

Preferential recognition of primary protein structures of α -casein by IgG and IgE antibodies of patients with milk allergy

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We studied the binding activities of IgE and IgG antibodies in patients with allergy to cow milk proteins, against different α -casein preparations: α -casein treated with urea, hydrochloric acid, sodium hydroxide, or sodium dodecyl sulfate (SDS); or heat-denatured α -casein. The binding activities of IgE and IgG antibodies to these denatured α -casein preparations were compared with those to native α -casein. The binding activities of IgE and IgG antibodies to these denatured α -casein preparations were similar to those to native α -casein although the binding activities of IgG antibodies to these denatured α -casein preparations were relatively heterogeneous compared with those of IgE antibodies. Since modifications of α -casein did not alter the ability of α -casein to react with these antibodies, IgE and IgG antibodies to α -casein in sera from patients with allergy preferentially bind to the antigenic determinants associated with primary protein structures.

INTRODUCTION

Milk is an important nutritious food for a growing child's diet, especially during infancy; however, it has also been implicated in gastrointestinal, cutaneous, and respiratory hypersensitivity reactions.^{1,2} IgE antibodies to milk proteins must always play some important roles in induction of milk allergy.³ IgG antibodies to food have been found in both food-allergic subjects^{4,5} and healthy children,⁶ and evidence for involvement of the IgG class of immunoglobulin in food sensitivity is much less convincing.

In cow milk, casein, β -lactoglobulin, α -lactalbumin, bovine serum albumin, and bovine immunoglobulins are major allergens related to

milk hypersensitivity.^{7,8} Among them, caseins are the most abundant, forming 80% wt/wt of the milk proteins.^{7,8} Protein allergens have numerous epitopes on their molecular surfaces. Protein antigenic determinants are divided into two categories, sequential and conformational, depending on whether the primary sequence or the three-dimensional conformation appeared to contribute most to binding. Generally, antibodies recognize the overall configuration of antigens, rather than their chemical compositions. Much less is known regarding the antigenic structure of casein recognized by IgE or IgG antibodies in sera of patients with milk allergy.

In this study, we undertook identification of reactivities of IgE and IgG antibodies in patients with allergy to sequential or conformational antigenic determinants of casein using physically and chemically denatured α -casein.

MATERIAL AND METHODS

Patients

Sera were obtained from 11 Japanese patients (eight boys and three girls) with cow milk allergy display-

ing clinical symptoms of atopic dermatitis, diarrhea, vomiting, or anaphylactoid shock after ingesting cow milk. They were diagnosed on the basis of food elimination and challenge tests,⁹ and had positive RAST scores for cow milk protein class 3-4. They ranged in age from 2 to 11 years (mean 5.4 years). No patient had been treated with systemic corticosteroids or desensitization therapy.

Antigens

Alpha-casein was purchased from Sigma (St. Louis, MO, USA) and was used without further purification.

Denaturation of α -Casein

Denaturation of α -casein was performed by the following five procedures: (1) α -casein solution (7.3 mg/mL of phosphate buffered saline: PBS, pH 7.4) was heated at 100 °C for three minutes. (2) Ten microliters of α -casein solution (10 mg/mL of distilled water) was incubated at room temperature overnight in alkaline condition (pH 11.2) by addition of 50 μ L 3M NaOH. (3) Ten microliters of 10 mg/mL of α -casein solution was treated with 5 mL 0.3% sodium dodecyl sulfate (SDS) for five hours at room temperature.

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(4) α -Casein solution (15 mg/mL of 8M urea) was incubated for two hours at 40 °C in the presence of 8M urea. (5) Ten microliters of α -casein solution (10 mg/mL of distilled water) was incubated at room temperature overnight in acid condition (pH 3.0) by addition of 110 μ L of 2N hydrochloric acid.

Sodium Dodecyl

Sulfate-Polyacrylamide Gel

Electrophoresis Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by Laemmli's method¹⁰ with some modifications. The samples (10 μ g of protein/lane) were placed on a SDS-polyacrylamide 10% to 15% gradient gel (Daiichi Pure Chemicals LTD, Tokyo, Japan). After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. Molecular weight protein standards (97.4, 66.2, 42.7, 31.0, 21.5, and 14.4 kD) were purchased from Bio-Rad Laboratories (Richmond, Calif., USA).

ELISA for Anti- α -Casein IgE, and IgG Antibodies

Quantitative immunoassay for IgG and IgE antibodies for α -casein was performed by ELISA, using a modification of the technique described previously.¹¹ The binding activities of IgG and IgE antibodies to α -casein reached a plateau when the plates were coated with more than 100 μ g/mL native α -casein. Disposable polystyrene plates with a flat-bottomed well (NUNC, Roskilde, Denmark) were coated by incubation with 100 μ L 100 μ g/mL native or denatured α -casein in PBS overnight at 4 °C. Alpha-casein preparations used in these experiments were diluted with PBS when used for coating the plates. The final pH of the α -casein preparation solution for coating the plate was 7.5 to 9.0. Sera from patients with milk allergy were tested at 1:20 dilution with PBS containing 0.1% gelatin and 0.05% Tween-20, and incubated overnight at 4 °C. An alkaline phos-

phatase-conjugated goat anti-human IgG antibody (Sigma) or an alkaline phosphatase-conjugated goat anti-human IgE antibody (Kirkgaard & Perry Laboratories Inc, MD, USA) was added to each well to a final dilution of 1:1000 or 1:800, respectively. The background of this assay was determined by use of a plate coated with 0.3% gelatin instead of α -casein preparations and was subtracted from all data. The background obtained by the detection reagent on different α -casein preparation-coated plates, with test samples omitted, showed no significant difference from the background of gelatin-coated plates. The binding activity of antibodies to the denatured α -casein is expressed as a percentage of binding compared to the binding activity to native α -ca-

sein. The percent binding activity was calculated when the mean absorbance of duplicate samples for native α -casein was higher than 0.65. The background optimal density (OD) values were lower than 0.1 in all the ELISAs.

Statistical Analysis

Statistical evaluation of the data was performed using Student's *t* test.

RESULTS AND DISCUSSION

There is little information about B cell epitopes on casein recognized by IgE or IgG antibodies in sera from patients with milk allergy. In order to evaluate the α -casein protein structures reactive with IgE and IgG antibodies of the patients, we studied the binding activities of antibodies to denatured α -casein by calculating the percentage of anti-

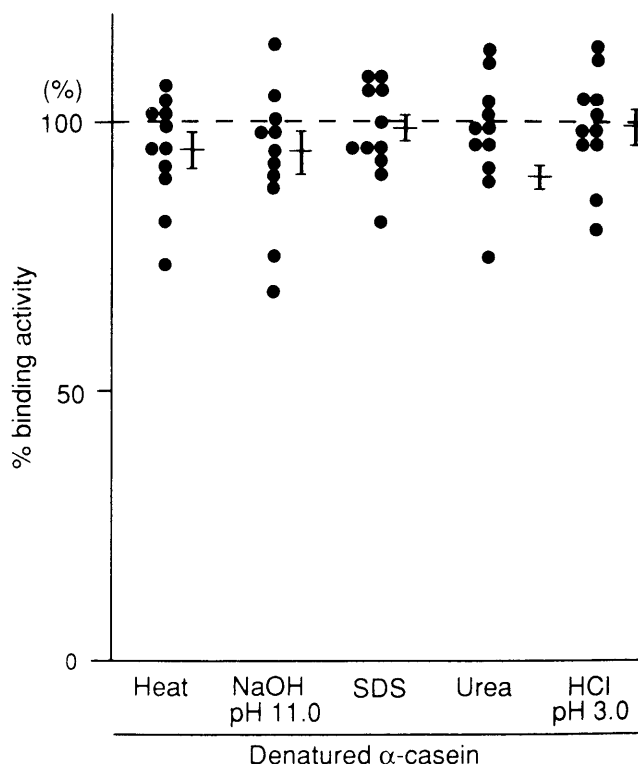


Figure 1. Percent binding activities of IgE antibodies in sera from patients with allergy to cow milk to denatured α -casein. The binding activities of IgE antibodies to each denatured α -casein were measured by ELISA and are expressed as the percentage of antibody binding to native α -casein, taken as 100%. Native α -casein was denatured as described in "Materials and Methods." The assay was performed in duplicate. The mean ($\pm$ SE) of absorbance readings produced by the sera to native α -casein is 1.16 ± 0.20 .

body binding to native α -casein, taken as 100%.

The binding activities of anti- α -casein antibodies of IgE and IgG classes in sera of 11 patients to α -casein denatured by the five treatments (1-5) were compared with those to native α -casein (Fig 1 and Fig 2). The percent binding activities to denatured α -casein of IgE antibodies were almost identical with those to native α -casein. Likewise, the binding activities of IgG antibodies to α -casein were similar to those to native α -casein. In gel electrophoresis using 10% to 15% polyacrylamide gel (PAGE) in SDS,

of denatured α -casein similarly displayed two bands with molecular weights of 19 and 26 kD, the former corresponding to κ -casein and the latter corresponding to α_s -casein (Fig 3). Alpha-casein is fractionated into α_s -casein and κ -casein.¹² These results indicate that degradation did not occur by these treatments and that the primary structure of α -casein was retained even after treatment. The caseins contain prolyl residues, which interrupt α -helical and β -sheet structures but produce a stable poly-L-proline helical structure.¹² The caseins are, therefore, relatively small, amphipathic, randomly-coiled, unstructured, open

molecules.¹² On the other hand, the binding activities of IgE and IgG antibodies, especially IgG, of some patients to denatured α -casein differed considerably from those to native α -casein, strongly suggesting that denaturation of α -casein modifies the conformational structure of casein. Accordingly, the binding of IgE and IgG antibodies to modified α -casein demonstrates that these bindings are preferentially associated with the sequential protein structure.

Recently, the existence of immunodominant IgE determinants was suggested by studies of codfish Gad cl and hazel Cor al^{13,14} which appear to elicit IgE that binds predominantly to discrete peptides. In addition, we also demonstrated that epitopes of OVA recognized by IgE antibodies differed from those by IgG or IgA antibodies.¹¹ In contrast, no differences in specificities were found between the binding of IgE and IgG antibodies to denatured α -casein; even the binding activities of IgG antibodies to these denatured α -casein were relatively heterogeneous compared with those of IgE antibodies. It is therefore not yet clear whether sequential antigenic determinants of α -casein recognized by IgE antibodies differ from those recognized by IgG antibodies.

In conclusion, IgE and IgG antibodies of patients with milk allergy predominantly bind to sequential antigenic determinants of α -casein. The primary structures of casein have been defined.⁸ These findings suggest that antigenic determinants recognized by IgE and IgG antibodies of patients can be determined by using synthetic peptides corresponding to α -casein. Elucidation of epitopes on molecules recognized by antibodies are important for studies concerning the pathological role of antibodies in the induction of allergy.

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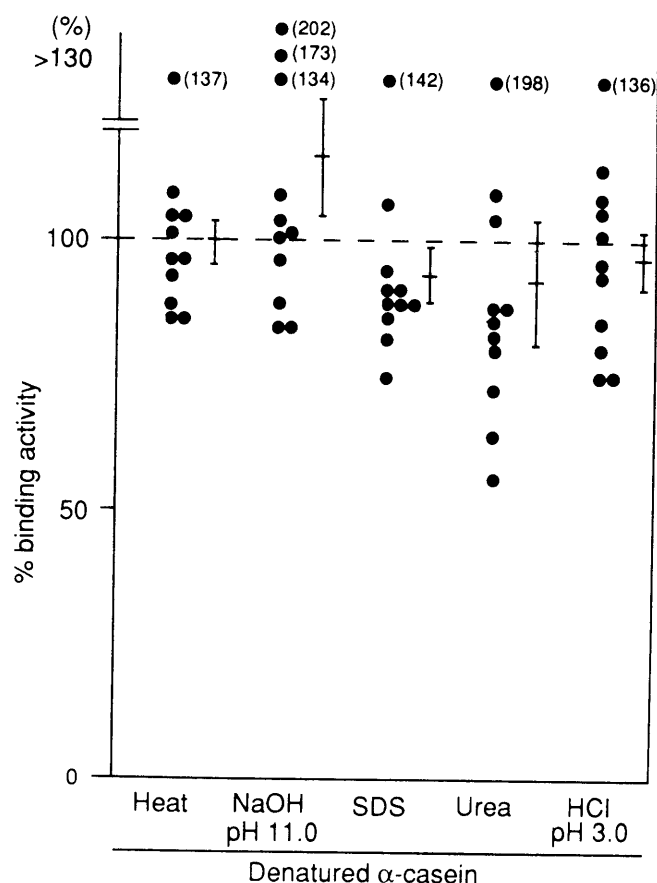


Figure 2. Percent binding activities of IgG antibodies in sera from patients with allergy to cow milk to denatured α -casein. The binding activities of IgG antibodies to denatured α -casein were measured by ELISA and are expressed as the percentage of antibody binding to native α -casein, taken as 100%. Native α -casein was denatured as described in "Materials and Methods." The assay was performed in duplicate. The mean ($\pm$ SE) of absorbance readings produced by the sera to native α -casein is 0.93 ± 0.07 .

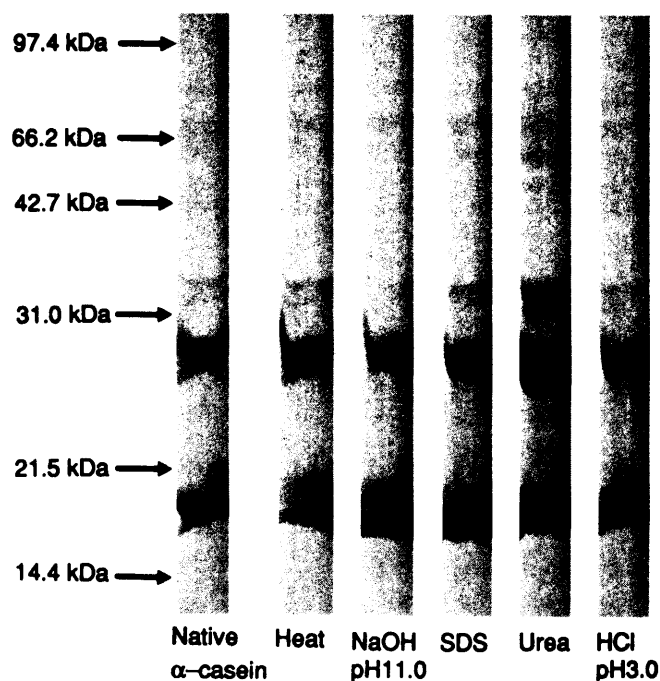


Figure 3. SDS-PAGE analysis of native α -casein and denatured α -casein. Native α -casein was denatured as described in "Materials and Methods."

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