

Putative allergic reactivity of casein phosphopeptide in severe cow's milk allergy patients

To the Editor,

Cow's milk (CM) is a common food allergen.¹ with high casein content, comprising approximately 80% of CM proteins.² Various processed foods and medicines include CM-derived processed products. Thus, the symptoms induced by an allergic reaction to the accidental ingestion of these products must be heeded.³

Casein phosphopeptide (CPP), a processed product, is derived from α s1-casein (E61–K79), α s2-casein (K1–Y20 and N46–K70), and β -casein (R1–R25) via tryptic digestion.⁴ It binds with amorphous calcium phosphate (ACP), increasing its ability to stabilize calcium phosphate in solution.⁵ Thus, CPP-ACP acts as a biologic calcium phosphate delivery vehicle and is used worldwide in various oral care products, including chewing gums, topical creams, and toothpaste, owing to its anticariogenic properties.⁶

Because the allergenicity of CPP has remained undetermined, whether CPP-ACP can induce allergic symptoms in patients with CM allergy (CMA) is unclear. Thus, this study aimed to validate the allergenicity of CPP and determine whether there is CPP-ACP-reactive CMA. Our study was approved by the Research Ethics Board of Aichi Children's Hospital (approval number: 2020114) and conformed to the Declaration of Helsinki.

First, we searched the electronic medical record database of Aichi Children's Health and Medical Center from February 2012 to March 2021 to identify any CPP-ACP-related episodes. Eight patients were identified in total (Table 1). Data on their characteristics, clinical information, episode details, and the associated results of the CM oral food challenge (OFC) test and blood tests were extracted from the medical records. The severity of the allergy-induced symptoms varied between patients. Although the amount of product used was small, of the six patients who reacted to the dental care product MI paste (GC Corp.), which contains 10% CPP-ACP, four developed anaphylaxis and required an intramuscular adrenaline injection. Two patients reacted to Recaldent gum (GC Corp.), which contains 0.18% CPP-ACP, and one of them experienced anaphylaxis. Some patients had relatively long intervals between the episode and OFC. However, all had severe CMA after exposure to <10 ml of CM during the OFC. Moreover, they presented with high casein-specific immunoglobulin (Ig) E titer levels and completely avoided CM during the episode.

Next, we performed ImmunoCAP inhibition assays and basophil activation tests using CPP or casein to examine the *in vitro*

allergenicity of CPP, as described in the Supplemental Methods. We recruited six patients with CMA whose severity was similar to that of CPP-ACP (reacted <10 ml of CM) and who presented with casein-specific IgE titer levels above two digits and completely avoided CM for the *in vitro* tests (Table S1). Written informed consent was obtained from the parents of the participants at enrollment.

The casein, CPP, and ovalbumin (FUJIFILM Wako Chemicals, Osaka, Japan) used in this *in vitro* study were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Supplemental Methods, and Figure S1). All the casein fractions (α s1-, α s2-, β -, and κ -casein) were detected in casein, and CPP showed a smear with a molecular weight <20 kDa.

Inhibition assay revealed that CPP inhibited casein-specific IgE binding in a concentration-dependent manner in all five patients (Figure 1). Although the rate at which casein-specific IgE was inhibited was low at lower concentrations, it was >80% when applied at the highest concentration (10 mg/ml). This inhibition was proven as antigen-specific because 10 mg/ml of ovalbumin did not interfere with the detection of casein-specific IgE (Figure 1), and 10 mg/ml of casein and CPP did not inhibit egg white-specific IgE using the identical sera (Figure S2). We further detected CPP-specific IgE binding by dot blot analysis (Figure S3).

Based on the basophil activation tests (Figure 2 and Figure S4), CPP activated basophils in a concentration-dependent manner comparable to that of casein in two of the patients (Figure 2a,b) but was less effective than casein in activating basophils at concentrations >10 μ g/ml in the remaining patient (Figure 2c).

These *in vitro* data clearly indicate that CPP possesses IgE-binding ability and induces basophil activation comparable to that of casein, supporting the fact that CPP-ACP causes severe allergic symptoms in CMA. Till date, many casein epitopes have been reported through IgE epitope mapping using synthetic peptides to predict CMA prognosis.^{7,8} However, only a few epitope sequences were located, and no common epitopes were detected in the CPP regions of the different casein components.⁹

We previously reported a strong correlation between the IgE levels specific to α s1- and β -casein¹⁰ and confirmed strong cross-reactivity between them using competitive enzyme-linked immunosorbent assays.⁹ Although α s1- and β -casein showed a fairly low level of homology of total amino acid sequences (4%–7%), we found

TABLE 1 Allergic events caused by casein phosphopeptide–amorphous calcium phosphate

Sex	Age (years)	Complications	Blood test				Oral food challenge test
			Total IgE (IU/ml)	Milk-sIgE (kUA/L)	Casein-sIgE (kUA/L)	Days from episode	Total dose (ml)
M	2	AD, AR, BA	356	61.4	53.5	393	0.11
F	8	AD	448	22.2	25.5	62	3.8
M	2	AD	1049	60.5	69	67	1.8
F	5	-	492	46.1	51.9	0	0.1
M	3	AD	305	41.6	32.8	42	1.5
F	3	AD	307	59.9	61.5	21	-
M	9	AD, AR	2267	100>	100>	133	1.6
F	7	AD, BA	-	100>	100>	388	1.7

Abbreviations: AD, atopic dermatitis; AR, allergic rhinitis; BA, bronchial asthma; CPP-ACP, casein phosphopeptide-amorphous calcium phosphate; F, female; G, gum; M, male; P, paste; sIgE, specific IgE.

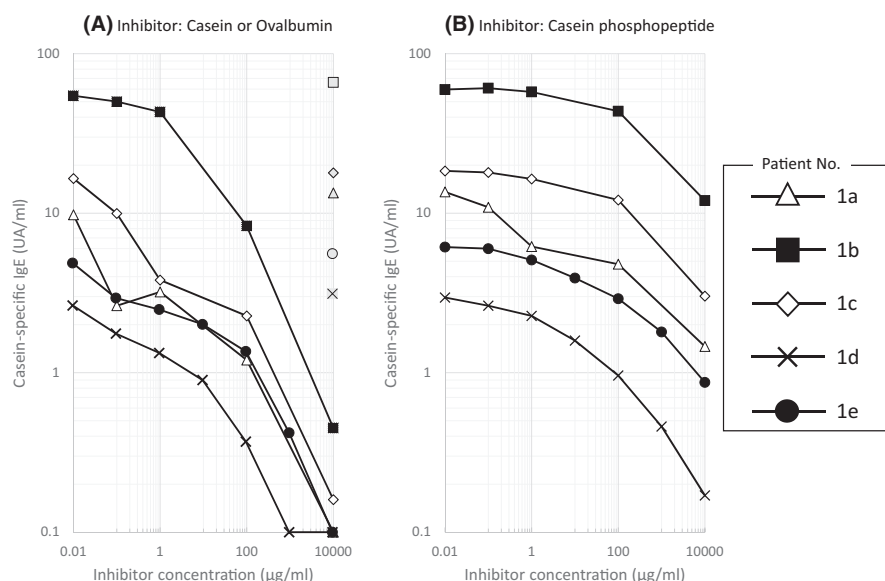


FIGURE 1 Inhibition assay of casein-specific IgE using the ImmunoCAP test. Different concentrations of casein, casein phosphopeptide, or ovalbumin (gray symbols) diluted with IgE sample diluent (Thermo Fisher Diagnostics, Tokyo, Japan) were mixed with the target serum at a 1:1 ratio and then incubated overnight at 4°C. Casein-specific IgE was measured using the ImmunoCAP test. Each line and symbol represents the results of patient numbers 1a, 1b, 1c, 1d, and 1e. For patient number 1d, a 40-fold serum dilution was used to evaluate reactivity in the low IgE antibody titer range. (A) Casein-specific IgE titers inhibited by casein or ovalbumin. (B) Casein-specific IgE titers inhibited by casein phosphopeptide

a partial similarity between the α s1-casein (E61–K70) and β -casein (I12–E21) amino acid sequences, both of which were included in the CPP region.⁹ Based on these findings, we hypothesize the existence of common and dominant IgE epitopes in these regions.

CPP contains highly phosphorylated serine residues in and around the core structure of the SSSEE motif. Therefore, the major phosphorylated epitopes in casein could have been missed in studies using non-phosphorylated synthetic peptides.⁹ Thus, phosphorylated serine residues in the CPP motifs could be critical in forming the dominant IgE epitopes in native casein components.

There are some limitations to our study. First, symptoms that developed after ingesting CPP-ACP containing paste or gum were possibly not induced by CPP-ACP because OFC using CPP-ACP was not performed, the time between OFC and episode was sometimes long, and *in vitro* tests were performed with CPP instead of CPP-ACP. All participants who experienced symptoms had severe CMA. Therefore, no patients with CPP-ACP-reactive non-CMA could be identified for over a decade of retrospective medical chart review. Moreover, we confirmed the strong antigenicity of CPP, which has an almost similar peptide part as CPP-ACP. Thus,

Symptoms	Days from episode	CPP-ACP induced episode		
		Culprit	Symptoms	Adrenaline use
pharyngeal discomfort, sneeze, runny nose	1095	P	generalized hives, difficulty in breathing, wheeze, desaturation ($\text{SpO}_2 < 80\%$)	+
oral discomfort, rash around mouth	148	P	lip swelling, pharyngeal discomfort, nausea, pallor of face	+
cough	501	P	generalized hives, cough	+
generalized hives, cough, runny nose, sleep, tachycardia, pallor	673	P	lip swelling, cough, wheeze	+
cough, wheeze, multiple hives, sleep	320	P	local hives	-
-	-	P	local hives, nausea, hoarseness	-
pharyngeal discomfort, cough, eyelid swelling	527	G	oral discomfort, lip swelling, cough	-
local hives, wheeze, diarrhea	173	G	oral discomfort	-

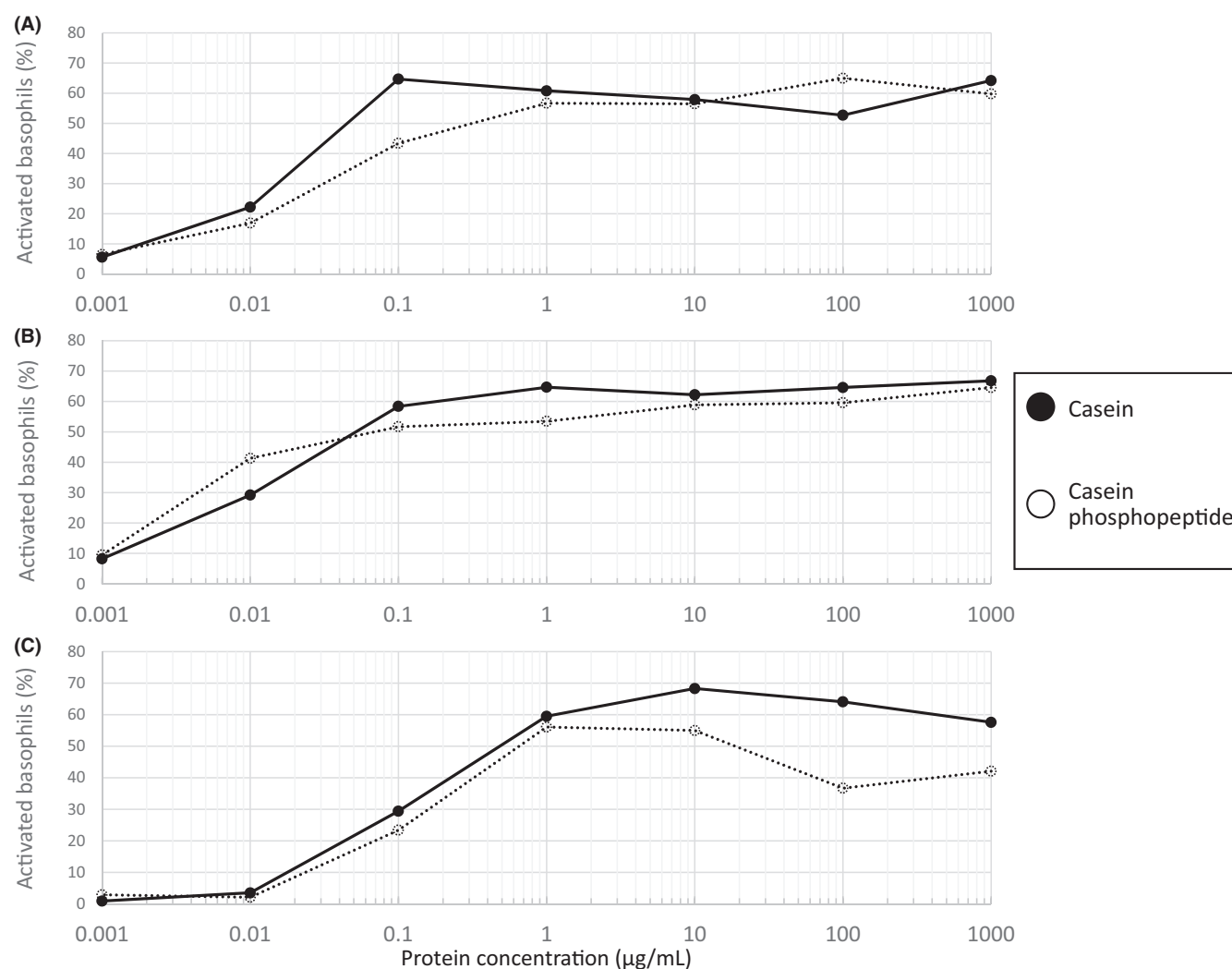


FIGURE 2 Basophil activation test stimulated by casein or casein phosphopeptide. The basophil activation test was performed using an Allergenicity Kit (Beckman Coulter). Different concentrations of casein (black circles) and casein phosphopeptide (white circles) were used as stimulators. The percentage of activated basophils was determined using a threshold CD203c fluorescence intensity based on a comparison between the positive and negative controls

assuming that CMA was likely to have reacted to CPP, rather than to other components was relatively reasonable. Second, the inhibition assay revealed that CPP had a lower inhibitory ability than casein, but showed an almost identical basophil activating ability to that of casein. Interpreting the difference between the methods was difficult, which could be partially attributed to the small sample size. Third, the CPP reagent used in our experiments was a mixture of trypsin-digested casein peptides, which might contain residual protein IgE epitopes, along with the CPP region in casein. Nevertheless, *in vitro* data and the clinical fact that an anaphylactic reaction was induced by a small amount of CPP-ACP suggest that CPP has strong allergenicity.

In conclusion, despite the fact that major epitopes of CPP have yet to be reported, CPP showed representative allergenic activity in casein. We are currently performing experiments using dephosphorylated CPP peptides and also planning to use synthetic phosphorylated CPP to confirm our study findings.

1 | IMPACT STATEMENT

CPP-ACP causes severe allergic reactions in patients with CMA. Moreover, CPP possesses IgE-binding ability and induces basophil activation comparable to that of casein.

KEYWORDS

basophils, casein, cow milk, food allergy, milk hypersensitivity, phosphopeptide

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CONFLICT OF INTEREST DISCLOSURE

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTION

Teruaki Matsui: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Investigation (lead); Writing – original draft (lead); Writing – review & editing (lead). **Michihiro Naito:** Conceptualization (equal); Investigation (equal); Writing – review & editing (equal). **Katsumasa Kitamura:** Data curation (equal); Writing – review & editing (equal). **Atsushi Makino:** Writing – review & editing (equal). **Yoshihiro Takasato:** Writing – review & editing (equal). **Shiro Sugiura:** Writing – review & editing (equal). **Hidehiko Izumi:** Conceptualization (equal); Writing – review & editing (equal). **Komei Ito:** Conceptualization (equal); Writing – review & editing (equal).

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/pai.13752>.

DATA AVAILABILITY STATEMENT

The datasets of this study are not publicly available to protect the participants' personal data but can be availed from the corresponding author on reasonable request.

Teruaki Matsui¹ 

Michihiro Naito²

Katsumasa Kitamura¹ 

Atsushi Makino¹

Yoshihiro Takasato¹

Shiro Sugiura¹ 

Hidehiko Izumi²

Komei Ito¹ 

¹Department of Allergy, Allergy and Immunology Center, Aichi Children's Health and Medical Center, 7-426 Morioka, Obu-City, Aichi, 474-8710, Japan

²Department of Nutritional Sciences, Nagoya University of Arts and Sciences, 57 Takenoyama, Nisshin, Aichi 470-0196, Japan

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Correspondence

Teruaki Matsui, Department of Allergy, Allergy and Immunology Center, Aichi Children's Health and Medical Center, 7-426, Morioka-cho, Obu, Aichi 474-8710, Japan.

Email address: terumatsu1981@hotmail.com

ORCID

Teruaki Matsui  <https://orcid.org/0000-0002-3660-1823>

Katsumasa Kitamura  <https://orcid.org/0000-0002-8962-851X>

Shiro Sugiura  <https://orcid.org/0000-0003-1048-2704>

Komei Ito  <https://orcid.org/0000-0002-1932-7973>

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SUPPORTING INFORMATION

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