Early induction of secretory immunity in infancy: specific antibody in neonatal breast milk

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SUMMARY Neonatal breast milk from 50 babies aged between 2 and 39 days was studied for the presence of antibody to the cows' milk protein beta lactoglobulin. Specific IgA antibody and specific secretory antibody to beta lactoglobulin were detectable towards the end of the second week of life in milk secreted by neonates fed cows' milk formula. Specific antibody concentrations were independent of total IgA concentrations. Babies receiving little or no cows' milk protein did not produce antibody in neonatal breast milk. Antibody specific mucosal immune responses develop in tissues distant from the site of primary mucosal exposure by the end of the second week of life in term human neonates, suggesting that prophylactic immunisation against enteric or other mucosal pathogens within a few days of birth may provide antibody responses in secretions, which may protect against mucosal infection.

The mucosal immune system has a twofold purpose: the prevention of infection at mucosal surfaces and the exclusion of antigens capable of causing an unwanted systemic response. There are many factors involved in this role in humans, one of the more important being the production of specific antibody within secretions.

A characteristic of the induction of a specific mucosal immune response is the ability of cells, primed by antigen at one site, to migrate selectively to distant mucosal sites, thereby allowing subsequent local production of specific antibody without the requirement for antigen exposure at the secondary site. This adaptive response is important in the provision of specific antibody in maternal breast milk against enteric antigens and is in part responsible for the observed protection against gastrointestinal infection seen in breast fed infants.

Milk is produced by the breast tissue of the term human neonate during the first weeks of life. Although it is known that IgA occurs in secretions such as saliva soon after birth, specific antibody in secretions has not yet been well shown in the neonatal period. Neonatal breast milk was studied to determine whether specific antibody to enteric antigen is detectable in non-enteric secretions within the first four weeks of life. Such antibody, if present, would provide evidence for the early induction of specific adaptive mucosal responses in the human infant.

Methods

Samples. Neonatal breast milk from 50 babies was collected into glass capillary tubes after gentle manual expression of breast tissue. Sufficient milk for assay (50 μl or more) was collected on a single occasion from 43 neonates of differing ages. Sequential samples were collected from seven babies. The gestational ages of the 50 babies studied ranged from 36 to 42.5 weeks; ages after birth were between 2 and 39 days. The mean birth weight for the study group was 3540 g (range 2000-4660 g). The babies were inpatients in special care nurseries at the time of study, having been admitted for observation. None were being ventilated, and all but one were being fed orally.

Parental consent was obtained before enrolment in the study. Of the 43 babies studied once only, five had not received any cows' milk protein containing formula. Of these, four received breast milk only until the time of sampling, while one had received only intravenous dextrose/saline solution. The remaining 38 babies had received commercial cows' milk formula for at least two thirds of their feeding days apart from two who received formula for only one third of their feeding days. All began milk containing feeds within 36 hours of birth.

The feeding regimens for the babies studied sequentially are detailed below in association with the results for their antibody assays.
Milk was collected from both breasts simultaneously, and samples for each breast were analysed separately when sufficient volume could be collected. Separate analysis of milk from each breast was possible for 28 of the 43 babies studied once only. The seven babies studied sequentially had 93 samples collected. On 38 occasions sufficient sample was present to analyse milk from each breast separately. If sample volumes from each breast were insufficient the two samples were pooled. Milk samples were centrifuged after collection and were stored at −70°C in sealed glass capillary tubes until tested. All samples were coded and were tested in random order; all assays were performed in duplicate.

**IgA antibody to beta lactoglobulin.** An enzyme linked immunosorbent assay (ELISA) was used to measure specific IgA antibody to beta lactoglobulin in all samples. Ninety six well microtitre plates (NUNC) were incubated for 90 minutes at 37°C with 300 μl of purified beta lactoglobulin (Sigma Chemical Co) in 0-05M bicarbonate buffer solution (final concentration 0-1 mg beta lactoglobulin/ml, pH 9-5) in each test well. The beta lactoglobulin had been crystallised three times and gave only one band on protein electrophoresis. After incubation and washing, 50 μl of a 1:10 dilution of centrifuged neonatal breast milk was added to each well. The plates were incubated for two hours at room temperature, left overnight at 4°C, washed, and 50 μl of a 1:600 dilution of peroxidase labelled, affinity isolated, goat antihuman IgA (TAGO) was added to each well. After two hours at room temperature the plates were washed, 50 μl of orthophenylenediamine substrate added, and incubated in the dark for 15 minutes. The reaction was stopped by adding 25 μl of eight times isotonic hydrogen sulphate to each well. Absorbance values were read at 492 nm; values were considered positive if they were greater than 2-5 times the mean of 15 replicates for sample free control wells.

Serial dilutions of a reference pooled human serum standard (Australian Serum Protein Standard, ASPS 78–1) derived from 240 healthy adult donors and containing 102 IU of IgA/ml were included in each test plate. The reference serum was assigned a value of 100 units of IgA antibody to beta lactoglobulin/ml. The lower level of sensitivity of the assay was 0-2 units of IgA antibody to beta lactoglobulin. Samples with values greater than 100 units/ml were not diluted further due to limitations on available sample volumes.

Specificity of the assay for antibody to beta lactoglobulin was shown by adsorbing known positive neonatal breast milk samples and pooled mature adult breast milk with a beta lactoglobulin immunoadsorbent precipitated by glutaraldehyde and comparing titres with aliquots of the same samples adsorbed with insolubilised human cord serum. Titres of beta lactoglobulin specific antibody decreased only with the beta lactoglobulin immunoadsorbent. The goat antihuman IgA antibody did not crossreact with purified IgM and IgG in ELISA assays, and when tested against serum, saliva, and adult breast milk samples fractionated by gel filtration on S300 (Pharmacia) reacted only with fractions at an appropriate molecular weight for IgA.

**IgM antibody to beta lactoglobulin.** Specific IgM antibody to beta lactoglobulin was measured in samples from the seven babies studied longitudinally and 13 babies studied once only. The ELISA assay was identical to the above but used a peroxidase labelled, affinity isolated, goat antihuman IgM antibody (TAGO), which was shown not to crossreact with purified IgA. The reference serum (ASPS 78–1) was again assigned a value of 100 units of IgM antibody to beta lactoglobulin/ml. The lower level of sensitivity of the assay for the reference serum was 0-18 units of IgM antibody/ml.

**Secretory antibody to beta lactoglobulin.** ELISA assays to detect the presence of specific secretory antibody to beta lactoglobulin employed a monoclonal antibody to human secretory component (Australian Monoclonal Development Pty Ltd), used as a 1:1000 dilution of purified ascitic fluid, followed by peroxidase labelled, goat antimouse immunoglobulin (TAGO) at a dilution of 1:250. The reference standard for secretory antibody to beta lactoglobulin was pooled unstimulated saliva from 10 healthy adult donors. This standard was tested in serial dilutions on each assay plate and was assigned an arbitrary value of 100 units of secretory antibody to beta lactoglobulin/ml. Values were considered positive if they were greater than 2-5 times the mean of 15 replicates for sample free control wells. The lower level of sensitivity of the assay for secretory antibody to beta lactoglobulin in pooled saliva was 1-2 units/ml of saliva.

**Total IgA concentrations.** ELISA assays for the measurement of total IgA concentrations employed affinity isolated, goat antihuman IgA (TAGO) at a 1:1000 dilution in bicarbonate buffer as precoat. The reference serum (ASPS 78–1) was tested in serial dilutions on each plate. The assay for total IgA was capable of detecting concentrations of IgA in the reference standard as low as 3-52x10^-6 IU/ml (0-05 ng/ml). The goat antihuman IgA antisera used as precoat and as conjugate were shown in separate
ELISA assays not to react with purified secretory component.

Results

Milk was able to be expressed readily from the breast tissue of neonates of 36 weeks' gestation or more but not from babies of less than 36 weeks' gestation. Total IgA concentrations and specific beta lactoglobulin antibody concentrations for milk from right and left breasts for individual babies were in very close agreement for the 66 occasions when sample collection from each breast was possible (no significant differences. Wilcoxon matched pairs signed ranks test). Therefore, the mean of results for simultaneously collected right and left breast milk samples was used for data analysis where appropriate sample volumes from each breast were available.

**Total IgA concentrations.** Sufficient milk was collected from 45 babies to measure total IgA concentrations. IgA was detected on all occasions, the range of concentrations being 0-02 IU/ml (0-028 mg/100ml) to greater than 11U/ml (1-42 mg/100ml). Breast milk total IgA concentrations rose during the first two weeks in the seven babies studied longitudinally then tended to remain constant during week 3. The IgA concentration in a pooled breast milk standard containing breast milk from five mothers who had been lactating for greater than three weeks was 40 IU/ml (57 mg/100ml), and in the pooled saliva standard was 2 IU/ml (2-84 mg/100ml).

**IgA antibody to beta lactoglobulin.** Specific IgA antibody to beta lactoglobulin was present in breast milk from 18 of the 50 babies. Specific IgA antibody was present in samples from 14 of the 43 babies tested once only and became detectable at the end of the second week of life (Fig. 1). IgA antibody to beta lactoglobulin was absent in all samples from the five babies who had never received feeds containing cows' milk, except for a low value (0-6 units/ml) in milk from one breast of a baby breast fed for 16 days, whereas no antibody was detected in milk collected simultaneously from the other breast. The ages of these five babies at the time of sample collection were between 6 and 16 days.

Four babies who received only cows' milk containing commercial formulas beginning within six hours of birth were studied longitudinally. In these babies IgA antibody to beta lactoglobulin also occurred during the second week of life (Fig. 2(a)). The rise in antibody concentration with increasing birth age was independent of the rise in total IgA concentration, as shown in Fig. 2(b) where the concentration of specific IgA antibody to beta lactoglobulin and the total IgA concentration has been expressed as a ratio for each sample.

Three babies who received minimal amounts of cows' milk protein in the first week of life were also studied longitudinally until the end of the third week of life. None developed IgA antibody to beta lactoglobulin in breast milk samples at any time during the three week study period. One neonate received small amounts of cows' milk formula until day 2 and was then placed on prolonged intravenous treatment because of sepsis. A second had no enteric feeds until day 6 and was then breast fed. A third received only breast milk except for 60 ml of cows' milk formula on day 3. Sequential maternal milk samples collected between days 2 and 17 from the mother of this baby had IgA antibody concentrations to beta lactoglobulin that ranged between 0-02 and 0-04 units/ml. The pooled adult mature breast milk contained 8-6 units/ml of IgA antibody to beta lactoglobulin, while the concentration in the pooled saliva standard was 102 units/ml.

**IgM antibody to beta lactoglobulin.** Specific IgM antibody to beta lactoglobulin was present in milk from five babies only of 20 tested. Four babies fed on cows' milk were studied longitudinally for production of IgM antibody. No IgM antibody was detectable before day 21 of life, and the highest titre was 1-3 units/ml. The remaining 16 babies were
studied on one occasion only. Again no IgM antibody was present before the third week of life, the maximum titre for the two babies with detectable concentrations of IgM antibody being 0-55 units/ml.

Secretory antibody to beta lactoglobulin. True secretory antibody to beta lactoglobulin as recognised by the presence of specific antibody to beta lactoglobulin associated with secretory component became detectable at the end of the second week of life and showed a rise to high titres with increasing age parallel to that seen for total IgA antibody to beta lactoglobulin. In three of the 50 neonates, however, secretory antibody was also detected in very low titre on one occasion during the first six days of life. Two of these babies were studied longitudinally. One was breast fed, and no secretory antibody was detected subsequently. The second was formula fed with reoccurrence of secretory antibody in week three.

There was no relation between birth weight or gestational age and total IgA concentrations in neonatal breast milk nor between birth weight or gestational age and specific beta lactoglobulin antibody concentrations (not significant. Spearman rank correlation coefficient).

Discussion

This study has shown that an IgA antibody response to an enteric antigen occurs in a non-enteric secretion, neonatal breast milk, towards the end of the second week after birth in human neonates. The rise in antibody concentrations with increasing birth age is independent of total IgA concentrations. The times of occurrence of total IgA antibody and secretory component associated antibody are similar, whereas specific IgM antibody occurs later and only in low titre, suggesting that the greater proportion of the specific antibody response is true secretory IgA. Specific antibody to beta lactoglobulin was not present in significant concentrations in babies who had little or no antigen exposure during the first two weeks of life but was present by the third week of life in all babies receiving most of their feeds as cows’ milk formula.

The human fetus is able to produce an antigen specific, systemic immune response at an early stage during gestation, and the premature neonate develops appropriate humoral antibody responses to extrinsic antigens soon after birth. The development of antigen specific mucosal immunity in the newborn, however, has not been well documented. Studies of mucosal responses in adults have suggested that antigen exposure at one mucosal site leads to the local formation of antigen committed IgA lymphoblasts, which circulate and selectively populate other mucosal tissues. After enteric immunisation with Escherichia coli 083 during the last trimester of pregnancy, Goldblum et al showed the presence of cells secreting IgA antibody to the same E. coli strain in breast milk in the post partum period. No detectable antigenaemia or systemic antibody response had occurred. Specific antibody responses have also been shown in tears after enteric immunisation of adults with a bacterial antigen. IgA antibody to respiratory syncytial virus occurs in adult breast milk after respiratory syncytial virus infection of the lower respiratory tract.

Previous evidence for the time at which endoge-
nous IgA is detectable at mucosal surfaces in the human infant has been conflicting, although more recent studies suggest that IgA production probably begins in the first two weeks of life. Cells containing immunoglobulin could not be found in bowel mucosa before 12 days of age in a study of biopsy and autopsy specimens from the gastrointestinal tracts of neonates and infants aged 2 hours to 6 months. Between 12 days and 1 month cells containing IgM were predominant, with a later increase in the numbers of cells containing IgA. Cultured rectal biopsy samples from 12 neonates whose mean age was 3 days, however, did contain cells producing IgG, IgA, and IgM, and immunoglobulin was present in the culture supernatants. During sequential studies of the time of occurrence of IgA in saliva in 25 full term neonates IgA was detected by immunodiffusion assays in one neonate only during the first week of life. IgA was present in saliva from half of the neonates by 14 days and in 80% by the end of the third week. Salivary IgA was present a few days before serum IgA was detectable, suggesting that serum and salivary IgA synthesis may develop independently. Subsequent studies by Gross and Buckley using a more sensitive double antibody radioimmunoassay showed endogenous IgA in the saliva of eight bottle fed term neonates in the first few days of life with a rise in concentration during the second week.

There have been few studies of the ability of the neonate or young infant to produce specific antibody in secretions. Cells containing IgA and IgM antibody specific for beta lactoglobulin and bovine serum albumin have been found in jejunal mucosa as early as 2 months of age in infants having biopsy examinations performed during the course of investigation for intolerance to cows' milk protein. Haemagglutinating coproantibodies to milk protein have been detected by 1 month of age. Gleeson et al reported that haemagglutinating salivary antibody to human red cells sensitised with pooled antigen from six common E. coli serotypes was not present in infants studied from birth until the end of the first year even though salivary IgA was detected by 3 weeks of age. Secretory antibody to E. coli antigens, however, was present in saliva in the first year of life in the study reported by Mellander et al, with secretory IgM antibody perhaps being important in the first months.

The above studies have investigated the ability of the neonate or infant to produce IgA and antibody in gastrointestinal tract secretions, where antibody is likely to result from antigen exposure within the gastrointestinal tract itself. Neonatal breast milk affords the opportunity to study the secretory antibody response at a distant site. Breast milk is secreted by many term neonates, although we have found milk is not obtainable from neonates of less than 36 weeks' gestation. Others have shown in cross-sectional studies that IgA is present in neonatal breast milk. Our studies confirm that IgA is present and show that the IgA has antibody specificity by the end of the second week of life. In several neonates IgA antibody to beta lactoglobulin was present in breast milk in much higher concentrations than in pooled adult breast milk.

Our study does not confirm that the antibody has been produced within the neonatal breast tissue itself, although as in the adult breast this seems to be the most probable explanation. The antibody present is not antibody from maternal milk in the gastrointestinal tract that has been absorbed into the systemic circulation and resecreted by the neonatal breast, as 12 neonates in this study with positive antibody titres had not received breast milk at any time. Furthermore, IgA antibody to beta lactoglobulin was not detected throughout the first three weeks of life in a neonate who was breast fed with milk containing moderately high titres of beta lactoglobulin antibody. The same findings provide evidence that antigen committed lymphocytes derived from maternal breast milk and homing to the neonatal breast are not the source of the antibody response in these babies.

The IgA antibody could be transported selectively into neonatal breast milk from the systemic circulation, although early humoral responses to ingested bovine antigens in neonates and infants seem to be mainly IgG antibody, with IgA antibody concentrations only reaching measurable concentrations after 8 weeks of age. In our study serum samples could not be collected to determine simultaneous serum concentrations of IgA antibody to beta lactoglobulin. The high titres of secretory component associated antibody, however, suggest local synthesis, and others have shown 11S IgA in neonatal breast milk, again suggesting local synthesis. There is little evidence to support direct immunisation of neonatal breast tissue with beta lactoglobulin absorbed from the neonatal gastrointestinal tract into the systemic circulation, as earlier studies have shown intact gastrointestinal mucosal barrier function in term neonates with no appreciable absorption of beta lactoglobulin after an oral load. A further possible explanation is that specific antibody in neonatal breast milk may arise as an anti-idiotype response to transplacentally acquired maternal antibody to beta lactoglobulin. If this were the case, however, antibody might be expected in breast milk from most neonates in the first week of life irrespective of the type of feeding, whereas in this study titres only rose during the second week and were only present...
at high concentrations in babies who were receiving cows’ milk formula.

This study shows that an antigen specific mucosal immune response can develop in tissues distant from the site of primary mucosal immunisation by the end of the second week of life in term human neonates. Indirect evidence suggests that the response may be due to selective homing of antigen committed cells to neonatal breast tissue from the gastrointestinal tract, resulting in the formation of antibody in secreted milk. If similar production of antibody occurs in the early neonatal period at other mucosal surfaces—for example, in the respiratory tract—or even within the gastrointestinal tract at sites distant to the site of antigen exposure, prophylactic mucosal immunisation against a variety of enteric or other mucosal pathogens within a few days of birth may be expected to provide adequate antibody responses in secretions. This knowledge will be important in the design of trials of immunising agents designed to provide protection against rotavirus, respiratory syncytial virus, and other mucosal infections of early infancy.

References


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Received 8 January 1986