

Original Article

Anaphylactic augmentation by epicutaneous sensitization to acid-hydrolyzed wheat protein in a guinea pig model

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ABSTRACT — Recent reports suggest that hydrolyzed wheat protein (HWP) variants such as Glupearl® 19S (GP19S) induce immediate-type hypersensitivity via epicutaneous (EC) sensitization. The identification of strong allergens is a key step in product assessment before commercial launch. However, few reports have described the estimation of actual and potential anaphylactic sensitizing capacity. In this study we assessed the strength of both the actual and potential anaphylactic sensitizing capacity by investigating the immediate-type hypersensitivity inducing potential of HWP compared with gluten. We assessed these strengths via the EC route using an EC or intradermal (ID) sensitization method. We quantified the strength of immediate-type hypersensitivity by evaluating the titer of serum antibodies isolated from sensitized subjects using passive cutaneous anaphylaxis (PCA) reactions. We also evaluated the cross-reactivity between GP19S and gluten. GP19S and gluten applied by both the sensitization methods induced obvious IgG1-mediated PCA reactions. GP19S had stronger sensitizing potential than gluten, according to the serum titers and dye spot diameters. The difference in antibody titers between GP19S and gluten was 16-fold for the EC method versus 2-fold for the ID method. GP19S cross-reacted with gluten. Acid hydrolysis of gluten increased anaphylactic sensitizing capacity in the EC method. To our knowledge, our study is the first to quantitatively confirm that HWP and gluten can induce immediate-type hypersensitivity through an intact skin. These findings suggest that acid-HWP imposes a higher risk of EC sensitization than gluten because of the ease with which the former confers a sensitizing effect through the intact skin.

Key words: Acid-hydrolyzed wheat protein, Cross-reactivity, Epicutaneous sensitization, Guinea pig, Passive cutaneous anaphylaxis

INTRODUCTION

Immediate-type hypersensitivity is a type I allergic response mediated by type 2 helper T cells. Certain food proteins have long been recognized as triggers for this potentially fatal allergic response. Recent reports have described the activation of immediate-type hypersensitivity by proteins formulated in cosmetics, hair-care products, and facial soap (Amaro and Goossens, 2008; Fukutomi *et al.*, 2011; Laurière *et al.*, 2006). Anaphylaxis elicited by hydrolyzed wheat protein (HWP) has been a particularly important problem in the Japanese population (Chinuki

et al., 2011; Chinuki and Morita, 2012; Fukutomi *et al.*, 2011). Anaphylaxis can occur in people sensitized to HWP via the epicutaneous (EC) route when there is cross-reaction with gluten in food. Much can be gained by taking pre-emptive steps to characterize the immediate-type hypersensitivity inducing potential of HWP via the EC route and to determine its cross-reactivity with other proteins. However, to date, no methods to identify the hazards of immediate-type hypersensitivity and cross-reactivity have been validated (Dearman and Kimber, 2009). Much could also be gained by elucidating the key mechanisms of skin sensitization to HWP. In previous reports,

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we and others have proposed that HWP was sufficient to induce anaphylaxis in mice (Adachi *et al.*, 2012) and that the enhanced allergic potential of HWP stems from the development of a novel epitope (Kroghsbo *et al.*, 2014; Nakamura *et al.*, 2013a). However, there is little information about the quantitative detection of allergic potential or cross-reactivity assessment.

The model of type I allergic response consists of sensitization and elicitation phases. Homologous passive cutaneous anaphylaxis (PCA) in guinea pigs has generally been used to study the elicitation phase of type I allergies (Ovary, 1958). The PCA test has proven to be extremely sensitive in identifying the hazards of type I allergy and is reported to be useful for assessing large-molecular-weight substances such as proteins, polypeptides, vaccines, and bacterial extracts (Verdier *et al.*, 1994). The EC sensitization method, which utilises a delayed-type contact hypersensitivity sensitization, was suitable for the sensitization phase. Guinea pig skin and human skin have similar percutaneous permeability (Barbero and Frasc, 2009) and numbers of Langerhans cells (Chen *et al.*, 1985; Wolff and Winkelmann, 1967). As such, a combination of PCA and EC sensitization in guinea pig models is potentially useful. However, other studies on the sensitization phase have focused more on the intradermal (ID), intraperitoneal, and inhalation routes than on the EC route. Several skin sensitization models for type I allergy have also been reported in animals other than guinea pigs. Mouse models and tape-stripped skin or adjuvant were used to investigate the allergic potential of proteins in most of these studies (Adachi *et al.*, 2012; Bartnikas *et al.*, 2013), but a few recent physiological models have used an intact skin without adjuvant (Stremnitzer *et al.*, 2014). At the time of writing this report, there have been no direct comparisons of the same allergens of potential cutaneous allergenic potency by ID direct injection and actual cutaneous allergenic potency by EC application via skin, assuming topical application of facial soap and cosmetics to the skin. In this study we used two routes to sensitize the guinea pigs to proteins derived from wheat: topical application (EC) and ID injection into dorsal skin. We quantified the type I allergic potential and assessed the cross-reactivity of HWP and gluten by the PCA test, a quantitative technique to evaluate local anaphylaxis.

MATERIALS AND METHODS

Animals

Female Hartley guinea pigs aged 4–6 weeks were purchased from Japan SLC Inc. (Shizuoka, Japan). All animals were housed with free access to standard pellets and

water. All experiments, procedures, and conditions for animal care were approved by the local Animal Care and Use Committee.

Preparation of gluten from wheat and hydrolyzed gluten

Glupearl® 19S (GP19S), a product produced from gluten by partial hydrolysis with hydrogen chloride (HCl) at 95°C for 40 min, was supplied by Katayama Chemical, Inc. (Osaka, Japan). GP19S and wheat gluten (Sigma-Aldrich, St. Louis, MO, USA) were suspended in a 1 M Tris (hydroxymethyl) aminomethane (Tris) solution (pH, 11.4). After standing at room temperature for 24 hr, the samples were neutralized with HCl through a previously reported method (Adachi *et al.*, 2012). Acid-hydrolyzed gluten (acid-HWP) was prepared by hydrolyzing gluten in Tris solution with 0.1 N HCl at 100°C for 0.5 or 9 hr, and then the solution was neutralized with sodium hydroxide. A control sample was prepared by adding wheat gluten in Tris solution to HCl and sodium hydroxide simultaneously without heating (0 hr). Coomassie Brilliant Blue-stained SDS electrophoresis gels of GP19S, acid-HWP and gluten are shown in online supplementary Fig. S1.

Intradermal sensitization

The dorsal skin of guinea pigs was shaved with electric clippers. Samples of 1% GP19S and 1% wheat gluten were mixed with matching volumes (v/v 1:1) of Freund's complete adjuvant (FCA). A 0.1 mL volume of each mixture was intradermally injected into five sites on the denuded dorsal skin of guinea pigs (day 0). Whole blood was collected on day 21 and sera samples were isolated from the collected samples. All sera were stored at -20°C until use.

Epicutaneous sensitization (modified Buehler's method)

The EC sensitization was performed by the method developed by Buehler (1965) with minor modification. The dorsal skin of guinea pigs was shaved with electric clippers before antigen application. First, 2 × 3 cm² cotton pads were loaded with 25 mg of GP19S, wheat gluten, or acid-HWP (hydrolyzed for 0, 0.5, or 9 hr). The pads were then applied to a denuded section of the dorsal skin with an occlusive patch for 6 hr on day 0, and the same procedure was repeated on days 2, 4, 7, 9, 11, 14, 16, and 18. To detect delayed-type hypersensitivity, an occlusive patch containing a non-irritating concentration of test sample was applied to untreated flank skin for 6 hr on day 28. Delayed-type hypersensitivity to GP19S,

gluten, and acid-HWP was judged when erythema was detected at 24 hr after removing the patch. Sera were isolated from whole blood collected on day 35. All sera were stored at -20°C until use.

Passive cutaneous anaphylaxis

The PCA testing was conducted according to the method developed by Ovary (1958). The dorsal skin of guinea pigs was shaved with electric clippers. A 0.1 mL volume of diluted serum isolated from animals sensitized using the EC or ID method was intradermally injected at a maximum of 13 sites. After 24 hr, the guinea pigs were intravenously injected with 0.05 mg of antigen in combination with 1 mL of 0.5% Evans blue dye. The diameter of the leaked dye was measured 30 min after antigen injection and dye spots of greater than 5 mm in diameter were defined as positive.

ELISA measurement of antigen-specific IgG1 and IgG2

Antigen-specific IgG1 and IgG2 levels were determined by a standard ELISA protocol. Goat anti-guinea pig IgG1 polyclonal antibody (GenWay Biotech, San Diego, CA, USA), goat anti-guinea pig IgG2 polyclonal antibody (GeneTex, San Antonio, TX, USA), and rabbit anti-goat IgG-horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) were used. Briefly, 1 µg of antigen was coated on a 96-well plate overnight at 4°C. After washing, the wells were blocked with blocking buffer (Sigma-Aldrich) for 1 hr at room temperature and then incubated with guinea pig serum (diluted to 1:250,000 for IgG1 and 1:1,000,000 for IgG2) for 24 hr at 4°C. The plates were then incubated with goat anti-guinea pig IgG1/IgG2 for 1 hr at room temperature. After washing, the wells were incubated with horseradish peroxidase-labelled anti-goat IgG for 1 hr at room temperature. The plates were reacted with BD OptEIA substrate (BD Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions, and absorption was read at 450-620 nm. Signals of specific antibodies were corrected against the mean signal from the sera of animals treated with vehicle.

Statistical analysis

Statistical analyses were performed using the Mann-Whitney U-test (Figs. 1, 2) and one-way ANOVA followed by Tukey's honestly significantly different test (Fig. 3). P-values of < 0.05 were considered statistically significant.

RESULTS

EC and ID sensitization to GP19S and gluten provoked an obvious PCA reaction

We evaluated the actual and potential anaphylactic sensitizing capacity of GP19S and gluten via the cutaneous route by applying these proteins to the dorsal skin of guinea pigs in two ways: the former, by repeated EC occlusive application and the latter, by ID injection with FCA. The specific antibodies to GP19S or gluten in serum were assessed by the PCA test. The sera from animals that were epicutaneously immunized with GP19S or gluten induced obvious dye spots in all cases following corresponding antigen elicitation (Fig. 1A). The dye spots induced by GP19S had an average diameter of 24.8 mm whereas those induced by gluten averaged 13.8 mm (Fig. 1C). The sera from animals intradermally immunized with GP19S or gluten induced obvious dye spots in all cases following corresponding antigen elicitation (Fig. 1B). The dye spots induced by GP19S had an average diameter of 21.0 mm whereas those induced by gluten averaged 16.2 mm (Fig. 1D). These results showed that EC exposure to GP19S had a significantly higher immediate-type hypersensitivity inducing potential than EC exposure to gluten.

GP19S induced stronger PCA reactions than gluten in the EC and ID sensitization models

The endpoint dilution technique was used to determine allergenic potency more precisely. Strong PCA reactions were elicited after the intravenous injection of GP19S in animals after they received injections of 10-fold- to 2,000-fold-diluted sera obtained from animals epicutaneously sensitized to GP19S (Table 1). The PCA reactions were diminished at higher dilution rates of 10,000-fold. In contrast, no PCA reactions were activated after the injection of 500-fold-diluted sera from animals epicutaneously sensitized to gluten. Serum titers of anti-GP19S and anti-gluten antibodies were 5,000-fold and 320-fold in EC sensitization models, respectively, and 5,000-fold and 2,500-fold in ID sensitization models, respectively. These values indicated that GP19S had a stronger EC allergic potential compared with gluten without adjuvant and artificial skin injury. In ID sensitization model, GP19S had a marginally strong allergic potential compared with gluten. However, the titer ratio of anti-GP19S compared with anti-gluten antibodies differed markedly between the EC (16-fold) and ID (2-fold) sensitization models.

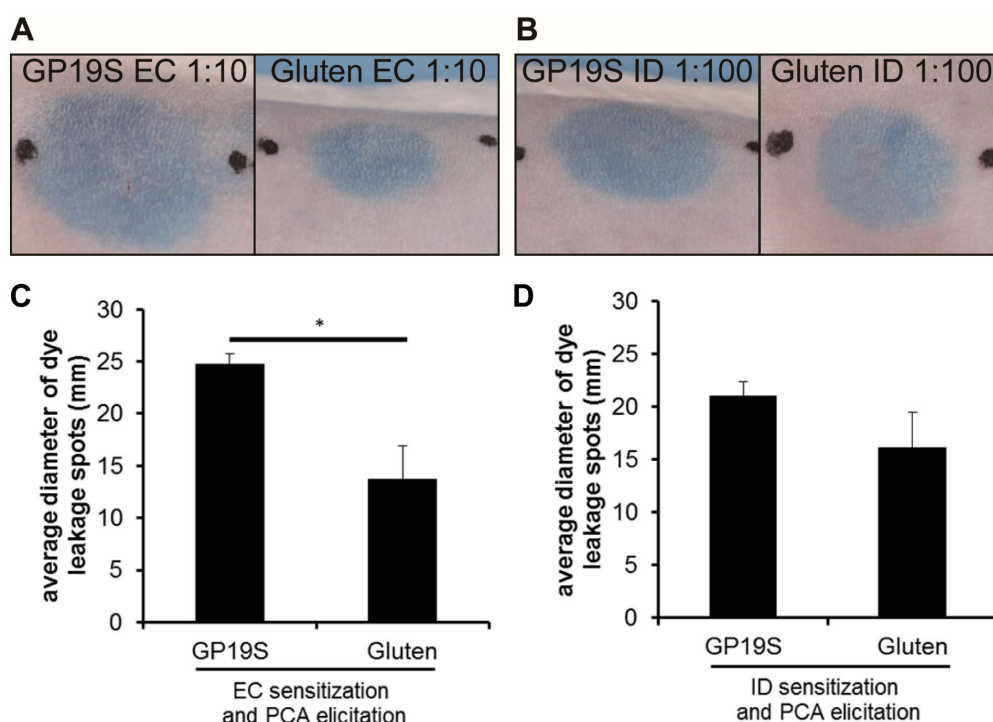


Fig. 1. Effect of acid hydrolysis as indicated by PCA reactions to GP19S and gluten. (A) The localized vascular hyperpermeability induced by the anti-sera of EC sensitized animals and corresponding antigen resulted in the extravasation of Evans blue into the skin. (B) The localized vascular hyperpermeability induced by the anti-sera of intradermally sensitized animals and antigen resulted in extravasation of Evans blue into the skin. The distance between black spots was about 25 mm. (C) The average diameter of the dye leakage spots caused by the 1:10 diluted sera derived from EC sensitized animals was quantified (GP19S: $n = 3$, Gluten: $n = 3$). (D) The average diameter of the dye leakage spots caused by the 1:100 diluted sera obtained from the ID sensitized animals was quantified (GP19S: $n = 4$, Gluten: $n = 6$). Values are shown as means $\pm$ S.D. * $P < 0.05$.

Acid hydrolysis augmented the immediate-type hypersensitivity inducing potential of gluten immunized by EC application

A previous study suggested that the allergenic potency of GP19S was augmented compared with that of gluten as a result of acid hydrolysis with heating (Chinuki and Morita, 2012). The SDS-PAGE patterns of gluten subjected to acid hydrolysis for 0.5 hr were similar to those of GP19S (supplementary Fig. S1). On the other hand, the molecular mass of gluten subjected to acid hydrolysis for 9 hr was much smaller than the molecular mass of gluten subjected to acid hydrolysis for 0 and 0.5 hr. We evaluated the EC anaphylactic sensitizing potentials of these acid-treated samples by conducting guinea pig PCA tests. In the animals given gluten treated for 0 hr, 10-fold- to 100-fold-diluted sera induced an obvious PCA reaction similar to the response to native gluten (Tables 1 and 2). In the animals exposed to gluten treated for 0.5 and 9 hr, 100-fold- to 1,000-fold-diluted sera induced an obvious PCA reaction. Vehicle induced no PCA reaction whatso-

ever (data not shown). Taken together, these results suggest that acid hydrolysis for 0.5 or 9 hr augments the EC anaphylactic sensitizing potentials of gluten.

Gluten cross-reacted with the sera of subjects epicutaneously immunized with GP19S

To assess the cross-reactivity between GP19S and gluten, gluten was administered for elicitation to animals sensitized with the sera from animals epicutaneously sensitized with GP19S. The PCA reaction was compared with the reaction to GP19S elicitation. The animals elicited by gluten showed an obvious PCA reaction to the sera from the animals epicutaneously sensitized with GP19S (Fig. 2A). However, the dye spot resulting from gluten elicitation was significantly smaller than that resulting from GP19S elicitation (Fig. 2A). The animals elicited by GP19S also showed an obvious PCA reaction to sera from the model animals epicutaneously sensitized with gluten. These results indicated that gluten cross-reacted with GP19S in the EC sensitized models. Because the

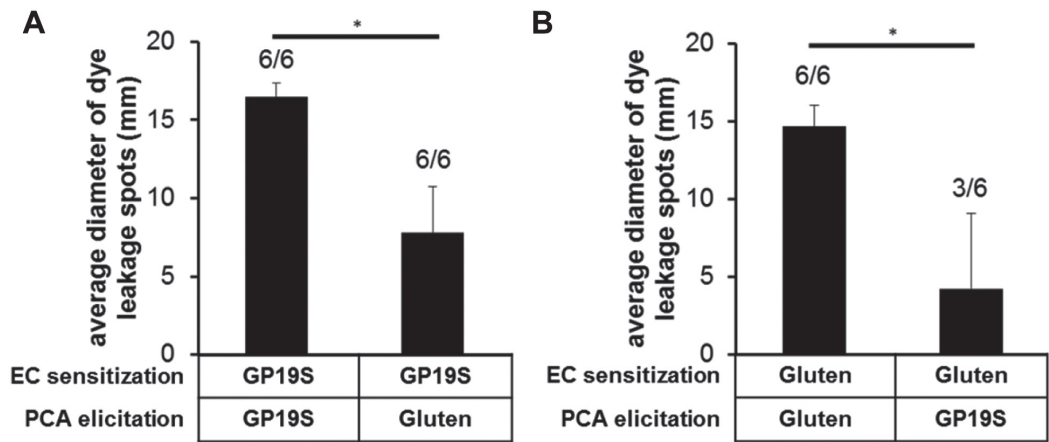


Fig. 2. Cross-reaction between GP19S and gluten as indicated by PCA reactions. (A) The average diameter of the dye leakage spots caused by the 1:100 diluted sera obtained from the animals EC sensitized to GP19S was quantified (GP19S: n = 6, Gluten: n = 6). The number of PCA-positive animals per sensitized animals is indicated above the bar. (B) The average diameter of the dye leakage spots caused by the 1:10 diluted sera obtained from the animals EC sensitized to gluten was quantified (Gluten: n = 6, GP19S: n = 6). The number of PCA-positive animals per sensitized animals is indicated above the bar. Values are shown as means $\pm$ S.D. *P < 0.05.

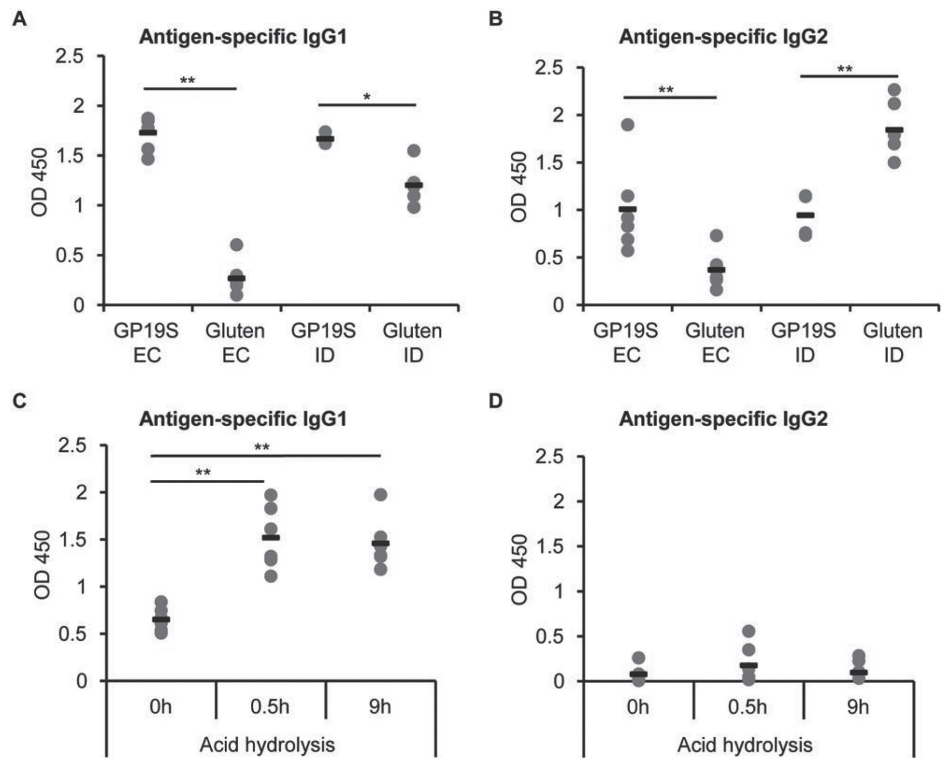


Fig. 3. Antigen-specific IgG1 and IgG2 levels. GP19S or gluten-specific IgG1 (A) and IgG2 (B) in the sera of animals subjected to EC and ID sensitization were determined using ELISA. Specific IgG1 (C) and IgG2 (D) antibodies to gluten subject to acid hydrolysis for 0, 0.5, and 9 hr in the sera of animals subject to EC sensitization were determined using ELISA. Bars represent the means of each group. *P < 0.05. **P < 0.01.

Table 1. Titers of anti-GP19S antibodies and anti-gluten antibodies as indicated by PCA reactions.

dilution series of serum	EC sensitization and PCA elicitation				dilution series of serum	ID sensitization and PCA elicitation			
	GP19S		Gluten			GP19S		Gluten	
10	+++	(3/3)	+++	(3/3)					
100	+++	(3/3)	++	(2/3)	100	+++	(3/3)	+++	(3/3)
160	+++	(3/3)	+	(1/3)					
320	+++	(3/3)	+	(1/3)	320	+++	(3/3)	+++	(3/3)
500	+++	(3/3)	-	(0/3)	500	+++	(3/3)	+++	(3/3)
1,000	+++	(6/6)	-	(0/3)					
2,000	+++	(5/6)	-	(0/3)	2,500	+++	(3/3)	++	(2/3)
5,000	+	(1/6)			5,000	+++	(3/4)	-	(0/3)
10,000	-	(0/6)			10,000	-	(0/4)		
titer	5,000		320		titer	5,000		2,500	

+++ : 6/6, 5/6, 3/4, 3/3, ++ : 4/6, 3/6, 2/3, + : 1/6, 1/3, - : 0/6, 0/4, 0/3 (PCA-positive animals/sensitized animals)

Table 2. Titers of anti-acid-HWP antibodies as indicated by PCA reactions.

dilution series of serum	EC sensitization and PCA elicitation		
	acid treatment for 0 hr	acid treatment for 0.5 hr	acid treatment for 9 hr
10	+++ (5/6)		
100	++ (4/6)	+++ (6/6)	+++ (6/6)
1,000	+	++ (4/6)	+
2,000	- (0/6)	+	+
5,000		- (0/6)	- (0/6)
titer	1,000	2,000	2,000

+++ : 6/6, 5/6, ++ : 4/6, 3/6, + : 2/6, 1/6, - : 0/6 (PCA-positive animals/sensitized animals)

strength of PCA reactions differed between GP19S and gluten, acid hydrolysis seemed to induce a new epitope in addition to the common epitope of native gluten.

The anaphylactic reactions in the skin sensitization models strongly correlated with the antigen-specific IgG1 level

To understand the antibody isotype responsible for anaphylaxis in guinea pig EC and ID sensitization, the antigen-specific IgG1 and IgG2 levels were determined using ELISA. The levels of GP19S-specific IgG1 and IgG2 exceeded the level of gluten-specific IgG1 and IgG2 in the serum of the epicutaneously sensitized animals (Fig. 3A, B). The level of IgG1 induced by GP19S exceeded the level of IgG1 induced by gluten in serum from the intradermally sensitized animals, whereas the level of IgG2 induced by GP19S was lower than the level of IgG2 induced by gluten. The levels of antigen-specific IgG1 in the animals treated with gluten subjected to acid hydrolysis for 0.5 and 9 hr exceeded the level meas-

ured in the control sample (0 hr). Antigen-specific IgG2 was unchanged. These results suggest that the increased IgG1 in response to GP19S provoked stronger PCA reactions than the increased IgG1 in response to gluten. Furthermore, the increased IgG1 in response to gluten subjected to acid hydrolysis for 0.5 and 9 hr also induced a stronger PCA reaction than the 0 hr acid-hydrolyzed gluten (control).

DISCUSSION

Our findings demonstrate that HWP and native wheat gluten both have sensitizing potential via the EC route in a high-sensitivity guinea pig model. GP19S and acid-HWP had stronger sensitizing potential than gluten. The difference in antibody titers between GP19S and gluten suggests that acid-HWP poses higher risks than gluten because of the potent sensitizing action of the former via the EC route. Furthermore, GP19S cross-reacted with gluten.

Many reports have shown that HWPs such as GP19S can induce immediate-type hypersensitivity through skin contact and/or food ingestion (Adachi *et al.*, 2012; Amaro *et al.*, 2008; Chinuki *et al.*, 2011; Chinuki and Morita, 2012; Fukutomi *et al.*, 2011; Kroghsbo *et al.*, 2014; Laurière *et al.*, 2006). Gluten from wheat, another well-known allergen in humans, contains major causative components such as omega-5 gliadin (Palosuo *et al.*, 1999). In a murine model, HWP induced the production of specific IgE and IgG1 and led to significant decreases in rectal temperatures and significant increases in plasma histamine levels (Adachi *et al.*, 2012). However, the only significant effect of gluten was the production of specific IgG1 (Adachi *et al.*, 2012). In the current study we detected immediate-type hypersensitivity inducing poten-

Acid-HWP augments anaphylaxis in guinea pigs

tial of HWP and gluten in guinea pigs without any artificial treatments. This conflicting result may be attributable to the more frequent application of allergen to the guinea pigs (nine times) or to a higher sensitivity to the allergen in guinea pigs compared with mice.

The allergenicity of HWP is strongly influenced by the deamidation and molecular weight of the protein (Chinuki *et al.*, 2013; Gourbeyre *et al.*, 2014; Nakamura *et al.*, 2013b). In addition, acid hydrolysis increases an aqueous solubility of the gluten. The higher allergenic potency of GP19S and 0.5 hr acid-HWP compared with gluten seems to stem from the deamidation and solubility increase with similar molecular weight (supplementary Fig. S1, Nakamura *et al.*, 2013b). Predictably, the allergenic potency of GP19S was higher than that of gluten in the EC sensitized guinea pig model. Furthermore, 0.5 hr acid-HWP was detected with higher sensitivity compared with the 0 hr acid-HWP control. The 9 hr acid-HWP also possessed strong immediate-type hypersensitivity inducing potential (Fig. 3C and Table 2). The 9 hr acid-HWP had a lower molecular weight than gluten. As such, this HWP could induce a stronger anaphylactic reaction than gluten. Another experiment showed that HWP treated for 9 hr retained its allergenicity even when HWP treated for 6 and 12 hr significantly responded in an *in vitro* elicitation test using serum from an HWP patient (Nakamura *et al.*, 2013a). Therefore, low molecular weight protein of the 9 hr acid-HWP may also contain highly reactive epitopes contained within GP19S and 0.5 hr acid-HWP. In addition, because 0.5 and 9 hr acid-HWP induced antigen-specific IgG1 at the same level, the 9 hr acid-HWP had as strong sensitizing potential as that of 0.5 hr acid-HWP. This may be due to higher aqueous solubility and/or higher skin permeability due to a smaller molecular weight than the gluten.

The GP19S had a substantially stronger allergic potential than gluten in our EC sensitized model but only a slightly stronger allergic potential than gluten in our ID sensitized model (Figs. 1, 2, 3, and Table 1). In other words, the immediate-type hypersensitivity inducing potential of GP19S was similar to gluten; however, the skin permeability of GP19S may be much higher. The increase in skin permeability may have stemmed from enhanced diffusibility in skin via increases in hydrophilic amino acid residues or via an invasion of a low molecular weight allergen. Further study is required to confirm this.

Our experiment is the first to demonstrate an anaphylactic cross-reaction between GP19S and gluten following EC sensitization in guinea pigs, animals that are similar to humans in terms of EC sensitizing capacity. Serum IgE is reported to react to gamma-gliadin in gluten in HWP-sen-

sitized patients (Fukutomi *et al.*, 2011). Its response was also enhanced by antigen deamidated by transglutaminase (Nakamura *et al.*, 2013b). In our model, we injected the antigen into a vein; after it leaked into the skin tissue, gluten may be deamidated by tissue transglutaminase and may change into an allergen with a higher IgE reaction.

IgG1 and IgE participate in the anaphylactic response in guinea pigs, whereas IgG2 does not. The IgG1 level in our study was correlated with the PCA observed in the reaction elicited 24 hr after the naïve guinea pigs were passively sensitized with anti-sera. The optimal latent time for anaphylactic response differs for each antibody type: 4 hr for IgG1a, 16–48 hr for IgG1b and more than 48 hr for IgE (Watanabe and Ovary, 1977). Our data were consistent with this. IgG2 reportedly acts as an inhibitory antibody for PCA (Ovary *et al.*, 1963), an antibody to enhance anaphylactic reaction (Neveu *et al.*, 1978), a cytophilic antibody for macrophages (Berken and Benacerraf, 1966), and a responsible antibody for fixing complements and complement-related phenomena (Bloch *et al.*, 1963). In our results, GP19S-specific IgG2 was more abundant than gluten-specific IgG2 in the EC model (Fig. 3B), and gluten-specific IgG2 was more abundant than GP19S-specific IgG2 in the ID model (Fig. 3B). However, we could not detect an obvious inhibition or enhancement effect on PCA reaction. The inhibition effect was seen when the injected IgG2 was more than a 100-fold that the injected IgG1 (Ovary *et al.*, 1963). One of the reasons for this may be the concentration of the antibodies. Although, sensitization route and FCA may contribute to the change in IgG1 and IgG2 antibody production, the exact mechanism of the change is unknown.

In summary, our IgG1-dependent guinea pig skin sensitization models for immediate-type hypersensitivity demonstrated that HWP had a stronger immediate-type hypersensitivity inducing potential and that HWP may have higher skin permeability than gluten. HWP may also cross-react with gluten. This highly sensitive animal model for type I allergy using immunization via EC routes is likely to become more important in the future. We expect this model to be widely applicable to detect potential and practical anaphylactic sensitizing capacity of various proteins and drugs via skin because it is sensitive, easy to use, and useful to evaluate cross-reactivity.

Conflict of interest---- The authors declare that there is no conflict of interest.

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