

RESEARCH ARTICLE

Total dose defines the incidence of percutaneous IgE/IgG1 mediated immediate-type hypersensitivity caused by papain

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Abstract

Assessment of human health risk requires an understanding of antigen dose metrics associated with toxicity. Whereas assessment of the human health risk for delayed-type hypersensitivity is understood, the metrics remain unclear for percutaneous immediate-type hypersensitivity (ITH) mediated by IgE/IgG1. In this work, we aimed to investigate the dose metric for percutaneous ITH mediated by IgE/IgG1 responses. Papain, which causes ITH via percutaneous sensitization in humans, was used to sensitize guinea pigs and mice. The total dose per animal or dose per unit area was adjusted to understand the drivers of sensitization. Passive cutaneous anaphylaxis (PCA) and enzyme-linked immunosorbent assay (ELISA) for papain-specific IgG1 enabled quantification of the response in guinea pigs. In mice, the number of antigen-bearing B cells in the draining lymph nodes (DLN) was calculated using flow cytometry, papain-specific IgG1 and IgE levels were quantified by ELISA. PCA positive test rates and the amounts of antigen-specific antibody corresponded with total dose per animal, not dose per unit area. Furthermore, the number of B cells taking up antigen within DLN also correlated with total dose. These findings indicate that the total antigen dose is the important metric for percutaneous IgE/IgG1-mediated ITH.

KEYWORDS

antibody-mediated immediate-type hypersensitivity, dose metrics, exposure assessment, IgE/IgG1, percutaneous sensitization

1 | INTRODUCTION

Allergic contact dermatitis (ACD) is a delayed-type hypersensitivity (DTH) response of the immune system to chemical allergens (Vocanson, Hennino, Rozieres, Poyet, & Nicolas, 2009). Following sensitization by a chemical allergen, the symptoms of ACD may continue for a lifetime because of immunological memory. Therefore, for a human health risk assessment, it is important to evaluate the sensitization potential and consumer exposure levels to the chemical. To assess this risk, a quantitative risk assessment (QRA) approach has been developed by the International Fragrance Association (Api et al., 2020, 2008; Basketter & Safford, 2016). The skin sensitization QRA approach is based upon determining the sensitization threshold,

as such limits exist for the induction of DTH (Boukhman & Maibach, 2001). Within the conduct of QRA, the exposure level is calculated as the dose per unit area. Historically, clinical diagnostic patch test data were evaluated as the incidence of contact dermatitis relative to concentration (%). However, Friedman and colleagues examined the dose metrics correlating with the induction of ACD using human clinical trials (Friedmann, 1990; White, Friedmann, Moss, & Simpson, 1986) and showed that the induction of ACD to DNCB was correlated with dose per unit area. Therefore, the widely accepted metric in skin sensitization QRA is dose per unit area (Api et al., 2008; Kimber et al., 2008; Robinson et al., 2000).

In immediate-type hypersensitivity (ITH) initiated in the skin, antigens are taken up by activated dendritic cells (DCs), which then

migrate to secondary lymphoid organs, such as draining lymph nodes (DLNs). Naïve T cells are primed by these DCs and differentiate into antigen-specific helper T (Th) cells (Brough et al., 2020; Shakib, Ghaemmaghami, & Sewell, 2008). In addition to the priming of Th cells via activated DC, activated B cells are also required to induce ITH, which is mediated by IgE/IgG1 (Figure 1). In this process, B cells capture antigens via the B cell receptor (BCR) and subsequently present these antigens bound to the major histocompatibility complex (MHC) class II molecules to antigen-specific Th cells in the DLN. On encountering the MHC class II–antigen complexes, the Th cells secrete cytokines directing the B cells to undergo class-switching and affinity maturation and differentiate into plasma cells (MacLennan et al., 1997; Pape et al., 2003; Vinuesa, Tangye, Moser, & Mackay, 2005). These plasma cells secrete antigen-specific IgE, which binds to the high-affinity Fc epsilon receptor 1 (FcεRI) on mast cells. Subsequent antigen binding triggers the cross-linking of IgE–FcεRI complexes, leading to mast cell activation and histamine release, inducing urticaria, asthma, rhinitis, and/or anaphylactic reaction (Burton & Oettgen, 2011). Protein allergens induce IgE-mediated ITH through multiple routes, including the respiratory (Boverhof et al., 2008) and gastrointestinal tracts (Yu, Freeland, & Nadeau, 2016). In addition to the aforementioned sensitization routes, several protein allergens, such as hydrolyzed wheat, house dust mites, peanut, and papain, induced IgE/IgG1-mediated ITH via percutaneous sensitization (Amaro & Goossens, 2008; Barbaud, 2020; Chinuki et al., 2011; Flohr et al., 2014; Kimber, Griffiths, Basketter, McFadden, & Dearman, 2014; Shimura et al., 2016; Stremnitzer et al., 2014). For example, Shimura et al. (2016) demonstrated that papain, a cysteine protease, induces the production of papain-specific antibodies (IgG1 and IgE) via percutaneous sensitization in mice. Furthermore, clinical evidence supports that papain can induce percutaneous ITH

(Barbaud, 2020). In addition, soap supplemented with hydrolyzed wheat protein can induce anaphylaxis via percutaneous sensitization (Chinuki & Morita, 2012). Therefore, the role of the percutaneous exposures during the induction of IgE/IgG1-mediated ITH is of great interest to investigators. These observations suggest that there is a need for a risk assessment approach for the induction of IgE/IgG1-mediated ITH via percutaneous sensitization. Currently, there is no generally accepted method for ITH hazard or risk evaluation. Some studies have reported potential approaches, such as the use of a passive cutaneous anaphylaxis (PCA) test, the Buehler guinea pig skin sensitization test method, and proteomics and protein sequence analyses (Krutz et al., 2019; Matsunaga et al., 2015; Maurer-Stroh et al., 2019). Furthermore, during an exposure assessment, the exposure amount should be calculated on the basis of the dose metric, correlating with the incidence of toxicity. However, the dose metric that correlates with the incidence of percutaneous IgE/IgG1-mediated ITH remains unclear. As mentioned above, a protein allergy can be induced through multiple physiologic routes (e.g., respiratory tract, gastrointestinal tract, or skin). However, in this study, we focused on the skin as a sensitization route. This study aimed to examine exposure metrics related to the incidence of percutaneous IgE/IgG1-mediated ITH and establish an exposure assessment method for percutaneous ITH.

2 | MATERIALS AND METHODS

2.1 | Animals and materials

Female Hartley guinea pigs aged 4–8 weeks were purchased from Japan SLC Inc. (Shizuoka, Japan). Female CBA/J mice aged 4–6 weeks

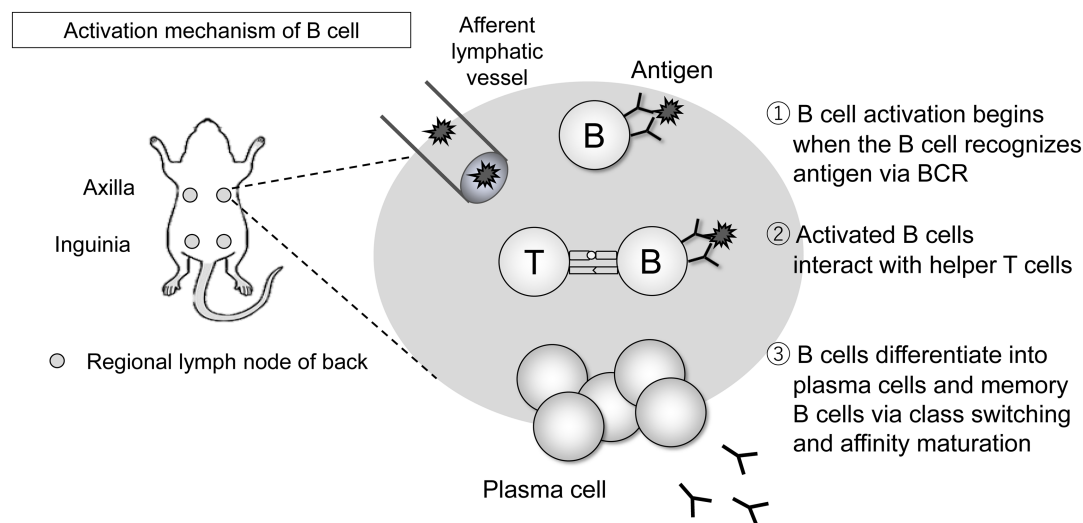


FIGURE 1 Schematic model of B cell activation. Antigens pass through the epidermal barrier, enter the dermal afferent lymphatic vessel, and flow into the draining lymph node. They are then captured by the corresponding B cell receptors (BCRs), after which the BCR–antigen complexes are internalized and the antigen presented on the B cell surface in a complex with major histocompatibility complex (MHC) class II molecules. In the same draining lymph node, antigen-specific helper T cells simultaneously interact with antigen–MHC class II complexes on B cells through the T cell receptors (TCRs). Finally, the T cells secrete cytokines, which induce the activation, proliferation, and differentiation of B cells into plasma cells via class-switching and affinity maturation [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/terms-and-conditions)]

TABLE 1 Sensitization induction conditions in the guinea pig experiment

Concentration (%)	0.0375	0.075	0.075	0.15
Volume (ml)	0.5	0.25	0.5	0.25
Exposed skin area (cm ²)	2 × 3	2 × 1.5	2 × 3	2 × 1.5
Total dose (μg/animal)	187.5	187.5	375	375
Dose per unit area (μg/cm ²)	31.25	62.5	62.5	125

were purchased from Charles River Japan Inc. (Kanagawa, Japan). All animals were housed with *ad libitum* access to standard feed pellets and water. Papain was purchased from Calbiochem (San Diego, CA, USA). All animal experiments were conducted at the Experimental Animal Facility of the Kao Corporation's R&D Department. The study was approved by the Kao's Animal Care Committee, and all experiments followed the guidelines of the committee.

2.2 | Animal sensitization

Percutaneous sensitization for guinea pigs was performed on the basis of the Buehler method (Buehler, 1965). Dorsal skin was shaved with electric clippers prior to antigen application. Under specific sensitization conditions (see Table 1), a 6-h occlusive patch was applied on Days 1, 8, and 15. The total dose per animal or dose per unit area was adjusted by altering sample concentration, exposed skin area, and application volume. Sera were isolated from whole blood collected from sensitized guinea pigs on Day 37 and were stored at −80°C until use.

The back skin of mice was shaved using electric clippers prior to antigen application. For analysis of the number of B cells taking up antigen, a 24-h occlusive patch was performed under the conditions described in Table 2. The total dose per animal or dose per unit area was adjusted by altering the sample concentration, exposed skin area, and application volume. The axillary and inguinal lymph nodes were harvested after a 24-h occlusive patch. For analysis of the amount of specific antibody production, 24-h occlusive patches (twice a week for 7 weeks) were performed under the conditions described in Table 2. The total dose per animal or dose per unit area was adjusted using the same method as above. Sera were isolated from whole blood collected from sensitized mice on Day 54 and stored at −80°C until use.

TABLE 2 Sensitization induction conditions in the mouse experiments

Concentration (%)	0.05	0.2	0.2
Volume (ml)	0.28	0.07	0.28
Exposed skin area (cm ²)	2 × 2	1 × 1	2 × 2
Total dose (μg/animal)	140	140	560
Dose per unit area (μg/cm ²)	35	140	140

2.3 | PCA

PCA test was performed according to the method developed by Ovary (1958). The dorsal skin of guinea pigs was shaved, and 0.1 ml volume of diluted sera was intradermally injected at a maximum of 10 sites. After 24-h, these guinea pigs were intravenously injected with 0.05 mg of papain in combination with 1 ml of 0.5% Evans blue (Fujifilm Wako Pure Chemical Corporation, Tokyo, Japan). The diameter of the leaked dye was measured 30 min after papain injection; dye spots >5 mm in diameter were scored as PCA test positive.

2.4 | Fluorescent dye protein labeling

Papain was labeled with Alexa Fluor 488 (AF488), using the AF488 protein labeling kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). To purify AF488-labeled papain, a solution of AF488-labeled papain was centrifuged (14,000×g, 10 min) using an Amicon Ultra-0.5 3 K centrifugal filter (Millipore, Bedford, MA, USA) and rinsed with Dulbecco's phosphate-buffered saline (DPBS). To confirm successful protein labeling, the labeling efficiencies were assessed using the extinction coefficient for AF488 at 492 and 280 nm. The dye to protein ratio was calculated to be 2.13.

2.5 | ELISA measurement of antigen-specific IgG1 and IgE

Antigen-specific IgG1 and IgE levels were determined via a standard enzyme-linked immunosorbent assay (ELISA) protocol. Goat anti-guinea pig IgG1 (Genetex, Irvine, CA, USA), rabbit anti-goat IgG horseradish peroxidase (HRP)-conjugated (Careforde, Chicago, IL, USA), goat anti-mouse IgG1 antibody HRP-conjugated (Bethyl Laboratories, Montgomery, TX, USA), rat anti-mouse IgE antibody biotin-conjugated (BD Bioscience, San Jose, CA, USA), and streptavidin HRP-conjugated (GE Healthcare, Amersham, UK) antibodies were used. Briefly, papain (10 μg/ml) was used to coat a 96-well Maxisorp plate (NUNC, Roskilde, Denmark) overnight at 4°C. Wells were washed with DPBS-Tween 20 (0.05%) and then blocked with Immunoblock (DS Pharma, Osaka, Japan) for 90 min at 37°C and incubated with guinea pig serum (1:50,000) or mouse serum (1:5,000 for IgG1; 1:50 for IgE) for 90 min at 37°C. Plates were then incubated with goat anti-guinea pig IgG1 or goat anti-mouse IgG1 HRP-conjugated for 90 min at 37°C or rat anti-mouse IgE biotin-conjugated antibody for 60 min at room temperature. Wells were washed with DPBS-Tween 20 (0.05%) and then incubated with HRP-labeled anti-goat IgG1 for 90 min at 37°C (for guinea pig IgG1) or HRP-labeled anti-rat IgE for 30 min at room temperature (for mouse IgE). Plates were treated with TMB Microwell Peroxidase Substrate (Seracare, Milford, MA, USA) according to the manufacturer's protocol, and absorption was read at 450 nm.

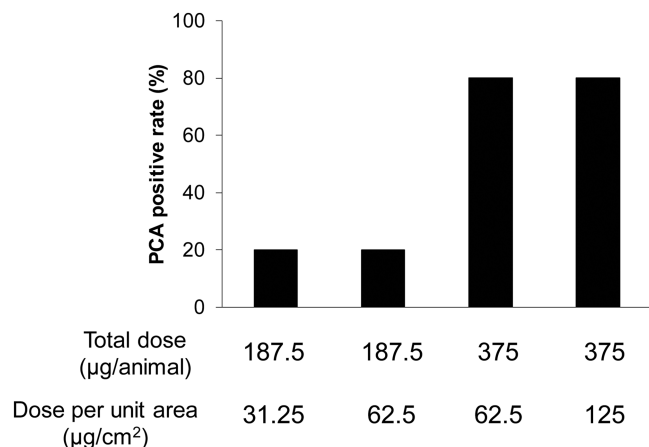


FIGURE 2 Passive cutaneous anaphylaxis (PCA) positive reaction rate was correlated with the total dose. Papain was applied onto the back skin of guinea pigs ($n = 5$ per group). Sera were collected on Day 37 after the initial sensitization treatment. Exposure conditions are defined in Table 1. The positive rate of PCA reaction resulting from sensitization was quantified

2.6 | Cell surface staining and flow cytometric analysis

Cell suspensions prepared from pooled DLNs (axillary and inguinal) were digested with 400-U/ml collagenase (Sigma, St Louis, MO, USA) for 30 min at 37°C and then filtered through a 70 µm cell strainer (BD Falcon, BD Biosciences, San Jose, CA, USA) and resuspended with RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and 50 µM 2-mercaptoethanol. To characterize the subsets of antigen-uptake B cells, staining was performed with PE-conjugated rat anti-mouse B220 (clone #RA3-6B2, BD Biosciences, San Jose, CA, USA) for 30 min at 4°C. All flow cytometry samples

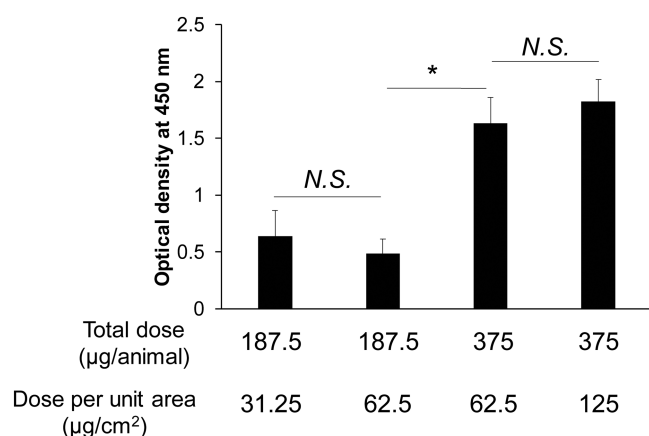


FIGURE 3 Papain-specific IgG1 levels were correlated with total dose. Papain was applied onto the back skin of guinea pigs ($n = 5$ per group), and sera were collected after sensitization. Exposure conditions are defined in Table 1. The amount of papain-specific IgG1 in the sera of sensitized guinea pigs was measured using enzyme-linked immunosorbent assay (ELISA). Data are shown as mean $\pm$ SEM, * $p < 0.05$

were scanned on BD FACS Verse (BD Bioscience, San Jose, CA, USA) and analyzed using FlowJo Software (Treestar, Ashland, OR, USA). Numbers of antigen-uptake B cells were calculated on the basis of the percentage of AF488/B220 double-positive cells and the total number of living lymphocytes.

2.7 | Statistical analyses

Data are presented as mean $\pm$ standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Tukey–Kramer post hoc test was used for statistical analyses. p values < 0.05 indicated statistical significance. All statistical analyses were performed using the software EZR version 1.40 (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (Kanda, 2013).

3 | RESULTS

3.1 | Positive PCA reaction rates were correlated with the total dose

Papain was used to sensitize guinea pigs in order to elucidate the dose metrics correlating with the incidence of percutaneous IgE/IgG1-mediated ITH. The total dose or dose per unit area was adjusted to elucidate the conditions contributing to sensitization. Sera from sensitized animals were collected to perform a PCA test. The group of guinea pigs exposed to a total of 187.5 µg papain administered over an area of 3 cm² (i.e., dose per skin unit of 62.5 µg/cm²) showed a positive PCA reaction rate of 20% (Figure 2). However, when the same dose per unit area was achieved by administering a total of 375 µg papain over an area of 6 cm², PCA positive reaction rate was found to be 80%. On the other hand, when the same total dose was achieved by administering a total of 187.5 µg papain over an area of 6 cm² or 187.5 µg of papain over 3 cm², the positive PCA reaction rates were both 20%. For the same total dose condition (375 µg papain over either 6 or 3 cm²), the positive PCA reaction rate totaled 80%. These results indicated that the PCA positive rate correlated with the total dose and not the dose per unit area.

3.2 | The amount of papain-specific IgG1 antibody was correlated with the total dose

In guinea pigs, ITH develops through the production of antigen-specific (largely IgG1) antibodies (Verdier, Chazal, & Descotes, 1994). Therefore, to clarify the dose metrics contributing to the production of antigen-specific antibody, papain-specific IgG1 levels were determined using ELISA. Consistent with the results of the PCA test, where the same dose per unit area (62.5 µg/cm²) was achieved either with 187.5 µg papain over 3 cm² or 375 µg papain over 6 cm², the amount of papain-specific IgG1 differed significantly between these conditions (Figure 3). In addition, when the same total dose was applied,

the amount of papain-specific IgG1 did not differ significantly between the groups receiving a total dose of 187.5 or 375 μg . These results support that the total dose is of importance in the induction of percutaneous ITH.

3.3 | The number of antigen-bearing B cells in the DLNs was correlated with the total dose

An interesting question arose from the guinea pig experiments regarding the mechanistic basis for the importance of total dose in the induction of percutaneous IgE/IgG1-mediated ITH. To elucidate the underlying mechanism contributing to the dose metric for percutaneous ITH, we focused on the activation mechanism of B cells controlling a specific antibody production. We hypothesized that the amount of antigen trafficking into the DLN and antigen exposure to B cells is important for B cell activation, thereby driving IgE/IgG1-mediated

ITH. To test this hypothesis, the relationship between dose metrics and the number of antigen-bearing B cells in DLNs was examined using a mouse model. To elucidate the sensitization conditions contributing to the number of antigen-bearing B cells, the total dose or dose per unit area was adjusted. After a 24-h exposure to a closed patch of Alexa488-labeled papain, the number of Alexa488-labeled papain-bearing B cells (Alexa488⁺B220⁺) in the DLNs was determined using flow cytometry (Figure 4A). When the same total dose was administered (i.e., 140 μg Alexa488-papain over either 4 or 1 cm^2), the number of antigen-bearing B cells was comparable (Figure 4B).

Indeed, in the condition of 140 μg Alexa488-papain over 4 or 1 cm^2 , the mean numbers of Alexa488-labeled papain-bearing B cells were calculated as 0.56×10^4 cells and 0.49×10^4 cells, respectively. On the other hand, the same total dose per unit area was achieved (i.e., 140 μg Alexa488-papain over 1 cm^2 or 560 μg over 4 cm^2), the number of antigen-bearing B cells differed by approximately fivefold. Indeed, in the condition of 140 μg Alexa488-papain over 1 cm^2 or

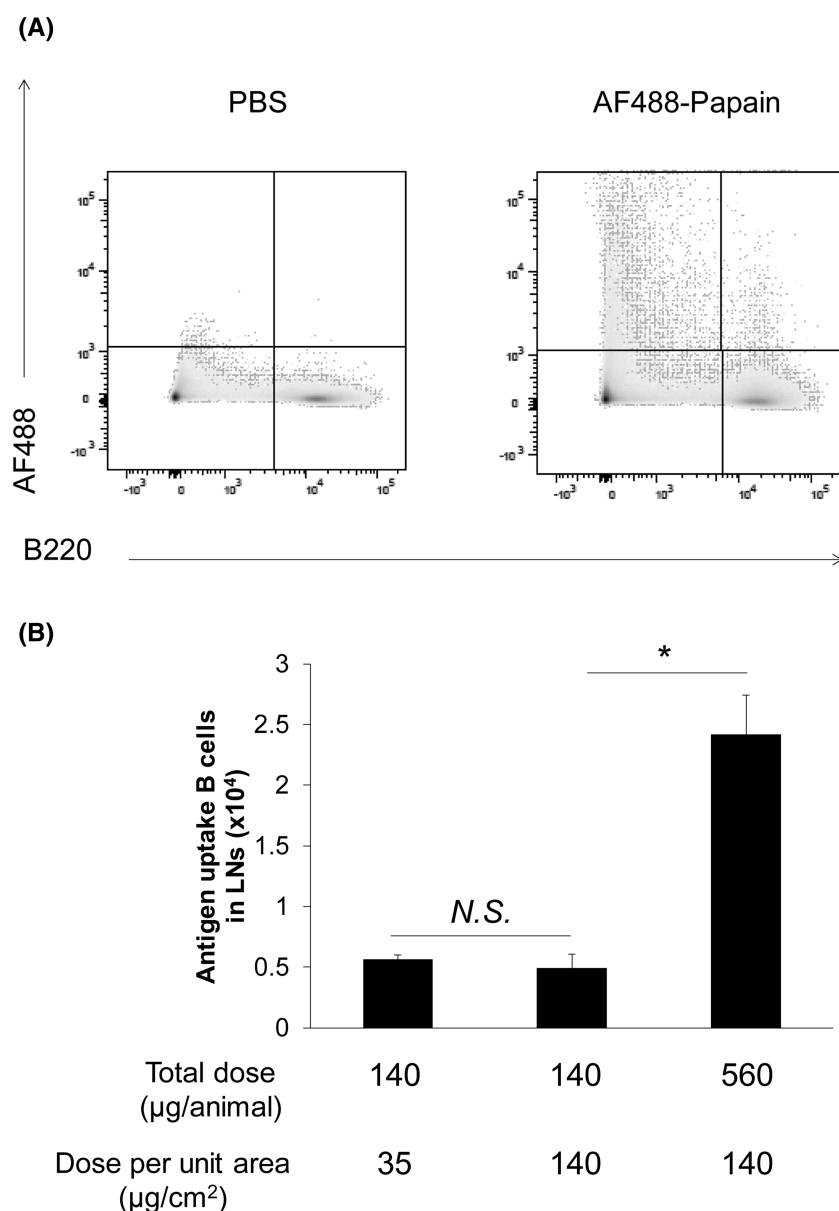


FIGURE 4 Correlation of the number of antigen-bearing B cells in draining lymph nodes (DLNs) with the total dose. Alexa 488-labeled papain was applied onto the back skin of mice (24-h closed patch, one time, $n = 5$ per group), and DLNs were collected. Exposure conditions are defined in Table 2. The number of Alexa488-papain-bearing B cells in the DLNs was calculated using flow cytometry. Data are shown as mean $\pm$ SEM, $*p < 0.05$. (A) Gating of Alexa 488-papain-bearing B cell for flow cytometry analysis. (B) Number of Alexa 488-papain-bearing B cells in DLNs

560 μg over 4 cm^2 , the mean numbers of antigen-bearing B cells were calculated as 0.49×10^4 cells and 2.42×10^4 cells, respectively. These results indicated that the number of antigen-bearing B cells within DLNs correlated with the total dose.

3.4 | Papain-specific IgG1 and IgE levels were correlated with the total dose

In the mouse model, it was demonstrated that the number of antigen-bearing B cells in DLNs correlated with the total dose. To examine the dose metrics corresponding with antibody levels under these conditions, we repeated percutaneous topical exposure with the same parameters. ELISA analysis of antigen-specific antibodies showed that when the same total dose was administered (i.e., 140 μg Alexa488-papain over either 4 or 1 cm^2), the amounts of both papain-specific IgG1 and IgE were comparable (Figure 5A,B). Meanwhile, when the same total dose unit area was achieved (i.e., 140 μg Alexa488-papain over 1 cm^2 or 560 μg over 4 cm^2), both papain-specific IgG1 and IgE were significantly different. In summary, both the amount of antigen to which B cells were exposed and the amount of papain-specific antibodies (IgG1 and IgE) were correlated with the total dose.

4 | DISCUSSION

These studies have demonstrated that, under these controlled experimental conditions, the incidence of papain-evoked percutaneous IgE/IgG1-mediated ITH correlates with the amount of total antigen dosage per animal, not dose per unit area. Moreover, the amounts of antigen-specific antibody and antigen-bearing B cells in DLNs were correlated with total dose. These results suggest that total dose exhibits a dominant impact on the effectiveness of percutaneous sensitization for IgE/IgG1-mediated ITH. On the other hand, it is well established that dose per unit area is the more important metric for skin sensitization efficacy (Friedmann, 1990; White, Friedmann, Moss, & Simpson, 1986). Therefore, it is of interest for the toxicological safety assessment of IgE/IgG1-mediated allergy that dose metrics driving the induction of sensitization vary depending on the type of allergy.

Friedman et al. and Kligman have shown the importance of dose per unit area for the induction of DTH within human clinical studies (Friedmann, 1990; Kligman, 1989; White, Friedmann, Moss, & Simpson, 1986), and information from guinea pig studies is consistent with this conclusion (Magnusson & Kligman, 1970). In these animal experiments, three different allergens (DNCEB, p-phenylenediamine, and p-nitroso-dimethyl dimethylaniline) were applied to different skin areas in various volumes so that the dose per unit area remained constant. These data showed that for all three allergens, the incidence of DTH was comparable when the dose per unit area was constant. These results suggest that an underlying mechanism of DTH is common across species. In relation to such an underlying mechanism of ITH, there is an important commonality that IgE binds to Fc ϵ RI on mast cells

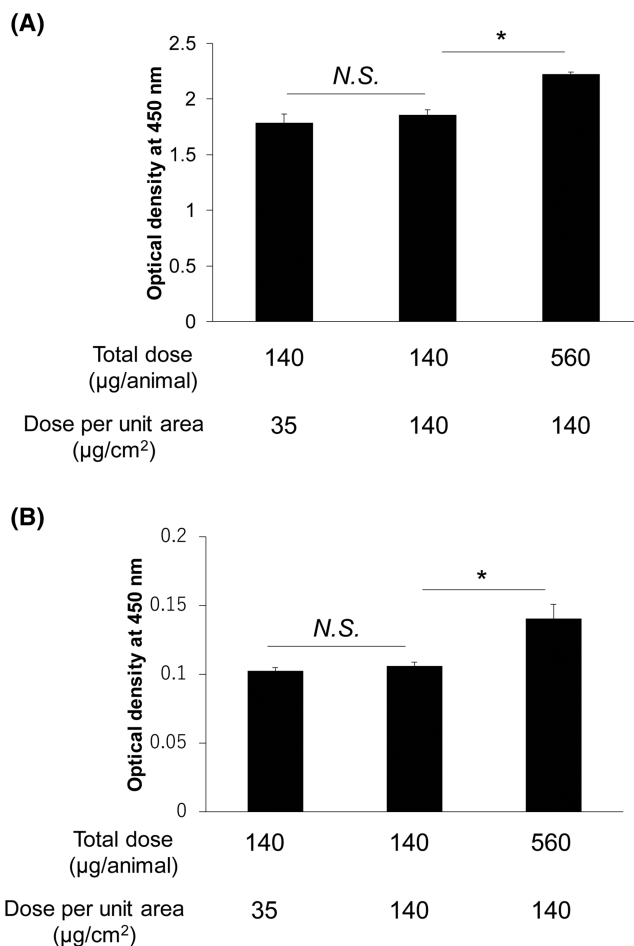


FIGURE 5 Papain-specific IgG1 and IgE levels were correlated with total dose. Papain was applied onto the back skin of mice (24-h closed patch, 14 times, $n = 5$ per group), and sera were collected after sensitization. Exposure conditions are defined in Table 2. Papain-specific IgG1 and IgE in the sera of sensitized mice were analyzed using enzyme-linked immunosorbent assay (ELISA). Data are shown as mean $\pm$ SEM, * $p < 0.05$. (A) Papain-specific IgG1 levels. (B) Papain-specific IgE levels

and leads to degranulation of mast cells in both human and guinea pig models (Simons, 2008; Verdier, Chazal, & Descotes, 1994). However, in addition to IgE, IgG1 also binds to mast cells and elicits an anaphylactic reaction in guinea pigs (Verdier, Chazal, & Descotes, 1994). Therefore, similar to DTH, it is assumed that the dose metric correlating with the incidence of percutaneous ITH is preserved between species, but clinical studies may also be required to confirm this in the future because of the complicated functionality of IgG1.

In a previous study, the utility of combining the Buehler skin sensitization method and PCA tests for evaluating the potential of percutaneous ITH was examined. Matsunaga et al. (2015) conducted a Buehler modified sensitization method and PCA tests to compare the potential of percutaneous ITH using gluten and Gluparl 19S, the latter being produced by hydrolyzing gluten. Several studies reported that the potential for induction of ITH with hydrolyzed wheat depends upon the protein molecular weight (Gourbeyre et al., 2012;

Nakamura et al., 2013). Consistent with these results, Matsunaga et al. showed that the PCA titer and PCA positive rate for Glupearl 19S were higher than those arising in response to gluten exposure. Consequently, it appears that a combination of a sensitization method based on that of Buehler and PCA testing has the potential to detect percutaneous ITH. Furthermore, in another study, Sarlo, Fletcher, Gaines, and Ritz (1997) performed a PCA test to compare the potential of ITH between enzymes such as Termamyl and Alcalase. From the PCA test results, the ITH potential of Termamyl was shown to be about 10-fold stronger than that of Alcalase. Thus, on the basis of these results, by reducing the concentration of Termamyl in the product to 1/10 that of Alcalase, the positive rate of the human skin prick test induced by both enzymes in an occupational setting was comparable. These studies suggest that the anaphylactic potential of allergens inducing IgE/IgG1-mediated ITH in humans is accurately evaluated with the PCA test. In our experiments, the PCA positive rate was correlated with total antigen exposure. Additionally, the level of IgG1 associated with the ITH response was also correlated with total exposure. Therefore, the dose metric that defines the incidence of percutaneous IgE-mediated ITH was indeed total exposure.

The underlying mechanism by which the incidence of DTH is defined by the amount of dose per skin area takes into consideration that the activation of DCs is important for the induction of DTH (Friedmann, 2007; Kimber et al., 2008). On the other hand, within the induction of IgE/IgG1-mediated ITH, there are some mechanistic differences relative to DTH. In the first step of IgE/IgG1-mediated ITH, DCs uptake antigen in the skin and migrate into the DLN, where antigen-specific Th cells are primed by the activated DCs. Simultaneously, B cells uptake antigens via BCR in the DLN and interact with antigen-specific Th cells, and then B cells differentiate into plasma cells, producing antigen-specific antibodies (MacLennan et al., 1997; Pape et al., 2003). Therefore, the number of antigen-bearing B cells in the DLN is thought to be an important factor in the induction of IgE/IgG1-mediated ITH. The results reported herein showed that the number of antigen-bearing B cells, as well as the amount of antigen-specific antibodies, was correlated with total exposure. Considering the data mentioned above, the amount of antigens to which B cells are exposed in the lymph nodes is important for the induction of IgE/IgG1-mediated ITH.

Because only papain was used as an antigen to induce percutaneous IgE/IgG1-mediated ITH, it is important to consider whether modifications of protein properties could influence the dose metrics of percutaneous IgE/IgG1-mediated ITH. For example, in the case of protein insolubilization, it is reported that the sensitization of insolubilized bovine serum albumin induced a larger number of responding animals and markedly higher antibody titer rather than that of soluble bovine serum albumin (Hirata & Sussdorf, 1966). These data suggest that modification of protein properties (e.g., insolubilization), which enables high tissue retention, would strengthen the ITH response by raising exposure to B cells. However, in regard to dose metrics, we concluded that total exposure defines the incidence of percutaneous IgE/IgG1-mediated ITH and would be the case for any protein or its modification. It is because

the amount of antigen that exposes the B cell would be correlated with total exposure irrespective of the kind of protein and modification of the protein properties.

In the case of DTH, it has been reported that when the area of the application site is very small, the relationship between dose per unit skin area and the acquisition of sensitization was lost (Rees, Friedmann, & Matthews, 1990). For percutaneous ITH, it is unclear as to whether the relationship between the total dose and incidence of percutaneous ITH is lost under similar conditions. However, for the induction of ITH, the activation of Th cells by migrating DCs is also required, in addition to the activation of B cells. Therefore, within sensitization conditions in which the application site is very small, it is assumed that the relationship between the incidence of percutaneous ITH and total exposure would be lost.

The current study demonstrated that the incidence of papain-evoked percutaneous IgE/IgG1-mediated ITH correlates with the amount of total antigen dosage. Although the process by which antigens activate DCs in the skin is important within DTH, the process by which antigens reach DLNs to activate B cells is important for percutaneous IgE/IgG1-mediated ITH. An appreciation of the dose metrics corresponding with toxicity is of vital importance in developing refined risk assessments for IgE/IgG1-mediated ITH. Therefore, irrespective of the detailed mechanistic bases for relevant dose metrics in IgE/IgG1-mediated ITH, it is suggested that the total dose will dictate the effectiveness and extent of percutaneous ITH. Because there remains no validated hazard assessment method for ITH, further studies are essential in order to establish an accurate risk assessment method for percutaneous ITH.

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CONFLICT OF INTEREST

We have no conflict of interest to declare.

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