

A prospective study of humoral immune responses to cow milk antigens in the first year of life

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Previous studies have shown that in cow milk allergy the specific immune response to dietary cow milk antigens is deficient. This study aimed at delineating the development of humoral immune response to cow milk antigens in healthy infants. Twenty-five healthy newborns were enrolled, and seen at scheduled visits at the ages of three, six and eleven months, and they formed two groups: those breastfed and those fed adapted cow milk formulae. The local immune response in the gut was approximated using the ELISPOT assay of circulating antibody secreting cells. At the age of three months, in the formula fed group, cells secreting specific IgA to cow milk antigens were detected despite low levels of IgA serum antibodies. The total number of IgA secreting cells increased with age ($p = 0.001$). The milk in the infant diet directly influenced this development so that the age related increase was significantly greater in the formula fed group ($p = 0.04$). The results indicate that diet has a significant effect on the developing immune system, and that healthy infants are able to respond in an antigen specific fashion to dietary antigens, which may be central in attaining clinical tolerance of such antigens.

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Despite the widespread use of adapted cow milk formulae, most studies have concentrated on the small minority of infants who develop overt symptoms of cow milk allergy as a result of aberrant immune responses to cow milk antigens (1). While some of these immunological aberrations are relatively well described (2–4), evidence on the development of oral tolerance is only available from animal studies (5, 6). Oral tolerance undoubtedly also exists in humans (5), and therefore the developmental chain of events in the majority of infants resulting in clinical tolerance to cow milk needs to be delineated, to provide a basis for new strategies in the treatment of those suffering from cow milk allergy (4).

The progress of the humoral immune response to cow milk antigens was investigated, using the ELISPOT assay of circulating antibody secreting cells, in a prospective study of healthy, full term infants during the first year of life.

Subjects and methods

Mothers of healthy newborns were approached at the maternity ward within 2 days of delivery at the Tampere University Hospital. They received written and oral information on the study protocol and gave their informed consent. The study was approved by the hospital's Ethics Committee.

Eligible infants came from non-allergic families, their delivery was at term and uneventful. The infants were born at mean gestational age 39 weeks (range 37 to 42) and their mean birth weight was 3550 g (SD 348). Eligibility of mothers was also ascertained: no dietary restrictions during pregnancy, and no plans to have any during lactation. One mother was delivered by cesarean section due to her antiepileptic treatment which had maintained her epilepsy well under control for years. The diets of the mothers contained in all cases cow milk products every day throughout the study. Two mothers tolerated only fermented milks because of lactose intolerance.

Table 1. Number of available samples and predominant milk at the follow up visits. Mean (SD) age at the time of blood sampling of the infants, their predominant milk with number of infants on each diet indicated, and the number of samples obtained for the analyses during the first year of life. Diet is breastmilk (b) or adapted cow-milk formula (f). Note that age is days in the newborn period, months thereafter.

	Newborn	3 mo	6 mo	11 mo
Age	3.2 (0.2) d	3.2 (0.3)	5.8 (0.4)	11.0 (0.5)
Diet	25b	19b/4f	14b/9f	1b/21f
Number of samples				
ELISPOT		22	23	22
Serum				
Infant	25	22	23	22
Mother	25	23		
Breastmilk	25	17		

Study design

Scheduled visits took place at infant ages three, six and eleven months (Table 1). Special attention was paid to the diets of the infants at each follow up visit, and the time of weaning determined. The infants were grouped according to the predominant milk at each visit; if weaned or nursed only twice daily, adapted cow milk formula was considered the predominant milk. For analysis of the development of the ISC response, infants were divided into two groups based on their predominant milk at the age of six months. Both recruitment and clinical follow up were conducted by one of the investigators (M.K.).

Samples

Blood samples were obtained from the infants for determination of serum cow milk antibodies at the maternity ward and at the successive follow up visits, while heparinized blood samples for the ELISPOT assay were obtained beginning at the first follow up visit at the age of three months (Table 1). Serum and breastmilk samples were obtained from mothers at the ward and at infant age three months (Table 1). All samples were taken in the morning between 8 and 10 am, and assessed blinded as to the dietary group.

Laboratory methods

Determination of serum antibodies – Serum antibodies of isotypes IgM, IgA and IgG to cow milk proteins beta-lactoglobulin (β -LG) A and B, alpha-casein (α -CN), alphasalalbumin (α -LA), bovine serum albumin (BSA) (all from Sigma Chemical Co., St. Louis, Mo) and total milk protein (dried milk powder, Valio Ltd., Helsinki, Finland), were determined by ELISA as previously described (7).

ELISPOT assay – The ELISPOT method was used to measure circulating antibody secreting cells, con-

sidered to give indirect evidence of gut local immune defense and based on the concept of common mucosal immune system as previously described (7, 8). Earlier studies have shown that circulating antigen specific cells become measurable in blood a week after antigen contact in the gut and remain measurable as long as the antigen is present in the gut (8, 9). The total number of ISC (antigen non-specific immune response) and the number of sASC (antigen-specific immune response) to cow milk proteins β -LG and CN were enumerated in isotypes IgM, IgA and IgG.

Mononuclear cells (mainly lymphocytes) were obtained by Ficoll-Paque (Pharmacia AB, Uppsala, Sweden) centrifugation of the blood samples. After washing the lymphocytes three times in Hanks buffered salt solution (Flow laboratories, Irvine, Scotland) they were then suspended in culture medium and adjusted to a maximum concentration of 2×10^6 cells/ml.

The cells were incubated in antigen-coated, flat-bottomed 96-well microtiter plates (Immunoplate RI, A/S Nunc, Roskilde, Denmark) and antibodies secreted by each cell reacted with the antigen. These antibodies were visualized by application of enzyme-labeled antisera, followed by a substrate agarose overlay. The colored spots were counted using a stereoscopic microscope after various times of storage at 4°C. The wells were coated with anti-human antisera (anti-IgM, -IgA and -IgG) for the determination of ISC, and with β -LG and CN for the determination of sASC. The sum total of sASC was calculated by adding together the numbers of sASC to β -LG and CN. As in previous studies, it was considered an immune response with >0.5 sASC/ 10^6 cells (10).

Determination of IgA in breastmilk – The level of IgA in breastmilk was measured with single radial immunodiffusion method (11) in which the α -chain specific antiserum is in an agarose gel layer (LC-Partigen IgA, Behringwerke AG, Marburg, Germany). Stabilized human serum was used as the standard (Behringwerke AG). Manufacturer's instructions were followed.

Statistical analysis

Because of skewed distribution of data, the numbers of ISC and sASC as well as the levels of serum antibodies were transformed to their natural logarithms, and are then reported as geometric means [95% confidence interval, CI]. Student's *t* test was used to compare differences between the dietary groups. Successive ISC measurements were studied by using analysis of variance for repeated measures. Spearman's Rank Correlation was used to correlate

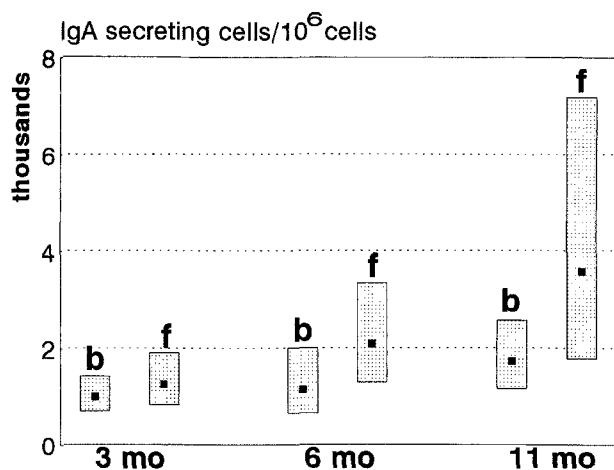


Fig. 1. The geometric mean [95% CI] number of IgA ISC/ 10^6 mononuclear cells is depicted at the follow up visits of 3, 6 and 11 months in breastfed (b) and adapted cow-milk formula-fed (f) infants. The dark square is the mean, the columns show the 95% CI. Analysis of variance for repeated measures showed that age significantly influenced the development of the IgA ISC ($p = 0.001$). Milk in the diet had an independent effect ($p = 0.04$): at successive points the age related increase was more significant in the formula-fed group.

the measurements of the mother and the infant, without the transformation.

Results

Clinical outcome

All 25 infants received breastmilk in the early post-natal period; formula supplementation was commenced in three infants within the first week. The total duration of breastfeeding varied from two to eleven months, three infants still receiving breastmilk twice daily at the last follow up. Table 1 gives the number of infants feeding adapted cow milk formulae or breastmilk at these times. Solid foods were introduced according to current recommendations: mashed fruits and vegetables from three

months, cereals and minced meat and fish from six months.

The infants remained generally healthy during their first year, though eight infants were diagnosed with acute otitis media altogether twenty times and received antibiotics. Acute diarrhea, flu and urinary tract infection were diagnosed in one infant each, and altogether three had exanthema subitum. The infections did not require hospital admission, and the infants were healthy at the follow up visits. The infants did not show signs of atopic predisposition, except one acute urticaria after first known ingestion of hen's egg after the six months visit.

Antigen non-specific immune response (ISC)

Fig. 1 depicts the developmental pattern in the number of IgA ISC from three months of age until the end of follow up at eleven months of age. At the first visit at three months of age, the numbers of IgA ISC were similar in the two groups; this was also true for IgM and IgG ISC. Analysis of variance for repeated measures clearly demonstrates that age has a significant effect on the development of the IgA ISC response ($p = 0.001$). In addition, the milk in the diet had an independent effect ($p = 0.04$). This means that at successive measurement points the age related increase in IgA ISC was more significant in the adapted cow-milk formula-fed group. The analysis gave a similar result for IgG ISC: both age ($p = 0.002$) and the milk in the diet ($p = 0.005$) significantly affected the development in the level of IgG ISC. In the adapted cow-milk formula-fed group the IgG ISC response developed from 1918 [1371, 2681] IgG ISC/ 10^6 cells at three months of age, to 5172 [3378, 7911] IgG ISC/ 10^6 cells at six months of age, to 4528 [2639, 7778] IgG ISC/ 10^6 cells at 11 months of age. In the breastfed group the number developed from 1463 [1009, 2119] IgG ISC/ 10^6 cells at three months of age, to 1953 [1143, 3338] IgG ISC/ 10^6 cells at six months of age, to 3258

Table 2a. The geometric means [95% confidence intervals] of antibodies to cow milk proteins [g/l] at 3 and 6 months of age. B = breastfed, F = formula fed.

		β -LG	α -LA	α -CN	BSA	TM
3 mo						
IgM	B	0.05[.04,.07]	0.09[.07,.12]	0.08[.06,.11]	0.04[.03,.06]	0.07[.05,.10]
	F	0.09[.03,.22]	0.15[.07,.33]	0.21[.07,.67]	0.08[.03,.21]	.23[.14,.36]
IgA	B	0.02[.01,.03]	0.02[.01,.03]	0.02[.01,.03]	0.01[.01,.02]	0.02[.01,.03]
	F	0.04[.005,.38]	0.02[.01,.03]	0.06[.01,.48]	0.01[.006,.02]	0.07[.01,.75]
IgG	B	0.10[.05,.21]	0.13[.07,.24]	0.20[.12,.35]	0.07[.04,.11]	0.22[.13,.38]
	F	0.51[.04,6.7]	0.36[.10,1.3]	0.55[.02,18.9]	0.14[.02,.78]	0.72[.06,9.2]
6 mo						
IgM	B	0.08[.05,.11]	0.12[.09,.15]	0.11[.08,.15]	0.07[.05,.08]	0.13[.09,.20]
	F	0.11[.08,.14]	0.19[.14,.27]	0.19[.12,.28]	0.10[.07,.15]	0.23[.17,.32]
IgA	B	0.02[.01,.07]	.02[.01,.03]	0.02[.01,.04]	0.01[.01,.02]	0.03[.02,.05]
	F	0.08[.07,.15]	0.05[.02,.10]	0.10[.05,.23]	0.06[.02,.10]	0.14[.06,.30]
IgG	B	0.19[.10,.36]	0.22[.13,.36]	0.28[.17,.45]	0.12[.07,.19]	0.37[.23,.59]
	F	1.38[1.0,1.9]	1.14[.74,1.7]	1.5[.93,2.3]	0.58[.31,1.1]	1.7[1.5,2.3]

Table 2b. The geometric means [95% confidence intervals] of antibodies to cow-milk proteins (g/l) at the newborn period and at 11 months of age. All 25 infants were breastfed (B), and 21 formula-fed, respectively (F).

	β -LG	α -LA	α -CN	BSA	TM
<i>Newborn</i>					
IgM	0.03[.02,.03]	0.06[.05,.07]	0.05[.04,.06]	0.02[.02,.03]	0.03[.03,.07]
IgA	0.0004[0,.001]	0.003[0,.007]	0.002[0,.004]	0.001[0,.003]	0.001[0,.002]
IgG	0.11[.10,.14]	0.15[.13,.18]	0.47[.31,.71]	0.11[.10,.15]	0.46[.31,.68]
<i>11 mo</i>					
IgM	0.13[.11,.15]	0.20[.17,.24]	0.19[.16,.22]	0.12[.11,.15]	0.18[.16,.21]
IgA	0.04[.03,.07]	0.04[.02,.05]	0.07[.05,.10]	0.06[.03,.05]	0.08[.06,.11]
IgG	0.47[.29,.76]	0.43[.29,.65]	1.0[.70,1.4]	0.38[.23,.62]	0.91[.66,1.3]

[2269, 4675] IgG ISC/10⁶ cells at 11 months of age. By contrast, neither age nor diet significantly affected the levels of IgM ISC during the follow up.

The antigen specific immune response (sASC and serum antibodies)

In the newborn period, the level of IgM antibodies to all the assessed cow milk proteins was very low, and that of IgA antibodies was negligible (Table 2b). The level of IgG antibodies was of a distinctly greater order.

At three months of age, serum antibodies of all isotypes had increased since the first measurement, those of IgA still being lowest (Table 2a). The differences in the levels of serum antibodies between the two dietary groups were inconsistent. By contrast, the number of IgA and IgG sASC differed between the adapted cow-milk formula-fed and breastfed groups (Table 3). It is worthy of note that IgA sASC were measurable while the serum IgA antibody level was still low.

At six months of age, the levels of serum IgA and IgG antibodies to the assessed cow milk proteins were invariably higher in formula-fed than breastfed infants (Table 2a). The trend persisted from the previous visit of more sASC in the formula-fed than breastfed infants (Table 3). Between the visits at six and 11 months, the mean numbers of sASC of all

three isotypes decreased in the formula-fed infants (Table 3). At 11 months of age, the one remaining breastfeeding infant was negative in all isotypes.

Correlation between the immune responses of the mother and the infant

Breastmilk IgA level in the after delivery period was a mean 0.80 g/l (range 0.14–1.97), and at infant age three months 0.26 g/l (range 0.13 to 0.55 g/l).

In the newborn period, the levels of serum IgG antibodies to cow milk antigens were significantly correlated between the mother and her newborn ($p = 0.001$). Apart from this, there were no correlations between serum antibodies of the mother to those of her infant (data not shown). The breastmilk IgA level did not correlate with the number of cells secreting antibodies specific to cow milk antigens.

Discussion

The results of this study show that diet has a strong impact on gut immunological events in the first year of life. The healthy infants produce an antigen-specific immune response to dietary antigens, while dietary antigens also influence the antigen non-specific response. Oral tolerance has been defined on the basis of animal studies as antigen specific mucosal immune responsiveness concurrently with systemic hyporesponsiveness (5). An adequately responding immune defense system as a component of gut mucosal barrier may therefore favor the development of oral tolerance.

Gut immune system acts in concert with various non-immunologic defense mechanisms, providing a well-functioning mucosal barrier against the influx of harmful substances (12). During the development of these barrier functions, antigen handling in the gut may be defective, which may be related to the fact that infants are at greatest risk for developing food allergic reactions (12). Mucosal production of IgA is deficient in the first months of life, and adult levels are attained as late as by school age (13). Functionally, gut mucosa is permeable early in life (14). Considering these shortcomings in the mucosal bar-

Table 3. Numbers of sASC during the follow up. The geometric mean [95% CI] total number of sASC of isotypes IgA, IgM and IgG at the ages of 3 mo, 6 mo and 11 mo according to the dominant milk at the time. b = breastmilk, f = adapted cow-milk formula.

	IgM	IgA	IgG
<i>3 mo</i>			
b (n = 18)	1.5[.3,8.8]	0.06[.01,.5]*	0.02[.003,.1]*
f (n = 4)	1.6[.001,4465]	5.4[1.5,19.4]	2.5[1.0,5.9]
<i>6 mo</i>			
b (n = 13)	6.8[3.1,14.0]*	0.02[.001,.4]	0.03[.003,.3]
f (n = 9)	39.2[19.0,80.6]	0.6[.01,26.5]	0.6[.03,11.5]
<i>11 mo</i>			
f (n = 21)	4.2[1.5,12.1]	0.05[.01,.4]	0.05[.01,.3]

* $p < 0.05$.

rier functions, surprisingly few infants show symptoms of food allergy, while it is unknown, what is the basis of oral tolerance.

Several previous studies have demonstrated that the level of serum antibodies to dietary cow milk antigens is higher in formula-fed than in breastfed infants, while contradictory evidence is scarce (15–21). However, serum antibodies do not correlate with the local immune responses in the gut, at least when microbial antigens are used (22). The present study took advantage of ELISPOT assay to approximate gut immune responses (8, 9). It has been shown that the circulating cells appear in peripheral blood several days before the respective antibodies in the gut that need to be assessed invasively (22).

Despite the low serum IgA concentrations, the present results show that the healthy infant is capable of generating an antigen-specific humoral response of the IgA isotype, when evaluated using the ELISPOT assay of circulating antibody secreting cells. The sASC response of healthy infants in the present study increased until six months of age and decreased thereafter. This indicates stimulation of the sASC response upon first contacts with an antigen, and its attenuation with development of oral tolerance. This is in contrast to infants who have cow milk allergy (10). On formal clinical challenge with cow milk, cow milk-allergic infants generate an impressive antigen non-specific response, while the antigen-specific response is small and inconsistent. It is of interest that with clinical tolerance to cow milk the character of the response evolves towards specificity and an alleviation of the non-specific response (7). According to the present results the development of oral tolerance proceeds in humans as in experimental animals: via stimulation of an antigen-specific response towards attenuation of this response on continued antigen exposure.

The antigen-non-specific ISC response was influenced by the diet of the infant: those breastfeeding had lower numbers of circulating ISC than the infants who were fed adapted cow milk formulae. In contrast to this, the effect of diet could not be demonstrated by measuring serum total immunoglobulin levels in an earlier follow up study (15). Parenteral alimentation has been shown to result in decreased numbers of antibody producing cells in gut mucosa of premature infants compared to oral feeding (23). In a previous study, substituting adapted cow milk formulae in the diet of healthy infants with an extensively hydrolyzed whey protein hydrolysate was associated with decreased numbers of circulating ISC (24). The number of ISC may reflect antigen load on the immune system. It may be speculated that the immune system of healthy formula fed infants is stimulated by dietary antigens. By contrast, breast-milk may provide the infant gut with an extra bar-

rier, so that the immune responses of the infant are attenuated.

Taken together, the results of the present study indicate that healthy infants respond in a relatively antigen-specific fashion to cow milk antigens, which possibly enables them to acquire clinical tolerance. We, therefore, suggest that also in humans, oral tolerance is associated with development of an antigen-specific immune response in the gut.

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