

Regular Article

Comparison of the Allergenic Potency of House Dust Extract and House Dust Mite Allergen Extract for Subcutaneous Allergen Immunotherapy

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Subcutaneous allergen immunotherapy (SCIT) with non-standardized house dust (HD) extracts has been used in Japan since 1963 for house dust mite (HDM)-allergic patients. Since the potencies of HD extracts are unknown, the allergenic potency of HD extracts was examined by comparing with a standardized HDM allergen extracts. The major allergen content of HDM in the extracts was measured using a sandwich enzyme-linked immunosorbent assay (ELISA). The immunoglobulin E (IgE) inhibitory activities of the extracts were measured by a competitive ELISA. The extract concentrations giving 50% inhibition of IgE binding ($\log_{10}$ IC₅₀) were determined from dose–response curves and defined as inhibitory activities. A linear regression line was constructed from the $\log_{10}$ IC₅₀ values of the standardized HDM extract to interpolate the relative potency of the HD extract with strength of 1:10 w/v (HD 1:10). The amounts of major allergens (*Der f* 1, *Der p* 1 and *Der 2*) were 116.3 μ g/mL in the HDM allergen extract (100000 Japanese Allergy Units [JAU]/mL) and 0.77 μ g/mL in the HD 1:10. The inhibitory activity ($\log_{10}$ IC₅₀ values) of HD 1:10 was 2.389 ± 0.078 , indicating the allergenic potency was between 200 and 2000 JAU/mL. Based on regression analysis ($R^2 > 0.99$), the allergenic potency of HD 1:10 was estimated to be 842 ± 128 JAU/mL. The present study determined the major allergen content of HD extract, which contributes to its allergenic potency. The allergenic potency of HD 1:10 was *ca.* 100-fold less than that of HDM allergen extract.

Key words allergen immunotherapy; allergenic potency; house dust mite; standardized allergen

INTRODUCTION

House dust mite (HDM) allergy is the most common inhalant allergy and causes symptoms of rhinitis and asthma.¹⁾ *Dermatophagoides pteronyssinus* (*Der p*) and *Dermatophagoides farina* (*Der f*) are the major sources of HDM allergens.²⁾ A standardized HDM allergen extract containing a mixture of *Der p* and *Der f* extracts is currently in use for HDM-allergic patients with subcutaneous allergen immunotherapy (SCIT).³⁾ This formulation has been used for decades in the United States and Europe, and it has been demonstrated to be clinically effective.^{4,5)}

Manufacturers are responsible for the quality control of allergen extracts, such as the batch-to-batch consistency, but allergen extracts should be further standardized by regulatory authorities.⁶⁾ The Center for Biologics Evaluation and Research (CBER) of the U.S. Food and Drug Administration (FDA) validated the competitive enzyme-linked immunosorbent assay (ELISA) method to determine the potency of allergen extracts.⁷⁾ This method measures the allergen-specific immunoglobulin E (IgE)-binding inhibitory activity of the allergen extracts, and the relative potency of each extract is calculated by comparison with a standardized reference extract.

In contrast to HDM allergen extracts, house dust (HD) extracts are non-standardized and poorly characterized due to a lack of quality control of profiles for both the protein and the allergen. HD extracts have been used since the 1960s for SCIT to treat HDM-induced allergic rhinitis and asthma.^{8–10)} However, in the late 1990s, the WHO published allergen-

specific immunotherapy (AIT) guidelines advising that standardized allergen extracts from HDM, not from HD, should be used for HDM-allergic patients.^{11,12)}

In Japan, HD extract was the only treatment option for HDM-allergic patients before HDM allergen extract was approved to use for HDM-allergic patients in 2014.³⁾ The HDM allergen extract is a standardized SCIT formulation and possesses allergenic potency with Japanese Allergy Unit (JAU), which is a potency unit defined by a task force of the Japanese Society of Allergology (JSA).¹³⁾

Currently two allergen extracts (HDM allergen extract and HD extract) are available for use in SCIT, and physicians need to understand the differences in quality between these extracts and to be able to switch patient treatment from HD extracts to HDM allergen extracts. Even after the HDM allergen extract was launched in 2014, it is estimated that approximately 10000 patients are still receiving SCIT with HD extracts in Japan. Despite the long-standing use of HD extracts, these extracts have not been fully characterized. Due to unknown allergenic potencies with JAU, the HD extract is unable to compare with the standardized HDM extract. This issue seems one of reasons not to switch from HD extract to HDM allergen extract in clinical practice. The goal of the present study was to determine the allergenic potencies with JAU of HD extracts by comparison with the standardized HDM allergen extracts using a competitive ELISA.

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MATERIALS AND METHODS

HDM Allergen Extract and HD Extract (HD 1:10)

Three batches each of HDM allergen extract (batch A, 2F00362; B, 2G00502; C, 2G00512; Torii Pharmaceutical Co., Ltd., Tokyo, Japan) and HD 1:10 (batch D, A3AL90; E, A3AL91; F, A3AL92; Torii Pharmaceutical Co., Ltd.) were used in this study. The HDM allergen extract was a standardized SCIT formulation extracted from two HDM species, *Der p* and *Der f*, and its allergenic potency was 100000 JAU/mL. The HD 1:10 was extracted from house dust in 10-fold volumes of extraction solvent and it is indicated as strength of 1:10 w/v.

Measurement of Major Allergen Content The major allergen content (Der p1, Der f 1, and Der 2 [total of Der f 2 and Der p 2]) in the extracts was measured using a sandwich ELISA.

Briefly, 96-well microplates (Costar 3590, Cambridge, U.K.) were coated with mouse monoclonal antibody (mAb) to Der f 1, Der p 1, or Der 2 (prepared in-house) for 18 h at 4°C and then blocked with phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA)/0.9% NaCl for 1 h at room temperature (r.t.). A series of diluted HDM allergen or HD 1:10 were added to the wells, and the plates were incubated for 1 h at r.t. Rabbit polyclonal antisera to Der p 1, Der f 1, or Der 2 (prepared in-house) were added for 1 h at r.t., the plates were washed, and a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (Merck Millipore, Tokyo, Japan) was added for 1 h at r.t. Finally, a colorimetric enzyme substrate, *o*-phenylenediamine (KEM-EN-TEC Diagnostics A/S, Copenhagen, Denmark) in hydrogen peroxide solution (Sigma-Aldrich, Japan), was added to the wells, and the plates were incubated for 30 min at r.t. in the dark. The reaction was stopped by addition of 2 M H₂SO₄, and the absorbance at 490 nm was measured with a plate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). The major allergen content of the extracts was calculated from a standard curve constructed with the JSA reference HDM extract.¹³⁾

Measurement of IgE-Binding Inhibitory Activity Inhibitory activities of HDM-specific IgE-binding of the extracts were measured by a competitive ELISA. Each batch (A–C) of HDM allergen extracts was diluted in 1% BSA/PBS to the three concentrations, 200, 2000 and 20000 JAU/mL, and used as sample solutions along with undiluted HDM allergen extract (100000 JAU/mL) and HD 1:10 (batches D–F). A two-fold serial dilution with PBS was then prepared for each sample solution to produce test solutions. Specially, the dilution rates were 1/1000–1/128000 for 100000 JAU/mL of the HDM allergen extract, 1/200–1/25600 for 20000 JAU/mL, 1/20–1/2560 for 2000 JAU/mL, 1/2–1/256 for 200 JAU/mL, and 1/10–1/1280 for each batch (D–F) of HD 1:10. A pooled human serum sample was prepared from volunteers with HDM allergy. The serum HDM-specific IgE level of each volunteer was ≥ 3.5 kU/L based on results from ImmunoCAP Phadia 100 (Thermo Fisher Diagnostics Inc., Uppsala, Sweden). The test solutions of the extracts were mixed with an equal volume of the pooled serum (diluted 5-fold in 1% BSA/PBS) and incubated overnight at 25°C. As a no-extract control, 1% BSA/PBS was mixed with the diluted serum pool.

For the ELISA, 96-well microplates (Immulon 2HB; Thermo Fisher Scientific, Tokyo, Japan) were pre-coated with an

in-house reference solution of a mixed extract of *Der f* and *Der p*. After washing the plates, the control buffer/serum or extract/serum samples were added to the wells and incubated for 5 h at 25°C. A sample of β -galactosidase-conjugated anti-IgE mAb (Thermo Fisher Diagnostics Inc.) was then added to the wells and incubated overnight at 25°C. The enzyme substrate 4-methylumbelliferyl- β -D-galactoside (Sigma-Aldrich) in dimethyl sulfoxide was diluted with buffer (10 mM phosphate buffer, pH 7.0, 1 mM MgCl₂, 100 mM NaCl, 0.1% BSA, and 0.1% NaN₃) to 0.1 mM and added to the wells. The plate was incubated for 2 h at 25°C, and the reaction was stopped by addition of 0.1 M glycine in NaOH (pH 10.3). Fluorescence intensity (FI) was measured at 460 nm using a microplate reader (Molecular Devices).

All procedures with human serum were approved by the Ethics Committee of Torii Pharmaceutical Co., Ltd. according to the Clinical Research Ethical Guidelines.

Calculation of IgE Inhibitory Activity Each test solution was assayed in technical triplicate. Percent inhibition was calculated as: % inhibition = $(1 - [A1 - A2]/[A3 - A2]) \times 100$, where A1 is the FI of the extract sample, A2 is the FI of dilution buffer alone, and A3 is the FI of the control buffer/serum lacking extract. The percent inhibition of the dilution buffer alone and control buffer/serum were set as 100 and 0%, respectively.

Three experiments were performed for each extract (batches A–F). The mean $\pm$ standard deviation (S.D.) values for % inhibition were calculated and plotted against log₁₀ of the sample dilution. Four-parameter logistic (sigmoidal) curve fitting was then performed to generate dose-inhibition curves using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, U.S.A.). Hill slopes and log₁₀ IC₅₀ values (dilution factor resulting in 50% inhibition of maximum) were then obtained from the inhibition curves. The log₁₀ IC₅₀ values were defined to represent the IgE-binding inhibitory activities of the extract samples.

Calculation of HD 1:10 Potency The log₁₀ IC₅₀ values and log₁₀ potency values of the HDM allergen extract (200–100000 JAU/mL) were plotted to construct a regression line. The potency of HD 1:10 was then estimated by interpolation of its log₁₀ IC₅₀ value.

Statistical Analysis Data are presented as the mean $\pm$ S.D. of three technical triplicates. Variance equality of the Hill slopes was analyzed using an *F* test. If the variance was equal, curve parallelism was determined using Student *t*-test. If the curves were parallel, the log₁₀ IC₅₀ values were compared using an *F* test and Student *t*-test. Curves that were not parallel were not compared. A *p* value of <0.01 was considered significantly different.

RESULTS

Major Allergen Content of HDM Allergen Extract and

HD 1:10 The major allergen content (Der f 1, Der p 1, and Der 2 [total of Der f 2 + Der p 2]) in the HDM allergen extract (100000 JAU/mL) was 25.0 ± 1.7 , 22.5 ± 2.0 , and 68.9 ± 6.2 μ g/mL, respectively, totaling 116.3 μ g/mL (Table 1). Similarly, the Der f 1, Der p 1, and Der 2 content of HD 1:10 was 0.69 ± 0.03 , 0.02 ± 0.01 , and 0.06 ± 0.01 μ g/mL, respectively, totaling 0.77 μ g/mL. Thus, major allergen content of the HDM allergen extract was *ca.* 150-fold higher than that of the

Table 1. Major Allergen Content of the HDM Allergen Extract of 100000JAU/mL and HD 1:10

Allergen	HDM allergen extract of 100000JAU/mL ($\mu\text{g/mL}$)	HD 1:10 ($\mu\text{g/mL}$)
Der f 1	25.0 ± 1.7	0.69 ± 0.03
Der p 1	22.5 ± 2.0	0.02 ± 0.01
Der 2 [†]	68.9 ± 6.2	0.06 ± 0.01
Total of Der f 1, Der p 1, and Der 2 [†]	116.3	0.77
Ratio of total allergen content	151:01:00	

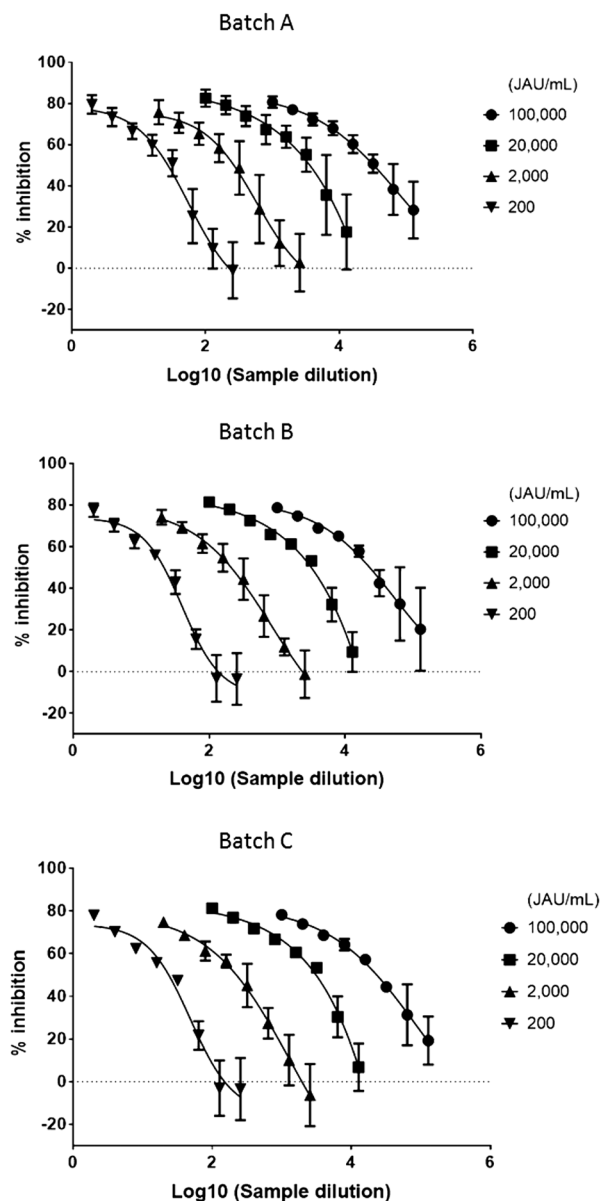
[†] Der f 2 + Der p 2. Data are the mean $\pm$ S.D.

Fig. 1. Dose-Response Curves for IgE Inhibitory Activity of HDM Allergen Extracts

Three batches of HDM extract (batches A–C) were tested at 200, 2000, 20000 and 100000JAU/mL. Data are the mean $\pm$ S.D. of three experiments.

HD 1:10 (Table 1).

IgE Inhibitory Activity of the HDM Allergen Extracts

All three batches (A–C) of HDM allergen extract showed

Table 2. Comparison of the Hill Slopes and IgE Inhibitory Activity ($\text{Log}_{10} \text{IC}_{50}$) for Three HDM Allergen Extracts

HDM allergen extract (JAU/mL)	Batch-to-batch comparison	Hill slopes		$\text{Log}_{10} \text{IC}_{50}^{\dagger}$	
		<i>p</i> Value		<i>p</i> Value	
		<i>F</i> Test	<i>t</i> -Test	<i>F</i> Test	<i>t</i> -Test
200	A vs. B	0.958	0.127	0.549	0.299
	B vs. C	0.391	0.533	0.679	0.366
	A vs. C	0.365	0.533	0.847	0.818
2000	A vs. B	0.308	0.414	0.488	0.803
	B vs. C	0.84	0.473	0.865	0.448
	A vs. C	0.232	0.473	0.595	0.48
20000	A vs. B	0.523	0.477	0.189	0.428
	B vs. C	0.503	0.494	0.937	0.656
	A vs. C	0.974	0.494	0.211	0.544
100000	A vs. B	0.069	0.534	0.825	0.925
	B vs. C	0.236	0.471	0.413	0.714
	A vs. C	0.423	0.471	0.54	0.59

[†] $\text{Log}_{10} \text{IC}_{50}$ represents IgE-binding inhibitory activities.

IgE-binding inhibitory activities in a concentration-dependent manner when tested at 200, 2000, 20000, and 100000JAU/mL (Fig. 1).

To evaluate whether the inhibition curves for batches A–C differed, the Hill slopes and $\text{log}_{10} \text{IC}_{50}$ values calculated from the curves were analyzed using an *F* test and Student *t*-test. The variance of the Hill slopes and the $\text{log}_{10} \text{IC}_{50}$ values (Table 2) of the inhibition curves were parallel at each concentration (200–100000JAU/mL). The $\text{log}_{10} \text{IC}_{50}$ values for batches A, B, and C were not significantly different, indicating that they had equivalent IgE-binding inhibitory activities.

IgE Inhibitory Activity of the HD 1:10 The inhibition curves for the three batches (D–F) of HD 1:10 are shown in Fig. 2. Concentration-dependent inhibition was observed for each batch, indicating that the extract could inhibit HDM-specific binding to IgE. The variance of the Hill slopes and $\text{log}_{10} \text{IC}_{50}$ values for the curves were equal (Table 3). Thus, HD 1:10 batches D–F did not differ significantly in their IgE inhibitory activities.

Comparison of HDM Allergen Extract and HD 1:10 Inhibition Curves We next plotted the inhibition of HDM allergen extract and HD 1:10 using the mean values for the three batches (Fig. 3). The inhibitory activity of HD 1:10 was intermediate between those of the HDM allergen extract at 200 and 2000JAU/mL, indicating that the allergenic potency of HD 1:10 was in the range 200–2000JAU/mL. The variance of the Hill slopes for HD 1:10 and each concentration of HDM extract were equal, and the inhibition curves were parallel, as determined by an *F* test and Student *t*-test, respectively (Table 4).

These results confirm that the allergenic potency of the HD 1:10 can be estimated from the inhibitory activity of the HDM allergen extract.

Estimation of HD 1:10 Potency The $\text{log}_{10} \text{IC}_{50}$ values of the HDM allergen extracts at 200, 2000, 20000, and 100000JAU/mL were 1.678 ± 0.071 , 2.785 ± 0.020 , 3.849 ± 0.043 , and 4.825 ± 0.106 , respectively (Table 4). The regression line constructed from these data gave an R^2 value of >0.99 (Fig. 3B). Interpolation of the calculated IC_{50} of 2.389 ± 0.078 for HD 1:10 gave an estimated potency of

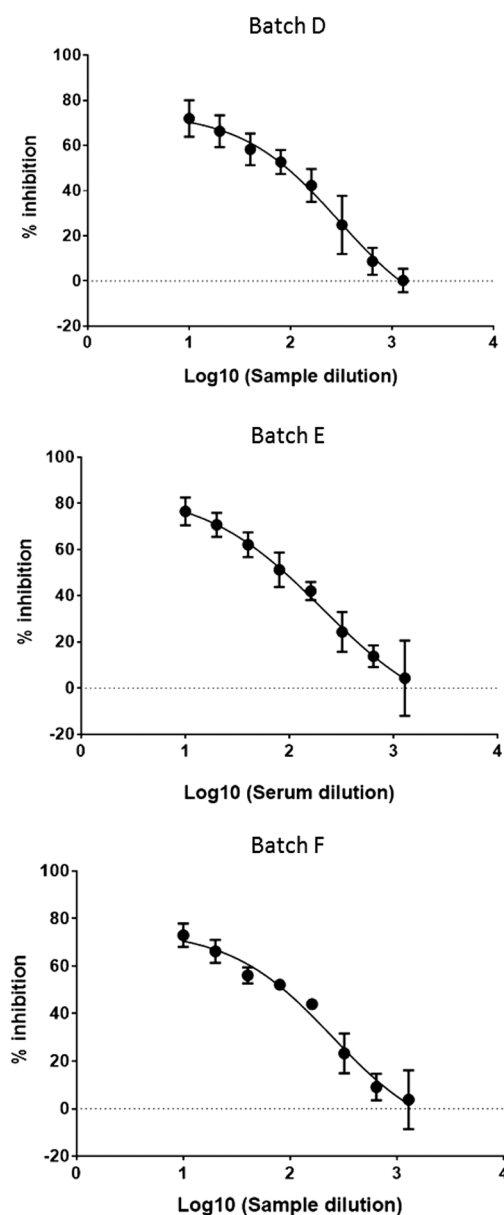


Fig. 2. Dose-Response Curves for IgE Inhibitory Activity of HD 1:10
Three batches (D-F) were tested. Data are the mean $\pm$ S.D. of three experiments.

Table 3. Comparison of the Hill Slopes for Three Batches of HD 1:10

HD extract	Batch-to-batch comparison	Hill slopes		$\text{Log}_{10} \text{IC}_{50}^{\dagger}$	
		p Value		p Value	
		F Test ‡	t -Test ‡	F Test ‡	t -Test ‡
HD 1:10	D vs. E	0.139	0.534	0.788	0.925
	E vs. F	0.502	0.471	0.999	0.714
	D vs. F	0.364	0.471	0.787	0.59

$^{\dagger} \text{Log}_{10} \text{IC}_{50}$ represents IgE-binding inhibitory activities. ‡ HDM allergen extracts vs. HD extracts.

842 $\pm$ 128 JAU/mL (Fig. 3B, Table 4).

DISCUSSION

AIT has been used for more than 100 years as a desensitiz-

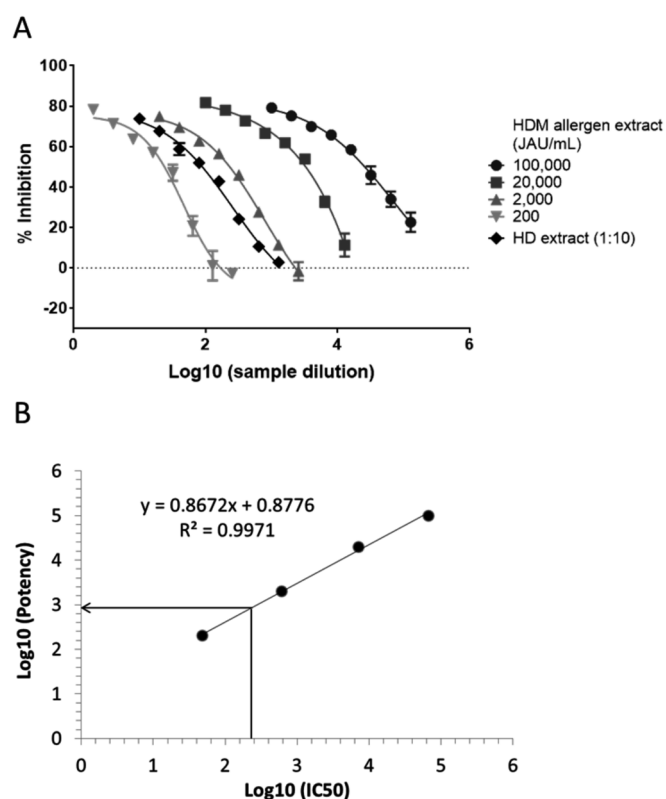


Fig. 3. Estimation of Allergenic Potency of HD 1:10

(A) Dose-response curves for IgE inhibitory activity of HDM allergen-specific IgE binding by HD 1:10 and HDM extracts. Data are the mean $\pm$ S.D. of three batches of each sample. (B) Regression analysis of $\text{log}_{10} \text{IC}_{50}$ and log_{10} potency of the HDM allergen extract (200, 2000, 20000, and 100000 JAU/mL). The allergenic potency of the HD 1:10 was 842 JAU/mL (log_{10} potency: 2.922) based on interpolation of a $\text{log}_{10} \text{IC}_{50}$ value of 2.389. $\text{Log}_{10} \text{IC}_{50}$ represents IgE-binding inhibitory activities.

ing therapy for IgE-mediated allergic diseases and represents the only potentially curative treatment.¹⁴⁻¹⁷) AIT is currently administered *via* either the subcutaneous (*i.e.*, SCIT) or sublingual (*i.e.*, SLIT)¹⁸⁻²²) route, and is recognized as the only treatment option with the potential to provide long-term post-treatment benefits and alters the natural course of allergic disease.²³⁻²⁶) In 2014, an HDM allergen extract was the first standardized formulation of SCIT for Japanese HDM-allergic patients,³) and the JSA established a standardized reference HDM allergen extract of 100000 JAU/mL.¹³) The JAU is similar to the allergy unit (AU) defined by CBER of the U.S. FDA.^{7,27}) The JSA also demonstrated that the *in vivo* biological activity of the HDM extract correlated with its *in vitro* allergenic potency, as determined by a competitive ELISA.¹³)

The WHO guidelines advocate the use of an HDM allergen extract, not an HD extract, for AIT of HDM allergy.^{11,12}) Although a standardized HDM allergen extract is available in Japan, non-standardized and poorly characterized HD extracts continues to be used for the treatment of HDM-induced rhinitis and/or asthma patients. It seems likely that long-standing familiarity with the use of HD extracts deters physicians from switching treatment to a standardized HDM allergen extract. Furthermore, little information is available about the major allergen content and allergenic potency of HD extract compared with HDM allergen extracts. Here, we investigated the allergenic potency of HD extracts compared with HDM allergen extracts.

Table 4. Comparison of the Hill Slopes for HD and HDM Allergen Extracts

Extract	Dose	Batch	Hill slope			Log ₁₀ IC ₅₀ [†]			
			Value	p Value		Value	Mean ± S.D.	p Value	
				F Test [‡]	t-Test [‡]			F Test [‡]	t-Test [‡]
HDM (JAU/mL)	200	A	-1.165	0.366	0.013	1.746	1.678 ± 0.071	0.915	0
		B	-1.532			1.604			
		C	-1.409			1.685			
	2000	A	-1.118	0.904	0.059	2.765	2.785 ± 0.020	0.119	0.001
		B	-0.951			2.786			
		C	-1.121			2.804			
	20000	A	-0.972	0.557	0.056	3.898	3.849 ± 0.043	0.468	0
		B	-1.143			3.833			
		C	-1.255			3.817			
	100000	A	-0.658	0.812	0.096	4.925	4.825 ± 0.106	0.698	0
		B	-0.783			4.714			
		C	-0.745			4.837			
HD	1:10	D	-0.763	NA	NA	2.309	2.389 ± 0.078	NA	NA
		E	-0.922			2.464			
		F	-0.911			2.393			

NA, not applicable. [†] Log₁₀ IC₅₀ represents IgE-binding inhibitory activities. [‡] HDM allergen extracts vs. HD 1:10.

All the extracts showed dose-dependent inhibitory activities of HDM-specific IgE binding, and there were no significant inter-batch differences in IgE inhibitory activity for the HD extracts and HDM allergen extracts. A regression line obtained by plotting the mean log₁₀ IC₅₀ values of the HDM allergen extract at doses between 200 and 100000 JAU/mL enabled the allergenic potency of the HD 1:10 to be estimated at 842 ± 128 JAU/mL. Thus, the HD 1:10 has a HDM allergenic potency approximately 100-fold lower than that of the HDM allergen extract at 100000 JAU/mL.

The content of major allergens (groups 1 and 2) was measured in both extracts. The major allergens, Der f 1, Der p 1, and Der 2, were all contained in the HDM allergen extract, whereas only Der f 1 was dominantly found in the HD extract. The total HDM major allergen content was 0.77 µg/mL in HD 1:10; the HDM allergen extract possesses a 151-fold higher major allergen content compared with the HD 1:10, which is consistent with the *ca.* 100-fold difference in their allergenic potencies based on inhibitory activities of HDM allergen-specific IgE binding.

According to the WHO position paper, a suggested maintenance dose for AIT is around 5–20 µg of allergen content.¹¹⁾ Since the maximum injection volume for SCIT is 0.2–0.3 mL in clinical practice, use of HD extracts (HD 1:10) for SCIT would produce an allergen content of only 0.154–0.231 µg. However, clinical trial data demonstrated that 200 or 300 JAU (1000 JAU × 0.2 or 0.3 mL) of HDM extract was selected as the appropriate maintenance dose for the majority of participants,²⁸⁾ and this amount corresponds to 23.26–34.89 µg of total major allergen content. Thus, it is impossible to reach the required major allergen content level by using the HD extracts (HD 1:10) for SCIT at a volume of 0.2–0.3 mL, suggesting that HDM allergen extract, rather than HD extract, needs to be the first choice for SCIT in allergic patients. Recently, it was reported that SCIT with HDM allergen extract showed more effectiveness in HDM-allergic patients compared to that

with HD extract (Tanaka *et al.*).²⁹⁾ This result suggests that SCIT with standardized HDM allergen extracts could provide patients with more benefits.

To our knowledge, this is the first study to report the allergenic potency (in JAU/mL) of an HD extract (HD 1:10) based on a comparison with the allergenic activity of a standardized HDM allergen extract *in vitro*. This study determined the major allergen content of HD 1:10, which contributes to the allergenic potency of this extract. Even though the use of HD extract is sometimes clinically effective in HDM-allergic patients, the WHO has strongly suggested that allergen extract should be standardized to guarantee the quality control of both protein and allergen contents in extracts, as this is associated with their safety and efficacy in clinical practice.

Our finding indicates that HDM allergen extracts should be the first choice for use in SCIT in Japanese HDM-allergic patients.

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Conflict of Interest W.D., C.F., M.Y. and K.O.-D. are employees of Torii Pharmaceutical Co., Ltd. K.M. received a lecture fee from Torii. T.M. received a research grant from Torii.

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