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IgG AND IgA SERUM ANTIBODIES TO COW MILK IN BREASTFED OR FORMULA FED HEALTHY INFANTS

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A b s t r a c t

Cow milk proteins belong to major food antigens in infancy. The aim of the study was to determine the levels of cow milk-specific IgG and IgA antibodies in healthy infants, both breastfed and formula fed, during the first six months of age. The initial levels of these antibodies were determined by ELISA in the cord blood samples of 75 healthy infants. Their concentrations were then determined during a follow-up study in the same cohort both at 3 and at 6 months of age (37 a 17 samples). IgG antibodies to milk were high at birth and decreased significantly from birth to 3 months and further from 3 to 6 months in breastfed infants. In formula fed infants, however, the levels increased during the first trimester and insignificantly decreased at 6 months. The milk-specific IgA antibodies, low in cord blood serum, increased continually from birth to 3 and then to 6 months regardless of milk feeding. The increase, however, was higher in formula fed infants. It is concluded that cow milk-specific IgG production is stimulated after cow milk formula feeding but not during exclusive breastfeeding. The production of specific IgA, however, is stimulated even during exclusive breastfeeding. These findings may be due to different mechanisms of immune response after low- or high-dose antigen exposure.

K e y w o r d s

IgA, IgG, Cow milk, Infant, Nutrition

A b b r e v i a t i o n s u s e d

BLG, beta-lactoglobulin; CMP, cow milk protein; EIU, enzyme immunosorbent unit; ELISA, enzyme-linked immunosorbent assay; IgA, class A immunoglobulin; IgG, class G immunoglobulin; SD, standard deviation; TGF, transforming growth factor

INTRODUCTION

Cow milk proteins belong to the strongest food antigens and allergens in infancy. After repeated exposure to milk proteins in infants fed cow milk formula, cow milk-specific antibodies are formed *(1,2)* and can be detected in serum using routine enzyme immunoassays *(3,4)*.

Excessively high levels of cow milk-specific antibodies are found in cow milk protein intolerance and other malabsorption syndromes of infants on cow milk formula *(5)*. For this reason, concentrations of cow milk antibodies of IgG and IgE classes are occasionally analysed in paediatric practice. As physiological

levels of cow milk antibodies during infancy are not clearly defined and standardized, their elevated concentrations were often misinterpreted. In practice, this leads to useless recommendation of a cow milk-free diet with all negative consequences for the infant.

The aim of our study was to investigate the systemic cow milk-specific humoral immune response in IgG and IgA immunoglobulin classes in healthy infants with different feeding during the first six months of life.

The following questions were of special interest: what are the concentrations of cow milk-specific IgG and IgA antibodies in umbilical cord serum? What is the development of these antibodies during the first 6 months of infancy in exclusively breastfed infants and in cow milk formula fed infants?

MATERIALS AND METHODS

Proband recruitment and follow-up

Subjects

Umbilical cord blood samples from 75 randomly selected newborns were obtained after cutting the umbilical cord in full-term vaginal deliveries with no perinatal pathology. Blood samples were let clot at 4°C and centrifuged, sera were stored at -20°C until further analysis, which was performed by 2 months from sampling.

Three days after delivery, a family history of the newborn was taken and informed consent for cord blood serum analyses and further clinical examinations including blood analyses were received from the mother. Exclusive breastfeeding was strongly recommended during the first six months. The infants were then invited to follow-up clinical examinations at 3 and 6 months of age.

The infants were seen at the outpatient department of our Department and history of cow milk formula intake in infants including the number of milk meals and the amount of milk drunk was assessed. Infants were weighed and clinically examined by an experienced paediatrician and a venous blood sample was drawn.

At the time of 3-month examination, the infants were divided into two groups according to the history of cow milk formula intake. The exclusively breastfed group never received any infant formula or any cow milk product. The cow milk fed infants received at the time of examination various amounts of cow-milk based formula.

Cow milk-specific IgG and IgA antibody analyses

The blood samples from infants were handled equally as those of cord blood. The serum concentrations of cow milk-specific antibodies were in all samples (cord blood and infant blood) determined using ELISA as described elsewhere *(3)*. Briefly, 96-well ELISA plates were coated with 100 µl fresh dairy low-fat milk, centrifuged to remove milk fat, at 4°C overnight. After washing, serum samples diluted in PBS 1:100 for IgG and 1:10 for IgA determination, respectively, were incubated at room temperature for 1 hour. After washing with PBS, a horseradish peroxidase-labelled swine-anti-human IgG (IgA) antibody (Sevac, Prague, Czechoslovakia) of 1:1000 dilution was incubated with o-phenylene diamine as chromogene and the absorbance was measured at 405 nm. The results were expressed as EIU (enzyme immunosorbent units); EIU= test serum absorbance*100/standard serum absorbance. The standard serum was a pooled serum from approx. 100 milk drinking toddlers showing no symptoms of cow milk intolerance.

Data handling, processing and statistical analyses

The data were stored in a Microsoft Access® Database ver. 97 (Microsoft Corp.) and statistical analyses were done using GraphPad Prism® ver. 3.0 (GraphPad Software, Inc.; San Diego, CA, USA).

For descriptive statistics Kologorov-Smirnov test was used to look for normality of the distribution (*P* value below 0.05 was considered statistically significant). In case of unnormal distribution, logarithmic transformation y'=ln y was performed. After normalisation of the data, and if variances were not significiantly different (F-test), parametric tests were used (two-tailed unpaired t-test or two-tailed paired t-test, respectively).

RESULTS

Infants

75 cord blood samples could be analysed. At 3 months of age, serum samples of 37 infants could be analysed. 16 babies were exclusively breastfed, 21 babies were formula fed. The formula fed infants had the reported relative daily cow milk protein intake (mean; SD) 2.23; 0.67 $g*kg^{-1}$ body weight. The body weight of breastfed infants was significantly lower than that of formula fed infants at 3 months (in grams; mean; SD): breastfed: 5 703.0; 710.7, formula fed 6 226.0; 764.8; p= 0.0372, unpaired t-test.

At 6 months of age, serum samples of 17 infants could be analysed. 6 babies were exclusively breastfed, 11 babies were formula fed. The formula fed infants had the reported relative daily cow milk protein intake (mean; SD) 1.54; 0,76$g*kg^{-1}$ body weight. The body weight values of breastfed infants did not significantly differ from those of formula fed infants at 6 months (in grams; mean; SD: breastfed: 7758.0; 970.8, formula fed: 8555.0; 892.3, p= 0.1085; unpaired t-test).

Antibodies

Cow milk-specific IgG antibodies

The cow milk-specific IgG antibodies at birth were relatively high, (EIU) median 125.6 (minimum 26.10; maximum 464.6; n=75).

In the exclusively breastfed infants, cow milk-specific IgG antibodies (*Fig. 1*) were significantly lower than those in the formula fed infants both at 3 months (EIU, mean; SD: breastfed: 44.44; 11.24; n=16; formula fed: 283.6; 180.7; n=21; p<0.0001, Mann-Whitney U-test) and at 6 months (EIU, mean; SD: breastfed: 32.23; 14.80; n=6; formula fed: 232.10; 135.10; n=11; p=<0.0001, unpaired t-test).

As to the development, cow milk-specific IgG antibodies markedly decreased in the exclusively breastfed infants, when comparing the respective cord blood levels (EIU: mean; SD: 215.80; 137.70) with those at 3 months (44.44; 11.24; two-tailed paired t-test; p<0.0001; n of pairs 16). Similar paired analysis was not done between the 3 and the 6-month antibody levels, respectively, because of the small number of infants at 6 months.

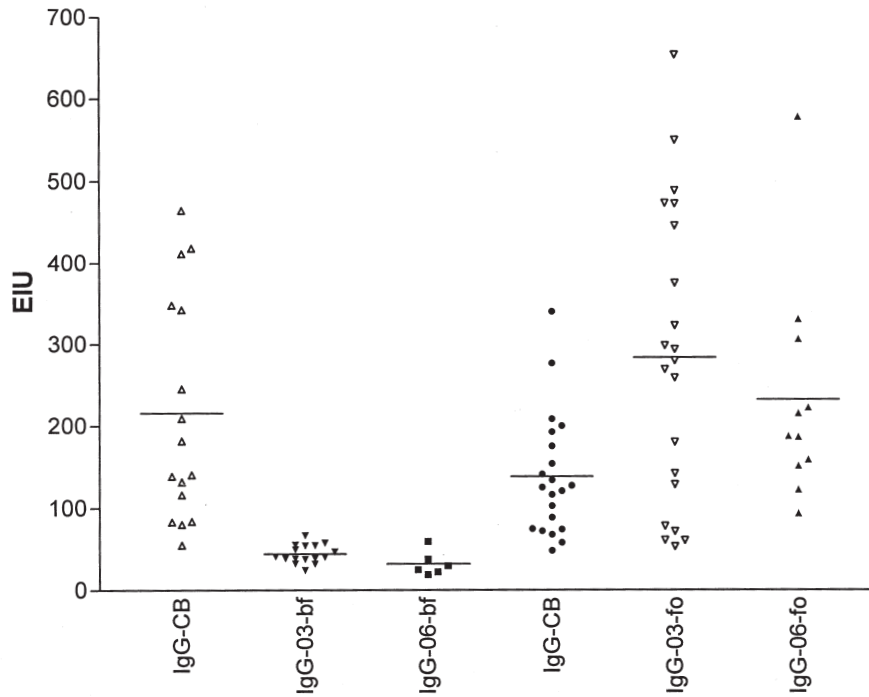

Fig. 1

Cow milk-specific IgG levels in infants from birth to 6 months of age. The horizontal lines give the means. Cord blood samples are presented only of those infants examined at 3 months. CB, cord blood; bf, breastfed; fo, formula fed; the numbers refer to the infant's month of age.

The cow milk IgG antibodies significantly increased in the formula-fed infants both when comparing the levels at birth (EIU: mean; SD: 138.20; 74.27) with the respective values at 3 months (283.60; 180.70; two-tailed paired t-test; p=0.0044; n of pairs 21). The levels decreased moderately but insignificantly from the 3rd (EIU: mean; SD: 292.40; 200.60) to the 6th month (232.10; 135.10; two-tailed paired t-test; p=0.2464; n of pairs 11).

Both groups, breastfed and formula fed at 3 months, respectively, did not differ in the cord blood cow milk-specific IgG concentrations (ln EIU; mean; SD): breastfed at 3 months: 5.169; 0.678; n=16 formula fed at 3 months: 4.801; 0.517; n=21; p=0.0690; unpaired t-test.

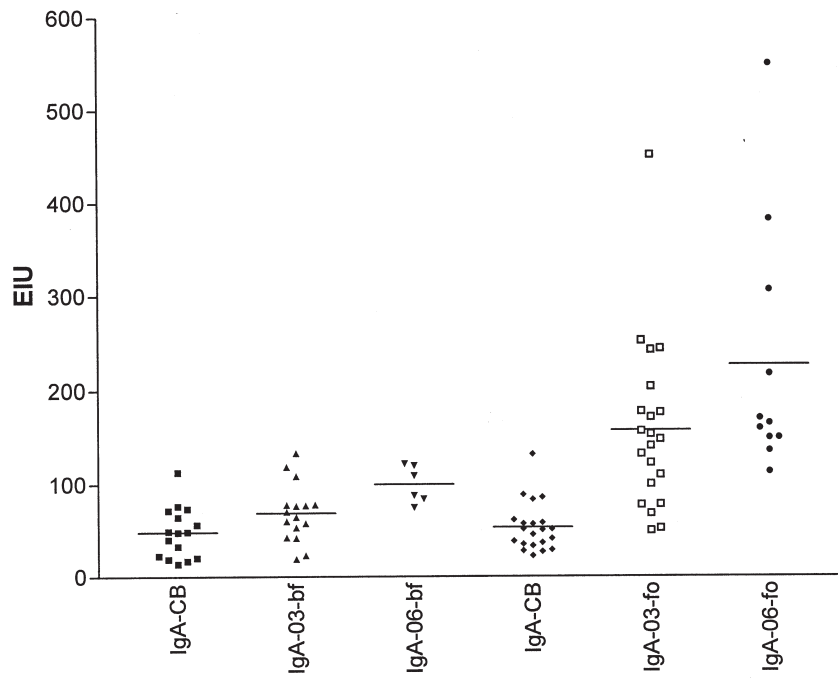

Fig. 2

Cow milk-specific IgA levels in infants from birth to 6 months of age. The horizontal lines give the means. Cord blood samples are presented only of those infants examined at 3 months. CB, cord blood; bf, breastfed; fo, formula fed; the numbers refer to the infant's month of age.

Cow milk-specific IgA antibodies

Cow milk-specific IgA antibodies (*Fig. 2*) were significantly lower in the exclusively breastfed infants than in the formula fed infants both at 3 months (EIU, mean; SD: breastfed: 69.02; 31.63; n=16; formula fed: 157.80; 90.84; n=21; $p<0.0001$, unpaired t-test) and at 6 months (median; minimum; maximum; breastfed: 98.80; 75.10; 122.30; n=6; formula fed: 165.4; 113.5; 550.3; n=11; $p= 0.0022$; the Mann-Whitney U-test).

As to the development, cow milk-specific IgA antibodies increased slightly and insignificantly in the exclusively breastfed infants when comparing cord blood levels (EIU, mean; SD: 48.16; 27.38) with those at 3 months (69.02; 31.63, two-tailed paired t-test, $p= 0.0563$; n of pairs 16). Similar paired analysis was not done between the 3 and the 6-month antibody levels, respectively, because of the small number of infants at 6 months.

Cow milk IgA antibodies significantly increased in the formula-fed infants when comparing the respective cord blood levels (EIU: mean; SD: 53.25; 26.40) with those at 3 months (157.80; 90.84; two-tailed paired t-test; $p<0.0001$; n of pairs 21). Similarly, the IgA antibody concentrations significantly increased from the 3rd (EIU: mean; SD: 154.40; 121.10) to the 6th month in formula fed infants (227.60; 134.10; paired t-test; $p=0.0430$; n of pairs 11).

Both groups, breastfed and formula fed at 3 months, respectively, did not differ in the cord blood cow milk-specific IgA concentrations (EIU; mean; SD): breastfed at 3 months: 48.16; 27.38; n=16; formula fed at 3 months: 53.25; 26.40; n=21; $p=0.5715$; unpaired t-test.

DISCUSSION

The quality and quantity of systemic humoral immune response to cow milk proteins depends on the time and duration of antigen exposure as well as the amount of antigen and way of application *(6)*.

There is an indirect evidence that foetus is exposed to cow milk proteins already in utero *(7)*. Transplacental passage of bovine beta-lactoglobulin has been experimentally proved *(8)*. It is probably the timing of the prenatal exposure to milk proteins that influences the postnatal immune response to these antigens. Earlier clinical studies failed to demonstrate any effect of milk protein avoidance in mothers' diet during last trimester of pregnancy on the levels of IgG to milk in infancy *(9)*. However, due to methodology problems, studies investigating the influence of prenatal antigen exposure on the development of milk-specific systemic humoral immune response in infants have not been done.

High levels of cow milk-specific IgG antibodies in cord blood are obviously of maternal origin due to placental transitivity of IgG antibodies. IgA antibodies, on the contrary, cannot pass the placental barrier, thus, cord blood levels would have developed after intrauterine antigen stimulation.

As expected, mechanisms of humoral immune response to cow milk proteins differs if the infant is breastfed or cow milk formula fed. Small amounts of cow milk proteins are regularly present in breast milk of most mothers drinking cow milk or dairy products throughout the whole pregnancy *(10)*. These amounts of antigen repeatedly presented to local immune system of the infant gut do not lead to sensitisation and probably induce systemic low dose oral tolerance. This effect is enhanced by interleukin-10 (IL-10) and transforming growth factor beta (TGF-beta), cytokines present in human milk *(11)*. Especially the TGF-beta induces the isotype switch and production of IgA *(12)*. This mechanism may lead to triggering of cow milk-specific IgA antibody production even in exclusively breastfed infants. There is no hitherto published study investigating the association between the bovine beta-lactoglobulin amount in breast milk and the cow milk-specific serum antibodies in breastfed infants. Nevertheless, the amount of bovine beta-

lactoglobulin, the major cow milk allergen, in breast milk does not correlate with the amount of cow milk ingested by the mother *(10)*. That is why any correlation of cow milk amounts ingested by the mothers with the anti-milk antibody levels in infants could have been hardly expected.

In formula fed infants, postnatal levels of cow milk-specific IgG and IgA are influenced by the time of introduction of cow milk to the infant diet and the amount of formula fed *(6)*. Early weaning and subsequent introduction of cow milk formula lead to higher IgG levels and often to sensitisation *(13)*. This risk is especially high when a newborn with allergic predisposition is fed cow milk formula immediately after birth before the onset of breastfeeding. High cow milk antigen load in the case of cow milk formula fed infants obviously leads to high levels of milk-specific IgG antibodies. Similarly, cow milk-specific IgA production is also stimulated.

We can conclude as follows: cow milk-specific IgG concentrations are higher than the IgA antibodies in umbilical sera. The IgG antibodies are stimulated by feeding cow milk formula and not by breastfeeding. The IgA antibodies are stimulated little during exclusive breastfeeding and much more intensively by feeding a cow milk formula.

Our results confirm the previously published data of *Tainio et al. (14)* that cow milk-specific plasma IgA antibodies are stimulated even during exclusive breastfeeding, while IgG antibodies are stimulated only with high amounts of antigen. The clinical significance of cow milk-specific IgG or IgA antibody levels in gastrointestinal allergies, however, remains low. Their concentrations reflect a physiological response to cow milk protein exposure through infant diet.

A c k n o w l e d g e m e n t s

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PROTILÁTKY TŘÍDY IgG A IgA PROTI KRAVSKÉMU MLÉKU V SÉRU KOJENÝCH NEBO UMĚLE ŽIVENÝCH KOJENCŮ

S o u h r n

Bílkoviny kravského mléka patří k hlavním alergenům v kojeneckém věku. Cílem naší studie bylo stanovit hladiny specifických protilátek IgG a IgA proti kravskému mléku u zdravých kojenců během prvních šesti měsíců života, a to jak plně kojených, tak i živených mléčným přípravkem. Protilátky proti kravskému mléku třídy IgG a IgA byly stanoveny metodou ELISA v pupečníkové krvi u 75 zdravých novorozenců. Koncentrace byly dále stanoveny ve venózní krvi u stejné skupiny kojenců ve 3. a 6. měsíci věku. Protilátky třídy IgG proti mléku významně klesaly od narození do 3. měsíce a dále od 3. do 6. měsíce u kojených dětí. U uměle živených kojenců hladiny vzrůstaly během prvního trimestru a poté nevýznamně poklesly. Specifické protilátky třídy IgA vzrůstaly průběžně od narození do 3. a dále do 6. měsíce nezávisle na typu výživy. Nárůst byl však silnější u kojenců živených umělým mlékem než u plně kojených. K tvorbě specifických protilátek třídy IgG proti bílkovině kravského

mléka dochází po podávání mléčného přípravku, nikoliv však během výlučného kojení. K produkci specifických protilátek třídy IgA však dochází i v průběhu výlučného kojení. Tyto nálezy mohou být podmíněny odlišnými mechansimy imunitní odpovědi po nízké resp. vysoké expozici antigenu.

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