B-cell epitopes as a screening instrument for persistent cow's milk allergy

Kirsi-Marjut Järvinen, MD, PhD, Kirsten Beyer, MD, Leticia Vila, MD, Pantipa Chatchatee, MD, Paula J. Busse, MD, and Hugh A. Sampson, MD *New York, NY*

Background: Cow's milk is one of the most common causes of food allergy in the first years of life. We recently defined IgE-binding epitopes of all 6 major cow's milk proteins (α_{s1}-, α_{s2}-, β-, and κ-casein; α-lactalbumin; and β-lactoglobulin) and had some evidence suggesting that IgE antibodies from patients with persistent cow's milk allergy (CMA) recognize different epitopes on cow's milk proteins than do those from patients who were likely to outgrow their allergy.
Objective: In this study we sought to assess whether recognition of IgE antibodies of certain epitopes of cow's milk proteins would clearly separate the patients with life-long CMA from those who will become clinically tolerant to cow's milk.
Methods: According to the known IgE-binding regions of cow's milk proteins, 25 decapeptides of α_{s1}-casein, α_{s2}-casein, κ-casein, α-lactalbumin, and β-lactoglobulin, comprising the core epitopes, were synthesized on a cellulose-derivatized membrane. Sera from 10 patients with persistent CMA and 10 patients who subsequently outgrew their milk allergy were used to investigate the differences in epitope recognition.
Results: Five IgE-binding epitopes (2 on α_{s1}-casein, 1 on α_{s2}-casein, and 2 on κ-casein) were not recognized by any of the patients with transient CMA but showed binding by the majority of the patients with persistent allergy. The presence of IgE antibodies against at least 1 of 3 epitopes (amino acid [AA] 123-132 on α_{s1}-casein, AA 171-180 on α_{s2}-casein, and AA 155-164 on κ-casein) identified all patients with persistent CMA.
Conclusions: The presence of IgE antibodies to distinct allergenic epitopes of cow's milk proteins can be used as a marker of persistent CMA. Prospective studies are needed to investigate the usefulness of these informative epitopes in predicting life-long CMA in young children. (J Allergy Clin Immunol 2002;110:293-7.)

Key words: *Cow's milk allergy, cow's milk proteins, cow's milk–specific IgE, B-cell epitope, oral tolerance, natural history, children, caseins, α-lactalbumin, β-lactoglobulin*

Abbreviations used
AA: Amino acid
CMA: Cow's milk allergy

Cow's milk is one of the most common causes of food allergy in the first years of life, with approximately 2% to 2.5% of newborns experiencing allergic reactions to cow's milk during this time.[1,2] Caseins account for about 80% of the total protein content in cow's milk, whereas whey proteins comprise the rest. Casein includes 4 protein fractions, α_{s1}-, α_{s2}-, β-, and κ-casein, comprising 32%, 10%, 28%, and 10% of the total milk protein, respectively. In solution, different caseins form complexes and ordered aggregates (ie, micelles). These globular complexes are composed of a peripheral hydrophilic layer and a hydrophobic core. In the core, caseins are assembled by means of intermolecular interactions between the colloidal calcium phosphate and the phosphoserine groups of the α_{s1}-, α_{s2}-, and β-caseins, whereas the C-terminal polar fragment of the κ-casein and the polar domains of the other caseins are exposed at the periphery.[3] In addition, α_{s2}- and κ-casein both contain one disulfide bridge per molecule.[3] Whey fraction contains essentially globular proteins, α-lactalbumin and β-lactoglobulin, containing 4 and 2 disulfide bridges and comprising 5% and 10% of total milk protein, respectively.[3]

The relative allergenicity of each cow's milk protein remains unclear, although data from recent studies[4,5] have emphasized the importance of the caseins as major milk allergens, and significant reactivity to the whey proteins (α-lactalbumin and β-lactoglobulin) was also noted. All these proteins share little primary structural homology.[3] We have recently mapped the major IgE- and IgG-binding epitopes on α_{s1}-, α_{s2}-, β-, and κ-casein; α-lactalbumin; and β-lactoglobulin.[6-9]

The majority of children outgrow their cow's milk allergy (CMA; become clinically tolerant) by 3 to 4 years of age. However, 15% of infants with IgE-mediated CMA retain their sensitivity into the second decade.[5] The mechanisms behind development of clinical tolerance to food proteins remain poorly understood. Our previous data suggested that IgE antibodies from patients with persistent CMA recognize certain epitopes of cow's milk proteins that were not recognized by IgE antibodies from patients who were likely to outgrow their allergy,[6-8] suggesting that the epitope specificity of IgE antibody

From the Division of Pediatric Allergy and Immunology and the Jaffe Institute for Food Allergy, The Mount Sinai School of Medicine, New York.
Supported by grants AI 44236 and AI 24439 from the National Institute of Allergy and Infectious Diseases, grant M01 RR00071 from the Division of Research Resources, the National Institutes of Health, and the Bunning Family Fund.
Received for publication November 29, 2001; revised April 23, 2002; accepted for publication April 23, 2002.
Reprint requests: Hugh A. Sampson, MD, Department of Pediatrics, Box 1198, The Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029-6574.

0091-6749/2002 $35.00 + 0 **1/87/126080**
doi:10.1067/mai.2002.126080

response might determine the clinical outcome of CMA. We confirmed this finding with a decapeptide of α_{s1}-casein (amino acid [AA] 69-78) in a population composed of children with either persistent or transient CMA.[10] The present study sought to investigate whether similar findings are true for other major cow's milk proteins. We also sought to determine whether a combination of certain allergenic epitopes of the major cow's milk proteins would clearly define the patients with lifelong CMA from those who will become clinically tolerant to cow's milk, which would be helpful in predicting the natural history of CMA.

METHODS

Subjects

The patient population consisted of 20 children with IgE-mediated CMA. The first group included 10 patients with persistent CMA who were 3.5 to 15 years of age (median, 8 years). Sera used for testing were drawn at the initial encounter, and milk-specific serum IgE antibody levels were greater than 100 kU_A/L, as measured with the CAP-System FEIA (Pharmacia Diagnostics, Uppsala, Sweden). The levels of specific IgE to cow's milk remained greater than 100 kU_A/L in 7 patients and decreased to 29, 62, and 91 kU_A/L, respectively, in the remaining 3 patients during the follow-up beyond the age of 7 years (median, 10 years; range, 7-16 years). Seven of the patients with persistent CMA had atopic dermatitis. All of them had multiple food allergies. CMA had initially been diagnosed on the basis of a positive double-blind, placebo-controlled food challenge result (n = 4) or a convincing history of severe allergic reaction to isolated ingestion of cow's milk (n = 6). Two of the patients reacted with anaphylaxis to ingestion of cow's milk, 6 had urticaria and abdominal pain–vomiting or throat tightness–respiratory distress, and 2 reacted with abdominal pain and pruritus or respiratory distress. During follow-up, all of these patients with persistent CMA were shown by means of challenge (n = 3) or accidental ingestion (n = 7) to retain their CMA beyond the age of 7 years. This age was used as a criterion for defining persistent CMA because development of clinical tolerance beyond this age is quite unlikely.[11,12] Epitope recognition of serum IgE antibodies was previously assessed in 5 of these patients.[6-9]

The second group included 10 patients who had outgrown CMA by the age of 7 years (median, 5 years; range, 1.5-7 years). At the time of testing, their median age was 4 years (range, 2-6 years), with a mean milk-specific IgE level of 11.3 kU_A/L (range, <0.35-46.4 kU_A/L). The serum samples used in the analysis were obtained at the time period of clinical reactivity to cow's milk. In 8 patients the IgE values had decreased at the time tolerance developed, and in 2 patients they had increased when tolerance was achieved. Median IgE level to cow's milk at the time tolerance was established was 1.2 kU_A/L (range, <0.35-20 kU_A/L). Seven of the patients with transient CMA had atopic dermatitis. Eight of the children had multiple food allergies, and 2 of them had allergy only to cow's milk. CMA had initially been diagnosed on the basis of a positive double-blind, placebo-controlled food challenge result (n = 3) or a convincing history of a severe allergic reaction (n = 7). One of the patients had urticaria, 1 experienced urticaria and vomiting after milk ingestion, 2 had an eczematous rash, and 3 had erythematous rash and vomiting or other gastrointestinal symptoms. Three children had vomiting and bloody diarrhea or pruritus.

Control sera were obtained from 5 atopic individuals without milk allergy with atopic dermatitis and IgE-mediated allergy to other foods (ie, shellfish, peanut, and nuts) who were aged 5 to 11 years. They had negative skin prick test responses to milk, milk-specific IgE antibodies of less than 0.35 kU_A/L, or both and clinical tolerance to cow's milk.

Informed consent was obtained, and the study was approved by the Institutional Review Board of the Mount Sinai School of Medicine.

Peptide synthesis

In previous studies IgE-binding epitopes for α_{s1}-, α_{s2}, β-, and κ-casein as well as α-lactalbumin and β-lactoglobulin were identified after synthesis of overlapping decapeptides by using the SPOTs membrane (Genosys Biotechnologies, Inc, Woodlands, Tex).[6-9] These articles compared the sequential epitope recognition of sera from older patients with persistent CMA and those of the younger children with decreasing milk-specific IgE antibody levels and therefore a high likelihood to outgrow their CMA. Twenty-five epitopes on cow's milk proteins (5 epitopes on α_{s1}-casein, 5 on α_{s2}-casein, 9 on κ-casein, 1 on α-lactalbumin, and 5 on β-lactoglobulin) were found to be recognized by IgE antibodies from children with persistent CMA but not by IgE from patients who were considered likely to outgrow their allergy. These epitopes were selected to be examined in this study. Major IgE-binding epitopes of β-casein have previously been examined by our group and were therefore not included in this study.[10]

The central decapeptide from each of these epitopes was selected for further synthesis on a SPOTs membrane as follows: for α_{s1}-casein, AAs 39-48, 91-100, 111-120, 123-132, and 157-166; for α_{s2}-casein, AAs 33-42, 87-96, 145-153, 159-168, and 171-180; for κ-casein, AAs 13-22, 17-26, 31-40, 55-64, 67-76, 97-106, 115-124, 139-148, and 155-164; for α-lactalbumin, AA 13-22; and for β-lactoglobulin, AAs 47-56, 67-76, 75-84, 127-136, and 141-150. SPOTs membrane (Genosys Biotechnologies, Inc), a derivatized cellulose membrane, was used to generate multiple decapeptides. Individual peptides were synthesized on the membrane by using the 9-fluorenyl-methoxycarbonyl method, according to the manufacturer's instructions. 9-Fluorenyl-methoxycarbonyl–AA derivatives were dissolved in 1-methyl-2-pyrrolidone and loaded on marked spots on the membrane. Coupling reactions were followed by acetylation with 4% acetic anhydride in N,N-dimethylformamide. The membrane was then stained with bromophenol blue to identify the location of the free amino groups. Cycles of coupling, blocking, and deprotection were repeated until the peptides of the desired length were synthesized. After the addition of the last AA in the peptide, the AA side chains were N-terminally acetylated and deprotected by using dichloromethane–trifluoroacetic acid–triisobutylsilane. Membranes were used for IgE-binding assays, as indicated below.

Identification of IgE binding

The membranes were blocked overnight with PBS containing 0.01% Tween-20, 1.5% human serum albumin (Sigma, St Louis, Mo), and 2% normal human serum (only for κ-casein). Serum samples were incubated with the membranes for 2 hours at room temperature or overnight at 4°C (for α_{s1}-casein, 1:20 dilution for patients who outgrew CMA and 1:50 dilution for patients with persistent CMA; for α_{s2}-casein, 1:5 and 1:30, respectively; for κ-casein, 1:20 and 1:50; and for α-lactalbumin and β-lactoglobulin, 1:20 and 1:20). IgE antibody binding was detected by using an immunoenzymatic method with biotinylated goat or mouse (α_{s2}-casein) anti-human IgE and peroxidase streptavidin conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, Md). Dilutions of sera and secondary antibody were based on preliminary experiments in which the intensity of the background was assessed separately for each protein to achieve a minimum of background that would not interfere with the interpretation of the results. The dilutions and incubation times for the sera of patients with transient CMA were adjusted to compensate for the lower specific IgE levels against cow's milk compared with the serum levels of patients with persistent CMA. In addition, different dilutions were required for the different milk proteins to avoid nonspecific background. Nonspe-

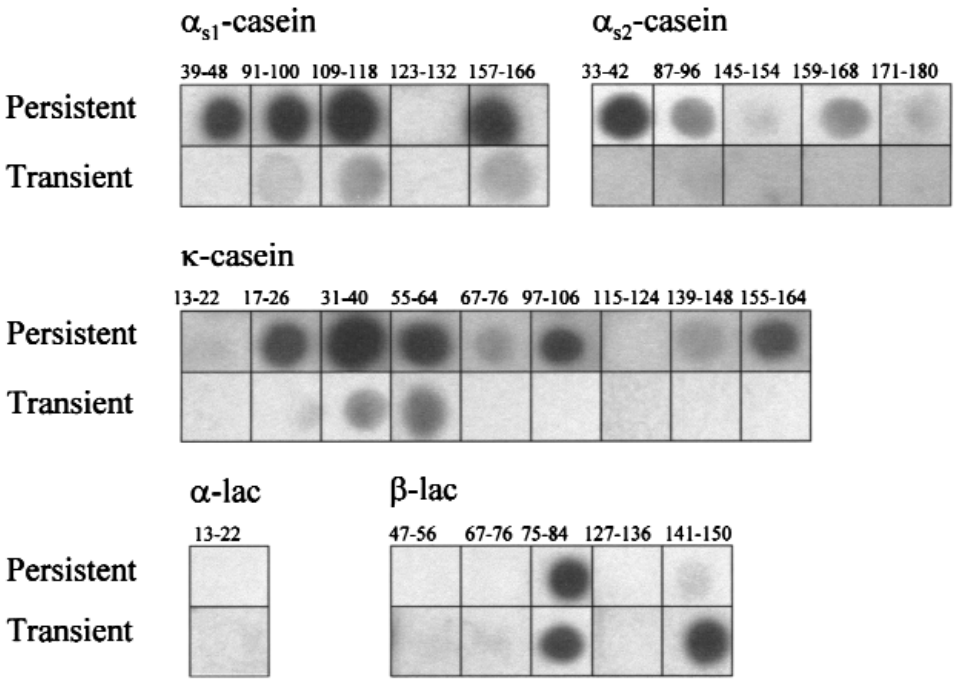

FIG 1. An example of binding to decapeptides of cow's milk proteins of serum IgE antibodies by a patient with persistent CMA *(upper rows)* and a patient with transient CMA *(lower rows).* Numbers indicate the residues corresponding to the peptide. α-*lac,* α-Lactalbumin; β-*lac,* β-lactoglobulin.

cific binding to the SPOTs membrane was ruled out by probing the membrane with sera from donors without milk allergy.

The membranes were subsequently developed with a chemiluminescent detection system (Amersham, Arlington Heights, Ill). After developing the x-ray film, the OD of each individual peptide spot was measured with reflection densitometry. The OD of each peptide spot was recorded as the difference between the OD of the peptide spot and the OD of the background.

RESULTS

Binding to 25 B-cell epitopes on 5 major cow's milk allergens were compared between children with persistent and transient CMA to identify informative epitopes for the prediction of persistence of CMA. Results from a representative patient from each group are shown in Fig 1. Fig 2 summarizes the data for all the patients. In general, the patients with persistent CMA showed binding to more numerous epitopes in the caseins (median, 15; range, 7-18) than the patients who outgrew their allergy (median, 8; range, 2-10). In contrast, a comparable number of epitopes were recognized in whey proteins (median of 3 [range, 0-6] and median of 3 [range, 2-6], respectively).

Seventeen of the 19 epitopes in the caseins and 4 of the 6 epitopes of the whey proteins were recognized by IgE antibodies from the majority (>50%) of the patients with persistent CMA (Fig 2). Five of these IgE-binding epitopes (2 on α_{s1}-casein, 1 on α_{s2}-casein, and 2 on κ-casein) showed no binding by any of the patients with transient allergy. In addition, at least 1 of these 5 epitopes is recognized by each of the children with persistent allergy. These 5 epitopes were therefore classified as the informative epitopes. Table I summarizes these 5 epitopes in addition to the one on α_{s1}-casein that was previously reported by our group to be recognized only by children with persistent CMA.[10] In a further attempt to define a smaller set of epitopes that could be used as a screening instrument for persistent CMA, we concluded that the presence of IgE antibodies against at least 1 of 3 epitopes (ie, AA 123-132 on α_{s1}-casein, AA 171-180 on α_{s2}-casein, and AA 155-164 on κ-casein) identified all of the patients with persistent CMA.

DISCUSSION

Cow's milk is a significant cause of food allergy in early childhood. The mechanisms leading to persistent (and transient) CMA are unknown. In this article we have shown that the presence of IgE antibodies against certain sequential epitopes on various milk proteins is associated with persistence of CMA. We further report that a combination of these epitopes might be used in identifying the patients who will have life-long CMA.

Previous studies looking for a marker to predict whether children will remain allergic or outgrow CMA have investigated IgE and IgG antibody levels against whole cow's milk and cow's milk protein fractions.[5,13-15] These studies have suggested that children with long-lasting CMA possess higher levels of total[5,13] and cow's milk–specific IgE antibodies[5,14] than those who became tolerant. In contrast, James and Sampson[15] found that initial milk-specific IgE antibody concentrations were not significantly lower in the patients ultimately becoming tolerant, but final casein and

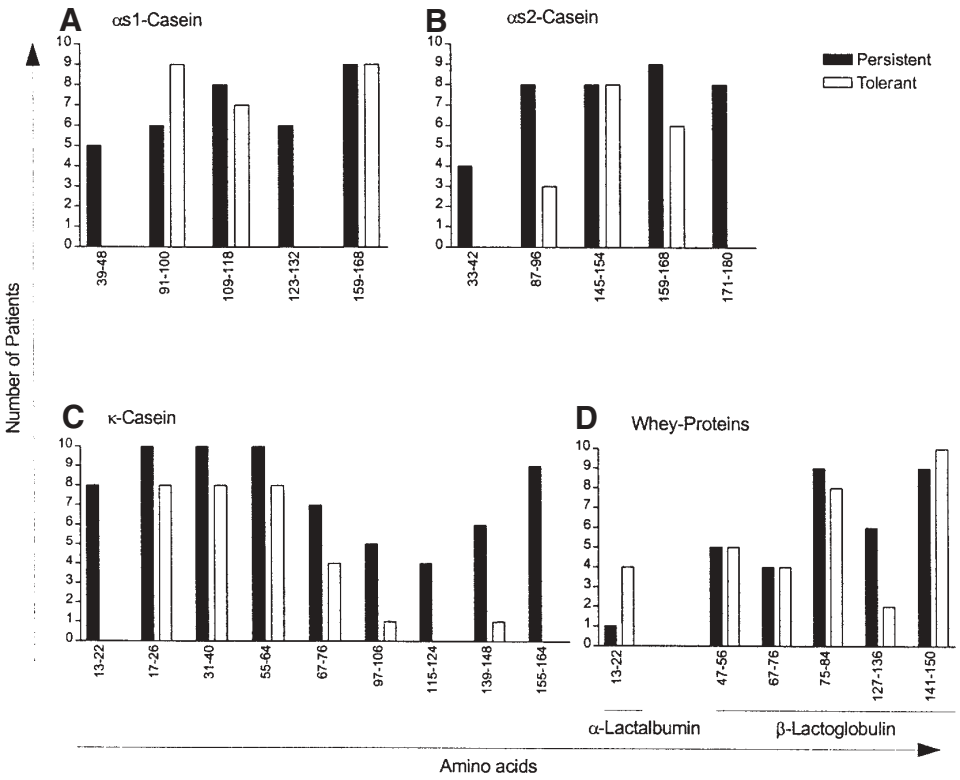

FIG 2. Numbers of patients showing binding to each major IgE-binding epitope (represented as AA residues) of α_{s1}-casein **(A)**, α_{s2}-casein **(B)**, κ-casein **(C)**, and whey proteins **(D)**. Patients with persistent CMA are shown in *filled bars*, and those with transient CMA are shown in *open bars*. The total number of patients in each group is 10.

TABLE I. Residues and corresponding AA sequences of the informative epitopes that were recognized by serum IgE-binding epitopes of the majority of the 10 children with persistent CMA but by none of the 10 children with transient CMA

Protein	AA	Sequence	No. of patients
α_{s1}-casein	39-48	ELSKDIGSES	5/10
	69-78*	EEIVPNSVEQ*	6/10*
	123-132	**MKEGIHAQQK**	**6/10**
α_{s2}-casein	**171-180**	**YQKFALPQYL**	**8/10**
κ-casein	13-22	KDERFFSDKI	8/10
	155-164	**SPPEINTVQV**	**9/10**

At least 1 of the 3 epitopes highlighted in bold were recognized by the patients with persistent CMA and would serve as a screening instrument.
*Data are from Vila et al,[10] Copyright 2000, Blackwell Science.

β-lactoglobulin–specific IgE levels were significantly lower. Significantly higher levels of IgG antibodies against certain milk proteins have been reported in children with persistent CMA[15,16] and adult milk-intolerant patients.[17] Finally, James and Sampson[15] observed that casein- and β-lactoglobulin–specific IgE/IgG ratios were significantly lower in children who lost milk reactivity, suggesting that monitoring those parameters might help in predicting the outcome of CMA. At this time there are no specific IgE levels that have been demonstrated to be highly predictive of the development of clinical tolerance in children with milk allergy. In the present study we identified IgE specificity to informative allergenic B-cell epitopes as a screening instrument for persistent CMA. It should be noted, however, that the quantity of milk-specific IgE antibody correlated with OD measurements of binding to the informative epitopes (Spearman rank correlation test). This is in part explained by the fact that the epitopes used as informative epitopes had to be recognized by greater than 50% of the persistent patients and 0% of the patients outgrowing CMA. Our data suggest that measurement of epitope-specific IgE might provide a more accurate tool for predicting the outcome of CMA, but further studies in bigger populations will be needed to confirm the present data.

Caseins are not significantly affected by heating but are very susceptible to many proteinases and exopeptidases, resulting in a great deal of modification after ingestion.[3] In comparison, β-lactoglobulin is relatively resistant to acidic pH values and to proteolytic enzymes, leaving its structure relatively unchanged during digestion and possibly allowing passage of intact protein into the circulation.[3] Consistent with this, the major IgE-binding regions, when projected onto the 3-dimensional structures of β-lactoglobulin, turned out to be located on the surface of the molecule, suggesting that the major IgE-binding sites of β-lactoglobulin are mainly confor-

mational structures. The same applied to α-lactalbumin. However, IgE-binding epitopes of the whey proteins on the basis of sera from children with long-lasting CMA did not differ from those detected with sera of patients with transient CMA, indicating that only the sequential epitopes of the caseins are associated with the persistence of CMA. The explanation for this phenomenon was not investigated in the present study but can be speculated about on the basis of the more spatial structure of epitopes of whey proteins. The immaturity of the newborn intestine allows the highest degree of absorption of intact α-lactalbumin and β-lactoglobulin immediately after birth, significantly declining over the next several months of life as the gut permeability decreases.[18] Therefore it might be hypothesized that hypersensitivity to whey proteins, which have mainly conformational epitopes, might attenuate during early childhood and might therefore not play a significant role in persistent CMA.

Understanding the mechanisms leading to persistent CMA is necessary to develop procedures that would interfere with this process. Immunotherapeutic interventions under investigation in our laboratory should be directed at those patients who will not outgrow their CMA, assuming this group of patients can be identified. We have evidence that the epitope-specific IgE antibodies are already present at an early age in patients with persistent CMA, suggesting that such a prediction could be made in the first years of life.[6] Furthermore, long-lasting food hypersensitivity is associated with subsequent allergic airway disease,[19] and early differentiation of these patients might affect preventive approaches. Finally, knowledge of the likelihood of outgrowing CMA will be beneficial in counseling patients and directing their treatment because many children with milk and egg allergy are able to tolerate small amounts of these foods in a cooked, but not in a raw, form.[20,21] During food processing and cooking, the native structure and many conformational epitopes of these proteins are modified or disrupted by heat, chemical treatments, or both, eliminating IgE binding to conformational epitopes and exposing sequential (linear) ones. Therefore a meticulous elimination diet and avoidance of cooked milk products might not be necessary in children who do not recognize sequential epitopes on cow's milk proteins or who will outgrow their CMA irrespective of their diet.

In conclusion, we were able to identify 6 IgE-binding sites on cow's milk proteins that differentiated between patients with persistent CMA and those with transient CMA. The presence of IgE antibodies against at least 1 of 3 of these epitopes (AA 123-132 on α_{s1}-casein, AA 171-180 on α_{s2}-casein, and AA 155-164 on κ-casein) might be useful as a marker of persistent CMA. Because IgE antibodies to these informative epitopes appear to develop at the time of initial sensitization and seem not to depend on the IgE concentration, this approach provides the advantage of allowing screening at the time of initial diagnosis in infants. Studies are under way linking 3 informative epitopes (peptides) to a commercial matrix to conduct prospective studies in larger patient populations.

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