

continued. As the patient's clinical condition remained stable, the fluconazole dose was reduced and fully tapered after 3 months of baricitinib. During 8 months of follow-up, no oral or vaginal ulcers reoccurred. Mucocutaneous candidiasis did not reappear, even without prophylactic antifungal therapy. Although (re) occurrence of viral infections, especially herpes zoster, and other opportunistic infections has been reported on treatment with JAK inhibitors,^{E1} our patient did not encounter any of these up to 8 months after initiation of treatment. Also, no other clinical or biochemical complications were reported.

Blood samples collected before the start of baricitinib and every 2 to 4 weeks afterward were examined for STAT1/STAT3 phosphorylation, STAT1-regulated gene expression, and cytokine production. Before treatment, pSTAT1 levels in T lymphocytes from the patient were greater than pSTAT1 levels in 5 age/sex/race-matched healthy control subjects, as reported previously.⁴ Baricitinib treatment reduced pSTAT1 levels at every time point examined (Fig 2, A). Although baricitinib did not clearly affect STAT3 phosphorylation in our initial *in vitro* studies (see Fig E1, B), IFN- α - and IL-6-induced STAT3 phosphorylation was reduced in T lymphocytes from the patient during baricitinib treatment (Fig 2, B). The remarkably high expression levels of STAT1-regulated genes (*CXCL9* and *CXCL10*) from patients' PBMCs before baricitinib treatment were strongly decreased on treatment (Fig 2, C). Before initiation of baricitinib treatment, PD-L1 (*CD274*) expression in PBMCs from the patient was approximately 3-fold greater compared with PBMCs from 5 healthy control subjects, but its expression level did not decrease during the treatment period (data not shown). PBMCs from the patient obtained during baricitinib treatment showed greater production of IL-17A, IL-17F, and IL-22 on stimulation with phorbol 12-myristate 13-acetate and ionomycin or HKCA than PBMCs obtained before treatment. However, these levels were much lower than those observed in healthy control subjects ($n = 5$; Fig 2, D, and see this article's Methods section in the Online Repository at www.jacionline.org).

This study provides the first evidence that baricitinib could be of value in the treatment of patients with *STAT1* GOF mutations. We demonstrate this at several levels. First, our patient showed remarkable clinical improvement with baricitinib treatment. Systemic prophylactic antifungal therapy and immunosuppressive treatment could be terminated without reoccurrence of candidiasis or ulcers. Second, baricitinib reduced STAT1 hyperphosphorylation and STAT3 phosphorylation and improved the capacity of PBMCs to produce IL-17A, IL-17F, and IL-22, cytokines crucial for antifungal immune responses. Third, baricitinib reduced expression of the IL-27/STAT1-regulated genes *CXCL9*, *CXCL10*, and PD-L1 (*CD274*) in long-term T-lymphocyte cultures from the patient. PBMCs obtained from the patient during 3 months of baricitinib therapy also expressed *CXCL9* and *CXCL10* at levels approaching those of the healthy control subjects. Overexpression of PD-L1 in naive T lymphocytes from patients with a *STAT1* GOF mutation is associated with enhanced IL-27/STAT1 signaling and contributes to inhibition of T_H17 differentiation.^{2,3} An anti-human PD-L1 inhibitory antibody was found to partially rescue IL-17A production in T lymphocytes from patients with a *STAT1* GOF mutation.² We found no reduction in PD-L1 (*CD274*) mRNA expression in unstimulated PBMCs from the patient during baricitinib treatment. However, PD-L1 is known to be upregulated on activated T lymphocytes,^{E2} and our data show that specific activation of T lymphocytes from the patient with IL-27 results in strong upregulation of PD-L1,

which can be abrogated by baricitinib (see Fig E1, D). Therefore the observed partial restoration of IL-17A production by PBMCs obtained from the patient during baricitinib treatment could result from less pronounced upregulation of PD-L1 on activated T lymphocytes under the phorbol 12-myristate 13-acetate and ionomycin and HKCA stimulation conditions.

In conclusion, for the first time, we show therapeutic benefit of the clinically available drug baricitinib in a patient with GOF *STAT1* mutation. Further studies are required to evaluate its clinical implications in other patients with *STAT1* GOF mutations.

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Hypoallergenic casein hydrolysate for peptide-based oral immunotherapy in cow's milk allergy



To the Editor:

Cow's milk allergy (CMA) is one of the most common food allergies and a critical public health problem in the case of children.¹ Oral immunotherapy and oral food challenges have been developed for treating and diagnosing food allergies, but

anaphylactic reactions occasionally occur during immunotherapy.^{E1} To avoid fatal allergic reactions during allergen-specific immunotherapy as well as oral food challenges, peptide-based approaches represent a safer option that might be used in the future with small peptides that contain T-cell epitope sequences, but avoid cross-reactions with IgE.^{E2} We previously identified an edible β -lactoglobulin, a common allergen component in cow's milk, in hydrolysate that contained T-cell epitopes and had reduced antigenicity.²

α _{S1}-Casein (Bos d 9) is the most common allergen in cow's milk, and T-cell epitopes have been screened using overlapping peptides from the entire α _{S1}-casein sequence.^{3,4} IgE epitopes of α _{S1}-casein have been identified in several studies.⁵ Therefore, attention must be paid to this essential region when hydrolyzing casein in order to reduce its antigenicity, but retain its ability to immunostimulate T cells. Because casein can be easily aggregated and precipitated at acidic pH,⁶ we used casein hydrolysate prepared at alkaline pH (aCNH) as well as casein hydrolysate prepared at neutral pH (nCNH), and we analyzed the impact of hydrolysis pH on peptide distribution in casein hydrolysates using Alcalase, a food-grade endoproteinase containing subtilisin Carlsberg derived from *Bacillus licheniformis*.^{E3}

Fig 1, A, shows the appearance and electrophoretic patterns of casein hydrolysate obtained using Alcalase at different pH values (see the Methods section in this article's Online Repository at www.jacionline.org). The hydrolysate was clear in aCNH, whereas it was partially precipitated at the beginning of hydrolysis in nCNH. The casein fraction (~20–30 kDa) was rapidly degraded regardless of the hydrolysis pH, whereas the bands observed differed according to the hydrolysis pH. The high-molecular-weight fraction above 10 kDa was hydrolyzed within 60 minutes in aCNH, but these bands remained visible throughout the hydrolysis in nCNH. Many bands corresponding to the middle-to-low molecular weight fraction below 10 kDa were observed for nCNH but not for aCNH throughout hydrolysis. Changes in the molecular weight distribution were also confirmed by chromatographic analysis (see Fig E1 in this article's Online Repository at www.jacionline.org) throughout hydrolysis at different pH values. These results suggested that the hydrolysis pH affected the formation and retention of high-molecular-weight peptides, which was accompanied by visible changes. At the beginning of hydrolysis, high-molecular-weight peptides were likely to undergo aggregation and to inhibit hydrolysis in the hydrophobic region of the hydrolysate.

Cleavage sites were analyzed from the deduced peptide patterns from the 2 hydrolysates (see Table E1 in this article's Online Repository at www.jacionline.org). Fig 1, B, shows the cleavage site patterns of α _{S1}-casein in the 2 hydrolysates, including the possible IgE epitopes and the probable T-cell epitopes from the literature.^{3,5,E4} In total, there were 59 cleavage sites in the IgE epitopes in aCNH and 39 in nCNH; 3 cleavage sites were located in the immunodominant T-cell epitopes in aCNH and 5 in nCNH. The total number of cleavage sites in the antigenic region in aCNH was 51% higher than that in nCNH, whereas the number of cleavage sites in the immunogenic region in aCNH was 40% lower than that in nCNH. Because MHC II molecules can present peptides as short as 3 to 10 amino acids present in other food allergens,^{E5-E7} we assessed lymphocyte proliferation using aCNH. The stimulation index of aCNH remained proliferative in the lymphocyte stimulation test using PBMCs from patients with CMA (1638 counts per minute [cpm] and a 2.1-fold change for

patient 1; 862 cpm and a 1.8-fold change for patient 2. In the case of unhydrolyzed casein, 3026 cpm and a 3.8-fold change for patient 1; 1247 cpm and a 2.6-fold change for patient 2). These results suggested that the enzymatic hydrolysis at alkaline pH preferably hydrolyzed the nonimmunogenic region of α _{S1}-casein, or that the hydrolysis at neutral pH tended to degrade immunogenic epitopes in α _{S1}-casein more effectively than at alkaline pH.

The antigenicity for IgG and IgE antibodies was characterized in the casein hydrolysates on the basis of B-cell reactivity measured by IgG inhibition ELISA and IgE Western blot. IgG inhibition ELISA was performed using anticasein rabbit sera to investigate the effect of hydrolysis pH on the antigenicity of casein hydrolysates (Fig 1, C). The binding capacity of aCNH for anticasein IgG antibody effectively decreased to approximately 10^{-4} to 10^{-5} of that noted for unhydrolyzed casein. In contrast, the antigenicity of nCNH showed a lesser reduction of around 10^{-2} . IgE Western blotting was performed with the antiserum from a patient with persistent CMA (Fig 1, D). The IgE-binding capacity was completely suppressed in aCNH; peptide fragments were clearly detected in the Coomassie Blue-stained gel. The unhydrolyzed casein substrate was also detected as IgE-binding bands corresponding to a molecular weight of around 30 kDa. For nCNH, bands indicating new IgE-binding capacity appeared at around 10 kDa, and smeared bands indicating weak remaining IgE-binding capacity were noted above the 10-kDa band. This 10-kDa peptide consistently remained throughout the enzymatic hydrolysis, especially at neutral pH (Fig 1, A). Hydrolysis principally decreases the antigenicity of the allergen component, whereas specific heat-induced hydrolysis of wheat protein leads to the formation of novel allergenic peptides.⁷ This result suggests that nCNH still has allergenic potency and that the hydrolyzing conditions of enzymatic hydrolysis may lead to the formation of an antigenic peptide against the antisera of patients with CMA.

The IgG-binding capacity in aCNH decreased to a greater extent than that in nCNH, and IgE binding was completely suppressed in aCNH. These antigenic responses were attributed to the distinctive degradation patterns noted at the hydrolysis pH. The hydrophobicity of casein and casein hydrolysate was inversely correlated with pH⁸; the hydrophobic fragment might contribute to the antigenic response to B cells. Although our results were limited to *in vitro* analysis, we believe that our study has provided a practical approach for a peptide-based edible vaccine against a major milk allergen using common food materials. We have performed a pilot phase I study with aCNH prepared on an industrial scale. Further clinical trials should shed light on the effect of casein hydrolysate used for CMA treatment.

In conclusion, casein hydrolysis at alkaline pH helped retain immunogenicity, but reduced antigenicity, which was associated with less aggregation and precipitation throughout the casein hydrolysis. The edible casein hydrolysate reduced antigenicity for casein-specific antibodies, but retained immunogenic epitopes. These results suggest that obtaining casein hydrolysate by using Alcalase at alkaline pH may be beneficial for hypoallergenic and peptide-based oral immunotherapy in order to safely induce immunologic tolerance to cow's milk.

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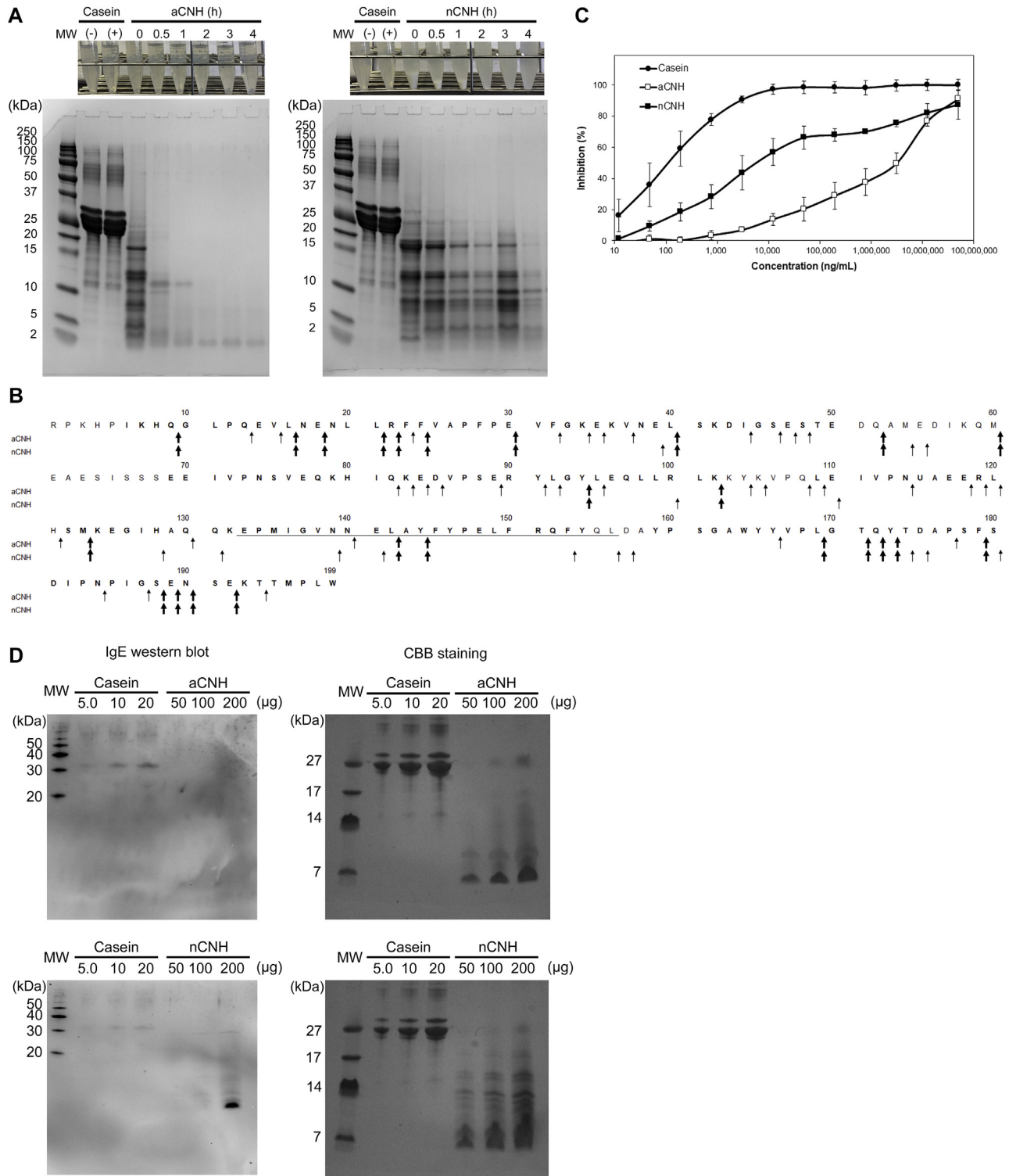


FIG 1. Casein showed reduced antigenicity, but retained immunogenicity when enzymatically hydrolyzed at alkaline pH. **A**, Appearance (*top*) and electrophoretic patterns (*bottom*) of casein hydrolysates. Casein was hydrolyzed at alkaline pH (aCNH; *left*) and neutral pH (nCNH; *right*) and loaded onto separate gels. pH adjustment of casein is indicated as $\pm$, and numbers indicate reaction time (h) for hydrolysis. **B**, Cleavage site distributions for α_{S1} -casein in casein hydrolyzed for 4 hours. Cleavage sites were determined for the identified peptides. The arrows indicate cleavage sites in aCNH (*top*) and nCNH (*bottom*); shared cleavage sites are indicated with **bold arrows**. IgE epitopes are shown in **bold**, and T-cell epitopes are underlined. **C**, Antigenicity of casein hydrolysates for anticasein IgG. The data represent the binding capacity of unhydrolyzed casein (*closed circles*) and casein hydrolysate in aCNH (*open squares*) and nCNH (*closed squares*). Data are shown as mean $\pm$ SDs from triplicate experiments. **D**, IgE Western blot analysis for casein hydrolysate. Electrophoretic patterns of hydrolyzed and unhydrolyzed casein on a membrane for an antigen-bound IgE against casein (*left*) and on a gel stained with Coomassie Blue (CBB; *right*) for proteins and peptides. Casein was hydrolyzed using Alcalase in aCNH (*top*) and nCNH (*bottom*).

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Screening for Wiskott-Aldrich syndrome by flow cytometry



To the Editor:

Wiskott-Aldrich syndrome (WAS) is a rare X-linked disease commonly associated with symptoms of immune deficiency. A diagnosis of WAS is suspected on the basis of clinical parameters including thrombocytopenia, eczema, recurring infections, and autoimmunity.¹ WAS protein (WASP) is exclusively expressed in hematopoietic lineages. Lowered levels are thought to be a feature of WAS.² However, the diagnostic accuracy of flow cytometric WASP measurement has not been determined, and optimal diagnostic cutoff values are lacking. We therefore reviewed results from 107 individuals with suspected WAS to calculate the diagnostic accuracy of a rapid whole-blood flow cytometry-based assay and found it reliable for predicting mutations in WAS.

WASP expression levels were determined in lymphocytes gated by CD45 versus side light scatter. A range of WASP protein expression was seen, from normal in healthy controls to those with reduced or poor WASP expression (Fig 1, A; see Fig E1, A-C, in this article's Online Repository at www.jacionline.org). Detailed methods can be found in this article's Online Repository at www.jacionline.org. The age at WASP staining ranged from 1 month to 32 years with a median of 1 year.

All 58 persons with normal WAS sequence displayed WASP expression at levels comparable to those in healthy controls (mean staining ratio, 0.94 ± 0.15) while 41 of 49 patients with a genetic abnormality were found with reduced staining ratios (mean staining ratio, 0.34 ± 0.28) (Mann-Whitney nonparametric test $P < .0001$). We further divided the patients with genetic abnormalities into 5 categories, that is, missense ($n = 30$), insertion ($n = 4$), deletion ($n = 8$), gross deletion ($n = 4$), and nonsense ($n = 3$) (see Fig E2, A, in this article's Online Repository at www.jacionline.org). Table E1 in this article's Online Repository at www.jacionline.org lists all patients in this study. Every patient with gross deletions had poor WASP expression. Eight patients from the other mutation categories had normal WASP staining despite having a variant in WAS. Three of these patients carried variants of unknown clinical significance (VUCS). Interestingly, the c.538C>A (p.H180N) variant (staining ratio, 0.98) has been implicated in patients with X-linked thrombocytopenia (SCV000053274).³ It was not possible to rule out mosaicism or the influence of maternal cells with the available data; however, no patient included here was observed to have bimodal WASP staining. In addition, of the 8 patients with normal WASP expression, 3 mutations were intronic and 3 located at the C-terminus. Because the WASP antibody recognizes an epitope in the region between aa.146-265, it may still bind proteins bearing missense or C-terminal-truncating mutations.⁴

Six patients with missense mutations displayed staining ratios between 0.3 and 0.5. This "intermediate" staining ratio was not observed in other mutation groups, possibly due to low sample numbers. All missense mutations were "probably damaging" on PolyPhen-2 except c.995T>C (p.V332A) (0.0, benign), a known VUCS with a 0.84 staining ratio. Fold stability change ($\Delta\Delta G$) predictions reported a range of slightly stabilizing to highly destabilizing effects (Fig 1, B).^{5,6} Certain damaging mutations might not influence the antibody epitope, leading to only slightly reduced antibody staining. Mutations that destabilize the protein could lead to degradation and poor detection, whereas stabilizing mutations could hinder protein function yet display normal levels of WASP staining. Highly accurate and reproducible staining is crucial for establishing diagnostic cutoff estimation. To evaluate staining consistency, we compared patients bearing similar WAS mutations. Here, the c.167C>T (p.A56V) mutation was found in 4 patients, c.356G>A (p.G119E) in 3, and c.559(+5) in 5. Results exhibited good reproducibility (see Fig E2, B). WAS scores were available for 28 of the 49 confirmed WAS cases (Table E1). Scores did not strongly correlate to WASP staining index or age of presentation (Fig 1, C; Fig E2, C and D).^{7,8} Interestingly, patients with autoimmunity (WAS score 5) were all low WASP expressers. No malignancies were reported in any of the 28 patients with available scores.

From the receiver-operating characteristic curve, when all entries were considered, a cutoff staining ratio of 0.60 returned a sensitivity and specificity of 82.6% and 100%, respectively.

Supplementary documents

METHODS

Materials

Casein of Hammarsten grade was obtained from MP Biomedicals (Santa Ana, CA, USA) and that of edible grade (93% [w/w] protein) was obtained from Tatua Co-operative Dairy (Morrinsville, New Zealand). Proteinase of food-additive grade was obtained from Novozymes (Alcalase 2.4L FG; Bagsværd, Denmark). Unless otherwise specified, the reagents used were of analytical grade.

Study participants

The study was approved by the Ethics Committees of the Graduate School of Medicine, Gifu University (Gifu, Japan), was registered with the Japanese University Hospital Medical Information Network Clinical Trials Registry (UMIN000002139), and was performed in accordance with the Declaration of Helsinki. Each participant provided written informed consent. CMA was diagnosed on the basis of clinical symptoms noted after ingestion, including the findings from an open-challenge test with bovine milk casein and measurement of cow's milk-specific IgE by performing the ImmunoCAP test (Phadia AB, Uppsala, Sweden). From the patient samples included in the ImmunoCAP test, samples from two patients with persistent CMA (patients 1 and 2) were used for the allergen-specific lymphocyte proliferation tests, and a sample from a third patient (patient 3) was used for IgE western blot analysis. Patient 1 was a 6-year-old boy with food allergy and a history of anaphylaxis with cow's milk. Patient 2 was a 6-year-old girl with atopic dermatitis, food allergy, and a history of anaphylaxis with cow's milk. Patient 3 was a girl aged 5 years and 10 months with asthma, atopic

dermatitis, allergic rhinitis, food allergy, and a history of anaphylaxis with multiple foods containing cow's milk. The IgE levels of patients 1, 2, and 3 were as follows: total IgE, 920, 545, and 804 IU/mL; for α -lactalbumin, ≤ 0.34 , 18.9, and 6.97 kU_A/L; for β Lg, 0.78, 1.5, and 9.44 kU_A/L; for casein, 5.19, 72.5, and 89.1 kU_A/L; and for milk, 5.18, 70.2, and 88.4 kU_A/L, respectively.

Protein digestion

Casein enzymatic hydrolysates were prepared as follows. In the experiment for determining the effect of hydrolysis pH on the immunogenicity and antigenicity of casein hydrolysate, casein (50 mg/mL) was hydrolyzed at alkaline pH (pH 10) and neutral pH (pH 7) and a shaking speed of 30 rpm by 0.5 mg/mL Alcalase at 55 °C for 4 h. After digestion, the enzymes were inactivated by heating the samples in boiling water for 10 min. They were then stored in the refrigerator at 4 °C until further analysis.

Chromatographic analyses

Chromatographic analyses were performed as per the procedure reported in our previous studies.^{2,E8} Briefly, the molecular-weight distribution of the digested peptides was analyzed by performing high-performance size-exclusion chromatography (HPSEC) on a Waters H-Class UPLC system (Waters, Milford, MA, USA) fitted with a Corona charged aerosol detector (Corona Ultra; Thermo Fisher Scientific, Waltham, MA, USA). The samples were applied to a size-exclusion column (TSKgel Super AW3000; Tosoh, Tokyo, Japan) and eluted with 0.1% (v/v) trifluoroacetic acid and 55% (v/v) acetonitrile in water. Molecular-weight calibration of the column was performed using the standard

proteins.

The hydrolyzed peptide fragments of α_{S1} -casein were identified by liquid chromatography (LC) separation and mass spectrometry (MS). Briefly, casein hydrolysates were alkylated with iodoacetamide and then subjected to LCMS analysis. Five hundred nanograms of alkylated samples were injected into a reversed phase column (L-column2 ODS; 0.2 mm ID $\times$ 150 mm; 3-mm particle size; Chemicals Evaluation and Research Institute, Tokyo, Japan) in solvent A (2% [v/v] acetonitrile and 0.1% [v/v] formic acid in deionized water) and solvent B (90% [v/v] acetonitrile and 0.1% [v/v] formic acid in deionized water). The proportion of solvent B was increased linearly from 0% to 45% over 90 min at 25 °C at a flow rate of 2 μ L/min. The mass spectrometer was set to the data-dependent MS/MS mode (m/z 400–2000), in which 1 full MS scan was followed by 5 MS/MS scans. MS/MS data analysis was performed using the Xcalibur 1.3 software and Proteome Discoverer 1.3 software (Thermo Fisher Scientific) with the SEQUEST algorithm. The search parameters used included a 2 Da tolerance for precursor ion masses and a 0.8 Da tolerance for fragment ion masses with no enzyme restraint. The cleavage site patterns of α_{S1} -casein were compared within the 2 hydrolysates, by referring to potential IgE epitopes and probable T-cell epitopes from the literature.^{3,5,E4}

IgG inhibition enzyme-linked immunosorbent assay (ELISA)

IgG-inhibition ELISA was performed as per the procedure reported in our previous study,² with minor modifications. Briefly, a 96-well microtiter plate (Maxisorp; Thermo Fisher Scientific) was coated with intact casein. After the wells were washed, they were blocked with 0.4% goat serum for 90 min at 25 °C. They were then washed and incubated

with horseradish peroxidase-conjugated anti-casein sheep sera (1:40,000 dilution; GeneTex, Irvine, CA, USA) and 50 μ L of casein, aCNH, and nCNH at concentrations ranging from 5 mg/mL to 12 ng/mL for 3 h at 25 °C. After washing, the color was developed by using *o*-phenylenediamine, and the percentage of inhibition was calculated as (total reactivity – remaining reactivity after absorption) $\times$ 100 / total reactivity.

Antigen-induced proliferative responses of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from heparinized blood from control donors and patients by gradient centrifugation in Ficoll-Paque (Pharmacia AB, Uppsala, Sweden). PBMCs were cultured with casein or digested peptides at a concentration of 20 mg/mL at 37 °C in a 5% CO₂-humidified atmosphere for 5 days. Proliferative responses to food antigens were determined as previously described^{E9}. Briefly, these assays were performed in triplicate in 96-well, flat-bottomed microtiter plates (Nunclon, Roskilde, Denmark) using 2×10^5 cells per well in a total volume of 200 μ L. The culture medium consisted of RPMI 1640 (Sanko Junyaku Co., Ltd, Tokyo, Japan) supplemented with 10% pooled human AB serum (Cambrex Bio Science Walkersville Inc., Walkersville, MD, USA), L-glutamine (2 mmol/L), penicillin (100 IU/mL), and streptomycin (100 μ g/mL). Proliferation was measured by [³H]-thymidine incorporation (0.5 mCi/well) during the last 16 h of culture. Proliferation response was measured as the stimulation index using the following formula: counts per minute (cpm) incorporated into antigen-stimulated cultures / cpm incorporated into control medium.

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE)

and IgE western blotting

SDS-PAGE and IgE western blotting were performed as per the procedures reported in our previous study,² with minor modifications. Casein (5.0–20 µg) and casein hydrolysates (50–200 µg) reduced with 2-mercaptoethanol were electrophoresed on 16.5% Tris-Tricine precast gels (Bio-Rad Laboratories, Hercules, CA, USA). A protein molecular weight marker (Polypeptide SDS-PAGE Molecular Weight Standard, Bio-Rad; or MagicMark XP Western Protein Standard, Thermo Fisher Scientific) was included for the corresponding detection system, and proteins and polypeptides were visualized using Coomassie Blue staining or IgE western blotting. For western blotting, proteins were transferred to polyvinylidene difluoride (PVDF) membranes and reacted with the serum from the patient with persistent CMA. The blots were incubated with horseradish peroxidase–conjugated human IgE goat antibody (Bethyl Laboratories, Montgomery, TX, USA), and the stained bands were visualized using the Amersham ECL Prime reagent (GE Healthcare, Little Chalfont, United Kingdom).

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Supplementary figure legends

FIG E1. Change in the molecular weight distribution of casein hydrolysates as determined by size-exclusion chromatography. Casein hydrolysates were prepared at alkaline pH (aCNH; left) and neutral pH (nCNH; right). Arrows indicate unhydrolyzed casein, salts, and molecular weight at 3 kDa. The peak corresponding to casein was largely hydrolyzed within the first 30 min in aCNH. After 4 h of hydrolysis, the peaks of aCNH were eluted from 8.1 min, corresponding to 3 kDa. In contrast, there were partially collapsed peaks at around 7 min in the chromatogram in nCNH, and these large peptides remained throughout hydrolysis for 4 h.