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Presence of functional, autoreactive human milk-specific IgE in infants with cow's milk allergy

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Summary

Background—Occasionally, exclusively breastfed infants with cow's milk allergy (CMA) remain symptomatic despite strict maternal milk avoidance.

Objective—To determine whether or not persistence of symptoms could be due to sensitization against endogenous human milk proteins with a high degree of similarity to bovine allergens.

Methods—Ten peptides representing known bovine milk IgE-binding epitopes [α -lactalbumin (ALA), β - and κ -casein] and the corresponding, highly homologous human milk peptides were labelled with sera from 15 breastfed infants with CMA, aged 3 weeks to 12 months, and peptide (epitope)-specific IgE antibodies were assessed. Nine of the 15 breastfed infants became asymptomatic during strict maternal avoidance of milk and other major food allergens; six infants remained symptomatic until weaned. Ten older children, aged 5–15 years, with CMA were also assessed. The functional capacity of specific IgE antibodies was assessed by measuring α -hexosaminidase release from rat basophilic leukaemia cells passively sensitized and stimulated with human and bovine ALA. Results A minimum of one human milk peptide was recognized by IgE antibodies from 9 of 15 (60%) milk-allergic infants, and the majority of older children with CMA. Genuine sensitization to human milk peptides in the absence of IgE to bovine milk was occasionally seen. There was a trend towards specific IgE being detected to more human milk peptides in those infants who did not respond to the maternal milk elimination diet than in those who did ($P = 0.099$). Functional IgE antibody to human ALA was only detected in infants not responding to the maternal diet.

Conclusions and Clinical Relevance—Endogenous human milk epitopes are recognized by specific IgE from the majority of infants and children with CMA. Such autoreactive, human milk-specific IgE antibodies appear to have functional properties *in vitro*. Their role in provoking allergic symptoms in infants exclusively breastfed by mothers strictly avoiding dietary milk remains unclear.

Keywords

atopic eczema; autoreactivity; cow's milk allergy; cross-reactivity; endogenous protein; human milk; IgE antibodies; infants; RBL assay; sensitization; SPOT method

Introduction

Cow's milk allergy (CMA) is due to an immunologically mediated adverse reaction to a single or multiple proteins found in cow's milk and can manifest as a wide variety of clinical symptoms. It can affect one or more systems within the body, with cutaneous manifestations such as urticaria and atopic dermatitis, gastrointestinal symptoms including colic, regurgitation, and bloody stools, as well as respiratory reactions such as wheezing [1–5]. Approximately 2–3% of infants and young children develop CMA [6, 7]. Symptoms usually begin within the first few months of life and may even develop while an infant is being exclusively breastfed [2, 8, 9]. In these cases, atopic dermatitis is often the main presenting symptom [2].

The major allergenic proteins in cow's milk include the caseins, α_1 -, α_2 -, β - and κ -caseins, which account for 80% of the total protein, and the whey proteins, α -lactalbumin (ALA) and β -lactoglobulin, which comprise 20% of the total protein. ALA, α_1 -casein, β -casein and κ -casein are also found in human milk, with ALA and β -casein being the most abundant (1.6 and 4.0 g/L, respectively) and α_1 -casein being least abundant (0.8 g/L) [4]. We have previously identified the specific IgE-binding epitopes on the major milk allergens [10–13]. The amino acid sequences of bovine and human ALA, β -casein and κ -casein, are 78%, 49%, and 52% identical, respectively, and 81%, 63%, and 58% homologous, respectively. Consequently, a high degree of similarity exists between the known IgE-binding regions on bovine milk and the corresponding endogenous human milk proteins.

Maternal cow's milk avoidance is often recommended in the management of breastfed infants who develop CMA. While some breastfeeding infants respond to the elimination of cow's milk antigens from the mother's diet, others continue to manifest symptoms which resolve only once the mother stops breastfeeding [8, 9]. Due to the high degree of similarity in amino acid sequence between the known IgE-binding epitopes of cow's milk allergens and endogenous human milk, as well as superimposed native structures [14], it could be postulated that cross-reactive IgE antibodies that recognize human milk proteins could exist in infants with CMA. In the present study, we sought to assess whether the presence of specific IgE antibodies against endogenous human milk proteins exists and whether they are indeed functional. Such IgE antibodies could hypothetically be responsible for the continuation of symptoms in breastfeeding milk-allergic infants despite strict maternal milk allergen avoidance. We also aimed to determine whether sensitization to human milk is due to cross-reactivity between bovine and human milk proteins or secondary to primary sensitization to endogenous human milk proteins.

Methods

Patients

The first patient population (Group 1) includes sera from 15 infants with confirmed CMA, aged 3 weeks to 12 months (median: 5 months), all clinically reactive at the time of serum collection. These infants were followed from birth due to a family history of food allergy. They all presented with symptoms of CMA, including atopic dermatitis or urticaria, which developed while breastfeeding within the first 4 months of life. The infants were evaluated by a clinician and skin prick (SPT) and serum IgE tests were performed as applicable. A stepwise, advancing restriction diet was recommended for the infants and their mothers in close collaboration with a dietician. This included avoidance of cow's milk, hen's egg, wheat, fish, shellfish, peanuts, and tree nuts, as well as other allergens as needed. This avoidance diet was implemented irrespective of the results of allergy testing in case symptoms persisted. The families were followed by a dietician at regular intervals to review their dietary intake to exclude the possibility of unintended exposure to allergens. Nine of

these infants became asymptomatic during a strict maternal elimination diet (Group 1, asymptomatic). These patients had milk-specific IgE antibodies ranging from <0.35 kU_A/L to 15.9 kU_A/L. Six of the 15 infants remained symptomatic despite restricted maternal diets, in several cases resulting in intake of as few as five foods by the mothers (Group 1, symptomatic). In all six, symptoms resolved after breastfeeding was discontinued. IgE-specific antibodies among these patients ranged from <0.35 kU_A/L to >100 kU_A/L. All 15 infants were brought in for a confirmative oral food challenge (OFC) with increasing doses (1, 10, 50, and 100 mL) of cow's milk-based formula given at 1-h intervals. They all reacted latest within 2 h of the last dose during the OFC suggestive of an IgE-mediated mechanism (Table 1). Table 1 summarizes the clinical characteristics of these infants.

The second patient population (Group 2) consists of sera from 10 children, 5–15 years of age, with persistent CMA and milk IgE levels >100 kU_A/L, who were clinically reactive at the time of serum collection, proven by a double-blind, placebo-controlled oral food challenge (DBPCFC) or recent allergic reaction to an isolated ingestion of milk protein. Sera from these patients were used to detect IgE binding to bovine ALA and β - and κ -casein peptides as well as to the corresponding homologous human milk peptides.

Informed consent was obtained from the patients and their parents and the study was approved by the Mount Sinai School of Medicine Institutional Review Board.

Peptides

The alignment between bovine and human milk proteins can be seen in Fig. 1. We have previously identified the IgE-binding epitopes from ALA, β - and κ -casein, which have a significant degree of amino acid sequence homology to human milk proteins. We selected 10 peptides (10–12 amino acids in length), representing the known bovine milk IgE-binding epitopes of ALA, β - and κ -casein [11, 12], being the three most abundant human milk proteins, and the corresponding, highly homologous human milk peptides (differing by 1–5 amino acids). Individual peptides were synthesized on derivatized cellulose membranes using the SPOT synthesis technique (JPT Peptide Technologies, Berlin, Germany). Membranes containing synthesized peptides were either probed immediately with serum IgE or stored at -20°C until needed.

IgE-binding assays

Membranes containing ALA, β - and κ -casein peptides were blocked overnight at 4°C with 1.5% human serum albumin (HSA) in TRIS-buffered saline (TBS) containing 0.1% Tween 20 and 5% goat serum (GS). Sera from the individual patients were diluted 1 : 10 with 1.5% HSA in TBS and incubated with the membrane-bound ALA peptides or, diluted 1 : 50 and incubated with the membrane-bound β - and κ -casein peptides for 3 h at room temperature. The use of 1 : 10 and 1 : 50 dilutions was based on conditions established by titration studies in previous experiments [11, 12]. Sera from a non-atopic patient and an atopic, non-milk-allergic subject with high IgE against unrelated food allergens, as well as detection antibody alone with no serum were used as controls. Membranes were then washed three times with TBS containing 0.05% Tween 20, and subsequently incubated at room temperature for 3 h with horseradish per-oxidase-conjugated biotin-labelled goat anti-human IgE. After washing the membranes three more times with TBS, the membranes were developed using Pierce ECL Western blotting substrate (Thermo Fischer Scientific Inc., Rockford, IL, USA) and exposed on an x-ray film. Binding with optical density of >0.03 was considered positive.

Inhibition studies

Inhibition studies were performed to examine the degree to which sensitization to human milk is due to IgE cross-reactivity between bovine and human milk proteins or secondary to

primary sensitization to endogenous human milk proteins. Due to the fact that the SPOT peptides represent the unfolded protein structure, the linear reduced and alkylated (R&A) forms of bovine and human ALA (3.5 mg in 1 mL of patient serum to label six spots) were used as inhibitors to detect binding to human and bovine peptides, respectively. R&A human and bovine ALA were prepared from native ALA utilizing a FOCUS™ Protein Reduction and Alkylation Kit (G-Biosciences, St Louis, MO, USA) that uses TCEP [Tri-(2-carboxyethyl)phosphine] for reduction of disulphide bridges and iodoacetamide for blocking of the thiols by alkylation. ALA is the only human milk protein commercially available and therefore was used for the inhibition studies. A change of conformation after R&A was confirmed by *tris*-glycine-*bis*-acrylamide gel electrophoresis using a 10–20% gradient gel (NuPage; Novex, San Diego, CA, USA), without reducing agents (native gel). After 1.5 h at 125 V constant voltage, staining was performed with Coomassie blue. Sera from one patient (patient 8, Group 2) with IgE >100 kU_A/L (diluted 1 : 10) was pre-incubated, for 2 h at room temperature, with three 10-fold dilutions of inhibitor in 1.5% HSA in T-TBS with 5% GS buffer. The mixture was then incubated with the membrane-bound ALA peptides and the remaining steps were carried out as stated before. Ovalbumin was used as an inhibitor control.

Rat basophilic leukaemia mediator release assay

Mediator release was assessed utilizing rat basophilic leukaemia (RBL)-2H3 cells transfected with and expressing the α -chain of the human Fc γ R1 receptor, as described previously [15]. These cells bind human IgE and have all the functional properties of mast cells. In brief, RBL cells are sensitized for 18 h with IgE from patient sera (diluted 1 : 40), washed, and then stimulated with antigen for 1 h, according to conditions established previously [15]. Native as well as R&A human and bovine ALA (Sigma, St. Louis, MO, USA) were used as antigens. Total β -hexosaminidase release was assessed by lysing cells with 1% Triton-X. Rabbit anti-human IgE polyclonal IgG (Bethyl Laboratories, Inc., Montgomery, TX, USA) was used as a positive control for IgE-mediated degranulation and stimulation with allergens alone without prior passive sensitization with patient IgE was used to assess non-specific mediator release. A non-milk-allergic patient with shrimp allergy and no milk-specific IgE (<0.35 kU_A/L) was used as a negative control. Release (%) was calculated as the difference between the allergen induced and spontaneous release of β -hexosaminidase divided by the total release. Assays were run in duplicate and an average was used for further calculations.

Statistics

Data were analysed using SigmaStat (Version 2.03; SPSS, Chicago, IL, USA). The Mann–Whitney rank sum test was employed for comparisons of medians and *t*-test for comparisons of means. The chi-squared test and Fisher's exact test were applied to determine differences in proportions. A *P*-value less than 0.05 was considered statistically significant.

Results

Milk epitope-specific IgE

Among the 15 cow's milk-allergic infants, IgE binding to at least one bovine peptide was seen in all subjects (Fig. 2). The majority of subjects showed IgE binding to at least one bovine peptide and its homologous human counterpart, with especially strong levels of binding in patients 4, 11, and 15 (Fig. 2). Interestingly, the pattern of recognition of homologous human milk peptides was not dependent on the degree of similarity between the bovine and human counterparts. Therefore, some patients had IgE binding to both the bovine and human counterparts, whereas others bound only to the bovine or human peptide even when the peptides were highly similar (e.g. the third peptide on ALA), but also when they

were not very similar (e.g. the first peptide on ALA (Fig. 2a). Occasionally, patients' IgE recognized the human milk peptide but had little or no binding to the homologous cow's milk peptide (patient 1, Fig. 2a; patients 11 and 15, Fig. 2b). Controls, including sera from a non-atopic patient and an atopic, non-milk-allergic subject with high IgE against unrelated food allergens, as well as detection antibody alone with no serum were all negative.

When comparing IgE binding in the two groups of infants with different responses to the maternal elimination diet, it was noted that at least one human milk peptide was recognized by IgE from five of the six symptomatic patients and four of the nine asymptomatic patients ($P = 0.68$). However, there was a trend towards specific IgE being detected to more numerous human milk peptides in those infants who continued to experience symptoms despite the elimination of maternal milk from their diet [19 (32%) of 60 peptides probed] than in those who responded to the maternal diet [14 (16%) of 90 peptides], $P = 0.099$.

To investigate whether autoreactive IgE antibodies are generally found in CMA patients, sera from older patients were used. Widespread IgE binding to both bovine ALA and β -casein peptides and the homologous human peptides was seen (Figs 3a and c). Occasionally, patients' sera bound to only the bovine peptide and not to the homologous human peptide. With β -casein, most patients showed binding to bovine peptides but not to the homologous human peptides. Interestingly, patients' IgE bound exclusively to the human β -casein peptides but not to the corresponding bovine peptides (patient 9, Fig. 3c).

Inhibition studies

To investigate the nature of such autoreactive IgE antibodies, inhibition studies were performed. Pre-incubation of serum from five patients (from Group 2) with milk-specific IgE $>100 \text{ kU}_A/\text{L}$ with bovine ALA resulted in variable degrees of reduction of peptide-specific IgE binding to the human ALA peptides among the patients (Fig. 4a). The degree of inhibition was less when human ALA was used in pre-incubation (Fig. 4b) suggesting that development of specific IgE was specific for bovine milk. Binding to human milk peptides seemed equally inhibited in part by pre-incubation with both bovine and human ALA (Fig. 4c). This may indicate that IgE autoantibodies to human ALA were due to cross-reactivity with bovine ALA. Ovalbumin which was used as a control inhibitor did not result in inhibition of IgE binding to milk peptides.

RBL mediator release assay

Finally, to assess whether or not such autoreactive IgE antibodies have functional capabilities, sensitized RBLs were stimulated with both bovine and human ALA. Older children with CMA (Group 2) and milk IgE $>100 \text{ kU}_A/\text{L}$ who possessed specific IgE to multiple epitopes on both bovine and human ALA showed good release of β -hexosaminidase following stimulation with bovine or human ALA (patient 6, Fig. 5a). Children who were sensitized to milk proteins other than ALA showed little β -hexosaminidase release (patient 2, Fig. 5b). Overall, R&A ALA provoked a greater response from sensitized RBLs than the native ALA. This may be due to the fact that R&A ALA more closely resembles digested ALA, which is the predominant form of a food protein absorbed from the gastrointestinal tract and therefore seen by the immune cells, compared with native ALA.

Likewise, infants who continued to have symptoms despite the initiation of a maternal elimination diet and who had epitope-specific IgE to human and bovine ALA also showed the IgE antibody to be functional in the RBL assay (patient 15, Fig. 5c). An infant who did not have any of the major IgE epitopes on ALA was found to have human and bovine ALA-specific IgE of functional quality (patient 12, Fig. 5d), which may have been due to specific

IgE against minor epitopes not assessed with the SPOT peptides. Serum IgE from infants whose symptoms resolved during the maternal elimination diet induced no release of α -hexosaminidase, even following stimulation with bovine ALA to which they were shown to have specific IgE by SPOT analysis (patients 3 and 4, Figs 5e and f).

Discussion

The present study shows that CMA infants possess specific IgE antibodies that recognize endogenous human milk proteins. We show that the presence of IgE specific to endogenous human milk proteins could be due to a high degree of sequence homology between the known bovine milk epitopes and the corresponding amino acid sequence on human milk proteins. These human milk-specific IgE antibodies were capable of mediator release in a functional assay in infants with high cow's milk-specific IgE levels. Such functional studies are necessary, although not sufficient, to support the presence of clinical reactivity. There was only a trend towards detection of a polyclonal IgE response to human milk peptides in those infants who continued to have symptoms despite a maternal cow's milk avoidance diet. However, a few such infants exhibited remarkably strong IgE binding to human milk peptides together with evidence that such autoreactive antibodies had functional properties *in vitro*. Our findings suggest that these antibodies could be clinically relevant in breastfed infants whose symptoms (eczema) cleared only after weaning. Similar antibodies were incapable of inducing mediator release (suggesting low-affinity antibodies) in infants who successfully responded to maternal milk restriction. Consequently, the role of these IgE antibodies in infants responding to maternal restriction and in older children with CMA remains unclear.

Significant clinical cross-reactivity exists between milk from ruminants such as cow, sheep, and goat, and to lesser extent between cow's milk and mare's or donkey's milk, which is based on a high degree of amino acid sequence homology between these proteins [4]. Similarly, based on the homology between certain cow's milk and human milk proteins, previous research has suggested that cross-reactivity could exist [16–19]. Clinical reactivity to human milk proteins was demonstrated in a case report describing an IgE-mediated allergic reaction in a male to human milk, but not cow's milk, which was found to be due to specific IgE antibodies to human, but not bovine ALA [20]. This case raises the possibility of a primary sensitization to human milk proteins. In a study by Schulmeister et al. [21], CMA subjects demonstrated IgE reactivity to cross-reactive as well as non-cross-reactive human milk proteins, and immediate type skin reactions were elicited using SPT with both native and boiled human milk. However, the clinical significance of these findings remains unclear. In the present study, we found that while some infants had specific IgE antibodies that recognize both bovine milk and the corresponding human milk peptides, demonstrating cross-reactivity or co-sensitization, some infants' IgE only recognized the endogenous human milk peptides suggesting primary sensitization to human milk proteins. On the other hand, binding to human milk peptides seemed equally inhibited in part by pre-incubation with either bovine or human ALA. This may indicate that the majority of IgE autoantibodies to human ALA were due to cross-reactivity with bovine ALA. It is also possible that full inhibition was not seen due to an insufficient amount of protein used in the inhibition assay. However, the R&A procedure poses limits to the maximum concentration of protein that can be processed. Unfortunately, due to small volumes of serum attained from infants, inhibition studies using their sera were not possible.

Prior studies have demonstrated that some patients with atopic dermatitis have IgE auto-antibodies to human proteins, such as manganese superoxide dismutase and calcium-binding proteins, suggesting auto-reactivity as a pathogenic mechanism in atopic dermatitis [22–26]. About 25% of adults with atopic dermatitis have IgE antibodies against self proteins [25].

IgE auto-antibodies can also be detected in patients with atopic dermatitis by their first birthday. These infants had higher total IgE levels and frequently were sensitized to food allergens [27]. It has been postulated that these IgE auto-antibodies may also be involved in the persistence of symptoms even when the initial inciting environmental allergens are absent [22–25]. Many of the patients with CMA in our study manifested symptoms of atopic dermatitis, and our findings raise the possibility that human ALA may represent a novel auto-antigen in infants with atopic dermatitis and CMA. Similar to the IgE auto-antibodies previously found to be associated with atopic dermatitis, human ALA may be responsible for the persistence of atopic dermatitis in milk-allergic infants who continue to have symptoms despite maternal avoidance of exogenous cow's milk antigens. Further studies are needed to confirm this possibility.

The mechanism(s) responsible for the generation of such IgE auto-antibodies is unclear. Medullary thymic epithelial cells (mTECs) express a highly diverse set of genes representing all tissues of the body (tissue-restricted antigens or TRAs) termed promiscuous gene expression (pGE) [28]. During the development of central tolerance, these mTECs are responsible for the negative selection of auto-reactive T cells, the development of tolerance to TRAs, and thus the prevention of auto-immune reactions. This development of self-tolerance occurs early during fetal development. Antigens expressed by the mTECs would result in deletion of thymocyte-specific antigen and tolerance of these self proteins. Human ALA is not expressed in the mTECs, thereby allowing the development/presence of human ALA-specific thymocytes potentially leading to auto-reactivity in infants exposed to human ALA via breast milk [28]. On the other hand, κ -casein is expressed in the thymus during mammary gland development, inducing negative selection (deletion) of autoreactive κ -casein-specific thymocytes [28]. However, the developmental delay in T regulatory cells maturation, which has been associated with CMA, may result in auto-reactivity despite adequate deletion of auto-reactive κ -casein-specific thymocytes.

One limitation of our study is that we did not perform clinical studies to verify reactivity to human milk in our subjects utilizing either skin testing with human ALA, as reported by Schulmeister et al. [21], or oral challenges with human milk after feeding with tolerated formula. However, the fact that symptoms subsided only after breastfeeding was discontinued in all six patients who failed to respond to maternal milk elimination further suggests that endogenous milk proteins may have played a role in the persistence of symptoms. Another possible limitation of the study is that the affinity or avidity of monoclonal or polyclonal antibodies to isolated and purified peptides may not reflect the strength of their interaction with the intact proteins.

Strengths of the study include a prospective format and sampling of a population of otherwise rarely encountered young subjects with severe CMA at an early age.

While breastfeeding has many benefits for both infants, in some cases in which the infant continues to display symptoms of CMA despite maternal dietary restrictions, it may be recommended that the mother stops breastfeeding. In rare cases, the continuation of symptoms in these infants may be due to continued passage of maternal dietary allergens into her breast milk or the presence of infant sensitization to endogenous human milk proteins. Further studies with larger sample sizes are needed to confirm these findings.

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(a)	
	1 20 40
BOVINE	<u>E</u> QLTKEVFRFLKDLKGYGGVSLPEWCTTFHTSGYDTQAI
HUMAN	KQFTKCELSQLLKDIDGYGGIALPELICTFHTSGYDTQAI
	60 80
BOVINE	VQNDSTGYGLFQINNKIWKDDQNPSSNICNISCDKFLD
HUMAN	VENNESTGYGLFQISNKLWCKSSQVPQSRNICDISCDKFLD
	100 120
BOVINE	DDLTDIMCVKKILDKVGINWLAHKALCSEKLDQWLCEKL
HUMAN	DDITDDIMCAKKILDIKIDYLAHKALCTEKLQWLCEKL
(b)	
	1 20 40
BOVINE	RELEELNVPGIIVESLSSEESITRINKKIEKFQSEEQQT
HUMAN	ACTVALALARETIESLSSEESITEYKQKVEKVKHEDQQQG
	60 80
BOVINE	EDELQDKIHFFAQTSLVYFPGPIHN-SLPQNIPLTQTP
HUMAN	EDEHODKIYFSFQPLIYFVEPIPYGFLPQNLPLAQA
	100 120
BOVINE	VVVPFPLQPEVMGSKVKEAMAPKHKEFPKYVVEPFTER
HUMAN	VVLP-VPQPEIMEVPAKDTVYTKGRVMFVLKSTIPFFDP
	140 160
BOVINE	QSLTLTDVENLHLPLPLLOSWMHOPHQLPPTVMFPQSVL
HUMAN	QIPKLTDLNLHLPLPLQLPLMQQVPQIPQTLALPPQPLW
	180 200
BOVINE	SLSSSKVLPVPKAVPYQORDMFIQAFLLYOEPVLPVVRGP
HUMAN	SVDPKVLPIQQVVPYQRAVQVQALLNQELLNPTHQI
BOVINE	FPIIV-----
HUMAN	YPVTQPLAPVHNPISV
(c)	
	1 20 40
BOVINE	<u>E</u> QNEQPIRCEKDERFFSDKIAKVIPIQVLSRYSYGLNY
HUMAN	VQNKQKQACHENDERPFYQKTAPYVPMYVVPNSYFYGTNL
	60 80
BOVINE	<u>Y</u> QOKPVALINNQFLPYYYAKPAAVRSPAQLQWVLSNTV
HUMAN	YQRRPAIAINNPNYVPTYANPAVVRPHAQIPQRYLPN--
	100 120
BOVINE	PAKSCQAQPTTMAHHPHLSFMAIPPKNQDKTEIPTINT
HUMAN	-----SHPTTVRRNLHPSFIAIPPKKIQDKIIPPTINT
	140 160
BOVINE	IASCEPTSTTEAVESTVAILEDSPVIE-SPPHINTVOV
HUMAN	IATVEPTPAFATEPTVDSVVTPEAFSEITSTPETTTAV
BOVINE	TSTAV
HUMAN	TPPTA

Fig. 1. Amino acid sequences of bovine and human α -lactalbumin (a), α -casein (b), and β -casein (c) aligned. Identical amino acids are shadowed with dark grey. Homologous amino acids are shadowed with light grey. Known IgE-binding epitopes for bovine proteins are underlined. Part (c) has been modified from Ref. [16].

Fig. 2. IgE binding to human and bovine peptides of α -lactalbumin (a), α -casein (b), and β -casein (c) utilizing SPOT. Binding of the sera from 15 breastfeeding infants with cow's milk allergy (Group 1) is shown. Nine of them became asymptomatic during maternal milk avoidance and six infants continued symptomatic despite maternal milk elimination. The known IgE-binding epitopes on bovine milk (Bov) and the homologous peptides on human milk (Hum) with their amino acid sequences are shown on the right. Identical amino acids are shadowed in dark grey and homologous ones in light grey. For the purpose of statistical comparisons, the circles highlight the IgE binding considered positive (OD > 0.03). A non-atopic subject is shown as a negative control.

Fig. 3. IgE binding to human and bovine peptides of α -lactalbumin (a), β -casein (b), and κ -casein (c) utilizing SPOT. Binding of the sera from 10 older cow's milk-allergic patients (Group 2) is shown. The known IgE-binding epitopes on bovine milk (Bov) and the homologous peptides on human milk (Hum) with their amino acid sequences are shown on the right. Identical amino acids are shadowed in dark grey. Homologous amino acids are shadowed in light grey. A non-atopic subject is shown as a negative control.

Fig. 4. Inhibition of specific IgE binding to cow's milk epitopes of β -lactalbumin by pre-incubation of the patient sera with bovine β -lactalbumin (a), or human β -lactalbumin (b). Similarly, inhibition of IgE binding to corresponding human milk peptides by pre-incubation with bovine β -lactalbumin (c), or human β -lactalbumin (d). Shown is the percentage of binding left after pre-incubation with β -lactalbumin when compared with IgE binding without inhibition. The known IgE-binding epitopes on bovine milk (Bov) and the homologous peptides on human milk (Hum) with their amino acid sequences are shown on the left. Sera from five individuals with cow's milk allergy (Group 2) are indicated by their order number, which corresponds to the patients shown in Fig. 3. Inhibition was performed using 3.5 mg of β -lactalbumin in 1 mL of patient serum to label six spots.

Fig. 5.

Percentage of total release when stimulated with either reduced and alkylated or native bovine (left panel) and human β -lactalbumin (right panel) in decreasing protein concentrations in six individual children with cow's milk allergy (CMA; a-f). Assays were run in duplicate and average was used for further calculations. Results are shown for two older children, patients 2 and 6 (Group 2; a, b), two breastfed infants who continued to have symptoms of CMA with maternal elimination diet, patients 12 and 15 (Group 1; c, d), and two breastfed infants who responded to maternal elimination diet, patients 3 and 4 (Group 1; e, f). Patients' IgE-binding to bovine and human milk β -lactalbumin peptides (as shown in Figs 2a and 3a) is also shown with binding to human peptides circled.

Table 1

Characteristics of breastfeeding infants with cow's milk allergy who either became asymptomatic after maternal elimination diet or remained symptomatic

Age (months) at serum sample	Milk IgE (kU _A /L)	SPT	Exclusive breastfeeding (months)	Age at symptom onset (months)	Persistent symptoms	Age at OFC (months)	Symptoms in OFC
Asymptomatic							
1	5	<0.35	–	3	–	1	Eczema
2	5.5	<0.35	–	0*	–	2	Eczema
3	4	15.9	–	4	–	1	Eczema+GI
4	6	4.0	+	7	–	1.5	Eczema
5	5	0.46	+	4	–	4	Eczema
6	7	<0.35	–	3	–	0.5	Eczema
7	6	<0.35	–	7	–	4	Eczema
8	1	<0.35	+	2.5	–	0.5	Eczema+Rhi
9	12	<0.35	+	3.5	–	2.5	Eczema
Symptomatic							
10	5	<0.35	–	5	–	0.5	Eczema
11	7	70.7	+	2	–	1	Eczema
12	4.5	>100	+	?	–	0.5	Eczema
13	1.5	<0.35	–	0*	–	1.5	Eczema+GI
14	4.5	<0.35	–	3.5	–	1.5	Eczema+GI
15	5.5	>100	+	0*	–	1.5	Eczema

*The subject was partially breastfed.

SPT, skin prick test; OFC, oral food challenge; GI, gastrointestinal symptoms (diarrhoea, vomiting, regurgitation); Rhi, chronic rhinitis; rash, eczema flare.