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ORIGINAL ARTICLE

A New Reliable Method for Detecting Specific IgE Antibodies in the Patients with Immediate Type Wheat Allergy due to Hydrolyzed Wheat Protein: Correlation of Its Titer and Clinical Severity

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ABSTRACT

Background: Immediate-type wheat allergy caused by a specific hydrolyzed wheat protein (HWP-IWA), Glupearl 19S (GP19S), typically develops food-dependent exercise-induced anaphylaxis (FDEIA), but is different from conventional FDEIA, or simple wheat allergy in many aspects. The skin prick test (SPT) is considered to be the most effective method for diagnosis of HWP-IWA. As SPT is a relatively qualitative method, we developed quantitative and high-throughput test method for HWP-IWA.

Methods: An enzyme-linked immunosorbent assay (ELISA)-based GP19S-specific IgE assay was tested using sera from 14 HWP-IWA and five conventional wheat-dependent exercise-induced anaphylaxis (CO-WDEIA) patients, as well as five healthy subjects. Then a validation study at five different institutions was carried out using sera from 10 HWP-IWA and five CO-WDEIA patients, as well as five healthy subjects different from the previous studies.

Results: The mean unit values converted from measured absorbance of ELISA were 68.3, 1.3 and 1.1 respectively. Furthermore, the validation study revealed reproducible results across all five institutions, with the standard deviation (SD) being 0.3-0.4 for the healthy group, 0.2-0.6 for the CO-WDEIA group, and 3.8-9.6 for HWP-IWA group except for one case. One case of HWP-IWA was excluded from analysis due to the high SD of 53.3 units, indicating that samples with a unit value > 100.0 will affect inter-laboratory reproducibility.

Conclusions: Our findings suggest that the ELISA-based GP19S-specific IgE assay can be used to test HWP-IWA using venous blood samples, except for those with a unit value > 100.0.

KEY WORDS

enzyme-linked immunosorbent assay, Glupearl 19S, hydrolyzed wheat protein, immediate-type wheat allergy, test method

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INTRODUCTION

Many cases of Immediate-type allergy after wheat intake have been observed in Japanese consumers using cosmetics that contain hydrolyzed wheat protein (HWP), HWP-IWA, who had used "Cha no Shizuku" soap (sold by Yuuka, Fukuoka, Japan) that contained Glupearl 19S (GP19S), a substance manufactured by Katayama Chemical Industries, Osaka, Japan. HWP-IWA patients, but not conventional wheat allergy patients, react to GP19S. Therefore, GP19S hypersensitivity is essential for diagnosis of HWP-IWA.[1,2]

HWP is a cosmetic ingredient specified in the Japanese Standards of Quasi-drug Ingredients, and which is a collective term for water-soluble materials that are produced by hydrolyzing wheat glutens with acid, alkali, enzymes or other substances. Cases of HWP allergy have been reported in Western nations; notably, cases are fewer and less severe than those in Japan.[3-6] In order to address the problem of HWP allergy, the "Special Committee for the Safety of Protein Hydrolysates in Cosmetics" was organized by the Japanese Society of Allergology to study the epidemiology, pathogenesis, and establish diagnostic criteria, among other activities.

HWP-IWA is different from conventional wheat allergy. In contrast to the onset of conventional wheat allergy in children, HWP-related allergy arise in adults with a history of cosmetic use.[7] Although both conventional and HWP-related wheat allergy in adults can cause wheat-dependent exercise-induced anaphylaxis (WDEIA), unlike conventional WDEIA (CO-WDEIA), HWP-IWA is not mediated by ω-5 gliadin.[8]

Currently, the skin prick test (SPT) by GP19S is considered the most useful method in diagnosing HWP-IWA.[7] It would be advantageous to develop a quantitative, high-throughput method for the laboratory diagnosis of HWP-IWA that gives consistent results across different institutions. For this purpose, we evaluated the utility of GP19S-specific IgE antibody detection by enzyme-linked immunosorbent assay (ELISA) for the diagnosis of HWP-IWA using sera from healthy individuals and from patients with CO-WDEIA or HWP-IWA. Five institutions were involved in this study to assess the reliability of this method.

METHODS

SUBJECTS

The HWP-IWA group consisted of 24 patients diagnosed with immediate-type wheat allergy induced by GP19S according to the diagnostic criteria by Special Committee for the Safety of Protein Hydrolysates in Cosmetics; CO-WDEIA group consisted of 10 patients with conventional WDEIA; and the healthy control group consisted of 10 individuals without wheat allergy (Table 1, 2). HWP-IWA patient 1 to 14, CO-WDEIA patient and healthy control 1 to 5 were used for utility evaluation of ELISA-based GP19S-specific IgE assay. HWP-IWA patient 15 to 24, CO-WDEIA patient and healthy control 6 to 10 were used for validation this method. Patients 1 to 14 in the HWP-IWA group were classified into the following four grades of severity based on symptoms after wheat ingestion: grade 1, eyelid swelling, symptoms limited to the face and nasal mucosa; grade 2, generalized urticaria in addition to grade 1 symptoms; grade 3, systemic symptoms (e.g. dyspnea, diarrhea) in addition to dermal swelling; and grade 4, anaphylactic shock. The presence of specific serum IgE was determined using ImmunoCAP (Thermo Fisher Scientific, Phadia AB, Uppsala, Sweden). Sensitivity to GP19S was evaluated using SPT.

ELISA-BASED GP19S-SPECIFIC IgE ASSAY

GP19S (Katayama Chemical Industries) at 1 mg/ml dissolved in phosphate-buffered saline (PBS) was centrifuged and the supernatant was recovered (GP19S solution). Next, 100 µl of GP19S solution was added to each well of a Nunc MaxiSorp flat bottom 96-well plate (Thermo Fisher Scientific, Waltham, MA, USA), and the plate was sealed and left overnight at 4°C. The plate was blocked with 1% skim milk/PBS with 0.1% Tween 20 (PBS-T) for 1 hour at room temperature, after which 100 µl patients' sera diluted to 20% in 1% skim milk/PBS-T were added to the wells, followed by a further incubation for 1 hour at room temperature. The plate was then washed with 1% skim milk/PBS-T. A total of 100 µl of 0.1 µg/ml anti-human IgE-HRP conjugate (KPL, Gaithersburg, MD, USA) in 1% skim milk/PBS-T was added to the wells, and the plate was incubated for 1 hour at room temperature. The plate was washed, and the colorimetric reaction was developed by adding 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific) and incubated for 15 min at room temperature. The reaction was stopped by adding 2 M H_2SO_4. Absorbance was measured by multi-plate optic densitometories, VersaMax (Molecular Devices, Sunnyvale, CA, USA), with a wavelength of 450 nm.

CONVERSION OF ABSORBANCE INTO "UNIT" VALUES

Serum taken from HWP-IWA patient 5 was chosen as the standard. Serial dilution was performed using 1% skim milk/PBS-T, starting at 40 times dilution, with subsequent doubling of the dilution factor up to 5120 times dilution. The GP19S-specific IgE ELISA was performed as described above. To create a curve for the conversion of absorbance values to "unit" values, the absorbance of the 40 times-diluted serum was defined as that corresponding to 100.0 units, with the absorbance of the 80 times-diluted serum as 50.0 units, and that of the 640 times-diluted serum as 6.3 units, and so forth, such that the absorbance at each dilution factor corresponds to a "unit" value. For each

Table 1 Clinical characteristics of the patients with HWP-IWA

ID	Age	Sex	Past allergic history	Severity	CAP-FEIA						GP19S Skin prick test
					wheat-sIgE		gluten-sIgE		ω-5 gliadin-sIgE		Threshold for positive prick reaction (%)
					(UA/mL)	(Class)	(UA/mL)	(Class)	(UA/mL)	(Class)	
HWP-IWA 1	47	F	Pollinosis	4	4.52	3	7.16	3	0.34>	0	0.001
HWP-IWA 2	38	F	Pollinosis, Graves disease	4	4.1	3	4.71	3	0.34>	0	0.0001
HWP-IWA 3	43	F	Non	4	2.21	2	1.94	2	0.34>	0	0.01
HWP-IWA 4	18	F	Atopic dermatitis, Asthma, Pollinosis	4	2.28	2	5.37	3	0.71	2	0.01
HWP-IWA 5	45	F	Atopic dermatitis (Childhood), Rhinitis, Pollinosis	4	25.1	4	57.3	5	0.68	1	0.001
HWP-IWA 6	61	F	Pollinosis	3	0.72	2	0.98	2	0.34>	0	0.001
HWP-IWA 7	62	F	Non	4	4.44	3	6.41	3	0.34>	0	0.001
HWP-IWA 8	33	F	Pollinosis	3	<0.35	0	<0.35	0	0.34>	0	0.01
HWP-IWA 9	44	F	Non	3	0.35	1	0.73	2	0.34>	0	0.001
HWP-IWA 10	49	F	Rhinitis	2	1.08	2	1.53	2	0.34>	0	0.001
HWP-IWA 11	43	F	Non	3	3.6	3	7.89	3	1.29	2	0.001
HWP-IWA 12	37	F	Non	2	0.67	1	1.41	2	0.34>	0	0.001
HWP-IWA 13	63	F	Contact dermatitis	1	<0.35	0	0.56	1	0.34>	0	0.01
HWP-IWA 14	30	F	Rhinitis, Pollinosis, Metal allergy	1	0.45	1	0.75	2	0.34>	0	0.10

sIgE, specific IgE.

Severity: 1: eyelid swelling, symptoms limited to face and nasal mucosa; 2: generalized urticaria besides Stage 1 symptoms; 3: general symptoms in addition to dermal disorders (diarrhea, dyspnea, etc.); 4: anaphylactic shock.

GP19S Skin prick test : GP19S was diluted to 100 μg/ml in sterile physiologic saline (PS) and then made into solutions at concentration from 0.00001% to 0.1%. Reactions were read at 15 min, a wheal at least half the size of that caused by histamine dihydrochloride (10 mg/ml) or 3 mm was considered a positive reaction.

serum, a "unit" value was obtained from the measured absorbance with 5 times-diluted serum samples according to this curve.

CORRELATION BETWEEN LABORATORY VALUES AND CLINICAL SEVERITY

Pearson's correlation coefficient (Pearson's product-moment correlation coefficient, represented by the letter r), between grades of severity and the following test values were calculated: wheat-specific IgE (UA/mL), gluten-specific IgE (UA/mL), ω-5 gliadin-specific IgE (UA/mL), GP19S SPT positive concentration (%), and GP19S-specific IgE (unit).

VALIDATION STUDY OF THE ELISA-BASED GP19S-SPECIFIC IgE ASSAY AT FIVE INSTITUTIONS

In order to validate and determine the inter-laboratory reproducibility of the ELISA-based GP19S-specific IgE Assay, the method was performed at five institutions affiliated with members of the Special Committee for the Safety of Protein Hydrolysates in Cosmetics. A manual was complied and distributed prior to the study to ensure common understanding of the technique and to allow discussion of uncertainties. All participating institutions used the same reagents and consumables that were prepared by members of Fujita Health University School of Medicine. The microplate reader for absorbance detection and other laboratory equipment were prepared by each institution. ELISA was performed using sera from 10 HWP-IWA and five CO-WDEIA patients, as well as five healthy subjects. Each sample was tested in duplicates to obtain absorbance and unit values. The absorbance and unit values obtained by all five institutions were examined to determine the validity of the test conditions. The standard deviation (SD) of absorbance and unit values was calculated to assess inter-laboratory reproducibility.

ETHICAL CONSIDERATION

This study was approved by the Ethics Committee of Fujita Health University (No. 11-210). Venous blood samples were collected with patients' informed consent.

RESULTS

ELISA-BASED GP19S-SPECIFIC IgE ASSAY

The range of measured absorbance was 0.01-0.09 optic density (OD) (mean, 0.04 OD) for the healthy control group (n = 5), 0.00-0.11 OD (mean, 0.05 OD) for the CO-WDEIA group (n = 5), and 0.21-3.92 OD

Table 2 Laboratory findings of the sera from patients and controls in the validation study

ID	Total IgE	CAP-FEIA						GP19S Skin prick test
		wheat-sIgE		gluten-sIgE		ω-5 gliadin-sIgE		
	(U/ml)	(UA/mL)	(Class)	(UA/mL)	(Class)	(UA/mL)	(Class)	
Healthy 6	8.16	0.34>	0	0.34>	0	0.34>	0	Nagative
Healthy 7	138	0.34>	0	0.34>	0	0.34>	0	Nagative
Healthy 8	NT	NT	NT	NT	NT	NT	NT	NT
Healthy 9	NT	NT	NT	NT	NT	NT	NT	NT
Healthy 10	NT	NT	NT	NT	NT	NT	NT	NT
CO-WDEIA 6	NT	0.57	1	2.65	2	0.34>	0	Nagative
CO-WDEIA 7	NT	2.37	2	1.19	2	9.73	3	Nagative
CO-WDEIA 8	148	0.46	1	2.51	2	13.3	3	Nagative
CO-WDEIA 9	NT	3.36	3	1.48	2	NT	NT	Nagative
CO-WDEIA 10	NT	0.55	1	3.85	3	9.39	3	Nagative
HWP-IWA 15	3650	8.91	3	NT	NT	NT	NT	Positive
HWP-IWA 16	36	0.34>	0	0.39	1	0.34>	0	Positive
HWP-IWA 17	101	0.85	2	2.91	2	0.34>	0	NT
HWP-IWA 18	285	0.77	2	1.84	2	0.34>	0	Positive
HWP-IWA 19	82	4.25	3	7.18	3	0.34>	0	Positive
HWP-IWA 20	738	13.1	3	24.3	4	0.34>	0	Positive
HWP-IWA 21	148	4.44	3	6.41	3	0.34>	0	Positive
HWP-IWA 22	442	0.54	1	1.23	2	0.34>	0	Positive
HWP-IWA 23	2343	3.55	3	4.27	3	0.34>	0	Positive
HWP-IWA 24	67	0.4	1	0.6	1	0.34>	0	Positive

NT, Not tested; sIgE, specific IgE.

Skin prick test: Reactions were read at 15 min, a wheal at least half the size of that caused by histamine dihydrochloride (10 mg/ml) or 3 mm was considered a positive reaction.

(mean, 2.10 OD) for the HWP-IWA group (n = 14). The absorbance values of the healthy and CO-WDEIA groups were relatively low and no marked difference was observed between the two groups. On the other hand, a wide range of absorbance values were observed in the HWP-IWA group, and were markedly different from those in the healthy and CO-WDEIA groups (Table 3).

CONVERSION OF ABSORBANCE INTO "UNIT" VALUES

Serial dilution of the serum sample resulted in a decrease in the measured absorbance, producing a standard curve. After assigning arbitrary "unit" values to the measured absorbance at each dilution factor, the following unit values were obtained from the measured absorbance: a range of 0.2-2.1 (mean, 1.1) for the healthy group, 0.0-2.5 (mean, 1.3) for the CO-WDEIA group, and 5.2-115.5 (mean, 59.5) for the HWP-IWA group (Table 3).

CORRELATION BETWEEN LABORATORY VALUES AND CLINICAL SEVERITY

The correlation coefficients of clinical severity and wheat-, gluten- and ω-5 gliadin-specific IgE antibodies were 0.43, 0.36 and 0.24, respectively. The correlation coefficient of clinical severity and GP19S SPT positive concentration was -0.53, which is high enough for quantitative diagnosis of HWP-IWA, but not high enough to indicate a correlation with severity. The correlation coefficient of severity and GP19S-specific IgE was 0.76, which was higher than all other parameters (Table 4).

VALIDATION STUDY OF ELISA-BASED GP19S-SPECIFIC IgE ASSAY AT FIVE INSTITUTIONS

The results for GP19S-specific IgE were obtained by each institution. Notably, the absorbance and unit values were low in the healthy and CO-WDEIA groups but high in the HWP-IWA group across all institutions. All samples were tested in duplicates and similar absorbance and unit values were obtained. The respective SD for absorbance and unit values were 0.02-0.05 OD and 0.3-0.4 in the healthy group, and 0.03-0.04 OD and 0.2-0.6 in the CO-WDEIA group. In the HWP-IWA group, the SD ranged from 0.19-0.31 OD and 3.8-9.6 for HWP-IWA 16-24, and it was as high as 0.93 OD and 53.3 for HWP-IWA 15. It was observed that the SD became higher as the GP19S-specific IgE level increased. We consider that a high inter-laboratory reproducibility is achieved only when the "unit" value is below 100.0 (Table 5).

Table 3 Results of the ELISA-based GP19S-specific IgE assay

ID	Absorbance	Unit
Healthy 1	0.01	0.2
Healthy 2	0.01	0.2
Healthy 3	0.05	1.2
Healthy 4	0.07	1.6
Healthy 5	0.09	2.1
CO-WDEIA 1	0.11	2.5
CO-WDEIA 2	0.06	1.4
CO-WDEIA 3	0.00	0.0
CO-WDEIA 4	0.03	0.7
CO-WDEIA 5	0.07	1.7
HWP-IWA 1	3.92	115.5
HWP-IWA 2	3.89	114.5
HWP-IWA 3	2.60	71.3
HWP-IWA 4	3.89	114.5
HWP-IWA 5	3.89	114.5
HWP-IWA 6	3.60	104.0
HWP-IWA 7	2.54	69.7
HWP-IWA 8	0.28	6.9
HWP-IWA 9	0.43	10.8
HWP-IWA 10	1.49	39.0
HWP-IWA 11	1.20	30.9
HWP-IWA 12	1.10	28.3
HWP-IWA 13	0.35	8.7
HWP-IWA 14	0.21	5.2

Absorbance, absorbance at 450 nm.

Table 4 Correlation between clinical severity and laboratory findings

	r	*p*
wheat-specific IgE (UA/mL)	0.43	0.12
gluten-specific IgE (UA/mL)	0.36	0.20
ω-5 gliadin-specific IgE (UA/mL)	0.24	0.41
GP19S SPT positive concentration (%)	-0.53	0.052
GP19S-specific IgE (unit)	0.76	0.0015

Positive concentration, threshold for positive prick reaction.

Correlation coefficient *r* were calculated by Pearson's product-moment correlation coefficient.

Table 5 Combined ELISA results from the five institutions

ID	Absorbance		Unit	
	Mean	SD	Mean	SD
Healthy 6	0.08	0.05	0.8	0.4
Healthy 7	0.07	0.04	0.7	0.3
Healthy 8	0.07	0.03	0.6	0.3
Healthy 9	0.08	0.02	0.8	0.3
Healthy 10	0.08	0.05	0.8	0.3
CO-WDEIA 6	0.11	0.03	1.5	0.5
CO-WDEIA 7	0.10	0.03	1.3	0.5
CO-WDEIA 8	0.08	0.03	0.8	0.2
CO-WDEIA 9	0.08	0.03	0.9	0.2
CO-WDEIA 10	0.10	0.04	1.2	0.6
HWP-IWA 15	4.36	0.93	154.1	53.3
HWP-IWA 16	3.22	0.31	97.0	9.2
HWP-IWA 17	3.06	0.23	90.1	9.6
HWP-IWA 18	3.10	0.19	92.0	8.8
HWP-IWA 19	2.60	0.28	72.0	5.9
HWP-IWA 20	2.59	0.30	71.8	5.7
HWP-IWA 21	2.38	0.24	63.6	6.1
HWP-IWA 22	1.55	0.25	36.7	3.8
HWP-IWA 23	1.73	0.24	42.3	3.9
HWP-IWA 24	1.32	0.24	30.1	4.5

Absorbance, absorbance at 450 nm.

DISCUSSION

In Japan, approximately 4.7 million people bought 46.7 million cakes of "Cha no Shizuku" soap that contained GP19S. According to an epidemiological study released online by the Japanese Society of Allergology on 20 November 2013, there were 2026 cases of HWP-IWA, of which 95.9% were females mainly in their 40s. About half of these cases experienced anaphylactic symptoms, of which half experienced anaphylactic shock. Many of these patients developed WDEIA suddenly, manifesting as eyelid edema after eating wheat-containing food, even though no symptoms appeared while using the soap.[7] This phenomenon was also noted in patients in our study (Table 1). Unsuspectingly, patients with oral wheat allergy continued to use the offending soap, highlighting the possibility of a large number of patients who are unaware of their condition.

As a result of sensitization to GP19S contained in the soap, the produced IgE cross-reacts with orally ingested wheat protein.[8] SPT using GP19S is considered a fast and sensitive method for the diagnosis of HWP-IWA; the condition is ruled out if the SPT using 0.1% GP19S solution is negative. However, some patients decline SPT, which causes discomfort and can induce a severe allergic reaction. In addition to SPT, the Special Committee for the Safety of Protein Hydrolysates in Cosmetics recommends other immunological methods such as dot blotting, ELISA, Western blotting (patient is considered HWP-IWA-positive if GP19S-specific IgE is detected in the blood), or basophil activation test that uses GP19S as the antigen (a positive result suggests HWP-IWA).

Reports exist regarding the diagnosis of wheat allergy using various immunological methods. Western blotting for GP19S using serum IgE antibody has been employed at many institutions.[1,2,8] Using patients' basophils, Hiragun *et al.* conducted the histamine release test[9] and Chinuki *et al.* performed the

CD203c expression-based basophil activation test.[10] Nakamura *et al.* described the EXiLE (IgE Cross linking-induced Luciferase Expression) method, which uses a rat mast cell line expressing human IgE antibody receptors.[11] Though useful, the abovementioned methods have drawbacks. Quantitative evaluation is difficult in Western blotting. Basophil-based tests and the EXiLE method are not widely available, and samples cannot be preserved in the former.

Continuing investigation into HWP-IWA will be required to assess the incidence, natural clinical course, allergenicity, treatment, and appropriate patient education. We believe that the most important issue was the development of a quantitative, high-throughput, GP19S-specific IgE diagnostic test that can provide consistent results at any institution. For these reasons, we tested an ELISA-based assay that would satisfy these conditions.

First, we compared the results of GP19S-specific IgE measurement by ELISA between healthy controls (five subjects), CO-WDEIA (five patients) and HWP-IWA patients (14 patients). Under the described test conditions, the measured absorbance was high in the HWP-IWA group but low in the healthy and CO-WDEIA groups, suggesting that ELISA is effective for the diagnosis of HWP-IWA (Table 3). Using the absorbance results from one patient's serum sample (HWP-IWA 5), "unit" values were assigned to absorbance values in order to improve inter-test and inter-laboratory reproducibility. Using one patient's serum as a standard is disadvantageous, as the created standard cannot be reproduced at other institutions. Therefore, dilution series using sera from other HWP-IWA patients were performed to determine whether the resulting curves are comparable to that of HWP-IWA 5; similar curves were obtained in each case (data not shown). This suggests that it is unnecessary to use the sample of HWP-IWA 5 as the standard if the relative concentration of antibodies against GP19S to HWP-IWA 5 can be determined.

The ELISA results differed widely among HWP-IWA patients (1 to 14); therefore, we examined the correlation between clinical severity and laboratory values to analyze the nature of these differences. GP19S-specific IgE had a higher degree of correlation with severity than gluten and wheat (Table 4), suggesting that it may be predictive of symptoms experienced by HWP-IWA patients. We believe that such data will be useful in gauging the effect of treatment and for patient education. The ELISA-based GP 19S-specific IgE assay provides quantitative results that are meaningful in predicting disease severity.

In the validation study conducted at five different institutions, repeat tests demonstrated reproducible results, with the HWP-IWA group consistently showing higher values. This suggests that the test conditions were appropriate. However, it was noted that one sample (HWP-IWA 15) had a high SD of 53.3, which was different from the SD of the HWP-IWA (<9.6), CO-WDEIA (<0.6) and healthy groups (<0.4) (Table 5). The absorbance of HWP-IWA 15 sample exceeded the maximum measurable limit of the microplate reader at each institution, thus causing the large SD. To ensure inter-laboratory reproducibility, we practically set the maximum "unit" value at 100.0.

A total of 10 healthy subjects, 10 CO-WDEIA and 24 HWP-IWA patients were examined in the present study. The respective maximum unit values in the healthy and CO-WDEIA groups were 2.1 and 2.5 units, while the minimum unit value in the HWP-IWA group was 5.2 units. Therefore, taking between 2.5 to 5.2 the cut-off value, the diagnostic criterion was set as follows: <3.0, negative; 3.0-5.0, suspected; >5.0, positive. Results based on this criterion were consistent with available SPT results. Cases (HWP-IWA 8, 16 and 24) with negative ImmunoCAP results (class 2 and above were considered positive) were positive based on this criterion. These observations suggest that our ELISA-based GP19S-specific IgE assay is a sensitive diagnostic method.

Our findings suggest that the ELISA-based GP19S-specific IgE assay is a useful quantitative and high-throughput method for the diagnosis of HWP-IWA. This method enables the examination and diagnosis of cases nationwide using a laboratory-based, measurable criterion. Hiragun *et al.* studied wheat- and gluten-specific IgE antibodies with CAP-FEIA, and glutenin-specific IgE with histamine-release test, concluding that HWP-IWA may get better over time.[9] We believe that the results of the ELISA-based GP19S-specific IgE assay accurately reflect the clinical situation. We envisage that this method will be useful not only for patients and doctors, but also for medical researchers and cosmetics makers. Many challenges remain regarding this disease, but we expect our test method to advance the diagnosis of this condition.

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COMMITTEE REPORT

Guide for medical professionals (i.e. dermatologists) for the management of Rhododenol-induced leukoderma

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ABSTRACT

Because some users develop depigmentation after the use of melanogenesis-inhibiting products containing the quasi-drug ingredient Rhododenol, Japanese Dermatological Association (JDA) established a Special Committee on the Safety of Cosmetics Containing Rhododenol on July 17, 2013 and management guide for dermatologists has been updated on the website in order to delineate the diagnostic criteria for Rhododenol-induced leukoderma and provides a broad guide for standard treatment based on current knowledge. This guide is produced on the basis of the guide (version 7) updated on June 20, 2014 in the website. Rhododenol-induced leukoderma refers to depigmentation of varying severity that develops after the use of cosmetics containing Rhododenol, mainly at the site of use. In most cases, repigmentation of part or all the affected area is evident after discontinuation. Histopathologically cellular infiltration around the hair follicles and melanophages are present in most cases. The number of melanocytes in the lesion is declined but not totally absent in most cases. Rhododenol itself is a good substrate for tyrosinase, resulting in the formation of Rhododenol metabolites (e.g., Rhododenol quinone). Melanocytes are damaged by Rhododenol metabolites during the subsequent metabolic process. The continued use of cosmetics containing Rhododenol thus induces tyrosinase activity-dependent cytotoxicity in melanocytes in the epidermis at application sites, resulting in decreasing the amount of melanin produced by melanocytes; the addition of some other factor to this process is believed to subsequently cause the decrease or disappearance of melanocytes themselves from the epidermis.

Key words: **cosmetics, leukoderma, Rhododenol, skin-lightening agent, ultraviolet light irradiation.**

INTRODUCTION

Because not a few users developed depigmentation after the use of melanogenesis-inhibiting products manufactured and sold by Kanebo Cosmetics (Tokyo, Japan), Lissage (Tokyo, Japan, merged into Kanebo Cosmetics Inc. as of January 1, 2014) and L'Equipe (Tokyo, Japan) that contain the quasi-drug ingredient Rhododenol (a brand name of rhododendrol, 4-[4-hydroxyphenyl]-2-butanol), a voluntary recall of cosmetics containing Rhododenol was launched on 4 July 2013.

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A large number of the patients have consulted dermatologists throughout Japan, but many issues regarding Rhododenol-induced leukoderma remain unclear, including the causal relationship between Rhododenol and clinical symptoms as well as its clinical forms, incidence, prognosis and pathogenesis. Thus, dermatologists are struggling to deal with these issues in clinical practice.

In accordance with its position of responsibility, the Japanese Dermatological Association (JDA) established a Special Committee on the Safety of Cosmetics Containing Rhododenol on 17 July 2013 in order to survey the actual situation of patients, provide correct information for medical professionals (i.e. dermatologists) and patients, elucidate the pathogenesis of this phenomenon, and urgently establish diagnostic and treatment methods; this committee has already initiated its activities.

First, information about the features of the cases collected so far including laboratory data were provided for doctors who are members of the JDA. Given the inadequacy of currently available clinical information, we are currently aggregating the cases in order to provide necessary and update information for use in clinical management. The guide herein only constitutes preliminary information, and it should not be regarded as drawing any conclusions.

This guide (version 7) was produced on 20 June 2014. In addition to data from the preliminary questionnaires sent by dermatologists to the Special Committee, information from emails and other sources, and survey results provided by Kanebo Cosmetics, this guide also includes the results of the nationwide secondary questionnaire. Moreover, it describes the currently emerging knowledge of the pathogenesis of this condition and provides new information on matters such as treatment policy. Thus, we hope it will be a useful reference for management.

STATUS OF THIS MANAGEMENT GUIDE

The Special Committee on the Safety of Cosmetics Containing Rhododenol, which was established by the JDA on 17 July 2013, immediately began deliberations involving its members and collaborating researchers in committee meetings and by discussions through emails. The resultant management guide delineates the diagnostic criteria for Rhododenol-induced leukoderma and provides a broad guide for standard treatment based on current knowledge.

DISCLAIMER

This management guide summarizes the opinions Special Committee members based on the data available at the time of writing; its content may be revised without warning in the future in accordance with new research findings. Doctors engaged in treatment may deviate from this management guide in accordance with the conditions of individual patients; in fact, such deviations may be preferable. Therefore, we are unable to accept any liability for negligence arising solely from the fact that doctors engaged in treatment have complied with this management guide; furthermore, any deviation from this management guide should not necessarily be regarded as negligence.

Q1. What symptoms have been reported?

A1. Disease concept: Rhododenol-induced leukoderma

What is Rhododenol-induced leukoderma?

Rhododenol-induced leukoderma refers to depigmentation of varying severity that develops after the use of cosmetics containing Rhododenol, mainly at the site of use. In most cases, repigmentation of part or all the affected area is evident after discontinuation.

Diagnostic criteria

Essential criteria

1. History of the use of cosmetics containing Rhododenol.

Note: This was determined on the basis of evidence from patient reports, purchase history and collection records.

2. No history of depigmentation prior to the use of cosmetics containing Rhododenol and the presence of complete or incomplete depigmentation that is mostly coincident with sites of use.

Minor criteria

1. Depigmented areas (item 2 of the essential criteria) stopped increasing in size within approximately 1 month of discontinuation.
2. Pigment in at least part of the depigmented areas (item 2 of the essential criteria) regenerated after discontinuation.

Note: This was determined on the basis of visual inspection by doctors using severity assessment criteria with reference to records including photographs, medical records and dermoscopic examination.

Reference criteria

1. Inflammatory symptoms such as erythema may be apparent prior to the appearance of depigmentation.
2. Depigmentation may not be of uniform severity and is often uneven with irregular margins.
3. It tends to occur if multiple cosmetics containing Rhododenol have been used together and at sites of repeated application.

Diagnoses by exclusion

Leukoderma due to the following must be excluded: Vogt–Koyanagi–Harada disease, Sutton nevus, infection-associated leukoderma (e.g. pityriasis versicolor, syphilis, leprosy and HIV), pityriasis simplex, leukoderma senilis, vitiligo vulgaris, postinflammatory depigmentation due to other causes, drug-induced melanoleukoderma, occupational leukoderma, piebaldism, Waardenburg syndrome and congenital pigmentary disorders such as tuberous sclerosis.

Assessment

Confirmed cases exhibit both items of the essential criteria and at least one minor criterion.

Patients who exhibit both items of the essential criteria but do not meet either items 1 or 2 of the minor criteria are regarded as suspected cases at that point.

Suspected cases should undergo continued careful follow up to watch for repigmentation.

Note: In rare cases, patients may be complicated with vitiligo vulgaris or have vitiligo vulgaris triggered by Rhododenol-induced leukoderma. It may be difficult to distinguish between vitiligo vulgaris and Rhododenol-induced leukoderma on the basis of clinical characteristics and histopathological findings. Thus, the diagnosis must be meticulous.

Specific example 1 (patients with prior vitiligo vulgaris and concomitant Rhododenol-induced leukoderma): Patients who exhibited depigmentation diagnosable as vitiligo vulgaris prior to the use of cosmetics containing Rhododenol, but in whom depigmentation appeared and spread mainly at locations where cosmetics containing Rhododenol had been used and repigmentation of these areas occurred when the use of these cosmetics was discontinued should be regarded as patients with vitiligo vulgaris complicated by Rhododenol-induced leukoderma.

Specific example 2 (patients with Rhododenol-induced leukoderma and concomitant vitiligo vulgaris): Patients who have developed depigmentation at locations where cosmetics containing Rhododenol have been used, and either these areas had stopped expanding after the use of these cosmetics was discontinued or the depigmentation had partially improved, but depigmentation had spread at locations where these cosmetics had not been used should be regarded as patients with Rhododenol-induced leukoderma complicated by vitiligo vulgaris.

The diagnosis of such patients must be based on the judgment of the individual attending physician in accordance with factors including the patient's clinical presentation, use of cosmetics concerned, timing of discontinuation and course of subsequent depigmentation.

Clinical classification

(1) Predominantly complete leukoderma.

Solely or predominantly complete leukoderma (i.e. complete leukoderma accounting for at least 60% of the total area of depigmentation).

(2) Mixed complete and incomplete leukoderma.

Mixed complete and incomplete leukoderma in approximately equal proportions.

(3) Predominantly incomplete leukoderma.

Solely or predominantly incomplete leukoderma (i.e. incomplete leukoderma accounting for at least 60% of the total area of depigmentation).

Characteristics of clinical and prodromal symptoms

1. Incomplete leukoderma distributed on the face, neck, backs of the hands, and forearms between 2 months and 3 years after the use of cosmetics containing Rhododenol. The depigmentation is mottled and varies in severity. It may be mild and with poorly demarcated margins, meaning it is not obvious at first observation but is discernible on close examination. However, this may progress to well-demarcated complete leukoderma in some patients. Even if it is difficult to clearly determine if pigmentation is complete or incomplete, dermoscopic examination can often reveal if the hairs in depigmented areas are colored.
2. Itchy erythema may be present in locations where these cosmetics have been applied. Some patients may develop depigmentation following inflammation, while others exhibit inflammatory vitiligo with inflammation at the boundaries between areas of depigmentation and healthy skin. Meanwhile, some patients exhibit no inflammation at all. Regardless of the development of inflammation, patients may react positively to Rhododenol in patch tests. However, patients having leukoderma associated with inflammation tend to have a higher positive rate on patch tests.
3. Repigmentation mostly occurs approximately 6 months after the use of cosmetics containing Rhododenol has been discontinued. Patients may recover from complete leukoderma via incomplete leukoderma, and follicular punctate repigmentation may also occur.
4. Temporary excess repigmentation may occur during the recovery process in some cases; such transient excess pigmentation is usually improved.
5. A large majority of patients are women, but some men have also developed this condition after using cosmetics containing Rhododenol on the advice of family members.

Definitions

1. Complete leukoderma: Whitish depigmentation with near complete or complete absence of melanin pigment and loss of normal color.
2. Incomplete leukoderma: Depigmentation with a decrease in melanin and a comparatively whitish appearance compared with normal color but not amounting to a total loss of normal color.

Note: these conditions are distinguished on the basis of visual examination and may be mixed, continuous or vary over time.

3. Inflammatory vitiligo: Depigmentation with erythema or infiltration along the rim of the vitiligo.

Histopathology

We are currently investigating the results of biopsies mainly from patients at institutions with which members of the Special Committee are affiliated. The histopathology is as diverse as the clinical profile, with melanocytes having disappeared in some patients and decreased in others, and some patients exhibiting inflammatory cell infiltration and others with only scattered melanophages in the superficial dermis. The following points may be useful for distinguishing from vitiligo vulgaris: (i) cellular infiltration around the hair follicles; and (ii) melanophages are present in almost all patients. At this point, if melanophages are not evident, it can be surmised that there is a high possibility the patient has vitiligo vulgaris. Most patients with vitiligo vulgaris completely lose all melanocytes in areas of complete leukoderma, whereas in this particular condition, this was the case for only a few patients in areas of clinical complete leukoderma, even if the number of melanocytes in those locations had declined; their presence was observed in some parts of specimens, including hair follicles.

Prognosis

The secondary questionnaire carried out in January 2014 indicated that 1339 cases were "under observation". Of these,

depigmentation had virtually disappeared in 7%, decreased to less than one-quarter of its initial area in 11%, decreased to between one-quarter and one-half of its initial size in 16%, remained more than half its initial size in 38%, remained unchanged in 25% and increased in size in 2% (n = 26); the outcome was unknown in 0.9% and 0.1% (n = 1) could not be evaluated.

Q2. Which cosmetics are covered by this guide?

A2. Melanogenesis-inhibiting products containing the quasi-drug ingredient Rhododenol (4-[4-hydroxyphenyl]-2-butanol). It is suspected that some people who have used cosmetics for inhibiting melanogenesis may develop an overinhibitory reaction or inflammation.
The products concerned are melanogenesis inhibitors manufactured and sold by Kanebo Cosmetics, Lissage and L'Equipe that contain the quasi-drug ingredient Rhododenol. A list of product names is available on the following websites:
Ministry of Health, Labor and Welfare website: http://www.mhlw.go.jp/stf/houdou/2r98520000035xv0.html.
Kanebo Cosmetics website: http://www.kanebo-cosmetics.jp/information/#products_name.

Q3. What sort of substance is Rhododenol (4-(4-hydroxyphenyl)-2-butanol)?

A3. (1) Which products contain Rhododenol?
Rhododenol is a substance that inhibits melanogenesis that was developed in-house by Kanebo Cosmetics. As a substance with a "whitening effect", it has been incorporated into numerous products produced by Kanebo Cosmetics that promise to whiten skin. As it was developed in-house and patented by Kanebo Cosmetics, it is not contained in cosmetic products produced by other manufacturers.

(2) How was it developed?
According to Kanebo Cosmetics, they focused on a substance called 4-(4-hydroxyphenyl)-2-butanol after screening several plant-derived natural substances for melanogenesis-inhibiting effects. Subsequent in-depth research revealed Rhododenol is an extremely potent melanogenesis inhibitor. It was approved in 2008 by the Ministry of Health, Labor and Welfare for its efficacy in preventing skin blotches and freckles by inhibiting melanogenesis.

(3) How does Rhododenol work to whiten skin?
Blotches develop on the skin as a result of excessive melanin deposition. Melanin is synthesized in melanocytes within the skin by an enzyme called tyrosinase, which plays the most important role in melanogenesis. The starting point for the series of reactions whereby melanin is formed is the oxidation of tyrosine by tyrosinase, which is the rate-limiting enzyme for the melanin-synthesis process; if this reaction does not occur, then no melanin is produced at all. Rhododenol binds to the active site of tyrosinase, where ordinary tyrosine should bind, because the chemical structure of Rhododenol is similar to tyrosine. This prevents tyrosine, the substrate for the reaction, from binding to tyrosinase, reducing melanin production. Rhododenol itself is a good substrate for tyrosinase, resulting in the formation of Rhododenol metabolites (e.g. rhododendrol quinone). It is also believed that melanocytes are damaged by rhododendrol quinone and further metabolites during the subsequent metabolic process. The continued use of cosmetics containing Rhododenol thus induces tyrosinase activity-dependent cytotoxicity in melanocytes in the epidermis at application sites, resulting in the formation of large quantities of the metabolites and decreasing the amount of melanin produced by melanocytes; the addition of some other factor to this process is believed to subsequently cause the decrease or disappearance of melanocytes themselves from the epidermis. A mild decline in the capacity for melanogenesis acts to whiten the skin, but a pronounced decrease in melanocytes results in depigmentation. These findings corroborate the previous report of the Special Committee, specifically histopathological tests of affected sites showing a decrease in melanocytes in areas of depigmentation, leading to the conjecture that some sort of melanocyte-damaging action is involved.
According to a study by Kanebo Cosmetics, depigmentation has occurred in approximately 2% of people who have used cosmetics containing Rhododenol. Why some people develop depigmentation whereas others do not remains unclear; we are currently investigating the reason for this.

Q4. How many people are affected?

A4. Kanebo Cosmetics is currently visiting all customers who have reported problems to them and confirming their symptoms. A media announcement by the company on 9 June 2014 states that the confirmed number of people affected stood at 14 612 as of 31 May 2014.
An estimated 800 000 people have used cosmetics containing Rhododenol, meaning that approximately 2% of users have developed depigmentation.
According to the 9 June announcement, of the 14 612 affected individuals, 1613 had developed "obvious leukoderma across a wide area of the face, hands, or elsewhere", and 4649 had either "leukoderma in at least three places", "leukoderma at least 5 cm in size" or "obvious leukoderma on the face", while 8350 exhibited symptoms milder than this. Among all patients, 4297 (29.4%) have recovered either completely (according to either a doctor's diagnosis or the patient's own report) or almost completely. Kanebo Cosmetics has collated cases of patients whose symptoms could be compared between the first, second and subsequent visits. They reported that symptoms improved between the first and second visits in 784 of 942 patients (83.2%) with "obvious leukoderma across a wide area of the face, hands, or elsewhere", and in 1848 of 2002 people (79.7%) with either "leukoderma in at least three places", "leukoderma at least 5 cm in size" or "obvious leukoderma on the face".
According to Kanebo Cosmetics, almost 100% of products containing Rhododenol have been recovered in the voluntary recall.

The JDA Special Committee on the Safety of Cosmetics containing Rhododenol had received primary questionnaires referring to 1338 cases as of 7 September 2013. As such, we would like to thank you for your cooperation.
We have collated the results of secondary questionnaires from institutions with which Special Committee members and collaborators are affiliated with, and we have formulated diagnostic guidelines and a severity-grading sheet based on current knowledge about the pathogenesis of this condition. We hope it will prove useful in clinical management.
It is important that severity is evaluated over time. We request that you keep accurate and detailed medical records as well as photographs, if possible (with the patient's consent).

Q5. A patient has asked me to provide a medical certificate. What should I do?

A5. At this point, a medical certificate is unnecessary for the voluntary product recall or to make a report to Kanebo Cosmetics. Should Kanebo Cosmetics or another organization require a medical certificate in the future, please describe patients as either confirmed or suspected cases of Rhododenol-induced leukoderma with reference to the diagnostic criteria proposed by the Special Committee in section A1 above.
If a patient needs to take time off work for a patch test or any other reason, they may need to submit a medical certificate to their employer. Please provide such medical certificates on your own authority as a doctor. Before a definitive diagnosis has been reached, the reason may be described as "suspected contact dermatitis" or "contact dermatitis" if this has been confirmed by a patch test.

Q6. How can Rhododenol-induced leukoderma be distinguished from vitiligo vulgaris?

A6. Please refer to the vitiligo vulgaris guidelines. Segmental vitiligo can be excluded, as it does not develop bilaterally and co-localize with the site where the cosmetics concerned have been used. It may be difficult to distinguish it from generalized vitiligo. Check whether the depigmentation developed following the use of the cosmetics concerned, whether it occurred in the same areas where they were used, whether it constitutes complete leukoderma clinically and histologically, and if required, investigate whether thyroid dysfunction, connective tissue diseases, diabetes, Addison's disease or hair loss is present, because it may be necessary to exclude these.
According to the results of the secondary questionnaire, pigmentation recovered at the site of use in 72% of patients after they discontinued using the cosmetics concerned. If repigmentation is observed over time, this indicates a high probability of Rhododenol-induced leukoderma.
See the diagnostic criteria given in section A1 above.
Some specialists consider that the possibility of progression to vitiligo vulgaris because of the use of the cosmetics concerned cannot be ruled out. At present, this possibility can be neither confirmed nor denied, because vitiligo vulgaris of generalized form is an autoimmune disease and the mechanism whereby this autoimmunity arises is unknown. When such patients are encountered, it is envisaged that a thorough examination will enable some of these cases to be distinguished from vitiligo vulgaris, while others must be treated as indeterminate. This is a question for future study.

Q7. Is not Rhododenol-induced leukoderma a type of drug-induced photosensitivity?

A7. Some patients taking thiazide antihypertensives have been diagnosed with drug-induced melanoleukoderma, but they exhibited no changes in clinical symptoms despite being instructed to discontinue taking these medications; they were later found to have been using the cosmetics concerned. It normally takes approximately 6 months after discontinuing medication for the melanoleukoderma rash to improve; therefore, careful follow up is important. As it is clinically difficult to differentiate these diseases, and it is also difficult to verify their causal relationship, it is vital to check what medications patients are taking. If one of their medications is a potential cause of melanoleukoderma (Table 1), they should preferably be instructed to discontinue it or to switch to a different drug.

Table 1. Drugs that may induce melanoleukoderma

Thiazide antidiuretics	Hydrochlorothiazide, chlorothiazide
Other antidiuretics	Meticrane
Antibiotics	Tetracycline, fleroxacin, griseofulvin
Muscle relaxants	Afloqualone
Non-steroidal anti-inflammatories	Tiaprofenic acid
Beta-blockers	Pindolol

Q8. Should patients be asked about their history of phenol and phenol compound use in order to distinguish Rhododenol-induced leukoderma from occupational one?

A8. Always ask patients about their occupation and history of exposure. The occurrence of similar depigmentation has been reported in employees of factories handling phenol and phenol compounds as well as hydroquinone; these chemicals are found in adhesives, inks, varnishes, various types of synthetic resin-modifying agents, raw materials for perfumes, insecticides, bactericide, rubber antioxidants, raw materials for vinyl chloride stabilizers, surfactants and other antioxidants, and oil additives.

Q9. Are the patch test and photopatch test necessary?

A9–1. To date, we have been sent the results of patch tests from 199 individuals from 52 institutions, including preliminary tests. After excluding 14 patients for whom only evaluations after 48 h were recorded, meaning that a positive determination could not be made, we analyzed the results of 185 cases.
(1) Of the patients, 13.5% (25/185) reacted positively to 2% Rhododenol in a white petrolatum base.
(2) The positive rate for patients with inflammation was 20.0% (20/100).

(3) The positive rate for patients without inflammation was 6.8% (5/74).
(4) The positive rate for patients for whom the presence of inflammation was unknown was 0% (0/11).
(5) One patient was recorded as "displaying leukoderma" and another as "possibly faintly white" in areas to which 2% Rhododenol in white petrolatum had been applied (to healthy skin) on assessment after 1 week. These two patients were reported by different institutions and accounted for approximately 1% of the total (2/199).

A9–2
(1) We investigated the positive rate from a pilot study of 5% Rhododenol in a white petrolatum base performed before the 2% Rhododenol in a white petrolatum base was distributed nationwide. This was applied to 73 patients; 26 of 54 patients who were negative to 2% Rhododenol were tested for 5% Rhododenol and four (15.4%, 4/26) exhibited a positive reaction.
(2) In the pilot study, we also investigated the photosensitivity of Rhododenol and performed photopatch tests on 52 patients. Only one patient exhibited a new or increased positive reaction after ultraviolet A irradiation; this patient did not constitute a confirmed case of photoallergy.
Patch tests should be performed in the future when possible. We are able to send out 2% Rhododenol in a white petrolatum base. However, please be aware that supplies are limited.
Caution: Before performing this test, patients should be informed that depigmentation may occur at the site of the patch test and their consent should be obtained beforehand.

Notes

Application site: Even if only a few applications are performed, they should be made to an unobtrusive site such as the back rather than the outside of the upper arm. If the test is performed in summer, it should be done in a cool environment or while the patient is hospitalized to avoid sweating. Performing the patch test once the weather has cooled should also be considered.
Timing of assessment: In addition to usual examinations at 48 h, 72 h and 1 week, the application site should also be examined until 1–2 months later to determine if depigmentation has occurred. See Q10 for information on how to obtain the test material for the patch test.

Q10. How can I obtain the test material for the patch test?

A10. We are distributing 2% Rhododenol in a white petrolatum base as a test material for diagnostic patch tests. Institutions that would like to be sent this test material should contact us at the following address. Please be aware that supplies are limited: JDA Administration Team, 4-1-4 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Email: gakkai@dermatol.or.jp; Fax: 03-3812-6790.

Q11. Is a skin biopsy required?

A11. The diversity of clinical symptoms is becoming clearer as the cases of Rhododenol-induced leukoderma will be accumulated. Biopsies that have already been performed show a decline in the number of melanocytes. Some patients who exhibit severe inflammation exhibited a lichenoid reaction. Performing a biopsy may help elucidate unclarified points such as whether or not symptoms vary depending on the stage of the disease and if there is any recovery of depigmentation; in addition, it may provide valuable information for the management of individual patients. Nevertheless, patients must be informed that a biopsy is an invasive procedure and does not always lead to a definitive diagnosis. Therefore, informed consent must be obtained before a biopsy is performed. The choice of harvesting site and immobilization method should be determined by the attending physician's discretion.

Note: Depending on the features of the case concerned, potential harvesting sites may include areas of depigmentation and erythema, healthy areas and areas of pigment deposition. A biopsy should be performed when you consider it essential for diagnosis. As they are covered by health insurance, they are basically performed by the attending physician's discretion.
If undifferentiated cells in hair follicles (i.e. melanocyte stem cells) are maintained and melanocytes in the epidermis, which are differentiated to some extent, have been affected, the color may recover at the area consistent with pores and surrounding the depigmentation. Therefore, some specialists believe biopsies of the depigmented area should include hair follicles. The number of melanocytes can be investigated by hematoxylin–eosin staining of paraffin-embedded slices, which can be combined with immunostaining such as Melan-A.
The Special Committee is undertaking histopathological analysis using special staining. Any useful information gained from this will be published; this will be followed by further studies by individual medical institutions.

Q12. Are blood tests required?

A12. If blood tests are required to distinguish Rhododenol-induced leukoderma from other disorders that cause depigmentation, they should be performed at the attending physician's discretion. At present, there have been a few cases of concomitant thyroid disorder. There is a tendency for patients to test positively for autoantibodies (including antithyroglobulin antibody and anti-thyroid peroxidase antibody), which often exhibit abnormal levels in autoimmune thyroid disorders, even though thyroid dysfunction may not be evident. Concordant with the primary questionnaire, blood tests should be performed at the attending physician's discretion if they are required for diagnosis. Please freeze and store any leftover sera. Should future studies identify any diagnostically valuable autoantibodies or other factors, we will publish this information.

Q13. Will the depigmentation recover?

A13. The nationwide secondary questionnaire carried out in January 2014 showed that 1339 cases were "under observation". Of these, depigmentation had virtually disappeared in 7%, decreased to less than one-quarter of its initial area in

11%, decreased to between one-quarter and one-half of its initial area in 16%, remained more than half its initial size in 38%, was unchanged in 25%, had increased in size in 2% (n = 26), outcome was unknown in 0.9% and could not be evaluated in 0.1% (n = 1).

As this survey was performed 6 months after the media story broke, most patients had discontinued the use of cosmetics containing Rhododenol 6 months prior; however, 118 had already stopped using these cosmetics at least 1 year prior, and in 60% of these patients, the area of depigmentation was reportedly contracting during follow up at medical institutions. At present, most patients have discontinued use less than 1 year ago. Therefore, continued follow up is necessary. A comparison of whether or not patients have undergone treatment showed that the area of depigmentation is decreasing in 77% of the patients who have received some sort of treatment but in 67% of those who are simply being followed up without treatment.

Q14. Should the use of skin-whitening cosmetics be prohibited in future?

A14. As the pathogenesis of this condition remains unexplained, no convincing evidence is currently available. Patients should be informed that it is impossible to completely rule out the risk of the same symptoms appearing from other skin whiteners and their use left to their own discretion. Individual patients should be instructed what to do according to the condition of their own blemishes and depigmentation.

Q15. Are other skin-whitening products safe?

A15. There have been reports of a few cases of the occurrence of similar depigmentation after the use of other skin-whitening cosmetics. As there is currently no available information on the safety of skin-whitening cosmetics with the same mechanism of action, it is impossible to state if they are safe or dangerous. When examining patients, it is always necessary to ask whether they have a history of using other skin-whitening products as well as cosmetics containing Rhododenol. Regardless of the product, resultant skin problems should always be treated individually by a dermatologist. The Ministry of Health, Labor and Welfare has also requested cosmetics companies to investigate whether any cases of similar symptoms of depigmentation due to skin-whitening cosmetics have occurred. See A26.

Q16. How should I treat patients who ask for any remaining blemishes to be treated with hydroquinone?

A16. There have been multiple reports of the simultaneous appearance of symptoms of depigmentation and chloasma, although the causal relationship is presently unknown. Hydroquinone has been used in some of these cases. There have been no reports of depigmentation when hydroquinone is used at a concentration of approximately 5%. Hydroquinone exhibits weaker cytotoxicity than hydroquinone monobenzylether, and its action is reversible. However, if users are exposed to strong ultraviolet light during the day, their blemishes may actually darken. If they use hydroquinone in the morning, they are advised to also use a product containing a sunblock of at least skin protection factor 30 and apply hydroquinone only once in the evening if they are exposed to strong ultraviolet light over long periods or are unable to protect themselves against ultraviolet light. Hydroquinone is a highly effective compound but must be used correctly. Doctors must carefully check that patients are using it correctly; and if patients are unable to use it properly, they should be instructed not to apply it. A study of 52 patients at 13 institutions who simultaneously applied Rhododenol and hydroquinone found that 11 reacted positively to a patch test with 2% Rhododenol in a white petrolatum base but all 11 reacted negatively to 1% hydroquinone in a white petrolatum base. Thus, it appears that no cross-reaction occurs.

Q17. Should patients be instructed to protect themselves from light?

A17. As the pathogenesis of this condition remains unexplained, there is currently no convincing evidence that photoprotection is necessary. There are reports of patients who developing Rhododenol-induced leukoderma when they used the cosmetics concerned after sunburn overseas or noticed it after becoming sunburned, suggesting that there may be some connections among onset, the use of the cosmetics concerned and exposure to ultraviolet light. Although it is unclear how long the effect of these cosmetics persists after their use has been discontinued, in most cases, melanocytes do not disappear entirely even though their number has declined; this suggests that the ability to produce melanin is preserved by the remaining melanocytes. Some patients have also been observed to suffer from transient pigment intensification during repigmentation. In the opinion of specialists, although the color will eventually match, it may be better to use photoprotection to encourage cosmetically satisfactory pigment regeneration without an obvious contrast between depigmented and repigmented areas. As the facial area is vulnerable to photoaging and prone to the development of blemishes, it may be a good idea to advise patients to avoid sunburn. In addition, patients can be advised to use sunscreen while checking that they are not developing contact dermatitis or photocontact dermatitis against sunscreens. It may be safest to perform a patch test or a test application twice daily for 1 week on an area approximately 2 cm in diameter exposed to ultraviolet light.

Q18. What treatments are effective?

A18. The only first-choice treatment is to discontinue the use of the cosmetics concerned, employ thorough photoprotection and monitor the patient's condition without treatment. However, the national secondary questionnaire in January 2014 revealed that 57% of the 1341 patients being followed up were receiving treatment while 43% were not. Improvement was evident in 67% of those who did not undergo treatment compared to 77% of those who did. The types of treatment include oral medication including vitamin C, tranexamic acid, vitamin E and anti-allergy drugs; topical medications including tacrolimus

ointment, topical steroids and topical vitamin D3; and ultraviolet light therapy. The efficacy of topical therapy was compared among patient groups comprising monotherapy with tacrolimus, steroids or vitamin D3. A greater proportion of patients receiving topical tacrolimus monotherapy reported that the depigmented area had decreased to less than half its initial area (assessed as either virtually disappeared, decreased to less than one-quarter its initial area size, decreased to between one-quarter and one-half its initial size, still more than half its initial size, no change or increased in size) compared with those receiving monotherapy with other topical agents and those not undergoing treatment.

Regarding overall assessment (i.e. recovered, greatly improved, improved, somewhat improved, no change or worse) including hyperpigmentation, a greater proportion of patients undergoing monotherapy with steroids was evaluated as "improved" or better.

In May 2014, a reassessment of the type of treatment used and its effectiveness in 59 of the 74 patients reported to be undergoing ultraviolet light treatment showed that 54 of 59 patients who responded regarding its effectiveness stated that it was from somewhat to very effective. Although we have received several opinions that its effect is difficult to distinguish from that of discontinuing the use of the cosmetics concerned, only one patient reported that the area of depigmentation spread as a result of ultraviolet light treatment, two reported no change, and one reported that although it improved, it was not due to the therapy; these findings suggest that this therapy may be worth trying for patients who do not exhibit any improvement. Although some effect was observed, great care is required, because there are also reports that irritation tends to occur, prompting the exposure to be set to a low dose; furthermore, there are reports of treatment discontinuation after hyperpigmentation developed around the area of depigmentation. As some patients improved without treatment, it is difficult to precisely differentiate between the effects of discontinuing cosmetic use and topical agents or ultraviolet light treatment on areas of Rhododenol-induced depigmentation. However, the results of the secondary questionnaire suggest that the routine treatment for vitiligo vulgaris may be effective to some extent. The type of treatment method will also vary depending on the area of application and symptoms as well as the question of whether to use any sort of treatment other than monitoring the patient's course. Ultimately, the attending physician's discretion should be respected.

Q19. Is phototherapy effective?

A19. In May 2014, a reassessment of the types of treatment used and their effectiveness in 59 of the 74 patients who were reported to be undergoing phototherapy (i.e. ultraviolet light treatment) in the nationwide secondary questionnaire revealed that most were being treated with excimer light, narrowband ultraviolet B, VTRAC, or a combination of these once or twice weekly, or depending on the patient's schedule. Moreover, 54 of 59 patients who responded regarding its effectiveness stated that it was from somewhat to very effective. Ten had finished treatment, because the depigmentation was no longer obvious. Four patients discontinued phototherapy because of hyperpigmentation of the irradiated area around the area of depigmentation. This hyperpigmentation was sufficiently severe such that it obviously contrasted with the depigmentation. Although we have received several comments that the effect of ultraviolet light treatment is difficult to distinguish from that of discontinuing cosmetic use, only one patient reported that the area of depigmentation spread as a result of ultraviolet light treatment, two reported no change, and one reported that although it improved, they did not feel it was due to the effect of ultraviolet light. These results suggest that this treatment method may be worth attempting in patients who do not exhibit repigmentation over the long term. However, great care is required, as there have also been reports of a tendency for irritation to occur, prompting the exposure to be set to a low dose. Furthermore, there have been reports in which the treatment was discontinued when hyperpigmentation developed around the area of depigmentation.

Q20. Is topical treatment effective?

A20. As described in A18, the nationwide secondary questionnaire carried out in January 2014 showed that improvement was evident in 77% of those who were undergoing treatment as well as in 67% of those who had been monitored without treatment after discontinuing the use of the cosmetics concerned. The first-choice treatment is to stop using the cosmetics concerned, employ thorough photoprotection and follow up without treatment. However, in severe cases, no repigmentation may be apparent in some patients who are only monitored without treatment; alternatively, if recovery is slow, topical treatment may tend to increase the rate of improvement. Topical agents may be effective for the treatment of patients with delayed repigmentation or severe inflammatory cell infiltration on histological examinations, erythema or suspected contact hypersensitivity to the cosmetics concerned. As described in A18, in the secondary questionnaire, a greater proportion of patients treated with topical tacrolimus reported that the area of depigmentation had decreased, while a greater proportion of patients treated with topical steroids were evaluated as having improved in the overall assessment, including hyperpigmentation.

When using a topical agent, proceed cautiously at your discretion as a specialist dermatologist by applying it to part of the depigmented area and observing whether there is a greater effect there than in the untreated area as well as monitoring for side-effects. Meanwhile, tacrolimus ointment should be applied once in the evening, and patients should be instructed to use sunscreen during the day. Few patients have been treated with topical vitamin D3 ointment; although its effectiveness has not been clearly established, no patient's condition has been reported to have worsened.

Q21. Is vitamin D3 ointment effective?

A21. Few patients have been treated with this agent; although its effectiveness has not been clearly established, no patient's condition has been reported to have worsened as a result. It may be worth trying in patients who have difficulty using tacrolimus or steroid ointment.

Q22. What kind of patient information is required?

A22. Many issues regarding Rhododenol-induced leukoderma remain unclear, including the causal relationship between Rhododenol and clinical symptoms as well as its clinical forms, incidence, prognosis and pathogenesis. Resolving these issues will require gathering information from numerous clinical case studies. Furthermore, the JDA is conducting epidemiological surveys mainly via its Special Committee. First, it is recommended that the dermatologists record the patient's past history and clinical findings according to the primary questionnaire (that can be downloaded from the JDA website). This questionnaire also includes sections for information on tests, treatment, and the patient's course; it is designed so as not to interfere with patient examinations. The deadline for returning the secondary questionnaire passed on 31 January 2014, but a third questionnaire is being planned to be sent out in approximately 1 year. To track the severity of individual cases over time, we would like to request diligence when recording information including the location of depigmentation and its clinical classification in medical records as well as the provision of photographs whenever possible. Please download and use the severity assessment sheet for tracking the severity of depigmentation over time that we have developed.
See Q26 for patients with depigmentation suspected to be due to cosmetics other than Rhododenol.

Q23. What is the JDA planning to do in future?

A23. As an immediate measure for investigating the cause of Rhododenol-induced leukoderma, the JDA set up its Special Committee on the Safety of Cosmetics Containing Rhododenol on 7 July 2013 and appointed Professor Kayoko Matsunaga of the Department of Dermatology of Fujita Health University School of Medicine as its chair. The Special Committee held its first meeting on 19 July 2013 and embarked on activities including survey studies, elucidating the pathogenesis of this condition, diagnosis and treatment, and providing information. A case review meeting was held on 11 August 2013. The second Special Committee meeting was held on 7 September 2013, resulting in the production of version 5 of the management guide. The Special Committee met for the third time on 2 November 2013 and continued its discussions toward the formulation of this management guide. It also implemented a secondary questionnaire for institutions with which members of the Special Committee and collaborating researchers are affiliated; version 6 of this management guide was produced on 12 December 2013 on the basis of the collated results. The fourth, fifth and sixth Special Committee meetings were held on 10 January, 14 March and 31 May 2014, respectively; during these meetings, the committee gathered and considered information from studies designed to help elucidate the pathogenesis of Rhododenol-induced leukoderma. The results of the nationwide secondary questionnaire implemented in January had been collated at this point and were incorporated into version 7 of this management guide, which has been published and uploaded. Further information divided into information for doctors as well as patients and the general public will continue to be published on the JDA website. Version 6 of the management guide has been published in the *Japanese Journal of Dermatology*, and will later be published in the JDA's English-language journal, the *Journal of Dermatology*. The date of publication will be announced on this website once it has been decided.

Q24. Are medical costs covered by health insurance?

A24. Contact dermatitis and depigmentation caused by cosmetics are skin disorders that are covered by health insurance in Japan. Please leave any issues such as corporate compensation for copayments to the negotiations between patients and their employers. There is no need for doctors to get involved.

Q25. Please explain what is known about why this depig mentation occurs?

A25. Rhododenol is believed to be associated with the development of depigmentation. The evidence for this is as follows:
(1) According to a press release by Kanebo Cosmetics, dated 24 November 2013, depigmentation has been confirmed in 16 864 individuals who had used cosmetics containing Rhododenol. A questionnaire of doctors revealed over 1200 patients with evident depigmentation.
(2) Repigmentation of depigmented areas occurs after discontinuing the use of cosmetics containing Rhododenol, and most patients are recovering.
(3) According to a survey by Kanebo Cosmetics, although numerical data have been produced on the basis of inaccurate information and are thus inaccurate themselves, the incidence among patients tended to increase in the order of those who used only toner containing Rhododenol; both toner and lotion; and toner, lotion and cream.
(4) In some patients, depigmentation occurred after itchiness or erythema developed after using cosmetics containing Rhododenol; some of these patients had positive patch test results with the cosmetics concerned and Rhododenol.
(5) It is now known that Rhododenol is metabolized into a cytotoxic substance by tyrosinase in melanocytes (see Q3).
(6) The number of melanocytes in the epidermis decreases in areas of depigmentation.
(7) It was found that the model mice with mimic human skin developed leukoderma similar to Rhododenol-induced leukoderma after the application of Rhododenol.
However, it has not been fully clarified why some people develop leukoderma and some do not. At present, the reason is under investigation by the Special Committee members. The new findings will be informed when we obtain the reliable information in the future.

Q26. I have examined patients who believe that depigmentation may have been caused by other cosmetics. What is the situation regarding this?

A26. There have been a few reports of similar depigmentation occurring because of the use of skin-whitening cosmetics other than those containing Rhododenol. The Ministry of Health, Labor and Welfare is investigating whether any notifications have been received by cosmetics companies from dermatologists. Regarding the diagnostic criteria for a medical institution to provide information to a company, there is a legal obligation to report serious cases (including patients who require treatment for ≥30 days) to companies if the possibility of a side effect of a cosmetic or other product cannot be ruled out; this should be used as a guideline for when to provide information. However, it should be noted that medical institutions are not necessarily obliged to report cases to corporations.

If the manufacturer's contact details are unknown, the matter may be reported directly to the Pharmaceuticals and Medical Devices Agency (PMDA) as a patient safety report; the PMDA may request the company to both provide information and instruct it to gather additional information if necessary. In this process, record the patient's responses to the medical interview and findings on clinical examination in accordance with the "Questionnaire on Cases of Depigmentation due to the Use of Cosmetics Not Containing Rhododenol". This questionnaire also includes sections for information on tests and treatment, and it is designed so as not to interfere with patient examinations. The Special Committee is not engaged in investigating depigmentation that develops after the use of cosmetics that do not contain Rhododenol or the elucidation of its pathogenesis.

Members of the Japanese Society for Dermatoallerology and Contact Dermatitis are gathering information via the Case Information Network of Skin Safety on Cosmetics and Other Products (http://jsac-public.sharepoint.com/). We would be grateful for cooperation with this network when submitting a report to a manufacturer or the PMDA.

The JDA website contains a report from the Ministry of Health, Labor and Welfare on leukoderma and other skin disorders developed on users of topical quasi-medications and cosmetics other than cosmoceuticals containing Rhododenol (https://www.dermatol.or.jp/modules/guideline/index.php?content_id=8). Please see this article for more information.

CONFLICT OF INTEREST: None.

APPENDIX 1

PRIMARY QUESTIONNAIRE (TO BE COMPLETED BY DOCTOR)

Name of Institution

Address of Institution Tel:

Date Completed (mm/dd/yyyy) / /20 Name of Doctor

E-mail:

□Patient attributes

Patient ID: Age: years Sex Female/Male

□Occupational history Current occupation: Housewife/Company employee/Other ( )

Previous exposure to phenol/phenol compounds (Note 1): No/Yes/Unknown

□Family history: *Vitiligo vulgaris*: No/Yes

Other family history: No/Yes ( )

□Previous medical history: Vitiligo vulgaris: No/Yes Hay fever: No/Yes (in which month(s)?)

Hives: No/Yes (caused by:) Allergic dermatitis: No/Yes

Contact dermatitis: No/Yes (caused by:) Asthma: No/Yes

Alopecia: No/Yes Diabetes: No/Yes (Type I/Type II)

Psoriasis vulgaris: No/Yes Addison's disease: No/Yes

Pityriasis versicolor: No/Yes Connective tissue disease: No/Yes ( )

Thyroid disorder: No/Yes (if yes, name of disorder)

History of use of medications that may induce melanoleukoderma: No/Yes

□History of previous cosmetic use

1) Hydroquinone products: No/Yes (hydroquinone concentration %, period of use from (year) to (year))

→If Yes, were there any skin problems? No/Yes

2) Skin-whitening cosmetics other than Rhododenol No/Yes (Product name: , period of use from (year) to (year))

3) History of use of hair dyes: No/Yes (period of use since years ago, frequency once every)

→If Yes, were there any skin problems? No/Yes

4) History of use of hair growth promoters: No/Yes (product name: , period of use from (year) to (year))

→If Yes, were there any skin problems? No/Yes

□Photographs

1) Clinical photographs of areas of pigment loss: No/Yes

2) Dermoscopic photographs of areas of pigment loss: No/Yes

3) Clinical photographs of erythema or other areas of inflammation: No/Yes

4) Clinical photographs of areas of pigment deposition: No/Yes

5) Permission to submit photographs to the Special Committee: No/Yes

*Please obtain the patient's permission to submit photographs.

□Clinical symptoms

1) Month of onset: /20 (age years) (as reported by patient)
2) Type of pigment loss (clinical): total leukoderma/incomplete leukoderma/mixed type

3) Site(s) of pigment loss: face/neck/backs of hands/forearms/upper arms/other

( )

4) Area of pigment loss: Face: approximately 0–25%/approximately 25–50%/almost entire area

Neck: approximately 0–25%/approximately 25–50%/almost entire area

Backs of hands: approximately 0–25%/approximately 25–50%/almost entire area

Forearms: approximately 0–25%/approximately 25–50%/almost entire area

Upper arms: approximately 0–25%/approximately 25–50%/almost entire area

5) Site(s) where the product was used: face/neck/backs of hands/forearms/upper arms/other ( )

6) Inflammatory symptoms such as erythema, itching, or scaliness at sites where the product was used: No/Yes

7) Coincidence between sites of use and sites of inflammation: Mostly consistent/Not consistent

→If not consistent: inflammation in areas where the product was not applied/Widespread inflammatory symptoms from the margins of areas where the product was applied

8) Coincidence between areas where the product was applied and areas of pigment loss: Mostly consistent/Not consistent

→If Not consistent: Pigment loss in areas where the product was not applied/Widespread pigment loss from the margins of areas where the product was applied

9) Pigment loss from areas where the product has not been used: No/Yes (area:)

10) Photosensitivity (clinical, subjective on the part of the patient): No/Yes/Unknown