

Development of immune response to cow's milk proteins in infants receiving cow's milk or hydrolyzed formula

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Background: Development of humoral and cellular immune responses to orally administered antigens in human beings is poorly understood, although antigen administration has been suggested as a treatment for hypersensitivity disorders and autoimmune diseases.

Objective: The purpose of the study was to investigate the development of systemic immune response in infants fed with formula containing whole cow's milk proteins or hydrolyzed formula containing casein peptides.

Methods: In a double-blind trial, 10 infants received cow's milk-based formula, and 10 infants received a casein hydrolysate formula until the age of 9 months. Blood samples were taken at the ages of 6, 9, and 12 months. Cellular responses were assessed by proliferation assay of peripheral blood mononuclear cells to cow's milk proteins (β -lactoglobulin, bovine serum albumin, and α -casein). Humoral responses to the same proteins were measured by ELISA for IgG antibodies.

Results: Feeding infants with cow's milk-based formula induced systemic humoral and cellular responses to cow's milk proteins. T-cell response later declined, supporting the concept of oral tolerization. Exposure to cow's milk proteins after the age of 9 months resulted in depressed cellular and humoral responsiveness to these proteins.

Conclusion: Our results support the view that induction of oral tolerance in human beings is an age-dependent phenomenon. (*J ALLERGY CLIN IMMUNOL* 1995;96:917-23.)

Key words: Oral tolerance, β -lactoglobulin, bovine serum albumin, casein, insulin-dependent diabetes mellitus, cow's milk allergy, formula

Immunity to orally administered proteins has been studied intensively during recent years. However, the development of immune response to oral antigens in human beings, particularly cellular immunity, is poorly understood. Studies performed in animal models suggest that early exposure to orally administered proteins initiates an immune

Abbreviations used

BLG:	β -Lactoglobulin
BSA:	Bovine serum albumin
CAS:	α -Casein
CM:	Cow's milk
IDDM:	Insulin-dependent diabetes mellitus
PBMCs:	Peripheral blood mononuclear cells
SI:	Stimulation index

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Supported by grants from the Foundation for Diabetes Research, Finland (O. V., H. K. Å.), the Foundation for Nutrition Research (O. V.), the Sigrid Juselius Foundation (H. K. Å., E. S.), the Juvenile Diabetes Foundation International (H. K. Å., no. 192612), the Dorothea Olivia, Karl Walter and Jarl Walter Perklén Memorial Fund (H. K. Å.), and the Nordisk Insulin Foundation (H. K. Å.).

Received for publication Nov. 7, 1994; revised Feb. 15, 1995; accepted for publication Feb. 16, 1995.

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0091-6749/95 \$5.00 + 0 1/1/64643

response to them, whereas in adult animals the same exposure leads to tolerance.¹

The induction of oral tolerance has been studied in animal models of human cell-mediated autoimmune diseases, such as insulin-dependent diabetes mellitus (IDDM), multiple sclerosis, and rheumatoid arthritis, by feeding the animals with insulin, myelin, and collagen, respectively.²⁻⁵ In patients with multiple sclerosis, oral administration of bovine myelin antigens suppressed the symptoms of the disease and even decreased the number of T

cells that reacted with myelin basic protein.⁶ Similarly, administration of collagen to patients with rheumatoid arthritis relieved the symptoms in a considerable proportion.⁷ These studies suggest that oral administration of a specific protein to adults suppresses cell-mediated immunity to the protein. Recently, it was reported that in adults, an oral antigenic stimulus led to T-cell tolerance, but not to B-cell tolerance.⁸

A few studies of humoral immune response in healthy infants fed with cow's milk (CM)-based formula have demonstrated that oral introduction of CM proteins in early infancy elicits clear-cut antibody production to these antigens in all infants.^{9, 10} This immune response is strongest when CM proteins are introduced early during the neonatal period. The reactivity decreases with age, and at the age of 1 year there is no difference in antibody levels to CM proteins between those infants who received CM in early infancy and those who began receiving CM later.¹⁰ It has also been reported that humoral immune response to bovine serum albumin (BSA), which is a component of CM, declines with age.^{11, 12} These findings support the view that in human beings, a systemic hyporesponsive state (tolerance) to oral antigens also develops with age.

In this study we monitored the development of both humoral and cellular immunity to CM antigens in infants who received CM-based formula or hydrolyzed formula. The results indicate that the oral administration of proteins during early infancy resulted in systemic humoral and cellular immune responses. The later decline of the T-cell responses supports the concept of oral tolerization. Delayed exposure to oral proteins depressed both cellular and humoral responsiveness to these proteins.

METHODS

Subjects

Twenty newborn infants whose mothers had IDDM were recruited for the study, which was the pilot stage of a trial for primary prevention of IDDM by elimination of CM proteins during early infancy. Exclusive breastfeeding was encouraged; thereafter, 10 infants (group I) received adapted CM-based formula (Enfamil; Bristol-Myers Institutional Products, Evansville, Ind.) and 10 infants (group II) received a casein hydrolysate formula (Nutramigen, Bristol-Myers Institutional Products) until the age of 9 months. The trial was a double-blind, randomized study of two different nutritional supplements: the CM formula (group I) contained whole CM proteins, and to disguise the taste 25% of the powder was replaced with Nutramigen, whereas the other formula, Nutramigen, contained hydrolyzed casein peptides

of a molecular weight less than 1200 kd (group II). The mothers of all infants were advised to eliminate infant food products containing CM or beef until the age of 9 months. At the age of 9 months the dietary manipulation ended, and both groups started to receive ordinary CM-based formula containing whole CM proteins. The infants and their mothers visited the outpatient clinic of the Children's Hospital, University of Helsinki, when the infants were 3, 6, 9, and 12 months old; in addition, the dietary advisor had phone contact with the mothers. Blood samples were studied at the ages of 6, 9, and 12 months. In one child in group II manifest diabetes developed at the age of 14 months, and in one child in group I CM allergy developed at the age of 7 months.

Protocol

BSA, β -lactoglobulin (BLG), and α -casein (CAS) were all purchased from Sigma Chemical Co. (St. Louis, Mo.). Antibodies to BSA, BLG, and CAS were detected by ELISAs. The details of the methods for detection of antibodies to BLG¹³ and BSA¹⁴ have been described previously. The method for detection of antibodies to CAS was a modification of the latter. Briefly, polystyrene microtiter plates were coated with the antigen (2 μ g/ml in phosphate-buffered saline), and nonspecific binding was blocked by 1% gelatin. Duplicate plasma samples (diluted 1:50 to 1:100 in phosphate-buffered saline) were incubated for 1 hour at 37° C in parallel in wells coated with the antigen and in wells coated with the blocking solution only. Plates were washed with distilled water–0.05% Tween-20, and alkaline phosphatase-conjugated monospecific swine anti-human IgG (Orion Diagnostica, Espoo, Finland) was used as a second antibody. After incubation with p-nitrophenyl phosphate substrate, the absorbances were measured at a wavelength of 405 nm. A dilution series of a standard serum pool, which was obtained from patients with intestinal CM allergy and a high level of antibodies to CM proteins, was run in each plate; and the levels of antibodies were expressed as percentages of absorbance of the standard. Intra-assay and interassay coefficients of variation were 9.3% and 15.8% for antibodies to BSA, 10.0% and 21.0% for antibodies to BLG, and 6.0% and 10.0% for antibodies to CAS, respectively. Mononuclear cells were derived from the infants' peripheral blood by Ficoll-IsoPaque (Pharmacia, Uppsala, Sweden) density centrifugation (1400 g). For the proliferation tests, peripheral blood mononuclear cells (PBMCs) (1×10^5 cells/well) were incubated with various concentrations (2, 20, 200 μ g/ml) of the antigens (BSA, BLG, CAS) in RPMI 1640 medium (Sensitive Ltd., West Sussex, U.K.) containing 5% human AB+ serum (Finnish Red Cross Blood Service, Helsinki, Finland) and 4 mmol/L L-glutamine for 6 days. The cultures were pulse-labeled with 1 μ Ci of thymidine (Amersham International, Ltd., Little Chalfont, Bucks, U.K.) and harvested 18 hours later. Proliferation response was expressed as stimulation index (SI): tritiated thymidine incorporation in the presence of

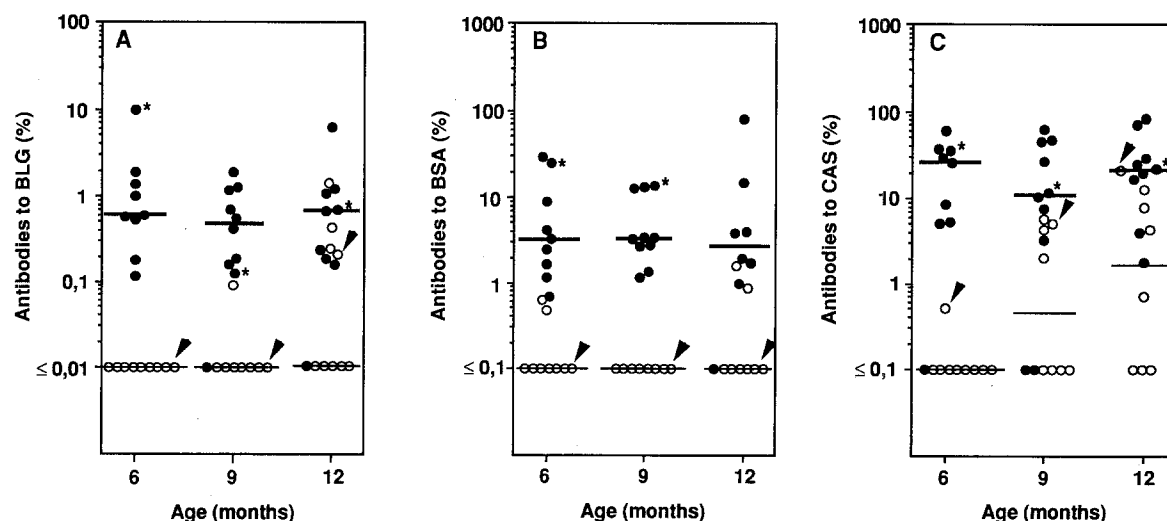


FIG. 1. Distribution of IgG class antibodies to BLG (A), BSA (B), and CAS (C) by age in infants receiving CM formula (●) and in infants receiving hydrolyzed formula (○). The infant in whom IDDM developed at the age of 14 months is indicated with an *arrowhead*, and the infant in whom CM allergy developed at the age of 7 months is indicated with an *asterisk*. *Thick line* (—) shows median value for group I (CM formula); *thin line* (—) shows median value for group II (hydrolyzed formula).

the antigen divided by thymidine incorporation in the absence of the antigen. Tetanus toxoid without thiomersal (National Public Health Institute, Helsinki, Finland) (8 µg/ml) was used as a positive control for proliferation, and only results of experiments in which a clear-cut proliferation for tetanus toxoid was observed were accepted as valid. All children received a tetanus vaccination at 3, 4, and 5 months of age. An SI of 3 was considered as significant proliferation.

For fluorescence-activated cell sorter analysis, 2×10^6 mononuclear cells were cultured for 7 days in 24-well tissue culture trays (Nunc, Copenhagen, Denmark) in the presence of BLG (20 µg/ml). On day 7, human interleukin-2 (20 IU/ml) (Janssen Biochimica, Geel, Belgium) was added, and the cells were cultured for another 7 days. The cells were incubated with fluorescein-conjugated antibodies to T-cell receptor α/β , CD4, CD8, CD3, and CD19 for 20 minutes at room temperature, washed, and analyzed by a fluorescence-activated cell sorter.

RESULTS

All of the infants except one received the test formula before the age of 6 months (mean age, 1.2 months; range, 0 to 7.5 months). No difference was observed in the duration of breast-feeding and the start of the substitution formula between the two groups. The infant in group I exposed to the CM formula for the first time at the age of 7.5 months was excluded from the analyses performed at the age of 6 months.

IgG class antibody levels to BLG and BSA in individual cases by age are shown in Fig. 1, A and

B. No significant antibody response was detectable until the age of 9 months in infants who received casein-based hydrolyzed formula before 9 months of age (group II).

The difference in the IgG antibody levels to BLG between the groups was significant at the ages of 6 and 9 months ($p = 0.0004$ and $p = 0.001$, respectively; Mann-Whitney U test) (Fig. 1, A). After the age of 9 months, infants in group II started to receive CM formula, and at the age of 12 months, the difference between the two groups was smaller and not statistically significant ($p = 0.09$; Mann-Whitney U test). Similarly, IgG antibody levels to BSA were significantly higher in group I than in group II ($p = 0.0004$ at 6 months of age, $p = 0.0003$ at 9 months of age; Mann-Whitney U test) (Fig. 1, B). At the age of 12 months, the BSA antibody level remained significantly lower in group II than in group I, despite the exposure to CM proteins after 9 months of age ($p = 0.006$; Mann-Whitney U test).

Some similarities were also seen in the proliferation of PBMCs to BLG (Fig. 2, A). No significant proliferation to BLG (SI ≥ 3.0) was detectable until the age of 9 months in group II; and at the age of 9 months, a significant difference in SIs to BLG appeared between the two groups ($p < 0.04$; Mann-Whitney U test). The SIs to BLG correlated with the level of IgG antibodies to BLG at 9 months of age ($r = 0.577$, $p = 0.02$; Spearman's

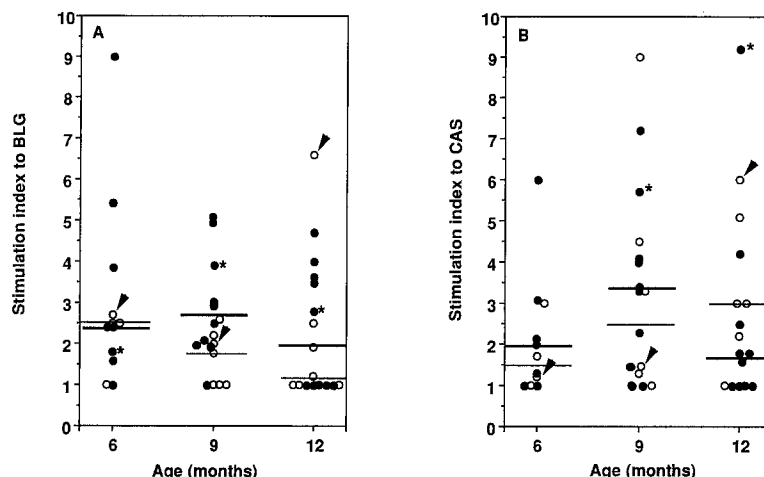


FIG. 2. Distribution of SIs to BLG (**A**) and CAS (**B**) according to age in infants receiving CM formula (●) and in infants receiving hydrolyzed formula (○). The infant in whom IDDM developed at the age of 14 months is indicated with an *arrowhead*, and the infant in whom CM allergy developed at the age of 7 months is indicated with an *asterisk*. Thick line (—) shows median value for group I (CM formula); thin line (—) shows median value for group II (hydrolyzed formula).

correlation test). At the age of 12 months, however, the difference between the groups disappeared, and the median SI to BLG decreased in group I from 9 months to 12 months of age. The SIs to BLG by age, for each infant in group I, are presented in Fig. 3.

In six of nine infants there was a decrease in SI from 9 months to 12 months of age, in two children there was an increase in SI, and in one child the SI remained the same (SI = 1). Thus a decreasing tendency in reactivity to BLG seemed to take place in the majority of the children who received CM proteins continuously.

Proliferation of PBMCs to BSA was low (SI < 2.0) in all infants except one throughout the follow-up period (data not shown). The infant in whom CM allergy developed at the age of 7 months had SIs of 3.9 to BSA at 9 months and 2.8 at 12 months of age. The reactivities to CM proteins in the infant with CM allergy are shown in Figs. 1 and 2. A plasma sample for measurement of IgG antibodies to BSA was not available at the age of 12 months, and proliferation test to CAS at the age of 6 months was not done. Decrease in the level of IgG antibodies to CM proteins was seen between 6 and 9 months of age. In vitro stimulation of PBMCs to BLG and CAS was, however, still demonstrated at the age of 9 months.

Significant production of IgG class antibodies and proliferation of PBMCs to CAS was seen at the age of 9 months in some infants who received hydrolyzed formula containing casein peptides

with molecular weight less than 1200 kd (Figs. 1, C and 2, B). No significant difference was seen in mean SIs to CAS between the two groups. However, the infants in group I had higher levels of IgG antibodies to CAS at 6, 9, and 12 months of age ($p = 0.009$, $p = 0.02$, $p = 0.03$, respectively; Mann-Whitney U test).

The infant in whom IDDM developed at the age of 14 months received CM only after the age of 9 months, and increases in both antibody level and proliferation of PBMCs to BLG and CAS were observed between 9 and 12 months of age (Figs. 1 and 2). No reactivity to BSA was seen during the study.

Flow cytometry analysis was performed for BLG-stimulated PBMCs from two 9-month-old infants in group I. The majority of stimulated cells appeared to be CD4-positive T lymphocytes bearing an α/β -receptor (data not shown).

DISCUSSION

Development of both cellular and humoral immune responses to CM-derived BLG was observed in the infants who received CM proteins in formula. Our results thus indicate that early exposure often leads to sensitization of both T and B cells. Despite continuous oral antigenic stimulus, cellular response declined from the time of the introduction of the CM formula up to the age of 12 months. The same kind of tendency has been previously reported in the follow-up studies of antibody levels to CM proteins in infants,¹⁰ but

there are no follow-up studies of cellular immune responses.

In our study the proliferation of PBMCs to BLG decreased with age in the majority of infants who received the CM-based formula from early infancy (under the age of 6 months), suggesting that suppressive responses could have taken place in the regulation of immune response. This kind of tendency was not observed in the production of antibodies to CM proteins. Similarly, Husby et al.⁸ have reported that in adults, continuous oral antigenic stimulus leads to induction of T-cell, but not B-cell, tolerance. Furthermore, immune response to CM proteins was weaker in the children who started receiving CM proteins after the age of 9 months. This phenomenon has been reported previously in animal experiments,¹ in which the same exposure to oral antigens leads to tolerance in older animals, whereas in young animals, it results in immunity. Our results could be interpreted as supporting the concept of oral tolerization, the development of which is age-dependent, indicating that delayed exposure to an oral antigen leads to a suppressed systemic immune response.

Oral tolerization is an intensively studied area in immunology, especially because it could offer a means of inhibiting cell-mediated immunity to autologous targets in autoimmune diseases.¹⁵ Although the mechanisms of oral tolerization are poorly understood, oral administration of autoantigen might lead to suppression of immune responses to those antigens. Miller et al.^{16,17} have suggested that oral tolerance to a specific antigen, when induced in experimental animal models, is mediated by soluble lymphokines secreted by antigen-stimulated T cells. These lymphokines may suppress the induction of cytotoxic immune response to a tolerizing antigen. Another mechanism for oral tolerization is an induction of anergic state in T lymphocytes. Oral administration of ovalbumin has been reported to lead to anergy in T lymphocytes.^{18,19}

On the other hand, it is possible that the reason that a less marked immune response to CM proteins was seen in those subjects who received these proteins only after the age of 9 months is that the permeability of the gut to antigenic structures decreases with age.²⁰⁻²² According to permeability studies, gut closure is not complete at birth; in human beings, a period of increased permeability exists until the age of 3 months.²⁰⁻²² However, in infants the concentration of orally administered protein in serum does not always correlate with the level of antibodies to oral proteins.²³ Thus even a

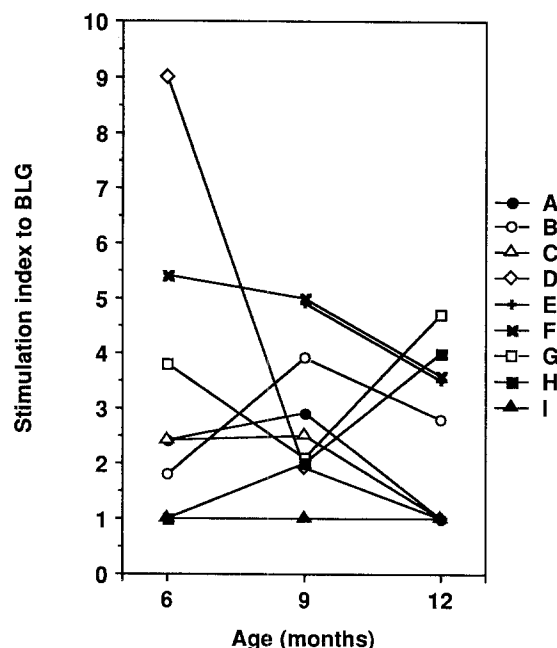


FIG. 3. Stimulation indexes to BLG in infants receiving CM formula throughout the study.

small amount of absorbed antigens might prove to be immunogenically active. Our observation that systemic immune response is low when foreign proteins are introduced to children after the age of 9 months has implications for treatment of food allergies in children and for understanding of the clinical peculiarity that food allergies mainly resolve themselves during childhood.

Interestingly, significant proliferation to CAS and IgG class antibody production was detectable in some infants who received only hydrolyzed formula. The formula is casein-based, and according to the manufacturer, contains peptides with a molecular weight of less than 1200 kD. In general, antigenic structures having such a low molecular weight are considered nonimmunogenic and have minor allergenic potential. Lack of reaction to Nutramigen formula in an oral challenge test in children with CM allergy has been demonstrated.^{24,25} However, even anaphylactic reactions to Nutramigen formula have been reported, suggesting that short chains of amino acids may also be allergenic.²⁶ Indeed, our results suggest that oral administration of peptides smaller than 1200 kD can induce production of IgG class antibodies and T-cell responsiveness.

The highest SI for BSA was 3.9, seen in a child in whom CM allergy developed during the study. In general, immune responses to BSA were low, suggesting that BSA is a less immunogenic protein

than BLG in CM. On the other hand, the stimulation of PBMCs to BSA could be blocked by human serum albumin, which is present at a high concentration in the culture medium and which bears a structural resemblance to BSA. Elevated antibody levels to BSA have been reported in patients with IDDM.^{14,27} BSA shows structural similarity to the pancreatic β -cell antigen islet cell antigen 69.²⁸ However, in the infant in whom IDDM developed at the age of 14 months, we could not demonstrate antibodies to BSA by ELISA or proliferation of PBMCs to BSA. Nevertheless, his antibody levels to BLG and CAS increased considerably from the age of 9 months to 12 months (Fig. 1, A and C). During the same period the proliferation of PBMCs to BLG and CAS increased (SI for BLG, from 2.0 to 6.6; SI for CAS, from 1.3 to 6) (Fig. 2). This kind of increase was not seen in the other infants who were exposed to CM proteins after the age of 9 months, all of whom remained nondiabetic. In this child, the immune response to these CM-derived proteins was thus exceptionally strong. It has been reported that a defect in suppressive immunoregulation occurs in patients with IDDM, and this kind of defect could explain the development of such a strong response. The permeability of the gut might also be greater in patients with IDDM than in normal children; our preliminary data suggest that permeability of the gut is enhanced in HLA-DR3-positive patients with IDDM (Savilahti et al. Unpublished data). In our case the exceptional increase in immunity to CM proteins preceded frank hyperglycemia and the diagnosis of IDDM.

In conclusion, our results suggest that orally administered proteins during early infancy (under the age of 6 months) provoke both a systemic cellular response and a humoral immune response. The proliferative cellular immune response later declined, supporting the appearance of tolerizing factors. Administration of these proteins only after the age of 9 months led to suppressed systemic immune response, which favors the view that induction of oral tolerance in human beings is an age-dependent phenomenon.

We thank Bristol-Meyers Squibb Company for providing infant formula. The skillful technical assistance of Sinikka Tsupari and Marjatta Viikari is greatly appreciated. We also thank Mrs. Tarja Tenkula, RN, and Mrs. Marja Salonen, MSc, for effective collaboration in the dietary intervention trial.

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