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Original Article

Cochineal dye-induced immediate allergy: Review of Japanese cases and proposed new diagnostic chart

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CE	cochineal extract
CA	carminic acid
SPT	skin prick test

ABSTRACT

Background: Cochineal dye is used worldwide as a red coloring in foods, drinks, cosmetics, quasi-drugs, and drugs. The main component of the red color is carminic acid (CA). Carmine is an aluminum- or calcium-chelated product of CA. CA and carmine usually contain contaminating proteins, including a 38-kDa protein thought to be the primary allergen. Severe allergic reactions manifest as anaphylaxis. The aim of this study was to review all Japanese reported cases and propose useful diagnostic chart.
Methods: All reported Japanese cases of cochineal dye-induced immediate allergy were reviewed, and newly registered cases were examined by skin prick test (SPT) with cochineal extract (CE) and measurement of CE and carmine-specific serum IgE test. Two-dimensional (2D) western blotting using patient serum was conducted to identify the antigen.
Results: Twenty-two Japanese cases have been reported. SPT and the level of specific IgE test indicated that six cases should be newly registered as cochineal dye allergy. All cases were adult females, and all cases except three involved anaphylaxis; 13 cases involved past history of local symptoms associated with cosmetics use. Japanese strawberry juice and fish-meat sausage, and European processed foods (especially macarons made in France) and drinks were recent major sources of allergen. 2D western blotting showed that patient IgE reacted to the 38-kDa protein and other proteins. Serum from healthy controls also weakly reacted with these proteins.

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2D PAGE Two dimensional polyacrylamide gel electrophoresis
BAT basophil activation test

Conclusions: SPT with CE and determination of the level of CE and carmine-specific IgE test are useful methods for the diagnosis of cochineal dye allergy.
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Introduction

Cochineal dye is a natural red colorant extracted from dried female cochineal insects (*Dactylopius coccus*), which are parasites of cacti native to Central and South America, particularly Peru. The main component of the red dye is carminic acid (CA), with a molecular weight of 492 Da. Carmine, which is a hydrated aluminum- or calcium-chelate product of CA, is also used as an insoluble red dye. Both carmine and cochineal dye are used worldwide as coloring agents in foods, drinks, cosmetics, quasi-drugs, and drugs.[1]

Allergic reactions induced by ingestion of processed foods containing cochineal extract (CE) or carmine are believed to be due to insect-derived contaminating proteins rather than CA. A suspected allergen protein is 38 kDa in size.[2]

Several cases of anaphylaxis induced by the intake of foods and drinks containing cochineal dye have been reported by the European Union (EU), prompting it in 2000 to mandate label declarations of potential allergic reaction associated with cochineal dye exposure (mandate E120, which includes CE, CA, and carmine).[3] In 2011, the United States Food and Drug Administration modified its labeling rules regarding indication as to whether CE and carmine are contained in food and cosmetic products.[4] In Japan, CE was approved for use as an existing food additive, according to a 1995 revision of the Food Sanitation Law. In 2011, after several severe cases of anaphylaxis were also reported in Japan, the Consumer Affairs Agency of the Japanese Government called attention to the potential for allergic reactions associated with the use of products containing cochineal dye.

The number of persons suffering from an allergic reaction to cochineal dye reported in the literature is probably an underestimation of the actual incidence because this type of allergy is not well recognized by physicians and because diagnostic methods are not well established. The aim of this study was to review all Japanese reported cases and propose new diagnostic chart. We first analyzed new cases of suspected cochineal dye-induced immediate allergy to propose adequate diagnostic chart. We then reviewed and analyzed all reported Japanese cases of this allergy and compared the data with those reported abroad. Finally, we examined the antigens associated with cochineal dye allergy using two-dimensional (2D) western blotting.

Methods

Subjects

All Japanese cases of cochineal dye-induced immediate allergy reported over a 17-year period (1999–2016) were identified from Japanese public databases (the Japan Medical Abstracts Society) and PubMed. New suspected cases from January through March 2014 were registered under “Epidemiological Research related to the Safety of Proteins included in Cosmetics” at the Department of Dermatology, Fujita Health University Hospital, or with us directly. The data from the other new cases, including clinical symptoms, source of cochineal dye, past history, and regional symptoms associated with cosmetics use, were registered between April 2014 and December 2016.

Ethics

All experiments were performed according to the ethical guidelines of Oita University, Faculty of Medicine (Permit Number: 610; opt-out URL: http://kenkyu-kokai.wp.med.oita-u.ac.jp/wp-content/uploads/sites/11/2018/02/610.pdf) and Fujita Health University School of Medicine (Permit Number: HM16-371; opt-out URL: http://info.fujita-hu.ac.jp/~allergy//research.html). The study was performed in accordance with the principles embodied in the Declaration of Helsinki.

Skin prick test (SPT)

A SPT using CE including CA was performed for all patients of newly registered cases as well as healthy volunteers as controls. Informed consent was obtained from all subjects. Samples for the SPT were prepared by us under guidance of the Division of Food Additives, National Institute of Health Sciences (Tokyo, Japan). Dried insects (*D. coccus*) were purchased online (http://www.cochinealdye.com/index.html, Birmingham, UK), pulverized with a homogenizer, suspended in 10-fold weight of tap water, and incubated at 80 °C for 15 min in a water bath, with shaking. Color values were measured in the presence of 0.1 N HCl by absorption spectrometry at a wavelength of 490–497 nm, in accordance with the Japanese Standards for Food Additives Handbook.[5] Because the color value of CE in foods and drinks is limited to <0.2 in Japan, samples for SPT were diluted to a color value of 0.1, 0.01, and 0.001 using phosphate-buffered saline (PBS). The protein concentration of SPT samples was measured using a Pierce 660-nm protein assay (Thermo Scientific, Rockford, IL, USA). A color value of 0.1 corresponded to a protein concentration of approximately 0.5 μg/ml. The final prepared samples were sterilized through filtration with 0.2 μm sterilized syringe filter (Steradisc 13, Kurabo, Osaka, Japan) before SPT. The results were evaluated based on criteria used in routine skin tests.[6] The first SPT was performed using a sample with a color value of 0.001, and when negative, the color value was stepped-up to 0.01 and 0.1. In brief, the result was defined as positive when the diameter of the wheal was larger than half of the diameter of the positive control using 10 mg/ml of histamine dihydrochloride (Wako Pure Chemical Industries, Osaka, Japan) or >3 mm.

Oral challenge test

Oral food challenge with cochineal dye-containing food was performed in several cases in which we could not diagnose the allergy by SPT with CE and CE and carmine-specific IgE test. During the challenge, we monitored patients' blood pressure and oxygen saturation. Small amounts of challenge sample were used at first, and if there were no symptoms, the amount was increased gradually.

ImmunoCAP assay with CE and carmine

The levels of serum IgE specific to CE and carmine were measured in patients of newly registered cases and healthy volunteers as controls. Informed consent was obtained from all subjects. Levels of IgE specific to a mixture of CE and carmine

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were measured using an established fluorescence-enzyme immunoassay (ImmunoCAP F340, Thermo Fisher Diagnostics, Tokyo, Japan). Levels of CE and carmine-specific IgE test were classified as follows: class 0 < 0.35 UA/ml; class 1, 0.35 UA/ml < 0.7 UA/ml; class 2, 0.7 UA/ml < 3.5 UA/ml; class 3, 3.5 UA/ml < 17.5 UA/ml; class 4, 17.5 UA/ml < 50.0 UA/ml; class 5, 50.0 UA/ml < 100 UA/ml; and class 6, ≤100 UA/ml. Patients determined to be class 2 or above (≥0.7 UA/ml) were considered to have been sensitized by CE.

Two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) and western blotting

Serum was obtained from patients and healthy controls after obtaining informed consent. Dried insects were purchased as described above. The methods for 2D PAGE and western blotting were as described previously.[7] In brief, pulverized dried insects were extracted with 30 mM Tris-HCl (pH 8.5), 2 M thiourea, 7 M urea, and 4% (w/v) CHAPS. The protein content was measured using a 2D Quant Kit (GE Healthcare, Buckinghamshire, UK), and samples were refined using a 2D Clean-Up Kit (GE Healthcare). Extracted proteins (20 μg) were separated in the first dimensional isoelectric focusing. In the second dimension, SDS-PAGE was performed. The gels were then stained with SYPRO Ruby (Life Technologies, Carlsbad, CA, USA) or after transfer of proteins onto a PVDF membrane. The membranes were blocked with 5% skim milk/PBS-T (Tween 20) for 1 h at room temperature. The membranes were then incubated for 1 h at room temperature with 5% serum diluted in 5% skim milk/PBS-T. The membranes were then washed with 5% skim milk/PBS-T and incubated for 1 h at room temperature with 0.4 μg/ml anti-human IgE-HRP conjugate (KPL, Gaithersburg, MD, USA) in 5% skim milk/PBS-T. The membranes were washed, and the chemiluminescence reaction was developed using Pierce™ ECL-Plus Western Blotting Substrate (Thermo Fisher Scientific). Fluorescence images were captured using a Typhoon FLA 9500 (GE Healthcare).

Results

Patient profiles

Demographic details of 22 cases reported in the literature or in abstracts of academic meetings are summarized in Table 1.[8–25] The diagnoses of those cases were mainly based on SPT with CE, carmine, or causative foods in addition to the level of CE and carmine-specific IgE test. Details of the seven newly registered patients with suspected allergy are summarized in Table 2.[26–29]

SPT with CE and ImmunoCAP assays specific for CE and carmine

We analyzed the results of examinations of five previously diagnosed cases (cases 5, 17, 19, 20, and 21; Table 1) and seven newly registered cases (cases 23–29; Table 2), and the results are summarized in Table 3. Samples for SPT with CE were supplied by us (see Methods section) for the newly registered cases. In order to confirm the reliability of the SPT, we performed SPT on 10 healthy volunteers using samples we prepared; all healthy volunteers showed negative reactions in the SPT. On ImmunoCAP assay with CE and carmine, 9 of 10 healthy volunteers were class 0 for specific IgE, with titers <0.1 UA/ml; the one exception was a man who was class 2 (2.24 UA/ml).

SPT with CE was performed in four of the five cases previously reported in the literature (cases 5, 19, 20, and 21, excepting case 17). Positive reactions were obtained in three of the four cases; in case 21, a positive SPT reaction with carmine was obtained, so this patient was diagnosed as having carmine allergy. By contrast, all five cases, including case 17, had CE and carmine-specific IgE, with titers >0.9 UA/ml and grade above class 2. In case 5, the titers in 2013 and 2014, were lower, but even 8 years after avoiding the causative foods and cosmetics, the titer remained at 0.17 or 0.10 UA/ml, indicating that antibodies against CE and carmine are retained for a long period even in the absence of antigen exposure.

In six of the seven newly registered cases (cases 23–25 and 27–29), SPT with CE was performed. Five of the six cases (cases 23, 24, 27, 28, and 29) showed positive reactions to CE, and the patients were therefore diagnosed as having cochineal dye allergy. The titers of CE and carmine-specific IgE test were measured in all seven of the newly registered cases. In three SPT-positive cases (cases 23, 28, and 29) and case 26, in which SPT was not performed, the levels were above class 2. These patients were therefore diagnosed as having cochineal dye allergy. In case 24, although the level of CE and carmine-specific IgE test was 0.257 UA/ml (class 0), the result of SPT with CE was positive. This patient was diagnosed as having cochineal dye allergy because the titer of CE and carmine-specific IgE test (0.257 UA/ml) was higher than that of healthy volunteers.

Oral challenge test

In cases 25, we could not diagnose cochineal dye allergy because the patient showed negative SPT results with CE, even though the titer of CE and carmine-specific IgE test was higher than that of healthy volunteers. In this case, an oral challenge test with cochineal dye-containing food (not the same original food) was performed and it was negative. So this patient was not diagnosed as having cochineal dye allergy.

Proposed diagnostic chart for cochineal dye allergy

Proposed diagnostic chart for cochineal dye allergy are shown in Figure 1. When SPT using CE or carmine is positive, the case would be defined as definite. SPT with CE was used to dilute to a color value of 0.1, 0.01, and 0.001 and carmine was diluted the commercialized reagent to 0.1% (w/v).[13, 25]

When the level of CE and carmine-specific IgE as determined by ImmunoCAP assay is ≥ 0.7 UA/ml (class 2), regardless of the SPT result, the case would also be defined as definite. When the level of CE and carmine-specific IgE is < 0.1 UA/ml, the patient would be said to have no allergy. When the level of CE and carmine-specific IgE is between and 0.1 and 0.7 UA/ml, the patient would be said to have suspected allergy, and it is necessary to refer the results of other examination (e.g. oral challenge test) for the diagnosis.

2D PAGE and 2D western blotting

The reliability of 2D western blotting for the detection of the potential protein antigen was examined. Prior to 2D western blotting, we confirmed the distribution of proteins from *D. coccus* using 2D PAGE, which showed a broad distribution of molecular weights and isoelectric points and a particularly high density of proteins in the 24–30 and 37–50 kDa ranges. The proteins were segregated into seven groups (Fig. 2a). On 2D western blotting, sera from 10 of 12 patients (cases 5, 17, 19, 20, and 24–29; 83%) reacted with a 38-kDa protein (Fig. 2b). However, sera from two of four healthy controls also reacted weakly with the 38-kDa protein (Fig. 2c). Case 17 reacted to purified CA in a basophil activation test (BAT) but did not react to extracted cochineal proteins,[21] nevertheless, strong reactions to the 38-kDa protein and moderate reactions to a 28-kDa protein were obtained on 2D western blotting (Fig. 2(b)). Case 28 and 29 showed a positive SPT reaction with purified CA and negative reaction to a sample enriched the 38-kDa protein, from

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Table 1
Summary of cochineal dye–induced immediate allergy cases reported in Japan.

Case	Age (years)	Gender (M/F)	Causative foods containing cochineal dye (country of manufacture)	Anaphylaxis	Time from intake until onset (min or hours)	Allergic history	History of local symptoms with cosmetics (country of manufacture)	Evidence for diagnosis	Author
1	28	F	Strawberry milk, Campari (Italy)	−	60 min	ND	ND	SPT with CE	Kume A et al, 1999[8]
2	26	F	Sponge cake (Japan)	+	5 min	ND	Eruption (unknown)†	Clinical data	Katada Y et al, 2006[9]
3	30	F	Campari (Italy)	+	ND	ND	Eruption (unknown)†	Clinical data	Katada Y et al, 2006[9]
4	33	F	Acerola drink (Japan) Red-and-white fish cake (Japan)	+	120 min	AR	ND	SPT with CE and carmine	Kotobuki Y et al, 2007[10]
5	23	F	Strawberry juice (Japan), Fish-meat sausage (Japan), Campari (Italy)	+	Several hours	AD	Itching with eye shadow (unknown)	‡	Takeo N et al, 2008[11]
6	35	F	Campari (Italy)	+	Immediate	AD	ND	SPT with CE, CE and carmine-specific IgE test WB 39–44, 24 (kDa)	Yamakawa Y, 2009[12]
7	44	F	Campari (Italy)	−	10 min	ND	ND	SPT with CE, CE and carmine-specific IgE test WB 40–45, 26 (kDa)	Yamakawa Y, 2009[12]
8	52	F	Strawberry-colored juice (Japan)	+	30 min	AD	ND	SPT with CE, WB 39–44 (kDa)	Yamakawa Y, 2009[12]
9	31	F	Salami (France), Red macarons (France)	+	Several hours	AR	Swelling with eye shadow (unknown)	Scratch test carmine	Toyonaga M et al, 2011[13]
10	26	F	Campari (Italy)	+	7 h	ND	ND	SPT with CE	Akiyama M et al, 2011[14]
11	49	F	Campari (Italy), Guava drink (importing)	+	Several minutes	ND	ND	SPT with CE	Yamakawa Y et al, 2012[15]
12	40	F	Chocolate macarons (France)	+	Immediate	−	Eruption with cosmetics (from abroad)†	SPT with CE, CE and carminie-specific IgE test	Saeki Y et al, 2012[16]
13	42	F	Acerola drink (Japan), Campari (Italy), Orange juice (Japan), Fish-meat sausage (Japan)	+	ND	AD	ND		Nomura K et al, 2012[17]
14	26	F	Wine (Japan)	+	ND	ND	ND	SPT with CE	Osawa N et al, 2012[18]
15	39	F	Strawberry-flavored macarons (France), Fish-meat sausage (Japan), Red-colored milk shake (Japan)	+	Several hours	−	Eruption with eye shadow (from abroad)†	SPT with CE	Kitabayashi T, 2012[19]
16	52	F	Red macarons (France)	+	ND	AR	ND	SPT with carmine	Ishikawa T et al, 2013[20]
17	39	F	Supplement drink (Japan)	+	Immediate	AR, BA	ND	‡,§	Sugimoto N et al, 2013[21]
18	30	F	Red macarons (France)	+	During intake	ND	−	SPT with CE and carmine WB 38–45, 70–75, 50–55, 23–26 (kDa)	Yamakawa Y et al, 2014[22]
19	49	F	Red macarons (France)	+	5–10 min	−	Itching and swelling with lipstick, cheek rouge (from abroad)	‡	Harada S et al, 2014[23]
20	33	F	Red macarons (France)	+	Immediate	−	Itching with lipstick (from abroad)	‡	Harada S et al, 2014[23]
21	29	F	Blood orange juice (Germany)	+	60 min	−	−	‡	Harada S et al, 2014[24]
22	40	F	Pink-colored macarons (Turkey), Raspberry chocolate biscuits (Australia), Strawberry juice (Japan)	+	1 h, 15min, 15min	AR	Itching and swelling with dark-red eyeliner, orange-eyeshadow (unknown)†	SPT with carmine BAT with carmine	Miyakawa M et al, 2015[25]

AD, atopic dermatitis; AR, allergic rhinitis; BA, bronchial asthma; BAT, basophil activation test; CE, cochineal extract; ND, not described; SPT, skin prick test; unknown, the country of the product was not recorded; WB, Western blotting.

† Obviously contained cochineal dye.

‡ Results of SPT with CE and/or CE and carmine-specific IgE test of these cases are shown in Table 3.

§ Basophil activation test results are shown in Table 3.

Table 2
Summary of newly registered cases, suspected cochineal dye–induced immediate allergy.

Case	Age (years)	Gender (M/F)	Causative foods containing cochineal dye (country of manufacture)	Anaphylaxis	Time from intake until onset (min or hours)	Allergic history	History of local symptoms with cosmetics (country of manufacture)	Registered year Author Reference
23	32	F	Fish-meat sausage (Japan)	+	ND	AD	Itching, redness, and swelling with eye shadow (Japan)[†]	2013
24	48	F	Fish-meat sausage (Japan), Red wine jelly (Japan), Strawberry milk (Japan)	+	15 min–10 h	–	–	2013 Ando A et al, 2013[26]
25	10	M	Pink-colored, Mashed and seasoned fish (Japan)	– High fever, vomiting, diarrhea; three times between 3 and 5 years old	5 h	–	–	2015
26	31	F	Blood orange cocktail (Japan), Strawberry milk (Japan), Macaroons (France)	+	1–2 h	ND	Contact dermatitis with eye shadow (Japan)	2016 Osako J et al, 2016[27]
27	44	F	Campari (Italy), Red macaroons (unknown), Syrup (unknown)	+ Mainly digestive symptoms	20–30 min	–	Itching and swelling with lipstick (imported), itching of cheek (Japan)	2015
28	65	F	Fish-meat sausage (Japan)	– Urticaria	1 h	–	–	2016 Saito K et al, 2016[28]
29	32	F	Hamburger (Japan), Pink-colored drink (Japan)	+	Immediate	–	Redness and swelling with cosmetics	2016 Hyodo Y et al, 2017[29]

Results of SPT with CE and CE and carmine-specific IgE test for these cases are shown in Table 3.
AD, atopic dermatitis; CE, cochineal extract; ND, not described; SPT, skin prick test.
[†] Obviously contained cochineal dye.

which CA was removed (data not shown), but these patient's serum reacted with the 38-kDa protein (Fig. 2(b)).

The 38-kDa protein was identified as CC38K by mass spectrometry.[7] This protein could be the major allergen, but further study is necessary to confirm whether the protein alone is allergenic or only becomes so upon binding with a hapten.

Clinical features of 28 Japanese cases of cochineal dye allergy with comparison to cases from abroad

A total of 28 cases, including 22 previous cases (Table 1) and 6 newly registered cases (cases 23, 24, and 26–29; Table 2), were analyzed. The number of patients with cochineal dye allergy has increased in Japan since 2006, although the first case was reported in 1999 (Fig. 3). By contrast, at least 56 cases of cochineal dye allergy were reported from abroad (Table 4).[30–42] These incidences distributed into four major peaks due to cumulated reports[33, 37, 39, 42] (Fig. 3).

All Japanese patients diagnosed with cochineal dye allergy were adult females in their 20s–60s, and all except cases 1, 7, and 28 had anaphylaxis. Liquor (Campari), which contained carmine until the replacement of red coloring in 2007, and red cake (macarons) containing carmine were the sources of allergen in ten cases. Both the Campari and macarons were European products, and the macarons were ingested while traveling abroad or carried into Japan as souvenirs. Among Japanese products, fish-meat sausage (cases 5, 13, 15, 23, 24, and 28) and strawberry milk or juice (cases 1, 5, 8, 15, 22, 24, and 26) were the major sources of allergen. Eight cases (cases 5, 9, 19, 20, 22, 23, 27, and 29) involved itching, redness, and swelling of the face following use of cosmetics. Case 26 involved a contact dermatitis–like eruption on the face. No detailed information regarding the eruption following use of cosmetics was available for cases 2, 3, 12, and 15. Major cosmetics allergens included eye shadow (cases 5, 9, 15, 22, 23, and 26) and lipstick (cases 19, 20, and 27). Six patients (cases 2, 3, 12, 15, 22, and 23) used cosmetics containing cochineal dye. Ten cases (cases 4, 5, 6, 8, 9, 13, 16, 17, 22, and 23) involved a history of allergic disorders, and five cases (cases 5, 6, 8, 13, and 23) involved atopic dermatitis.

Discussion

All Japanese patients with cochineal dye allergy were female, as reported previously. Cochineal dye allergy with local symptoms associated with cosmetics use has been also reported previously.[31, 34, 35] The preponderance of females with this allergy suggests that patients are sensitized by cosmetics containing cochineal dye, whereas immediate allergic reactions occur after ingestion of food containing cochineal dye.[43] With regard to cosmetics, there is no upper limit for cochineal contamination in Japan. The protein impurities in cochineal dye might induce sensitization in adolescent females; however, we could not determine how long or how often the patients used cosmetics.

In Japan, many persons exhibited an immediate type of allergy due to wheat protein after using a special facial soap in 2009.[44] The allergen was thought to be a 20- to 250-kDa hydrolyzed wheat protein in the soap. Most of those patients did not have a past history of atopic dermatitis. However, as the skin barrier is disturbed by the surfactant in soap, daily use could promote sensitization. By a similar mechanism, cosmetics, particularly eyeshadow and lipsticks containing cochineal dye, could promote percutaneous sensitization even in non-atopic individuals, especially when these products are applied along the border between the skin and mucous membranes.

The number of reports of food allergy associated with cochineal dye has been increasing in Japan, particularly since 2012. One reason for this increase is that the Consumer Affairs Agency of the

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Table 3
Results of examinations in 12 cases.

	Case	SPT with CE (minimum positive color value)	CE and carmine specific IgE test (UA/ml) (class)†	Diagnosis‡
Cases reported in the past	5	+ (0.2)§,¶	2.84 (class 2)¶ 0.176 (class 0)‖ 0.101 (class 0)#	Definite
	17	ND	19.2 (class 4)††	Definite
	19	+ (0.2)§	0.941 (class 2)	Definite
	20	+ (0.2)§	3.62 (class 3)	Definite
	21	−§,‡‡	0.929 (class 2)	Definite
Cases newly registered	23	+ (0.01)	0.734 (class 2)	Definite
	24	+ (0.1)	0.257 (class 0)	Definite
	25	−§§	0.420 (class 1)	Suspected
	26	ND	1.850 (class 2)	Definite
	27	+ (0.01)¶¶	0.143 (class 0)	Definite
	28	+ (0.001)##	3.53 (class 3)	Definite
	29	+ (0.1)##	1.30 (class 2)	Definite

CA, carminic acid; CE, cochineal extract; ND, not done; SPT, skin prick test.
† CE and carmine-specific IgE levels were classified as class 0: <0.35 UA/ml, class 1: 0.35 UA/ml < 0.7 UA/ml, class 2: 0.7 UA/ml < 3.5 UA/ml, class 3: 3.5 UA/ml < 17.5 UA/ml, class 4: 17.5 UA/ml < 50.0 UA/ml, class 5: 50.0 UA/ml < 100 UA/ml, and class 6: ≤100 UA/ml.
‡ When SPT with CE was positive, the grade of CE and carmine-specific IgE test was above class 2 (0.7 UA/ml or higher), or oral challenge test with cochineal dye-containing food was positive, we diagnosed the case as definite cochineal dye allergy.
§ Samples for SPT were supplied from a Japanese corporation providing food ingredients.
¶ In 2006.
‖ In 2013.
In 2014.
†† A basophil activation test with 96% purified CA was positive.
‡‡ Though SPT with CE was negative, SPT with carmine was positive.
§§ Oral challenge test was also negative.
¶¶ Though SPT with CE was negative by 15 min, it turned positive by 1 h. Oral challenge test was positive.
SPTs not only with CE, but also with CA solely were performed.

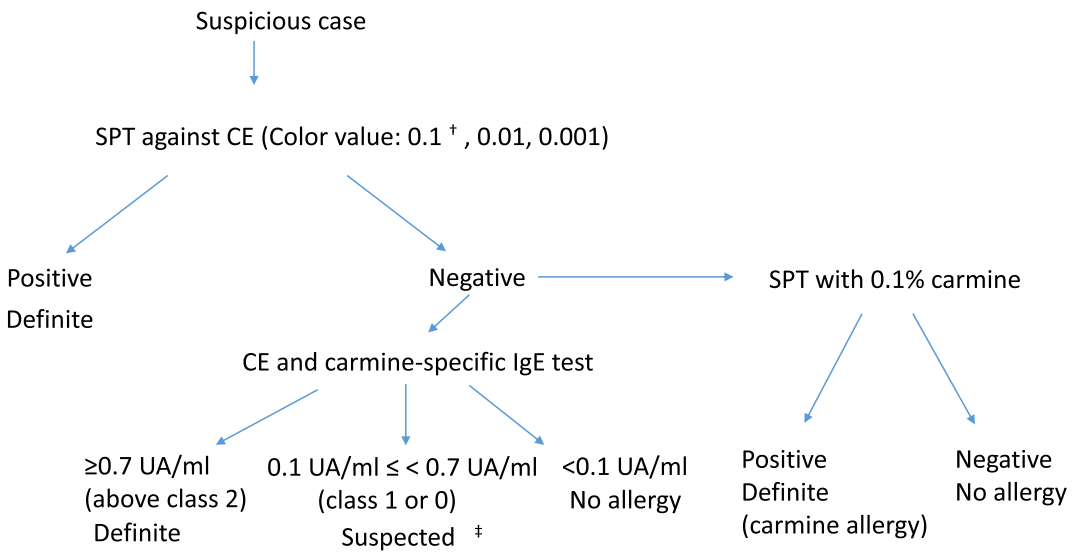


Fig. 1. When SPT using cochineal extract or carmine is positive, the case would be defined as positive. When the level of CE and carmine-specific IgE test as determined using the ImmunoCAP assay is ≥ 0.7 UA/ml, regardless of the SPT result, the case would also be defined as positive. When the level of specific IgE test is < 0.1 UA/ml, the case would be defined as no allergy. When the level of specific IgE test is between 0.1 and 0.7 UA/ml, the case would be defined as suspected allergy. CE, cochineal extract; SPT, skin prick test. † Protein concentration of color value 0.1 is approximately 0.5 μg/ml ‡ It is necessary to refer the results of other examinations for the diagnosis.

Japanese Government published an alert to call attention to cochineal dye allergy in 2011. Another possible reason could be a decline in the acquisition of immunotolerance to the cochineal dye allergen in foods during childhood combined with more frequent exposure to the allergen in adulthood.

Notably, the source of the allergen was more often European foods rather than Japanese foods. Japanese law limits the amount of CE protein as a food additive to <2.2%,[5] and carmine cannot be used as a food additive, although it can be used in cosmetics and pharmaceuticals. In Europe, there is no regulation for the upper limit of cochineal dye protein contamination in the E120.[45] The use of carmine as a red coloring in liquor (Campari) was banned in 2007, and it is interesting to note that the last report of possible cochineal dye allergy associated with Campari consumption (case 27) in Japan was in 2012. There have been no new reports since that date.

Macarons produced in France were the suspected source of allergen in eight cases in Japan. Compared with Japan, very few cases of cochineal dye allergy have been reported from EU countries, with the exception of Finland (Table 4).

Oral exposure to allergens is hypothesized to induce tolerance, whereas environmental exposure is hypothesized to induce allergy.[46, 47] If this hypothesis were true for cochineal dye, the

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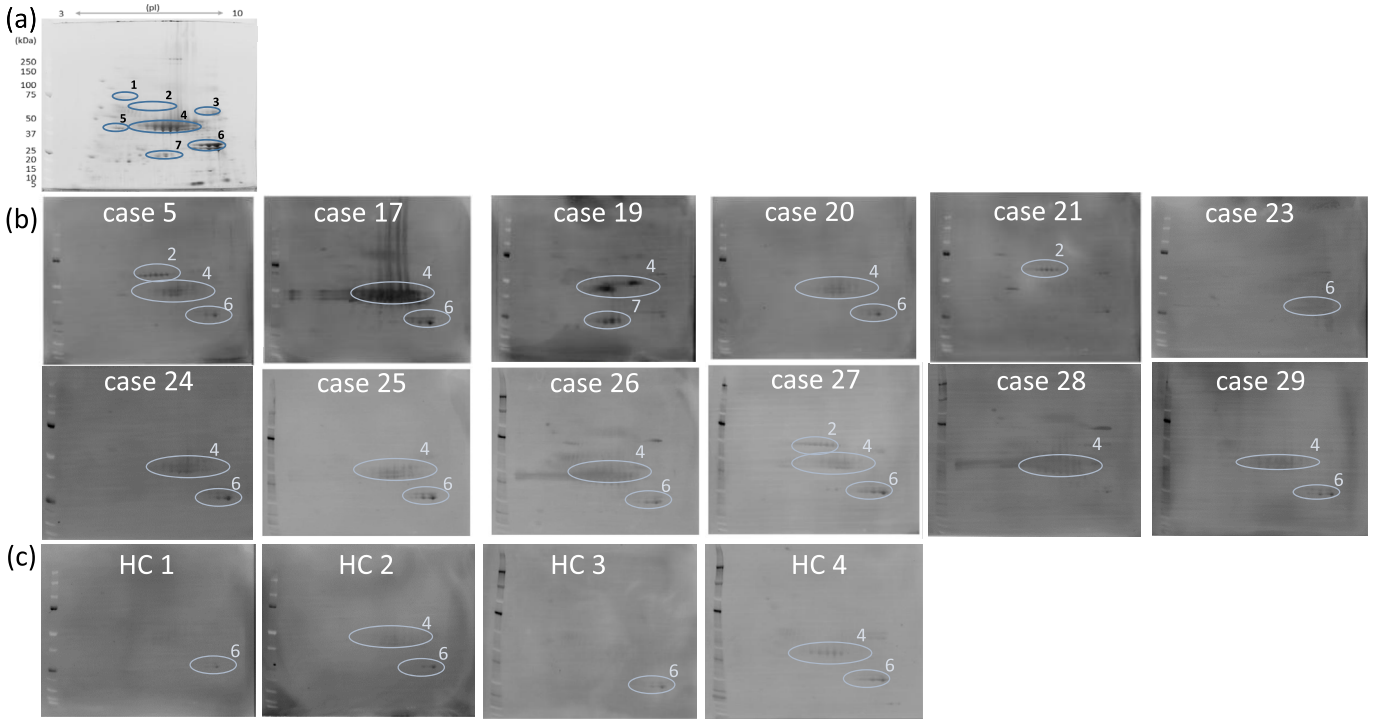


Fig. 2. **(a)** Distribution of proteins from *Dactylopius coccus* on 2D PAGE (SYPRO ruby staining). Proteins exhibited a broad distribution in molecular weight and isoelectric point and a high density in the 24- to 30-kDa and 37- to 50-kDa ranges. A total of seven proteins groups were observed, and numbered from 1 to 7. **(b)** On 2D western blotting, sera from 10 of 12 patients (cases 5, 17, 19, 20, 24–29; 83%) reacted with a 38-kDa protein (4). The serum from case 17 reacted with the 38-kDa protein strongly, although it reacted with carminic acid but did not react with extracted cochineal proteins in a basophil activation tests.[21] The serum from case 28 and 29 reacted with the 38-kDa protein but showed positive SPT reaction with carminic acid and negative reaction to a sample enriched the 38-kDa protein from which carminic acid was removed (data not shown). **(c)** 2D western blotting was carried out on samples from four healthy controls (HC), two of which (HC2 and 4) reacted weakly with the 38-kDa protein (4).

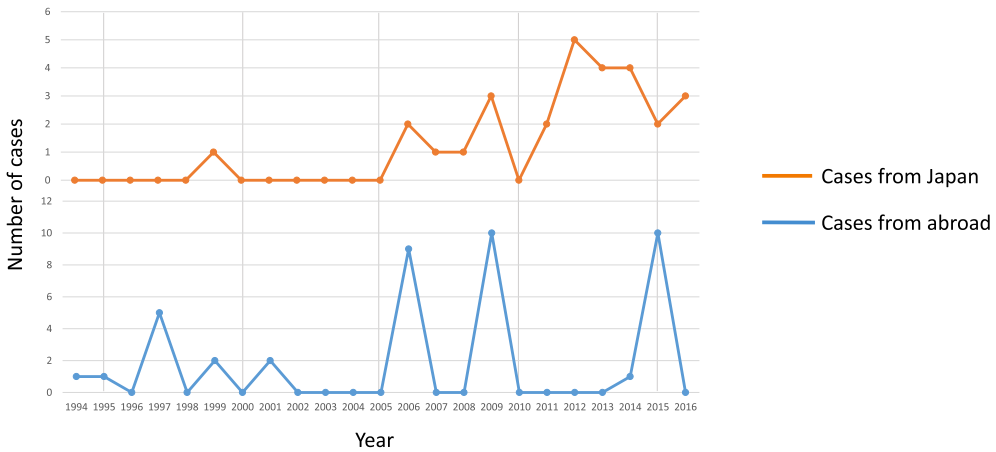


Fig. 3. The number of patients with cochineal dye allergy has increased in Japan, particularly since 2006. By contrast, the incidence of cochineal dye allergy abroad distributed into four major peaks due to cumulated reports.

following scenario would be plausible. In Japan, cochineal dye in which the allergen proteins have been removed, is widely used,[48] thus decreasing oral exposure to the allergen. Adult patients using cosmetics contaminated with cochineal dye allergen have a greater chance to be sensitized percutaneously. When these individuals then consume food containing the allergen, they would be at greater risk of cochineal dye allergy. The age of individuals using cosmetics is decreasing, and the potential for exposure to foods imported from Europe is increasing.

SPT combined with determination of the level of CE and carmine-specific IgE test using the ImmunoCAP assay was useful for diagnostic purposes. SPT with CE has been employed since the 1990s, but in this study, SPT using CE showed negative reactions in case 25 when CE and carimne-specific IgE test class was 1. Case 25 was negative for oral challenge with cochineal dye–containing food; however, this result is not absolutely reliable because the oral trial was not performed using the same food that prompted an anaphylactic response. This patient was categorized as a suspected

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Table 4
Summary of cochineal dye–induced immediate allergy, cases reported abroad.

Case	Age	Gender	Country	Ingestion exposure route	Anaphylaxis	Time from intake until onset	Allergic history	Topical exposure route	Evidence of the diagnosis	Author
1	19	M	UK	ND	+	ND	ND	Anaphylaxis (lipstick)	Clinical symptoms	Park et al, 1981[30]
2	34	F	Switzerland	Campari	+	15 min	AD, AR, BA	Itching in the eyes and burning skin (lipstick, mascara, eye shadow, other makeup)	SPT to Campari, E120 and cosmetic products IgE-RATS to Campari and E120 (class 2)	Kägi et al, 1994[31]
3	35	F	France	Yogurt with fruits	+	2 min–several hours	–	ND	SPT to yogurt and carmine Basophil histamine release to carmine	Beaudouin et al, 1995[32]
4	33	F	Switzerland	Campari, Mexican rice meal with shrimp and several sauces	–	30 min	AR, OAS	ND	SPT to carmine IgE-RAST to carmine (class 2)	Wüthrich et al,1997[33]
5	43	F	Switzerland	Campari	+	15 min	–	ND	Prick and scratch test to Campari IgE-RAST to carmine (class 2)	Wüthrich et al, 1997[33]
6	25	F	Switzerland (from USA)	Campari	+	30 min	AR	ND	SPT to carmine IgE-RAST to carmine (class 2)	Wüthrich et al, 1997[33]
7	39	F	Switzerland	Campari	–	30 min	–	ND	SPT to carmine from Campari	Wüthrich et al, 1997[33]
8	27	F	USA	Red popsicle	+	Several mins–3 hours	AR	Skin rash (blush)	SPT to carmine WB 23 kDa	Baldwin et al, 1997[34]
9	27	F	USA	Custard-style strawberry-banana flavored yogurt	+	30 min–2 hours	–	Skin rash and swelling (eye shadow)	SPT to carmine	DiCello et al, 1999[35]
10	42	F	USA	Crabmeat, Campari	+	30 min–2 hours	Food allergy	Eyelid swelling, facial urticaria, and rhinorrhea (eye shadow)	SPT to carmine	DiCello et al, 1999[35]
11	32	F	USA	Artificial crab, Ruby red grapefruit juice	+	3–5 h	AR	–	SPT and OCT to carmine WB 38 and 50 kDa	Chung et al, 2001[36]
12	30	F	USA	Artificial crab	–	4–5 h	ND	Immediate pruritus and erythema on the face (blush)	SPT and OCT to carmine WB 88 kDa	Chung et al, 2001[36]
13	30	F	USA	Tropicana Ruby Red Grapefruit Juice, Purple candy	+	ND	ND	Purple eye shadow	SPT to carmine	FDA, 2006[37]
14	26	F	USA	Custard-style strawberry-banana yogurt	+	ND	ND	ND	SPT to carmine	FDA, 2006[37]
15	50	F	USA	Fruit drink	+	Within 15 min	ND	ND	Clinical symptoms	FDA, 2006[37]
16	49	F	USA	Crab soup, Yogurt, Candy, Ruby red grapefruit juice, Pasta salad with artificial crabmeat	+	ND	–	ND	SPT to carmine	FDA, 2006[37]
17	50	F	USA	Jelly or Gelatin dessert	ND	ND	AD	ND	Clinical symptoms	FDA, 2006[37]
18	ND	F	USA	Custard-style yogurt	+	Immediate	ND	Sensitivity to eye shadow, other cosmetics	Clinical symptoms	FDA, 2006[37]
19	ND	F	USA	Foods	+	ND	ND	Cosmetics	Clinical symptoms	FDA, 2006[37]
20	ND	ND	USA	ND	–	ND	ND	Eyeliner	Clinical symptoms	FDA, 2006[37]
21	ND	ND	USA	Yogurt	+	ND	ND	ND	SPT to carmine	FDA, 2006[37]
22	47	F	USA	Generic azithromycin, Fruit-flavored yogurt, Colored pasta	+	Generic azithromycin-within 90 min	BA, AR	Facial rash and swelling (eye makeup)	SPT to carmine	Greenhawt et al, 2009[38]
23–31	ND	ND	Finland	Red-colored alcoholic/non-alcoholic beverages, Candies, Yogurt, Ice cream, Red juices, Jellies, Cakes coated in red icing	+-	ND	SPT positive to mites	Cosmetics	SPT to carmine E120	Liippo et al, 2009[39]
32	32	F	Italy	Protein-vitamin supplement	–	Immediate	–	ND	SPT to supplement f340 ImmunoCAP level 0.19KU/l	Voltolini et al, 2014[40]
33	52	F	Italy	Red fruit candy, Fruit juice	+	Immediate-a few min	–	ND	SPT to fruit juice f340 ImmunoCAP level 1.28 KU/l	De Pasquale et al, 2015[41]

34	39	F	Finland	Foods	–	30 min	SPT positive to pollen, mites	ND	SPT and OCT to carmine E120	Liippo et al, 2015[42]
35	62	F	Finland	Cake	+	1 h	SPT positive to pollen, animals	ND	SPT and OCT to carmine E120	Liippo et al, 2015[42]
36	57	F	Finland	Candies	–	ND	SPT positive to pollen	ND	SPT and OCT to carmine E120	Liippo et al, 2015[42]
37	33	F	Finland	Red-colored candies	–	ND	ND	Swelling of lips (red lipstick)	SPT and OCT to carmine E120	Liippo et al, 2015[42]
38	19	M	Finland	Foods	–	ND	SPT positive to pollen, cross-reactions, animals	ND	SPT and OCT to carmine E120	Liippo et al, 2015[42]
39–56	20–67	F (7) M(11)	Finland	Foods	-	ND	ND	In most cases, immediate-type symptoms on the face and lips	SPT to carmine (OCT negative to carmine)	Liippo et al, 2015[42]

AD, atopic dermatitis; AR, allergic rhinitis; BA, bronchial asthma; ND, not described; OAS, oral allergy syndrome; OCT, oral challenge test; SPT, skin prick test; WB, western blotting.

case, and further study would be necessary in order to make accurately diagnose such suspected cases.

The level of CE and carmine-specific IgE in case 5 might be a good marker of the clinical course, as it decreased after avoidance of the allergen for 7 years. In this study, we could not determine the antigen or antigenic epitope. In some cases (cases 17,28, and 29), CA was assumed to be the major antigen, but in another case (case 21), carmine was the candidate. The proteins that are contained in CE could be carriers, and 2D western blotting combined with other methods (e.g., BAT) could help confirm the role of specific proteins. BAT using highly purified protein components of cochineal dye would be a useful tool to determine the contribution of particular contaminant proteins to cochineal allergy. This method would be suitable for a number of patients.

In conclusion, SPT with CE and determination of the level of CE and carmine-specific IgE are very useful tools for diagnosing cochineal dye allergy. However, more specific and sensitive diagnostic methods are needed, and efforts to educate the Japanese people regarding the risks of cochineal dye allergy should continue.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors'contributions

NT and MN designed the study and wrote the manuscript. SN measured specific serum IgE to cochineal extract on ImmunoCAP assay. OO was an adviser of the research.

NSu offered purified carminic acid. SS created the database. NSa contributed to immunoblotting. SH, MY, NM, YK, KS, MT, AN and JSO contributed to data collection. YH, HA, AY and SF performed the statistical analysis and interpretation of the results. KM was a supervisor. All authors read and approved the final manuscript.

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