

Immune defense to dietary cow milk in healthy infants

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The healthy young infant is immunologically adapted to receiving vast amounts of antigens in diet. At the age of 6 months, nine infants were put on a CM elimination diet for 3 weeks and then challenged with CM. Gut immune response was evaluated indirectly with ELISPOT assay at 6 months, after CM elimination (Day 1) and challenge (Day 8), and at 11 months. CM elimination decreased the numbers of immunoglobulin secreting cells (ISC): in the IgM class from mean [95% CI] 4969 [2555, 9653] at 6 months to 1716 [1024, 2873]/10⁶ cells on Day 1 ($t = 3.14$, $p = 0.01$); and in the IgG class from 5547 [3562, 8630] to 2684 [1383, 5208]/10⁶ cells ($t = 3.29$, $p = 0.01$). CM challenge further reduced inter-individual variation, and at 11 months the scatter of ISC was comparable to that at 6 months. Specific antibody-secreting cells of the IgA and IgG class were seen at 6 months and again at 11 months, while specific IgM-secreting cells persisted throughout the dietary manipulation. The results indicate that diet profoundly affects the immune defense system, and further suggest that a focused immune response is vital in acquisition of tolerance to dietary antigens.

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During the first months of life, the newborn adapts to a constant peroral challenge with a multitude of foreign proteins, including dietary antigens. When breastfeeding is inaccessible, adapted cow milk (CM) formulae are recommended (1). Such an increase in the antigenic load is well tolerated by most infants.

The active process of adaptation includes suppression of any adverse immunologic reactions to antigens that are useful to the organism (2). Based on animal studies, it has been suggested that immune responses directed against dietary antigens regulate in part the development of gut mucosal barrier mechanisms (3, 4). In a small minority of infants, aberrant immune responses to CM result in clinical CM allergy (5). These responses have been studied extensively (6, 7). By contrast, the chain of immunologic events that leads to clinical tolerance to CM in healthy human infants remains poorly delineated.

The objective of the present study was to investigate the sequence of the immune defense in healthy infants, shown to tolerate CN, during CM elimination, CM challenge and prolonged CM administration.

Subjects and methods

Subjects and diet – Nine healthy infants were recruited at a mean (SD) age of 6 (0.1) months. These well-nourished infants had been born at term, and had no previous history nor current signs of allergy. One infant had slight constipation at the time of the study and a later diagnosis of anterior anus. The rest of the infants did not have any gastrointestinal problems. Parents gave written informed consent and the study was approved by Tampere University Hospital's Committee on Ethical Practice.

CM elimination was commenced at the age 6.8 (0.8) months. The idea was to study the effect of CM elimination and challenge with repeated analysis of the same infants so that they would in a way act as their own controls. The effect of the usual formula was assessed at six and eleven months. Weaning from breastmilk to CM formula was complete at a median age of four months (range two months to seven months and three weeks). One infant received breastmilk twice daily until the third day of CM elimination, but had been receiving CM formula daily from the age four months. The median time interval from com-

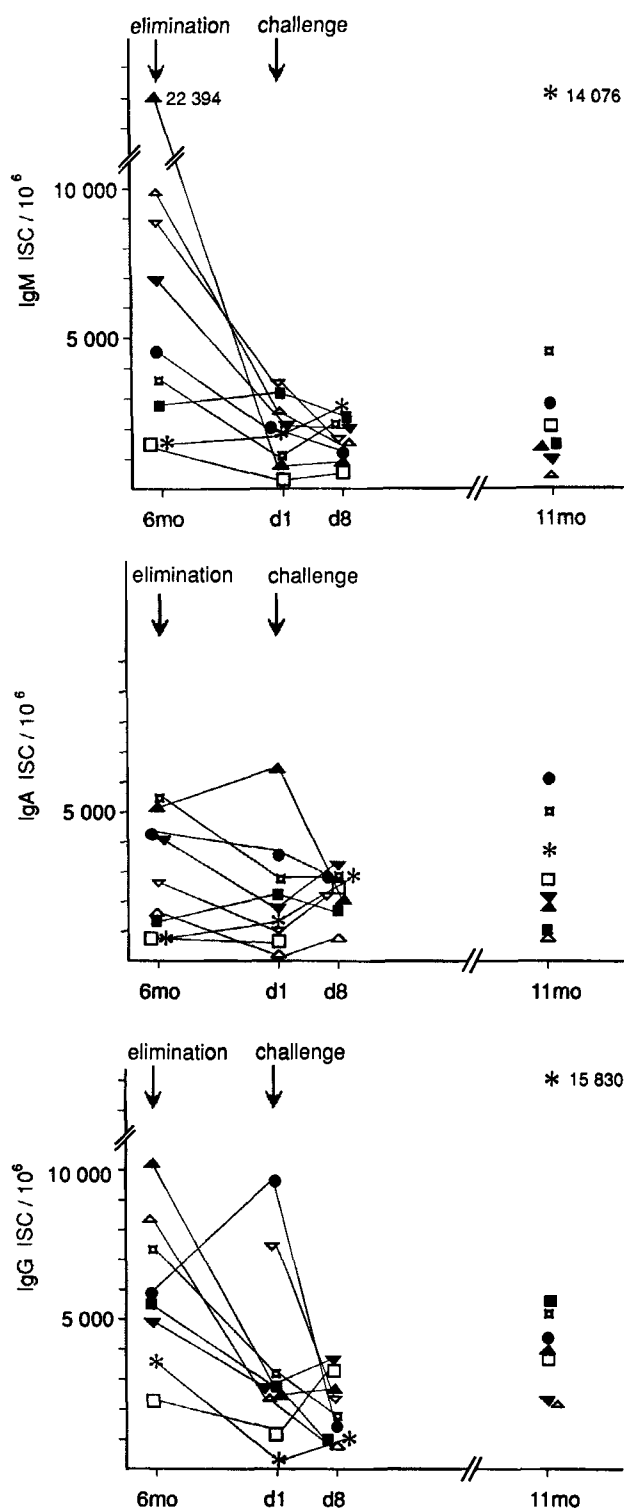


Fig. 1. The total number of (a) IgM secreting cells/ 10^6 cells, (b) IgA secreting cells/ 10^6 cells, and (c) IgG secreting cells/ 10^6 cells at the age of six months, after CM elimination (d1), after CM challenge (d8), and at the final visit at the age of 11 months. Each individual infant is indicated with a distinct symbol.

mencement of CM formula feeding to commencement of CM elimination was three months (range two to seven months).

Whey protein hydrolysate formula (Valio Finnish Cooperative Dairies' Association, Finland) was supplied for the infants for mean (range) 21 (14–26) days. A daily record was kept by the parents on any skin, gastrointestinal or other symptoms throughout the dietary manipulation. The bowel movements and the quality of stools (characterized as watery, loose or solid) were recorded. Except for advice to strictly avoid CM in the infant's diet, no dietary restrictions were instigated.

CM challenge – After the elimination period, the infants were given one standard dose of 10 ml of CM formula (Tutteli®, ready for use adapted CM formula, Valio, Finland) on Day 1. They then continued ingesting the whey hydrolysate, and were re-examined a week later on Day 8.

In a previous study a similar protocol was adopted, except that administration of CM antigens was continued until Day 8, when no symptoms of CM allergy appeared. These infants were thus negative on clinical CM challenge, and also hyporesponsive immunologically (8). A single dose was given in the present study to ensure that such hyporesponsiveness does not merely reflect fading of the ELISPOT response on continued antigen exposure. The dose of 10 ml was chosen since it has been shown an average dose to which CM allergic infants react (9, 10).

The infants resumed their respective CM formulae after the Day 8 visit, and eight of them were seen at the age of 11.2 (0.5) months.

Blood samples – Blood was drawn and heparinized for the ELISPOT assay at the six months' visit, on Day 1 before the CM challenge to assess the effect of CM elimination, and on Day 8 a week later to assess the effect of CM challenge. From earlier studies it is known that the maximal response after administration of antigen on Day 1 is measurable on Day 8 (11). A final sample was obtained at the 11.2 months' visit.

ELISPOT assay – The immune defense was assessed by the ELISPOT method as previously described (8, 11). The total number of immunoglobulin-secreting cells (ISC) and the number of cells secreting antibodies specifically (sASC) against CM proteins β -lactoglobulin and casein were enumerated.

Mononuclear cells (mainly lymphocytes) were obtained by Ficoll-Paque (Pharmacia AB, Uppsala, Sweden) centrifugation of the blood samples. Isolated cells were washed three times in Hanks buffered-salt solution (Flow laboratories, Irvine, Scotland) and were then suspended in culture me-

Table 1. The number of healthy infants showing specific antibody-secreting cells to CM proteins β -lactoglobulin and casein at the age of six months, before (d1) and after (d8) CM challenge, and at the final visit at 11 months.

	six months	d1	d8	11 months
IgM sASC	9/9	8/9	9/9	8/8
IgA sASC	5/9	2/9	1/9	5/8
IgG sASC	6/9	1/9	2/9	5/8

The infants were classified according to the level of sASC as follows: negative <0.5 sASC/ 10^6 cells, positive >0.5 sASC/ 10^6 cells.

dium and adjusted to a 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) so that the antibodies secreted by them could react with the antigen. These antibodies, and thus the cells, were visualized by application of enzyme-labelled antisera, followed by a substrate agarose overlay. The colored spots were counted with the help of a stereoscopic microscope after various times of storage at 4°C . The wells were coated with anti-human antisera (anti-IgA, -IgM and -IgG) for the determination of ISC, and with β -lactoglobulin and casein for the determination of sASC. The sum total of sASC was calculated by adding together the numbers of β -lactoglobulin-specific and casein-specific cells, and it was considered an immune response with >0.5 sASC/ 10^6 cells (8). The ISC and sASC responses were designated antigen non-specific and antigen-specific response, respectively.

Statistical analyses

Because of skewed distribution of data, natural logarithmic transformation was used, and results are expressed as geometric mean [95% confidence interval (CI)]. Paired *t* test was used to compare measurements at different time points.

Results

Clinical outcome – The infants accepted the protein hydrolysate well. No vomiting or diarrhea were recorded by the parents, but seven of the nine infants showed looseness of stools during CM elimination. All infants completed the dietary manipulation as planned. On CM challenge, they did not develop any symptoms indicative of allergy.

Antigen-non-specific immune defense – As depicted in Fig. 1, the numbers of ISC of all isotypes decreased during the period of CM elimination: in the IgM class from 4969 [2555, 9653] at six months to 1716 [1024, 2873]/ 10^6 cells on Day 1 ($t = 3.14$,

$p = 0.01$), and in the IgG class from 5547 [3562, 8630] to 2684 [1383, 5208]/ 10^6 cells ($t = 3.29$, $p = 0.01$). The trend in the IgA class was not statistically significant.

The CM challenge seemed to further reduce the interindividual variation in the levels of ISC of all isotypes (Day 8), though it did not result in any statistically significant changes in the antigen non-specific immune defense (Fig. 1). After resumption of the prior formulae, at the final follow up visit, the scatter of ISC in the IgA class was similar to that before the dietary manipulation.

Antigen-specific immune defense – As shown in Table 1, specific antibody-secreting cells in the IgM isotype were detected throughout the period of follow up. In contrast to this, specific IgA and IgG secreting cells were seen after prolonged administration of CM formula, both at the six months' visit and again at 11 months of age.

Discussion

The results of this study show that dietary manipulation significantly affects the immune defense system of the healthy infant, as evaluated with the ELISPOT method.

Measuring the number of immunoglobulin-secreting cells among circulating lymphocytes has been shown to give a good approximation of the gut local immune response (12, 13) and, moreover, is more consistent than the antibody response in either serum or duodenal fluid (14). In addition, direct sampling from the intestine with the help of a nasogastric tube twice within a week would seem practicable neither in healthy infants nor during diagnostic CM challenge.

The diagnosis of CM allergy is based on elimination of CM from the diet and a clinical challenge with CM, resulting in remission of symptoms during elimination and recurrence on challenge (15, 16). Our group has recently elucidated the immune response to CM challenge in CM allergic infants with the ELISPOT method (8). A strong antigen-non-specific response especially in the IgM isotype was compatible with CM allergy. At the same time, the antigen specific response was small and inconsistent. ELISPOT shows promise as an aid in diagnosing CM allergy.

In this study, one dose of CM formula did not result in an increase in the total number of immunoglobulin secreting cells. Previously a similar hypo-responsiveness was shown in infants who were given CM formula during the whole challenge period of one week since they clinically tolerated CM (8). It therefore seems that the immune response of healthy infants to oral antigen adminis-

tration is antigen specific, and results in clinical hyporesponsiveness.

It is interesting that substitution of the CM formula with a whey hydrolysate in the diet has such a profound effect on the total numbers of the circulating immunoglobulin-secreting cells, effecting a decrease in all isotypes. The dietary manipulation also seems to result in remarkably uniform levels of immunoglobulin-secreting cells in healthy infants of a narrow age distribution, irrespective of the premanipulation levels. These effects could be explained by a reduction in the antigenic load from the gut. This may be caused either by a lesser quantity of antigens in the hydrolysate itself compared with CM formula, or by a lesser effect on gut mucosal integrity compared with the CM formula. It has been shown that administration of CM, but not protein hydrolysate, enhances gut permeability of rat pups (17). Likewise, the gut of newborn infants has been shown more permeable during CM formula than breastmilk (18). The results of this study therefore support the idea that immune responses to dietary antigens in part regulate the development of gut mucosal barrier (3).

The healthy infants show antigen-specific cells in the IgA and IgG class after weaning, i.e. before any dietary manipulations, and again at the final follow up. This suggests that only prolonged exposure to CM induces specific IgA and IgG producing cells in addition to IgM. In accordance, CM allergic children have been shown to obtain the ability to produce antigen-specific IgA-secreting cells on becoming clinically tolerant to CM (9). As IgA is considered the most important antibody in the intestine (19), the in this study demonstrated sequence of events may represent one aspect of the healthy immune response to dietary antigens.

In conclusion, the results of this study indicate that a focused immune defense at the mucosal level is crucial in acquisition of clinical tolerance to dietary antigens. In immunologically healthy infants the early IgM response to dietary antigens evolves to IgA on prolonged exposure.

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