

Soon-Mi Kim
Atsushi Enomoto
Satoshi Hachimura
Kunio Yamauchi
Shuichi Kaminogawa

Department of Agricultural Chemistry,
The University of Tokyo, Japan

Serum Antibody Response Elicited by a Casein Diet is Directed to Only Limited Determinants of α s1-Casein

Key Words

Food allergy
B cell determinant
Oral immunization
Oral tolerance

Abstract

Feeding mice with bovine whole casein as a diet constituent elicited an anti- α s1-casein (α s1-CN) systemic humoral response. The mice fed with the casein (CN) diet responded poorly to subsequent parenteral immunization with the antigen, indicating that oral tolerance was induced in the animals. The serum antibodies elicited by feeding CN were tested for binding to a panel of synthetic peptides and are shown to recognize only a limited number of potential antigenic determinants on α s1-CN. Our results suggest that a similar mechanism may be involved in the onset of milk allergy.

Introduction

Food allergy is due to an adverse immune response to food antigens, and specific IgE antibodies to food protein allergens have been demonstrated in allergic patients [1]. Although IgG antibodies specific for food allergens are present in normal individuals as well as in patients [1], there is the possibility that certain characteristics of the IgG antibody response in the allergic individuals may be responsible for the disease. Therefore, it is important to examine the systemic immune response elicited by feeding protein, to elucidate the mechanism for the onset of food allergy.

In mice, it is difficult to induce a systemic response without the use of oral adjuvants [2–5], and the oral administration of protein antigens generally induces a state of unresponsiveness to subsequent parenteral immunization, termed oral tolerance [6]. In this paper, we report that we were able to elicit a significant systemic humoral response in mice to bovine α s1-casein (α s1-CN), a major

component of cow's milk and also a major allergen [7], by feeding a diet containing bovine whole casein (w-CN) as the only protein source. The conformation of α s1-CN is thought to be disordered, with no certain three-dimensional structure [8]. For this reason, this antigen is suitable for B cell determinant mapping using peptide fragments. In previous studies, we identified the B cell determinants on α s1-CN that were recognized by serum antibodies elicited by parenteral immunization [9–11]. In the present study, we localized the B cell determinants that were recognized by the serum antibodies elicited by feeding the casein (CN) diet. We will show that the orally induced antibody response was directed towards only a limited number of potential determinants.

Materials and Methods

Mice

C3H/He mice were purchased from Charles River Japan (Tokyo, Japan).

Correspondence to: Dr. Shuichi Kaminogawa
Department of Agricultural Chemistry
The University of Tokyo
1-1-1 Yayoi, Bunkyo-ku
Tokyo 113 (Japan)

Antigens

Bovine w-CN was purchased from Sigma (C-3400; St. Louis, Mo., USA), and was used without further purification. α s1-CN (genetic variant B) was prepared from fresh raw milk as described elsewhere [10].

Overlapping synthetic peptides of α s1-CN were synthesized with a peptide synthesizer (430A; Applied Biosystems, Foster City, Calif., USA), and purified by HPLC as previously described [11]. The peptides and their relation with the parent protein are shown in fig. 1. The peptides were 20 amino acid residues, except for peptide 181–199 of 19 residues, and overlapped their adjacent neighbors by 5 residues.

Induction of a Systemic Humoral Response with a Diet Containing w-CN

A diet containing bovine w-CN as the only protein source (CN diet) was prepared (Funabashi Farms, Funabashi, Japan). Mice (8–10 mice per group) were maintained on the CN diet, and individual serum obtained at 10-day intervals (unless otherwise indicated) was tested for specific antibodies by an enzyme-linked immunosorbent assay (ELISA). No abnormalities could be identified in the mice fed with the CN diet.

Induction of Oral Tolerance by the CN Diet

To examine whether oral tolerance would be induced in mice fed the CN diet, the animals were fed with the CN diet, subsequently immunized intraperitoneally (i.p.) with 100 μ g of w-CN in complete Freund's adjuvant (Difco, Detroit, Mich., USA) and then boosted with 100 μ g of antigen in incomplete Freund's adjuvant 2 weeks later. Serum collected 1 week after the second immunization was tested for its binding to α s1-CN by ELISA. The antibody titers from the mice fed on the CN diet were compared with the antibody titers of the control groups fed on a commercial diet (MF; Oriental Yeast, Tokyo, Japan) which did not contain milk proteins. Statistical testing was done using the Mann-Whitney test.

ELISA

Serum samples were tested for binding to α s1-CN, or the synthetic peptides of α s1-CN (fig. 1) by ELISA [11]. Microtiter plates were pre-coated with 100 μ g/ml of α s1-CN, or with each synthetic peptide by incubating overnight. The plates were washed, and the diluted serum samples were added to the wells. After 2 h incubation, the plates were washed, and an alkaline-phosphatase-conjugated antimouse Ig solution [specific for Ig(G+A+M) or class specific; Cappel, Durham, N.C., USA] was added to the wells. The plates were further incubated for 2 h, and after washing, an enzyme substrate solution (0.1% *p*-nitrophenylphosphate in diethanolamine buffer at pH 9.8) was added. Thirty minutes later, the reaction was terminated by adding NaOH, and the absorbance at 405 nm was measured. Background levels due to nonspecific binding were subtracted from the individual values.

Results

A Systemic Humoral Response Elicited by the CN Diet

We attempted to orally immunize mice by feeding protein antigen as a constituent of the diet, as this made it possible to feed a large amount of antigen, giving continuous

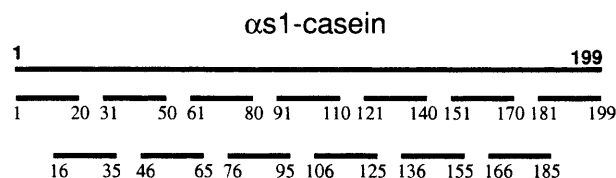


Fig. 1. The synthetic peptides corresponding to the amino acid sequence of α s1-CN.

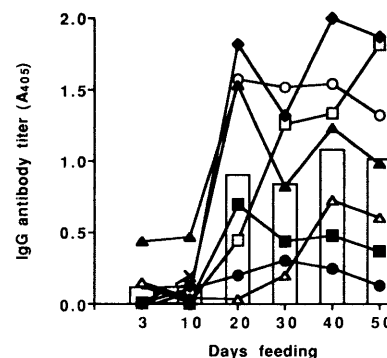


Fig. 2. Anti- α s1-CN serum IgG titers of C3H/He mice fed on the CN diet. Mice were bled at 10-day intervals, except for the first bleeding, which was on the third day of feeding. Individual serum was diluted 50-fold and tested for binding to α s1-CN by ELISA. Symbols indicate values from individual mice. The means are presented by the bars.

stimulation, compared to methods such as gastric intubation.

C3H/He mice (H-2^k, 3 weeks old) were maintained on the CN diet, and the serum of individuals were tested for the presence of anti- α s1-CN antibodies (fig. 2). There was considerable variation in antibody titer between individuals; however, α s1-CN-specific IgG antibodies could be detected in the serum of most of the mice 20 days after feeding started (fig. 2). IgM antibodies could also be detected, although the titers were low compared to the IgG titer (fig. 3). Specific IgA antibodies could not be detected in any of the individuals (data not shown). No α s1-CN-specific antibodies could be detected in mice fed a normal diet (data not shown).

Induction of Oral Tolerance by the CN Diet

The oral administration of a protein antigen generally results in tolerance of systemic immunity, which is known as oral tolerance [6]. It has been reported by other investi-

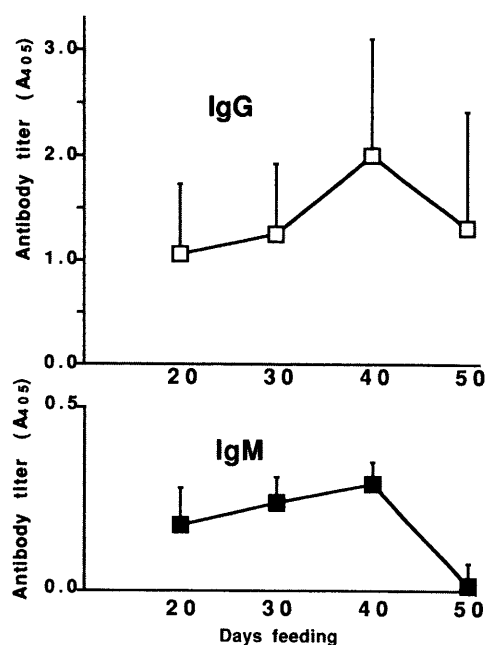


Fig. 3. Anti- α s1-CN serum IgG and IgM titers of C3H/He mice fed on the CN diet. Mice were bled at 10-day intervals. Individual serum was diluted 50-fold and tested for binding to α s1-CN by ELISA.

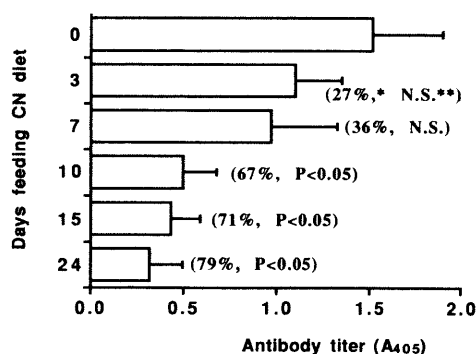


Fig. 4. Induction of oral tolerance by feeding the CN diet. Mice were fed on the CN diet for the periods indicated and subsequently immunized i.p. with w-CN. The animals received a booster immunization 2 weeks later, and were bled after another week. Individual serum was diluted 18,000-fold and assessed for α s1-CN-specific antibodies by ELISA. * Suppression (%) = $(1 - \text{antibody titer of the CN diet fed mice} / \text{antibody titer of the control mice}) \times 100$. ** Statistical testing was done using the Mann-Whitney test. N.S. = Not significant.

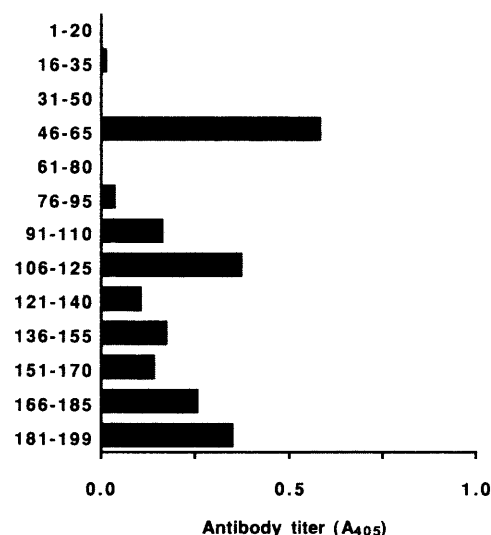


Fig. 5. B cell determinants of α s1-CN recognized by serum antibodies elicited in C3H/He mice by i.p. immunization of w-CN. Mice were immunized according to the protocols described for the controls in figure 4. Serum diluted 100-fold was tested for binding to the synthetic peptides shown in figure 1 by ELISA.

gators that oral tolerance could be induced by protein antigens in the diet [12, 13]. Although a systemic humoral response was elicited in the animals fed with the CN diet in our system, this did not exclude the possibility that feeding the CN diet induced oral tolerance. We, therefore, examined whether oral tolerance was induced in the CN-fed mice. Mice were fed with the CN diet for various periods and were subsequently immunized i.p. with w-CN, and the serum anti- α s1-CN antibody titer was compared with that of the controls which had not received the CN diet. The results show that oral tolerance was induced in the CN-fed animals (fig. 4). Feeding for 10 days suppressed the antibody response by 67% compared to the controls ($p < 0.05$). The degree of suppression was similar, or slightly greater in the case of mice fed the CN diet for longer periods (fig. 4). The results demonstrate that the mice fed a long-term CN diet, in spite of the presence of specific antibody in the serum, were in a tolerant state to subsequent parenteral immunization by the antigen.

Epitope Specificity of Serum Antibodies Elicited by the CN Diet

In previous studies, we have defined the B cell determinants of α s1-CN recognized by specific serum antibodies elicited by parenteral immunization of the antigen [9–11]. Typical data in C3H/He mice are shown in figure 5. Mice

were immunized i.p. with w-CN according to the same procedures as the controls in figure 4, and the antisera were tested for binding to the panel of synthetic peptides covering the entire sequence of α s1-CN presented in figure 1. The results show that the serum antibodies elicited by i.p. immunization of w-CN bound to 46–65, 91–110, 106–125, 121–140, 136–155, 151–170, 166–185 and 181–199, consistent with our previous studies [10, 11].

We next examined the antigenic determinants recognized in oral immunization to compare them with the determinants recognized in i.p. immunization. Serum samples showing a relatively high anti- α s1-CN titer (>0.7 at $\times 50$ dilution) were pooled and tested for binding to the panel of synthetic peptides. The results are shown in figure 6. The antibodies elicited by oral immunization reacted with 46–65 strongly and with 166–185 moderately, but bound poorly to the other peptides. These results indicate that the specific antibody response elicited by the oral administration of α s1-CN was only directed towards limited determinants on the antigen.

Discussion

One of the problems concerning investigations on food hypersensitivity with animal models is that, in the mouse and rat, which are the most popular laboratory animals in the field of immunology, the oral administration of protein antigens generally induces unresponsiveness of systemic immunity (oral tolerance) [6], and that only low levels of antibody response are elicited without the use of oral adjuvants, even by feeding considerably high doses [2–5]. In this study, we have shown that it was possible to elicit a humoral antibody response to a protein antigen in mice by feeding the antigen as a constituent of the diet, which enabled us to feed a considerably larger quantity of antigen compared to other administration methods such as gastric intubation. The CN diet contained 20.1% w-CN (w/w), and the mice ingested an average of 2.5 g/day, which resulted in a daily consumption of approximately 500 mg of w-CN (containing approximately 230 mg of α s1-CN) per mouse. The antibody response was mainly of the IgG class (fig. 2), which is consistent with reports from other investigators showing that an IgG response was preferentially produced in the serum by the oral administration of protein antigens [2, 3].

The results presented in fig. 4 suggest that the mice fed a long-term CN diet were hyporesponsive to subsequent parenteral immunization, as has been shown in other studies [12], indicating that the production of serum antibodies

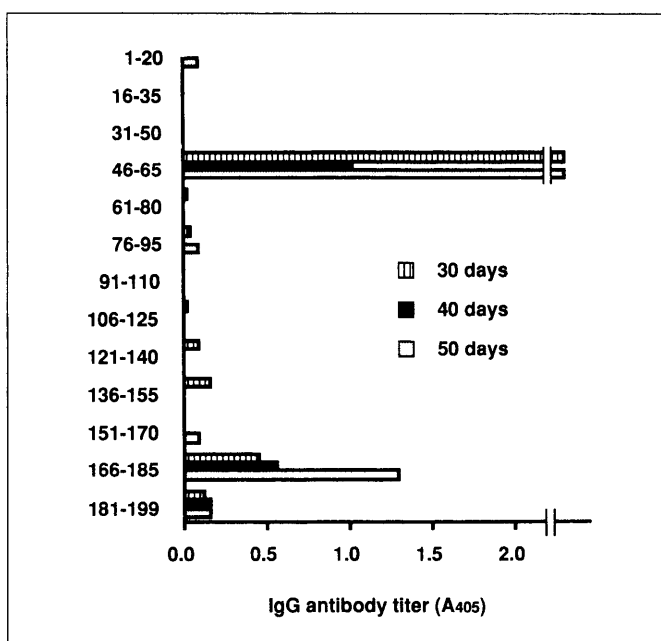


Fig. 6. B cell determinants of α s1-CN recognized by serum antibodies elicited in C3H/He mice by feeding the CN diet. Mice were fed the CN diet and serum samples with relatively high anti- α s1-CN titers were pooled, diluted 50-fold and tested for binding to the synthetic peptides shown in figure 1 by ELISA.

was not due to lack of oral tolerance. Feeding the CN diet was a very efficient method for inducing oral tolerance; feeding for 10 days suppressed the anti- α s1-CN antibody response to subsequent parenteral immunization by nearly 70% (fig. 4). In guinea pigs, a systemic response is rapidly elicited to dietary protein, and prolonged feeding is required for tolerance to be induced [14]. In contrast, in our system using mice, the induction of oral tolerance ensued rapidly, which is consistent with other studies in mice [4]. We note that, although lack of oral tolerance in C3H/HeJ mice has been reported [15], oral tolerance to CN was also induced to a similar degree in this strain (data not shown), suggesting that the lack of tolerance in the C3H/HeJ strain may be specific to the sheep erythrocyte system. Strain variation in the degree of immune responses to orally administered antigen has been reported [15–18]. We used our system to examine two other strains of mice: BALB/c (H-2^d) and C57BL/6 (H-2^b), and found strain variation in our system also. The variation in the degree of antibody responses was greater than that of oral-tolerance induction, and the C3H strain showed the highest antibody response among the three strains (data not shown).

α s1-Casein is a protein consisting of a single 199 amino acid residue polypeptide chain. The conformation of this antigen in solution is thought to be in a disordered state with no certain three-dimensional structure [8]. For this reason, this protein can be considered as a suitable antigen for B cell epitope mapping with peptides, as the specific antibodies produced by immunizing with this protein are expected to recognize only sequential epitopes on the antigen. In previous studies, we have localized the B cell determinants of α s1-CN recognized when the antigen was parenterally immunized [9–11]. Results obtained by immunizing w-CN are presented in fig. 5. Antibodies reactive to 46–65, 91–110, 106–125, 121–140, 136–155, 151–170, 166–185 and 181–199 were elicited by i.p. immunization of w-CN (fig. 5). Interestingly, of these only 46–65 and 166–185 were recognized when the antigen was orally administered (fig. 6).

We consider that enzymatic digestion of the antigen in the gastrointestinal tract is involved in determining the epitope specificity of the serum antibody response induced by oral immunization of α s1-CN. Determinants 91–110, 106–125, 121–140, 136–155, 151–170 and 181–199 were not recognized by the antibodies elicited by oral immunization (fig. 6). Enzymatic digestion of the antigen may result in the loss of a B cell determinant in the following two cases. Firstly, proteolytic cleavage may directly destroy certain antigenic determinants of the protein. Such determinants would not be able to stimulate the immune system when administered via the oral route. Secondly, even when an antigenic determinant is preserved on a peptide in terms of the amino acid sequence, the peptide may fail to elicit an antibody response when the peptide is the immunogen. We have previously reported peptides bearing antigenic determinants of α s1-CN which were not capable of mounting an antibody response when the peptide itself was used as the immunogen [11]. Although at present we do not know which fragments are actually formed by the *in vivo* enzymatic digestion of α s1-CN, we determined in previous studies the *in vitro* cleavage sites of three major digestive enzymes, pepsin, trypsin and chymotrypsin [9, 10], and showed that there is a considerable number of cleavage sites on this protein. Proteolysis at such sites may well result in the loss of antigenic determinants.

In addition to enzymatic digestion, there may be other factors determining the epitopic specificity of the antibodies elicited by feeding the CN diet. We have found in recent studies that there is variation in the degree of oral tolerance induced among different antigenic determinants of α s1-CN. The antibody response to 46–65 was not suppressed in orally tolerant mice [in preparation]. This may

be one of the reasons why antibodies directed to 46–65 were preferentially produced when the antigen was orally administered. The epitope specificity of oral tolerance will be the subject of another manuscript.

In the present study, we have shown that serum antibodies with distinct epitopic specificity were elicited by the oral administration of α s1-CN. We have recently reported that the sera of patients with milk allergy contain IgG4 antibodies which react to milk proteins in significantly high levels, and that such antibodies which bind to α s1-CN are preferentially directed to region 46–65 [19]. Our results suggest that a similar mechanism for determinant selection may be involved in the induction of food allergy.

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