

IgG and IgA antibody levels to cow's milk are low at age 10 years in children born preterm

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Background: Both innate and specific defenses of the preterm infant are even less developed than those of term infants, and the immune systems of preterm infants might be skewed differently at birth. Their immune responses to food antigens started early in life might therefore differ from those of term infants.

Objective: We sought to compare antibody levels to cow's milk, ovalbumin, and gliadin at age 10 years in children who had been born either preterm or at term.

Methods: IgG and IgA isotype antibodies to whole cow's milk, β -lactoglobulin, α -casein, and ovalbumin, as well as IgG antibody levels to gliadin and to tetanus and diphtheria toxoids, were measured for a group of 62 children born preterm and 61 control subjects born at term. These children were studied at the same time for atopy.

Results: Children born preterm had markedly lower levels of antibodies to cow's milk and to its protein fractions ($P < .0001$ for IgA and IgG antibodies to cow's milk and α -casein and IgG β -lactoglobulin antibodies). IgG gliadin antibodies were also significantly lower in the preterm group ($P = .03$), although the difference was not significant for IgG ovalbumin antibodies. In the preterm group both those born before gestational week 30 and those given cow's milk-based formula early (before day 50) had the lowest levels of cow's milk antibodies. In the preterm group atopy was associated with low levels of IgG cow's milk antibodies but with high levels of IgG ovalbumin antibodies.

Conclusions: Early introduction of food antigens into the immature gastrointestinal tract of preterm infants might result in tolerance. The presence of less atopy in these children might also be a result of tolerance development. (*J Allergy Clin Immunol* 2002;110:658-63.)

Key words: Food antigen, food antibodies, prematurity, atopy, allergy

Abbreviations used

BLG: Bovine β -lactoglobulin

OR: Odds ratio

The immune defense of the preterm infant is compromised at birth.^{1,2} However, repeated stimuli, such as those experienced in vaccination programs, have been observed to result in adequate antibody responses by the age of 4 years against most microbial antigens in vaccinations.^{1,3,4} Very little is known of the humoral responses of preterm infants against dietary antigens. The response to antigens in cow's milk are particularly interesting because these are usually the first foreign nutritional proteins for infants whose innate and specific immune defense of mucosa surfaces is not yet fully developed.⁵ In full-term infants antigens in cow's milk evoke both humoral and cell-mediated responses^{6,7}; comparison of infants having varying lengths of breastfeeding show that those briefly breast-fed for less than 90 days have higher titers than those breast-fed for longer than 150 days.⁶ Simultaneous feeding of breast and cow's milk might reduce the response.⁶

Responses require the permeation of antigenic material through the epithelium to reach the antigen-presenting cells of the gut and subsequently to provoke the immune response. A small amount of oral antigenic material reaches the newborn infant's circulation, with this proportion being markedly greater in preterm infants.^{8,9} We have demonstrated that prematurity is associated with the appearance of significantly less atopic sensitization by age 10 years than seen in an age-matched control group.¹⁰ A possible mechanism causing less sensitization might be the development of oral tolerance to food antigens in infants born preterm. Such tolerance development might result from the interaction of high antigen concentration with the immature, T_H2 -skewed immune system of the preterm infant.¹¹

In the present study we looked for evidence that oral tolerance develops to antigens received early in the diet by premature infants weighing less than 1500 g at birth.¹⁰ We compare IgA and IgG antibody levels to foods started at different ages during infancy in these preterm infants with levels in a similar group of children born at term. To rule out the possibility that children born preterm had a generalized defect in antibody production,

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TABLE I. Characteristics of study groups

	Preterm group (n = 62)	Term group (n = 61)
Male/female	38/24	32/29
Birth weight, g (mean $\pm$ SD)	1081 $\pm$ 261	3610 $\pm$ 477
Age at examination, y (mean $\pm$ SD)	10.1 $\pm$ 0.3	10.1 $\pm$ 0.4
Atopic symptoms, n (%) [*]	21 (34)	30 (49)
Obvious atopy, n (%) [†]	11 (17)	18 (29)
Gestational age, wk (mean $\pm$ SD)	28.5 $\pm$ 2.4	Not known
Exclusive breast-feeding, mo (mean $\pm$ SD) [‡]	3.0 $\pm$ 2.2	3.1 $\pm$ 1.5
Total breast-feeding, mo (mean $\pm$ SD) [‡]	6.7 $\pm$ 5.8	7.2 $\pm$ 4.6
Age at introduction of solid food, mo (mean $\pm$ SD) [‡]	4.7 $\pm$ 1.8	3.5 $\pm$ 1.0
Age at introduction of cow's milk-based formula, d (mean $\pm$ SD) [§]	57 $\pm$ 54	Not known

^{*}Any atopic symptom (ie, atopic eczema, allergic rhinoconjunctivitis, atopic asthma, or any combination of these).

[†]At least one atopic symptom and one of the following: at least one positive skin prick test response, serum total IgE level of greater than 320 kU/L, or at least one allergen-specific IgE level of greater than 0.8 kU/L.

[‡]Determined on the basis of history.

[§]Determined from hospital records.

we also measured IgG antibodies to diphtheria and tetanus toxoids in both groups.

METHODS

Study groups

Of 100 consecutive children born prematurely in 1987 and 1988 at Children's Hospital, University of Helsinki, weighing less than 1500 g, 72 took part in our study of atopy and lung function at a mean age of 10.1 years.¹⁰ A control group of 65 children born at term and with a similar environment was studied by using the same methods. For the present study, we obtained blood samples from 62 (86%) subjects in the preterm group and 61 (94%) subjects in the term group, all taken at the mean age of 10.1 years. The remaining children either refused to give a blood sample or the sample volume was insufficient for measurements. Group characteristics are given in Table I.

Data collection and definitions of atopy

Exact data on pregnancy and delivery and data on early medical problems, treatment, and nutrition were collected from the case records of those children born preterm. Both groups of children were invited at age 10 years to one visit at the outpatient clinic of the Hospital for Children and Adolescents, University of Helsinki. Parents were asked to fill in a questionnaire on possible risk factors and symptoms of atopy, and this questionnaire was checked at the outpatient clinic in detailed discussions with one of the authors. The same author (M.S.) carried out physical examinations of all the children.

When the history of atopic symptoms was validated with an objective finding of atopic sensitization, the child was classified as having obvious atopy.

Laboratory methods

Skin prick tests were performed with a panel of 8 standard Soluprick solutions from ALK (Allergologiska Laboratorium); histamine hydrochloride (10 mg/mL) served as the positive control, and 50% glycerol solution served as the negative control. The reaction was regarded as positive when the wheal diameter was 3 mm or larger and the negative control solution caused no reaction. The

serum total IgE concentration and the allergen-specific IgE antibodies to cow's milk, hen's egg, cat, and birch were measured by means of enzymatic UniCAP fluoroimmunoassay. Serum total IgE levels of greater than 320 kU/L, allergen-specific IgE antibody levels of greater than 0.8 kU/L, or a positive skin prick test response were considered to indicate atopic sensitization.¹⁰

Antibodies of IgA and IgG isotypes to cow's milk, bovine β -lactoglobulin (BLG), α -casein, and ovalbumin were measured with an ELISA, as described earlier, with modifications.^{7,12} Microtiter plates (Nunc Immunoplate, Nunc A/S) were coated with either diluted and defatted (1:500) adapted liquid cow's milk formula (Tutteli, Valio Ltd), with BLG (Sigma Pharmaceuticals) at a concentration of 1 μ g/mL, or with α -casein and ovalbumin (Sigma) at a concentration of 2 μ g/mL in carbonate buffer, pH 9.6, overnight. Diluted sera were applied in triplicate to the antigen-coated plates and in duplicate to wells of the same microtiter plates coated with blocking solution (1% sheep serum); the plates were incubated overnight at room temperature. After washing, 75 μ L of alkaline phosphatase-conjugated monospecific swine anti-human IgG, IgA, and IgM antisera (diluted 1:200, Orion Diagnostica) was added, and the plates were incubated for 60 minutes at 37°C. After washing, 75 μ L of *p*-nitrophenylphosphate substrate was added (2 mg/mL in diethanolamine buffer, pH 10.0, I. T. Baker Chemical). The reaction was stopped after 30 minutes with 75 μ L of 1 mol/L NaOH. The end product was measured at 405 nm in a semiautomatic photometer (Titertek Multiscan, Elflab Inc). We compared this result with that for 2-fold serial dilutions of a standard serum with a very high titer of the respective antibodies by using a computer. The standard serum was given a value of 100 AU. IgG gliadin antibodies were measured by using an ELISA, as described earlier.¹³

IgG antibodies to tetanus and diphtheria toxoids were measured with an ELISA. Toxoids were a gift from Dr Helena Käyhty (National Health Institute, Helsinki, Finland). Microtiter plates were coated with 100 μ L of either toxoid at a concentration of 0.125 μ g/mL (tetanus) or 0.25 μ g/mL (diphtheria) in 0.05 mol/L carbonate buffer, pH 9.6, and incubated overnight at 8°C. In the morning, these plates were washed with 0.05% Tween-PBS, and blocking was done with 100 μ L of 1% human serum albumin (Finnish Red

TABLE II. Levels of IgA and IgG antibodies to cow's milk and α -casein, IgG antibodies to gliadin and ovalbumin, and positive IgA and IgG titers to BLG in children at age 10 years born preterm or at term

	Born at term (n = 61)	Born preterm (n = 62)	P value	Born preterm					
				Born before gestational wk 30 (n = 40)	Born after gestational wk 30 (n = 22)	P value	Cow's milk-based formula before age 50 d (n = 36)	Cow's milk-based formula after age 50 d (n = 24)	P value
Cow's milk IgA (AU)	1.0 (0.7-1.5)	0.3 (0.2-0.5)	<.001*	0.2 (0.1-0.4)	0.6 (0.3-1.5)	.03*	0.2 (0.1-0.4)	0.5 (0.3-1.0)	.03*
Cow's milk IgG (AU)	9.2 (7.0-12.0)	2.4 (1.6-3.6)	<.001*	2.0 (1.3-3.2)	3.3 (1.5-7.3)	.08*	1.7 (1.0-3.1)	3.2 (1.8-3.2)	.16*
α -casein IgA (AU)	2.0 (1.3-3.1)	0.5 (0.3-0.8)	<.001*	0.4 (0.2-0.6)	0.9 (0.4-2.1)	.09*	0.3 (0.2-0.6)	0.8 (0.4-0.8)	.04*
α -casein IgG (AU)	2.4 (1.8-3.3)	0.48 (0.3-0.8)	<.001*	0.4 (0.2-0.7)	0.7 (0.3-1.9)	.72*	0.3 (0.2-0.6)	0.8 (0.4-1.6)	.54*
BLG IgA (no. with positive titer [%])	35 (57)	28 (45)	.3†	18 (45)	10 (48)	1†	14 (40)	13 (54)	.3†
BLG IgG (no. with positive titer [%])	55 (90)	36 (59)	<.001†	20 (50)	16 (76)	.05†	19 (54)	16 (67)	.3†
Gliadin IgG (AU)	3.2 (2.3-4.6)	1.7 (1.1-1.7)	.03*	1.4 (0.8-2.5)	2.4 (1.1-5.3)	.27*	ND	ND	
Ovalbumin IgG (AU)	74 (53-103)	48 (32-71)	.1*	46 (27-80)	49 (28-85)	.9*	ND	ND	

Values are given as geometric means (95% CIs) unless otherwise stated.

ND, Not done.

**t* Test.

† χ^2 Test.

Cross Blood Services) in PBS for 1 hour at room temperature. Sera were diluted in 0.2% human serum albumin in 0.05% Tween-PBS to a concentration of 1:400 for tetanus-coated plates and 1:200 for diphtheria-coated plates. Samples of 100 μ L were applied to washed microtiter plates in duplicate and incubated for 2 hours at room temperature. After washing, 100 μ L of alkaline phosphatase-conjugated anti-human rabbit IgG (Jackson Immuno Research) was added at a dilution of 1:3000 in 0.05% Tween-PBS with 0.2% human serum albumin. After incubation for 90 minutes at room temperature and washing, 100 μ L of phosphatase substrate (*p*-nitrophenyl disodium phosphate, Sigma) diluted in 0.05 mol/L carbonate and 0.001 mol/L $\text{MgCl}_2 \cdot 2\text{H}_2\text{O}$ buffer, pH 9.6 (1 tablet per 5 mL), was added. Microtiter plates were then incubated at room temperature for 30 minutes. The end product was measured at 405 nm in a semiautomatic photometer (Titertek Multiscan).

The standard was human immunoglobulin, another gift from Dr Helena Käyhty. It contained 65 IU/mL tetanus antibodies and 201 IU/mL diphtheria antibodies. The standard is annually calibrated at the National Health Institute with the International Standard of Tetanus Human Immunoglobulin of the World Health Organization and the International Standard of Diphtheria Equine Antitoxin of the World Health Organization.

Statistics

Logarithmic transformation was used before comparisons to correct for the fact that not all the levels of antibodies were normally distributed. Levels between the premature and full-term group were compared with a *t* test. Differences in the distribution of positive

antibody levels in various groups was tested by using the χ^2 test. The sum of 3 ranks in the group was calculated for both the IgA and the IgG isotype of cow's milk antibodies (whole cow's milk, BLG, and α -casein) to find associations between antibody levels and other data in either group. Odds ratios (ORs) for belonging to either the highest or lowest quartile for IgA and IgG antibodies were determined by means of logistic regression. In the analysis dichotomous variables for gestational age (cut-off point of 30 weeks) and introduction of cow's milk-containing formula to the diet (cut-off point 50 days after birth) were used for the preterm group. For both groups, a positive history of atopic symptoms and obvious atopy at age 10 years were used.¹⁰ We considered a *P* value of less than .05 to be statistically significant.

Ethics

The Ethics Committee of the Hospital for Children and Adolescents, University of Helsinki, approved the study.

RESULTS

Group characteristics are given in Table I. In the original series the incidence of both atopic symptoms and obvious atopy was significantly lower in the preterm group,¹⁰ but in the present series the OR for symptoms of atopy in the preterm group was 0.53 (95% CI, 0.26-1.1; *P* = .09) and that for obvious atopy was 0.52 (95% CI, 0.22-1.21; *P* = .13). The change in significance level in atopy

TABLE III. IgA and IgG antibodies to cow's milk and α -casein and IgG antibodies to ovalbumin in study groups divided on the basis of atopy findings

	Born preterm			Born at term		
	No symptoms of atopy (n = 41)	Obvious atopy (n = 11)*	P value†	No symptoms of atopy (n = 31)	Obvious atopy (n = 18)‡	P value†
Cow's milk IgA (AU)	0.4 (0.2-0.6)	0.4 (0.1-0.4)	1	1.4 (0.9-21)	0.8 (0.3-2.2)	.29
Cow's milk IgG (AU)	3.6 (2.5-5.3)	0.7 (0.2-2.8)	.04	9.0 (6.3-12.9)	9.0 (5.0-16.1)	1.0
α -Casein IgA (AU)	0.8 (0.4-1.2)	0.2 (0.1-0.8)	.1	2.8 (1.8-4.3)	1.4 (0.5-3.9)	.29
α -Casein IgG (AU)	0.8 (0.5-1.3)	0.2 (0.1-0.4)	.02	2.3 (1.6-3.5)	2.2 (1.0-4.9)	.94
Ovalbumin IgG (AU)	36 (22-61)	162 (114-230)	<.001	72 (50-106)	74 (33-168)	.94

*Ten children who had symptoms of atopy, negative skin prick test responses, total serum IgE levels of less than 320 kU/L, and allergen-specific IgE levels of less than 0.8 kU/L were excluded from this group.

†t Test.

‡Twelve children who had symptoms of atopy, negative skin prick test responses, total serum IgE levels of less than 320 kU/L, and allergen-specific IgE levels of less than 0.8 kU/L were excluded from this group.

incidence was due to disequilibrium in atopy among the dropouts in the preterm group: 1 of 10 dropouts had atopic symptoms. The ages of the children at the time of this study were similar. Durations of both exclusive and total breast-feeding were similar in both groups, but the age in the preterm group at introduction of solid foods was significantly higher ($P < .001$, Table I).

At a mean age of 10.1 years, children in the preterm group had significantly lower levels of antibodies of the IgG isotype to whole cow's milk and α -casein (Table II), with the geometric mean level being about one fourth that in the term group. Measurable IgG levels to BLG were significantly less frequent in the preterm group than in the term group (Table II). IgA antibodies to cow's milk and α -casein were also lower in the preterm group, whereas the proportion of children with measurable levels of IgA antibodies to BLG showed no difference between groups (Table II). IgG anti gliadin levels were lower in the preterm group, and the same tendency was evident for IgG ovalbumin antibodies (Table II). Measurable levels of IgE antibodies to egg (>0.1 kU/L) were equally frequent in the preterm and term groups (11/62 and 15/61). Specific IgE to cow's milk (>0.1 kU/L) could be measured in only 3 samples, 2 in the term group.

Within the preterm group, gestational age affected the level of cow's milk antibodies. Those born before the 30th gestational week had significantly lower levels of IgA cow's milk antibodies, and the proportion with positive IgG isotype antibodies to BLG among these patients was lower than among those born between 30 and 34 gestational weeks (Table II). Those children in the preterm group who had consumed cow's milk formula before age 50 days had significantly lower levels of IgA antibodies to cow's milk and α -casein than did those started on formula after day 50 (Table II).

Those children in the preterm group with obvious atopy had significantly lower levels of IgG cow's milk and α -casein antibodies than did those without atopic symptoms (Table III). In contrast, they showed higher levels of ovalbumin IgG antibodies than did children without atopic symptoms (Table III). In the term group no differences were apparent between children with obvious atopy and those with no symptoms of atopy. Comparisons of antibody levels of those with and without symptoms of atopy were similar to those in Table III, except that preterm children with atopic symptoms had significantly lower levels of IgA casein antibodies than did preterm children without symptoms (geometric means of 0.2 [95% CI, 0.1-0.5] and 0.8 [95% CI, 0.4-1.20], respectively; $P = .05$).

Logistic regression models showed a significant OR for those preterm children with atopic symptoms to be in the lowest quartile for IgG cow's milk antibodies (OR, 11.9; 95% CI, 1.6-88; $P = .002$). Significant ORs for being in the lowest quartile of IgA cow's milk antibodies were for early introduction of cow's milk formula (before day 50; OR, 13; 95% CI, 1.2-144; $P = 0.04$) and for gestational age less than week 30 (OR, 11; 95% CI, 1.1-120; $P = .04$). Among children born at term, long-term breast-feeding (>6 months) bore a significant OR for the low IgG cow's milk antibody quartile (OR, 12; 95% CI, 1.2-128; $P = .038$).

The preterm and term groups showed similar levels of IgG antibodies to tetanus (geometric means of $55.7 \text{ IU/mL} \times 10^{-3}$ [95% CI, 54.1-57.4 $\text{IU/mL} \times 10^{-3}$] and $45.3 \text{ IU/mL} \times 10^{-3}$ [95% CI, 33.4-61.5 $\text{IU/mL} \times 10^{-3}$], respectively) and diphtheria toxoids (geometric means of $20.0 \text{ IU/mL} \times 10^{-3}$ [95% CI, 15.4-26.1 $\text{IU/mL} \times 10^{-3}$] and 16.7 [95% CI, 12.9-21.7 $\text{IU/mL} \times 10^{-3}$], respectively).

DISCUSSION

The maturity of newborn infants has a definitive effect on their development of antibodies against food proteins ingested early during infancy. Children born preterm had, at the age of 10 years, markedly lower levels of IgG and IgG antibodies against cow's milk than did similar children born at term. Even within the preterm group, the most immature at birth had a lower level of antibodies than did the more mature. Therefore we infer that the immaturity of the innate and specific defense mechanisms of the intestines of preterm infants with a birth weight of less than 1500 g results in suppression of the immune response to oral antigens given early during life and makes the B cells of these children more tolerant to these antigens. The same preterm group showed less atopy,¹⁰ which might be another facet of their tolerance development. When the preterm group was divided according to time of introduction of cow's milk-based formula, those infants with earlier introduction had a lower level of antibodies. This speaks for the idea of tolerance development caused by high antigen concentration¹⁴ because the permeability of the intestines of preterm infants correlates with their degree of prematurity.⁹

When the food is introduced later, such as gluten in cereals at the age of 4 to 5 months,¹⁵ the difference between preterm and term groups is smaller, reflecting the maturation of the preterm infants' intestines and their immune systems. A significant amount of egg is usually introduced into the infant diet even later, after the age of 5 months,¹⁵ with no difference evident in the titers against that protein. This indicates rapid maturation of the mucosal immune responses of preterm infants. Children born preterm do not have a generalized B-cell defect, as shown by our groups' similar responses to repeated parenteral injections of bacterial toxoids in the form of vaccines, a finding also reported earlier.^{1,3,4}

The natural development of cow's milk antibodies has been well delineated, even though their pathogenetic role in food-related diseases is poorly recognized.^{16,17} Aberrant immune responses to food antigens might be important in the development of some autoimmune diseases, such as type I diabetes.¹⁸ Introduction of cow's milk-based formula results in the appearance of IgG and IgA antibodies to its fractions within weeks, and the peak of these antibodies is reached within a few months of their introduction, being different for different isotypes and fractions of cow's milk.^{6,7} The peak of the titer depends at least on the age of introduction of cow's milk. If cow's milk-based formula is introduced early, titers are higher.⁶ It was recently shown that even at age 8 years the mode of introduction still had an effect on antibody levels.¹⁹ We also found that children in the term group with long-term breast-feeding (>6 months) had lower levels of IgG cow's milk antibodies than did those with shorter-term breast-feeding.

IgE antibodies to ovalbumin have been found to be a good predictor of atopy.²⁰ In one study high levels of IgG antibodies to ovalbumin at age 1 year in a group of 264 full-term infants was a significant risk factor for atopy at

age 2 years, whereas the IgG cow's milk antibodies conferred no such risk.²¹ We found only the IgG isotype of food antibodies to be associated with atopy and only in the preterm group. In atopic children IgG antibodies to cow's milk were lower and those to ovalbumin were higher than in nonatopic children. Egg was started in the diet of the preterm group more than 3 months later than cow's milk. A simultaneous rapid change in intestinal permeability,⁹ changing the T_H1/T_H2 balance, and other developmental changes in immune responses during the early months of life²²⁻²⁴ might give rise to this contrasting development in the 2 food antibody levels.

Our study shows that the immune response of the gut to food antigens might be greatly influenced by the maturity of the intestine. A long-lasting effect on the titers to cow's milk proteins was caused in the term group only by the length of breast-feeding, whereas for the preterm group, in addition to time of introduction of cow's milk to the diet, the predisposition to atopy and the degree of prematurity also had effects.

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