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Anti-endotoxin antibodies in human milk: correlation with infection of the newborn

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A longitudinal study was performed to investigate the content of human colostrum and milk of antibodies against endotoxins of *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella minnesota* during the first 6 mo of lactation. The influence of the gestational age of the newborn and the prevalence of a systemic infection in the child on maternal antibody production were observed. Colostrum of mothers of term infants who had shown signs of systemic infection contained higher antibody concentrations compared to colostrum of mothers of healthy newborns. After the first week post partum, no difference in the milk's antibody content could be observed between these two groups. Antibody titres rose from 2 wk to 6 mo post partum ($p < 0.001$). Milk of mothers of preterm infants with signs of systemic infection contained higher antibody titres than milk of mothers of preterm infants without infection throughout the observation period. This difference reached statistical significance 3 wk after delivery ($p < 0.05$). The corrected endotoxin antibody levels against all tested antigens in milk of mothers of preterm infants with infection 6 mo post partum were 6 ± 3.5 times as high as 2 wk post partum.

Conclusions: **Breast milk contains anti-endotoxin antibodies. The particularly high levels of anti-endotoxin antibodies in cases of neonatal infection may present a special maternal protection for premature infants.**

Key words: *Endotoxin antibodies, human milk, neonatal infection, prematurity*

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The presence of immuno-protective factors in human milk supports the generally accepted opinion that breast milk is considered the best nourishment for infants (1, 2). Breast milk is especially rich in secretory IgA antibodies (3), which are directed against a wide variety of pathogens, including bacterial, viral and parasitic antigens (4–6). Secretory IgA antibodies are capable of neutralizing pathogens (7), preventing adherence of germs to the intestinal mucosa (8), inhibiting translocation of bacteria through the mucosa of the small intestinum (9), and assisting in phagocytosis of pathogens (10).

A marked number of studies have been done to prove and characterize these anti-infective properties of human milk (reviews in (5, 11, 12)). It has convincingly been demonstrated that the incidence and severity of intestinal and bronchial infections are reduced in breastfed infants (13–16).

Frequent causes of infection in the neonate and infant are Gram-negative rods, and so previous research has focused on the investigation of antibodies against Gram-negative bacteria. In the analysis of antibody titres against endotoxins in human milk, either a pool of antigens is used (17) or antibody levels against *E. coli* are compared to non-endotoxin antibodies (3, 18, 19). Although some investigators have used a broader spectrum of antigens, they have given less attention to the time course of antibody titres during lactation (20, 21).

In this study we aimed to analyse colostrum and milk samples of the first 6 mo of lactation from each mother. An enzyme-linked immunosorbent assay (ELISA) was used to study the milk's content of specific IgA antibodies against different bacterial endotoxins and to evaluate the time course of antibody titres during lactation. Our special interest focused on the differences in antibody content between milk of mothers of healthy preterm and term infants versus those who suffered from systemic infection.

Materials and methods

Milk samples

Up to seven colostrum and milk samples from 94 mothers were analysed. The women, who were admitted

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to the Department of Gynecology and Obstetrics of the University of Tübingen in 1995, showed no apparent signs of disease. All but one resided at the district of Tübingen and did not belong to a low socioeconomic group. Seventy-four of the women were primiparous and 17 infants were born by caesarean section. After maternal consent was obtained, milk samples were taken at the following dates post partum: 3 days, 1, 2, 3 and 6 wk, and at 3 and 6 mo. The milk was obtained by manual expression, or by the use of mechanical breast pumps (Medela AG, Baar, Switzerland), and collected in polypropylene tubes. After centrifugation (32,000 × *g* for 2 × 5 min) to remove the cellular and fatty components, the samples were stored at −20°C until analysed.

Grouping

Milk samples were prospectively divided into four groups:

	Term infants	Preterm infants
Median gestational age	40 wk	34 wk
Healthy newborns	$n = 65$	$n = 8$
Newborns with signs of systemic infection	$n = 9$	$n = 12$

Systemic bacterial infection was assessed by risk factors (premature rupture of membranes, prematurity, signs of maternal infection (23)), clinical symptoms of septicaemia, laboratory findings (C-reactive protein >1 mg/dl, leukopaenia, immature to total neutrophil ratio >0.2 and abnormally increased or decreased absolute numbers of neutrophils as defined by Manroe et al. (22)) and/or positive blood culture.

Determination of total IgA content

Total IgA content of the milk samples was determined turbidimetrically by using the Turbitime System (Behringwerke AG, Marburg, Germany). Recovery of added human IgA (Dako A/S, Glostrup, Denmark) was tested by augmenting the IgA concentration by 20, 50 and 100 mg/dl. Linearity of the method was controlled by assaying 10 different samples in dilution steps from 1:1 to 1:8 with 0.9% NaCl.

Antibody assay

Four antigens were chosen for the study: Lipopolysaccharide (LPS) of *Pseudomonas aeruginosa* serotype 10 and *Escherichia coli* serotype O111:B4, as well as lipid A of *E. coli* F583, Rd mutant and *Salmonella minnesota* Re595 (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany).

Coating of the ELISA plates (Maxisorp F8, Nunc A/S, Copenhagen, Denmark) was performed after a procedure described by Freudenberg et al. (24). Antigens were dissolved in a chloroform/ethanol (1:9, v/v) mixture. Phosphate-buffered saline pH 7.4 containing 0.25% Tween 20 (Serva Feinbiochemika GmbH, Heidelberg, Germany) was used to wash the plates.

For antibody quantitation, ELISA plates were incubated at 25°C for 120 min with the milk samples and a dilution series of a pool milk serum (25). After incubation with alkaline phosphatase-conjugated rabbit anti-human IgA antisera (Dako A/S, Glostrup, Denmark) and alkalization with substrate buffer (100 ml/l diethanolamine, 0.5 mmol/l magnesium chloride, pH 9.8), *P*-nitrophenylphosphate was added and the extinction measured at 405 nm (SLT Spectra ELISA reader, SLT Labinstruments, Crailsheim, Germany) by the endpoint method. A computer system was used to process the data (EasyFit, V 7.01, SLT Labinstruments). A standard curve with dilutions of a pool milk serum ranging from 1: 50 (400%) to 1: 3,200 (12.5%) was established in order to determine specific antibody levels of unknown milk samples. The median dilution of 1: 400 was arbitrarily defined as 100%. Sensitivity and cut-off for the negative controls were defined as concentration of the lowest standard + (3 × SEM).

Corrected antibody content

The corrected antibody content was calculated by dividing the specific IgA concentration according to the standard curve by the total IgA content of each sample.

Antibody specificity studies by inhibition

Milk sera were incubated with the corresponding antigens in the same concentration as used for optimal coating of the plates and 1/20 to 20× of this concentration. Inhibition was expressed as percent of the total activity of the unihibited sample.

Statistical analysis

The F-test was used to determine whether the variances of two random samples were equal or not. In case of equality of variances, Student's *t*-test was employed to compare mean values of the data, otherwise the Welch test was used. An analysis of variance was done to describe the trend of the antibody levels during lactation. The statistical analysis was performed with computer software JMP, V 3.1 (SAS Institute Inc., Cary, NC, USA).

Results

Assessment of systemic infection

Systemic infection was confirmed in 21 of the 94 infants. Only two presented with positive blood cultures (group B streptococci and *Pseudomonas aeruginosa*). All 21 infants showed clinical signs of septicaemia and positive laboratory findings as described in the Materials and Methods section. Bacterial swabs revealed infection with group B streptococci (8×), *Ps. aeru-*

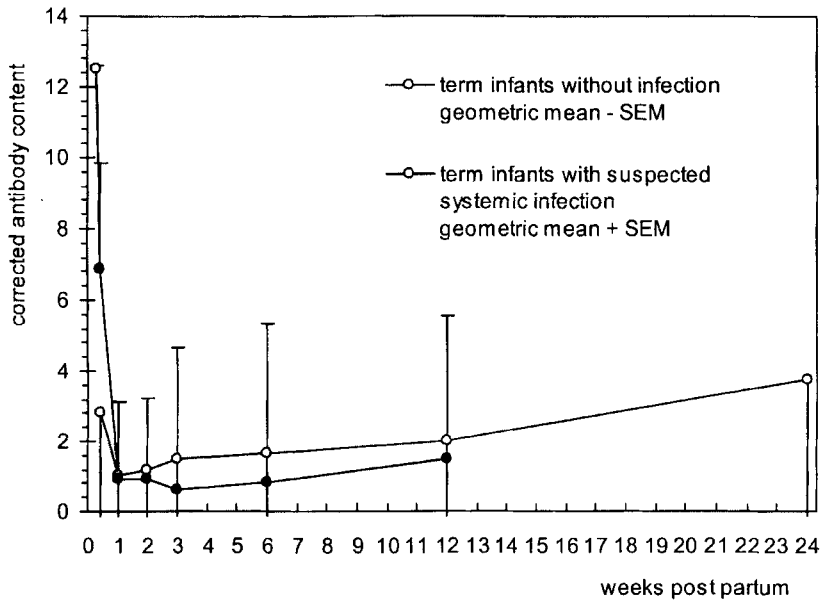

Fig. 1. Specific IgA antibodies against lipid A of *E. coli*. Comparison of corrected antibody content between milk of mothers of term infants without ($n = 8$) and with ($n = 12$) systemic infection.

ginosa (2×), *Staphylococcus aureus* (3×), *Stenotrophomonas maltophilia* (1×), *Haemophilus influenza* (1×) and *Herpes simplex* (1×). In three cases, determination of the causative organism failed.

Term infants with and without infection

The time course of absolute IgA antibody titres against all tested antigens followed a unique pattern. After a steep decrease of antibody levels during the first week, a nadir was reached 3–6 wk post partum. In milk of mothers of term infants without infection, specific antibody levels 6 mo after delivery were higher than 2 wk post partum ($p < 0.001$, all tested antigens).

The absolute content of specific antibodies did not differ significantly in milk of mothers with term infants with and without infection. However, the corrected antibody content was higher in colostrum of mothers of term children with infection. From the 7th day post partum on, no significant difference could be observed. This course was nearly identical for antibodies against all tested antigens. The time course of the corrected antibody content against lipid A of *E. coli* F583 Rd is depicted in Fig. 1.

Preterm infants with and without infection

In milk of mothers whose preterm children suffered from systemic infection, higher absolute and corrected antibody levels could be measured throughout lactation (Figs 2, 3). Absolute antibody titres against LPS of *E. coli* O111:B4 in milk of mothers of preterm children with infection were found to be 3.1 ± 1.6 times higher when compared with milk of mothers of preterm children without infection (Fig. 2, representative of all tested antigens).

The higher corrected antibody content in milk of mothers of preterm children with infection reached statistical significance 3 wk post partum ($p < 0.05$). An analysis of variance showed that the corrected antibody content in the 'infection' group was significantly rising from 1 wk to 12 wk post partum. The corrected antibody levels 24 wk post partum were 6 ± 3.5 times as high as 2 wk after delivery. In contrast, the corrected antibody levels in milk of mothers of preterm children without infection remained constant from 1 to 12 weeks post partum.

Correlation between specifc antibody content and total IgA

High concentrations of total IgA in colostrum and mature milk (≥100 mg/dl) did not correlate well with levels of specific endotoxin antibodies ($0.46 \leq r \leq 0.53$). However, good correlation was observed between different specific antibodies against the tested endotoxins ($0.62 \leq r \leq 0.90, p < 0.05$). In samples with high levels of specific antibodies against one endotoxin (≥200 arbitrary units), coefficients of correlation ranged from $r = 0.71$ to $r = 0.92$ ($p < 0.05$). The best correlation was observed between antibodies against lipid A of *E. coli* F583Rd and antibodies against LPS of *E. coli* O111:B4 ($r = 0.92$).

Discussion

The present study demonstrates that human milk

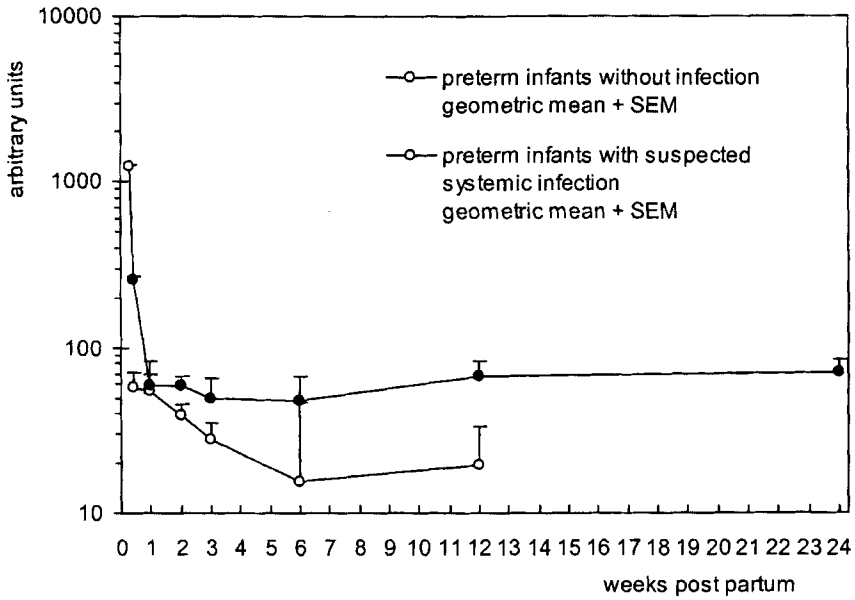

Fig. 2. Specific IgA antibodies against LPS of *E. coli*. Comparison of absolute antibody content between milk of mothers of preterm infants without ($n = 8$) and with ($n = 12$) systemic infection.

contains anti-endotoxin antibodies of the IgA class against different antigens of *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella minnesota*. Although the employed ELISA did not discriminate between monomeric IgA and dimeric antibodies containing a secretory component (SIgA), it has clearly been shown that more than 90% of the IgA antibodies in human milk are of the SIgA type (3).

When the milk's antibody content was investigated longitudinally, high colostral antibody titres against all tested endotoxins were found to be declining during the first week after delivery. These results were consistent with previous findings (18, 19, 26, 27), and did not depend on the gestational age of the newborn or the prevalence of signs of systemic infection in the infant.

However, we were able to demonstrate that antibody titres against endotoxins were remaining constant, or, under certain circumstances, were increasing during

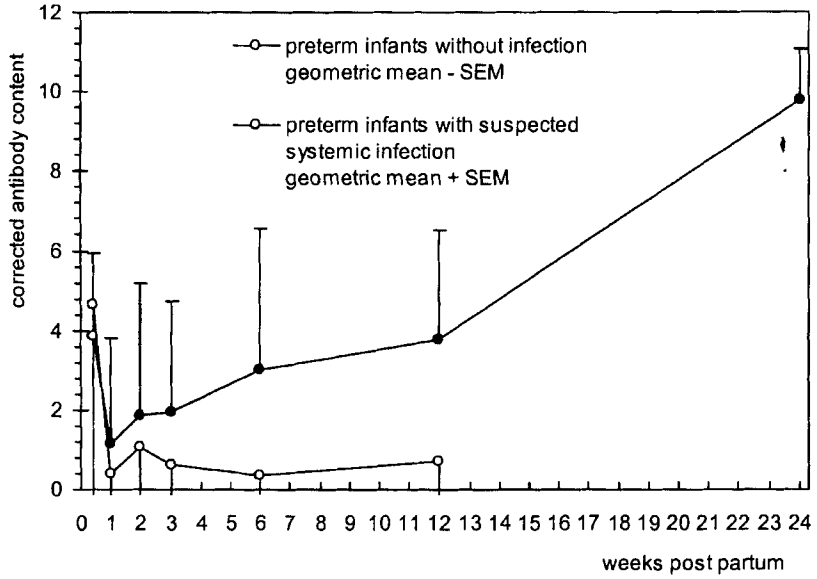

Fig. 3. Specific IgA antibodies against LPS of *Ps. aeruginosa*. Comparison of corrected antibody content between milk of mothers of preterm infants without ($n = 8$) and with ($n = 12$) systemic infection.

continued lactation, even when total IgA concentrations in breast milk were declining. So far, this observation has only been reported for antibodies against a pool of *E. coli* antigens in milk of some mothers of term newborns (3, 28).

To demonstrate the time course of specific anti-endotoxin antibody content of breast milk more precisely, we used a mathematical model: a corrected antibody content was calculated by dividing specific antibody levels by the total IgA content of each milk sample.

In milk of mothers of term children, the corrected antibody titres in colostrum were more elevated when the infants showed signs of systemic infection. No significant differences could be observed after the first week post partum. Corrected antibody titres against endotoxins reached a nadir 1–2 wk post partum, and were then rising until 6 mo after delivery.

It has been suggested that this rise in specific antibody production is caused by an activation of the maternal entero-mammary gland pathway (3), reflecting the acute response of the common mucosal defense system to intestinal and bronchial pathogens. Another explanation may be the increasing colonization of the infant's gastrointestinal tract after birth. Establishment of the normal intestinal flora takes place depending on the duration of breastfeeding and the postnatal environment of the infant. Preterm infants treated in intensive care units, in particular, are exposed to numerous potentially dangerous pathogens. It could be demonstrated that the mammary glands produce antibodies against antigens of the upper gastrointestinal tract of the infant. As indicated by carefully performed experiments in animals and humans, microbial colonization of the oral cavity of newborns induced a significant antibody response in breast milk (29). The anti-endotoxin antibodies provided by breast milk may therefore play an important role in maintaining an equilibrium of various bacterial strains in the infant's intestinum, along with other factors such as breast milk macrophages (30).

To our surprise, milk of mothers whose preterm infants had suffered from systemic infection contained higher antibody levels than milk of mothers of infants without infection. This difference reached statistical significance 3 wk post partum. It is certainly not justifiable to state that the higher corrected antibody content in its mother's milk is exclusively due to the infant's nosocomial infection. This fact could simpy reflect a selection of patients due to the relatively small number of non-infected infants in our study. However, we cannot exclude the possibility that certain pathogens may activate the maternal immune system and thereby increase the maternal antibody production in breast milk. In this context, the importance of special nursing practices, such as the kangaroo method (31), in stimulating specific immunoprotection by breast milk antibodies needs to be carefully evaluated.

In conclusion, the present study shows that breast milk contains anti-endotoxin antibodies throughout the lactational period. Breastfed preterm infants, and especially those who suffer from systemic infection, seem to be protected by even higher anti-endotoxin antibody concentrations in their mother's milk. Our results underline the immuno-protective properties of breast milk.

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