mL^{-1} in carbonate buffer. Standard wells were coated with 100 μL of serial dilutions of a standard human IgA derived from colostrum (Sigma); concentrations ranged from 5 to 10 000 ng mL^{-1}. Plates were incubated overnight at 4 °C and then washed four times. The plates were blocked for 1 h at 37 °C. After three washes, 100 μL samples of breast milk diluted in blocking buffer were loaded into the appropriate test wells. Blocking buffer (100 μL) was added to standard and control wells. Plates were incubated for 1 h at 37 °C. After four washes, 100 μL of HRP-labelled goat anti-human IgA (Calbiochem) diluted in blocking buffer was added to the test and standard wells and 100 μL of HRP-labelled sheep anti-SEB, HRP-labelled sheep anti-SEC or HRP-labelled sheep anti-TSST-1, diluted 1 in 300 in blocking buffer, was added to the appropriate control wells. The plates were incubated at 37 °C for 1 h. The experiment then continued as described previously.

Detection of toxin production by *S. aureus* isolates

Staphylococcus aureus isolates were cultured using the cellophane over agar technique (Newbould *et al.*, 1989) and the resulting supernatant samples were analysed by ELISA. Test and control wells were coated with 100 μL of anti-SEB, anti-SEC or anti-TSST-1 (Toxin Technology), diluted 1 : 1000 in carbonate buffer (Sigma). Plates were incubated overnight

at 4 °C and then emptied and washed four times with washing buffer. The plates were blocked with blocking buffer containing 2.5% (v/v) fish gelatin in PBST. Plates were washed four times. Supernatant samples and controls were Q2 diluted in PBS and 100 µL was loaded into each test well. Controls included purified SEB, purified SEC, purified TSST-1 (Toxin Technology, Sarasota, FL) and supernatant prepared from *S. aureus* isolates previously tested for the production of SEB, SEC and TSST-1 (Colindale Public Health Laboratory). Plates were then incubated for 1 h at 37 °C. After four washes, 100 µL of the corresponding biotinylated antitoxin (Toxin Technology) diluted 1 in 2000 in blocking buffer was added to the test and control wells. The plates were incubated for 1 h at 37 °C. After four washes, 100 µL of extravidin peroxidase (Sigma) diluted 1 : 2000 was added to each well and plates were incubated for 30 min at 37 °C. The experiment then continued as described previously.

Statistical analyses

Results were analysed using SPSS software (version 13.0). Nonparametric tests were used for data that were not normally distributed. These data were not normalized by log transformation because many nil values were detected. Spearman's correlation coefficient was used to assess the association between levels of antibody in antenatal and cord blood samples. Antitoxin levels were compared between antenatal and cord blood samples using the the Wilcoxon matched samples test. χ^2 Test, Mann–Whitney *U*-test and Kruskal–Wallis test were used to assess antitoxin levels in relation to other variables. Comparison of data is presented in Figs 1, 3 and 4 as box and whisker plots. The box represents the 25th–75th percentiles, and the median is represented by the line within the box. The whiskers represent the 5th–95th percentiles and the circles and stars depict outliers for each group.

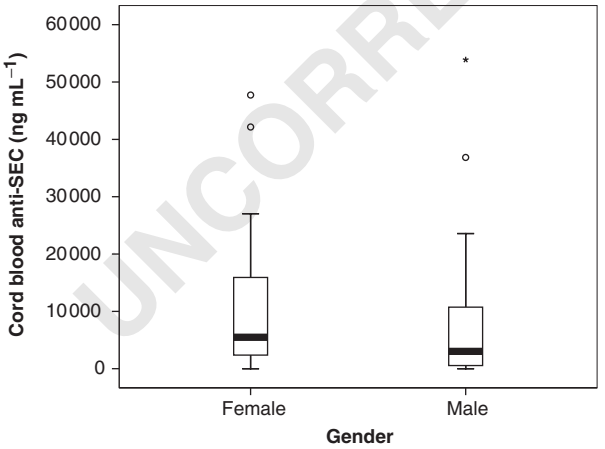

Fig. 1. Levels of anti-SEC IgG in the cord blood of male and female infants.

Results

Population characteristics

Ninety-six pregnant women were enrolled into the study. The mean gestation period was 281 days (range 257–296, SD ± 9.5). The average maternal age at birth was 31 years (range 18–42, SD ± 4.4). There was no significant association between length of gestation and maternal age.

Detection of IgG to staphylococcal pyrogenic toxins in blood samples

Ninety-two antenatal blood samples were available to test for the presence of IgG to SEB, SEC and TSST-1. The median level of antibody to SEB was 3799 ng mL^{-1} (mean: 6204 ng mL^{-1}; range: 0–35 588 ng mL^{-1}; SD ± 6846). Antibody levels to SEC ranged from 0 to 21 499 ng mL^{-1} (median: 5045 ng mL^{-1}; mean: 6248 ng mL^{-1}; SD ± 5858) and those for TSST-1 ranged from 0 to 16 412 ng mL^{-1} (median: 943 ng mL^{-1}; mean: 1674 ng mL^{-1}; SD ± 2333). Eight women (8.7%) had no detectable IgG to SEB, six (6.5%) had no detectable IgG to SEC and 10 (10.9%) had no detectable IgG to TSST-1.

Ninety-six cord blood samples were collected and tested for the presence of IgG to SEB, SEC and TSST-1. The median level of antibody to SEB was 4608 ng mL^{-1} (mean: 8987 ng mL^{-1}; range: 0–46 605 ng mL^{-1}; SD ± 11 190). Antibody levels to SEC ranged from 0 to 53 886 ng mL^{-1} (median: 4001 ng mL^{-1}; mean: 8970 ng mL^{-1}; SD ± 10 861) and those for TSST-1 ranged from 0 to 14 154 ng mL^{-1} (median: 1170 ng mL^{-1}; mean: 2512 ng mL^{-1}; SD ± 3084). Seven samples (7.3%) had no detectable IgG to SEB, three (3.1%) had no detectable IgG to SEC and seven (7.3%) had no detectable IgG to TSST-1. There was no significant association between maternal age and antibody levels or between length of gestation and antibody levels. However, 84% of pregnant women over the age of 30, but only 68% of pregnant women under the age of 30 had antibody to all three toxins (difference not significant).

Male infants ($n = 51$) had significantly lower levels of cord blood IgG to SEC than female infants ($n = 45$) ($P = 0.05$, Mann–Whitney *U*-test, Fig. 1). For anti-SEC IgG, the mean level in males was 7440 ng mL^{-1} and in females the mean level was 10 705 ng mL^{-1}. No significant differences were found between male and female infants for cord blood IgG levels to SEB or TSST-1.

Antenatal anti-SEB IgG correlated positively with cord blood anti-SEB IgG ($\rho = 0.815$, $P < 0.01$). Antenatal anti-SEC IgG correlated positively with cord blood anti-SEC IgG ($\rho = 0.856$, $P < 0.01$, Fig. 2). Antenatal anti-TSST-1 IgG

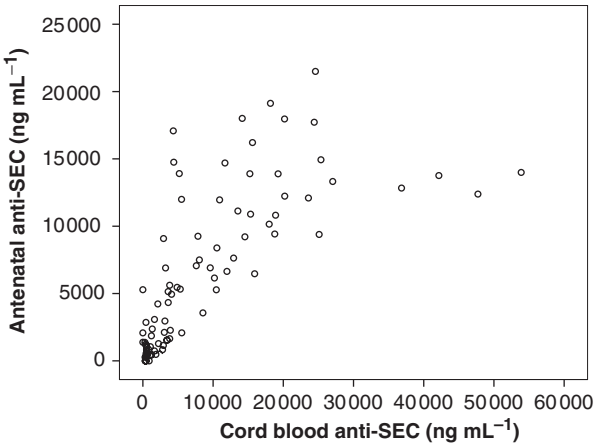

Fig. 2. Correlation between levels of IgG to SEC in antenatal and cord blood.

correlated positively with cord blood anti-TSST-1 IgG ($\rho = 0.721$, $P < 0.01$). Paired sample tests showed that specific IgG levels to each of the toxins were significantly greater in the cord blood samples than in the antenatal blood samples ($P < 0.01$).

Detection of IgA to staphylococcal pyrogenic toxins in breast milk

Breast milk samples were collected from 83 women in the first 7 days following the birth of their baby. The median level of antibody to SEB was 201 ng mL^{-1} (mean: 423 ng mL^{-1}; range: 0–10 000 ng mL^{-1}; SD $\pm$ 1135). Antibody levels to SEC ranged from 0 to 5370 ng mL^{-1} (median: 154 ng mL^{-1}; mean: 402 ng mL^{-1}; SD $\pm$ 787) and those for TSST-1 ranged from 0 to 3050 ng mL^{-1} (median: 0 ng mL^{-1}; mean: 146 ng mL^{-1}; SD $\pm$ 472).

Breast milk anti-SEB IgA showed a significant positive correlation with cord blood anti-SEB IgG ($\rho = 0.233$, $P < 0.05$). Breast milk anti-SEC-1 IgA correlated positively with cord blood anti-SEC-1 IgG ($\rho = 0.321$, $P < 0.01$). Breast milk anti-TSST-1 IgA showed no significant association with cord blood anti-TSST-1 IgG.

Carriage of *S. aureus* in pregnant women

Of the 96 women, 29 (30.2%) were colonized with *S. aureus*. Sixty-seven women (69.8%) had negative nasopharyngeal and stool cultures, four (4.2%) had positive nasopharyngeal and stool cultures, 16 (16.7%) were nasopharyngeal carriers only and nine (9.4%) were stool carriers only. Of the women with positive nasopharyngeal cultures, 50% were colonized with *S. aureus* on both sampling occasions. Of the women with positive stool cultures, 23% were colonized with *S. aureus* on both sampling occasions. The mean *S. aureus* count per gram of faeces was 1.99×10^4, (range: 1.2×10^3–1.2×10^5; SD $\pm$ 3.1×10^4).

Colonization with *S. aureus* was not significantly associated with IgG levels to the *S. aureus* toxins in antenatal or cord blood or with IgA levels to the *S. aureus* toxins in breast milk. There was no relationship between carriage of *S. aureus* and maternal age.

Detection of toxin production by *S. aureus* isolates

Staphylococcus aureus isolates were tested for the production of SEB, SEC and TSST-1. Thirteen per cent of isolates produced SEB, 13% produced SEC and 24% produced TSST-1. Some isolates produced more than one toxin.

Women colonized with an isolate producing the toxin SEB were significantly more likely to have greater levels of anti-SEB IgG in cord blood compared with women not carrying an SEB-producing isolate ($P < 0.05$, Mann–Whitney *U*-test, Fig. 3). This trend was also found for SEC and TSST-1 but was not statistically significant.

Breast milk anti-SEC IgA levels were significantly greater if the mother was colonized with a *S. aureus* isolate producing SEC ($P < 0.05$). Mothers colonized with a TSST-1-producing isolate had greater levels of anti-TSST-1 IgA ($P = 0.001$, Fig. 4). This association was also seen for SEB/anti-SEB IgA but was not statistically significant ($P = 0.078$).

Discussion

An assumption of the common bacterial toxins hypothesis is that healthy infants will mount an effective immunological response when first exposed to specific toxin antigens, or they will succumb to their effects if they lack sufficient passive neutralizing antibody (Morris *et al.*, 1987). Passive

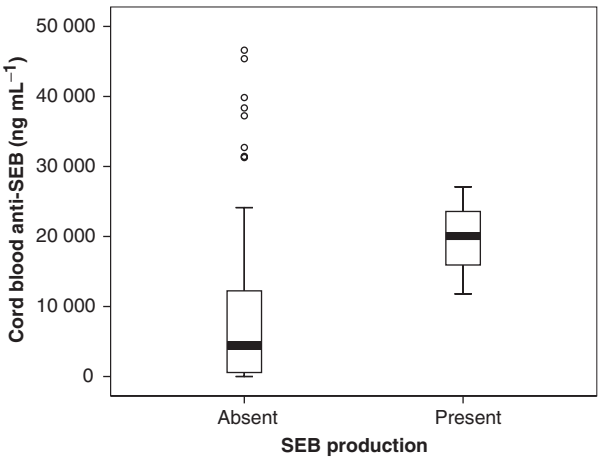

Fig. 3. Colonization with SEB-producing *Staphylococcus aureus* and its association with cord blood anti-SEB IgG levels.

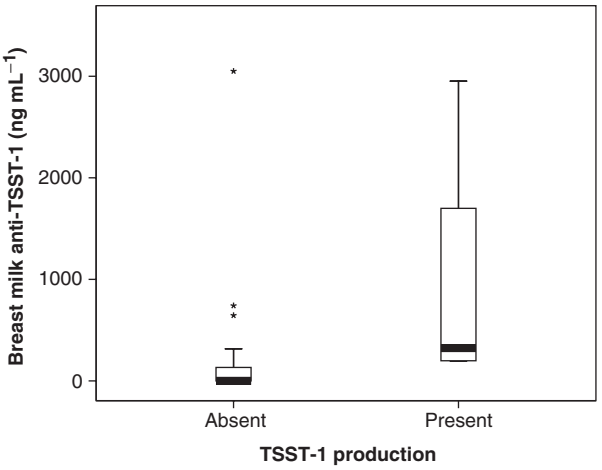

Fig. 4. Colonization with TSST-1-producing *Staphylococcus aureus* and its association with breast milk anti-TSST-1 IgA levels.

immunity is conferred on the infant by placental transfer of IgG from the mother. Thus, the mother's IgG profile will determine the infant's immunological status at birth.

In this study, analysis of antenatal serum detected IgG to SEB, SEC and TSST-1 in 92%, 93.5% and 89.1% of samples, respectively. Analysis of the cord sera detected IgG to SEB, SEC and TSST in 92.7%, 96.9% and 92.7% of samples, respectively. These proportions are very similar to those found in healthy adults from Europe and the United States (Notermans *et al.*, 1983; Vergeront *et al.*, 1983; Loch *et al.*, 1986; Parsonnet *et al.*, 2005). There are a small proportion of infants who are born with no detectable passive immunity to one or more of the toxins and could be at risk of death if, on exposure to the toxins, they fail to mount an effective immune response. A study by Siarakas *et al.* (1999), comparing serum antibody levels to SEB between SIDS cases and an age-matched comparison group of infants, found that a significantly smaller proportion of the SIDS cases had specific IgG and IgM. This implies that the SIDS infants had low levels of specific maternal IgG, or that on exposure to the toxin, antibody had been consumed and the infant had no time to mount an effective immune response. Factors associated with an increased risk of SIDS are also associated with the induction of bacterial pyrogenic toxins (Harrison *et al.*, 2004).

Cord blood samples from male infants born to women in this study had significantly lower levels of anti-SEC IgG compared with female infants (Fig. 1). Male infants are at an increased risk of SIDS (Leach *et al.*, 1999). Afro-American women are reported to have significantly lower serum levels of IgG to TSST-1 (Parsonnet *et al.*, 2005) compared with white American women and the SIDS rate for infants born to Afro-American women is twice that of white American women (Mathews & MacDorman, 2007). However, Japanese women also have lower levels of IgG to TSST-1 (Takahashi *et al.*, 2006) than European or white American women, but the SIDS rate in Japan is low (0.4/1000) (Fujita, 2002). Traditionally, Japanese infants have been placed to sleep in the supine position. At the end of 1998, only 20–30% of African American infants were placed to sleep supine. Sleeping supine greatly reduces the risk of SIDS (Leach *et al.*, 1999). Infants sleeping supine are less likely to be exposed to the factors that induce production of pyrogenic toxins in the upper respiratory tract (Blackwell *et al.*, 1999; Harrison *et al.*, 1999b; Molony *et al.*, 1999).

Transplacental transfer of IgG is upregulated in the last trimester of pregnancy (Holt & Jones, 2000), and in a full-term gestation, IgG concentration is usually higher in the cord serum than in the maternal serum (Wilmott-Pitcher *et al.*, 1980). Essery *et al.* (1999), analysing sera collected from pregnant women between 30 and 40 weeks gestation, found that levels of SEC- and TSST-1-specific IgG decreased with weeks of gestation but levels of SEB-specific IgG showed an increase. In our previous study (Harrison *et al.*, 2004), we reported that antibody levels to SEC and TSST-1 in cord blood samples showed a trend to increase with length of gestation. In this study, paired sample tests revealed that antibody levels to each of the toxins were significantly higher in cord sera than in the antenatal sera. The findings indicate active transport across the placenta, which might explain the decrease in the serum levels of some antitoxins in the latter stages of pregnancy reported by Essery *et al.* (1999).

Antenatal anti-SEC IgG showed a significant positive correlation with cord blood anti-SEC IgG (Fig. 2) and this was also the case for specific IgG to SEB and TSST-1. This relationship has also been found in a Japanese study (Takahashi *et al.*, 2006) and suggests that active immunity to the toxins is persistent or that low-level exposure to the antigens is common.

Humans become colonized with *S. aureus* soon after birth and some of the highest carriage rates are seen in the first few months of life (Blackwell *et al.*, 1999; Harrison *et al.*, 1999a; Peacock *et al.*, 2003; Lindberg *et al.*, 2004). Common sites of colonization include the upper respiratory tract and the intestinal tract. Carriage rates decline in infancy but both sites remain an ecological niche for *S. aureus* throughout life. Of the 96 women in this study, 30% were colonized with *S. aureus*, 21% had positive nasopharyngeal cultures and 14% had positive stool cultures. These carriage rates are similar to those demonstrated in previous studies (Peacock *et al.*, 2003; Harrison *et al.*, 2004; Lindberg *et al.*, 2004). Fifty per cent of the women had positive nasopharyngeal cultures on both sampling occasions and 23% had two positive stool cultures. Thirteen per cent of the isolates produced the toxin SEB, 13% produced SEC and 24% produced TSST-1. Intermittent carriage and undetected carriage of toxin-producing

S. aureus isolates from other body sites could explain as to why between 89% and 97% of the women have antibodies to one or more of the toxins even though, at any one time, carriage rates are only 30%.

There was a trend for women colonized with *S. aureus* toxin-producing isolates to have greater levels of antitoxin IgG in cord blood samples compared with women not colonized with a toxin-producing isolate. This relationship reached statistical significance ($P < 0.05$) for SEB.

Levels of passive immunity could be boosted through breast feeding. Women colonized with isolates producing TSST-1 had significantly greater levels of breast milk anti-TSST-1 IgA (Fig. 4, $P < 0.01$) and the same trend was shown for SEC/anti-SEC IgA ($P < 0.05$) and SEB/anti-SEB IgA ($P = 0.078$). Gordon *et al.* (1999) found that two-thirds of mothers colonized with *S. aureus* in the nose or throat had milk samples with levels of IgA to the toxins equal to or higher than the mean. There is conflicting evidence regarding the role of breast feeding in SIDS (Ford *et al.*, 1993; Fleming *et al.*, 1996) but some epidemiological studies report a protective, independent effect (Ford *et al.*, 1993; Alm *et al.*, 2002).

In conclusion, carriage of a *S. aureus* toxin-producing isolate in the later stages of pregnancy triggers the production of specific IgG and IgA, which in turn provides protection for the infant in the first few months of life. Experimental research is being carried out on the development of vaccines that induce IgG to the staphylococcal pyrogenic toxins (Cui *et al.*, 2005; Hu *et al.*, 2005; Narita *et al.*, 2008). Vaccination of pregnant women may be possible in the future and prevent infants being born with little or no passive immunity to the toxins.

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