

Case report

Identification of carmine allergens among three carmine allergy patients

Background: There have been several reports of carmine allergy; however, identification of the responsible carmine allergens has not been widely documented.

Methods: Three female patients presented with a history of anaphylaxis and/or urticaria/angioedema after ingestion of carmine-containing foods. All three patients had 4+ skin prick tests to carmine. Among them, two patients were confirmed to have carmine allergy by blinded, placebo-controlled food challenges to carmine. SDS-PAGE of cochineal insects and carmine, immunoblotting for IgE antibody with sera from all three patients, and immunoblotting inhibition with carmine were performed.

Results: SDS-PAGE of minced cochineal insects revealed several protein bands of 23–88 kDa. Several of these bands were variably recognized by our three patients' sera, and this reactivity was inhibited by carmine. Although no protein bands could be visualized on SDS-PAGE of carmine in Coomassie brilliant blue staining, three protein bands were recognized by two of the three patients' serum.

Conclusions: These results suggest that commercial carmine retains proteinaceous material from the source insects. These insect-derived proteins (possibly complexed with carminic acid) are responsible for IgE-mediated carmine allergy. Patient reactivity to these proteins may vary.

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Carmine is a natural red dye derived from the dried bodies of female cochineal insects and widely used as a coloring agent for food and cosmetics. Several cases of occupational asthma (1–4), extrinsic allergic alveolitis (5, 6), cheilitis (7), and food allergy (4, 8–12) to carmine have been reported. Some of those reports suggested that an IgE-mediated mechanism may be responsible for these events by demonstrating positive skin prick test (SPT) to carmine (1, 4, 8–12), as well as positive leukocyte histamine release (9), RAST (1, 8, 10), Prausnitz-Küstner test (P-K) (11), and immunoblotting for IgE (4). Positive inhalation and oral challenges (2–4, 6) were demonstrated in some reports of workers in dye manufacturing and other jobs involving the handling of carmine. However, the identification of responsible carmine allergens has not been widely documented. We describe three patients with food allergy to carmine who

were not workers who handled carmine. We previously reported one of these patients with positive SPT and P-K test to carmine in 1997 (11). In the past 2 years, we have identified two additional cases by history, SPT, and blinded, placebo-controlled food challenge to carmine. For identification of the possible allergen(s) responsible for carmine allergy, sera from these three patients were used in a series of immunoblotting and inhibition studies with carmine and cochineal insects.

Case report

Three female patients presented to the allergy clinic because of episodic urticaria/angioedema, and/or anaphylaxis 3–5 h after ingestion of foods containing carmine.

Patient 1, a 32-year-old woman, presented with a 4-year history of six episodes of urticaria and facial angioedema, and/or difficulty in breathing 3–5 h after ingestion of several pieces of artificial crab or a few sips of ruby red grapefruit juice. Her past medical history was significant only for allergic rhinitis. She did not use cosmetics. Her food allergy to carmine was diagnosed by history, SPT, and single-blind, placebo-controlled food challenge (SBPCFC) to carmine.

Abbreviations. SPT: skin prick test; DBPCFC: double-blind, placebo-controlled food challenge; SBPCFC: single-blind, placebo-controlled food challenge; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS: phosphate-buffered saline; TBS: Tris-buffered saline; P-K test: Prausnitz-Küstner test; CAS: Chemical Abstracts Services; BCIP: 5-bromo-4-chloro-3-indolyl phosphate; NBT: nitro blue tetrazolium.

Patient 2, a 27-year-old woman, suffered anaphylaxis requiring emergency treatment after eating ice on a stick colored with carmine. She experienced nausea within minutes and pruritus, urticaria, and hypotension with tachycardia within 3 h of ingesting this food item. The disorder responded to intravenous fluids, epinephrine, and diphenhydramine. Her past medical history was significant only for allergic rhinitis. The patient also recalled an immediate, pruritic, erythematous eruption after applying a blush, colored with carmine (Clinique®), directly to her facial skin, but not when the blush was used over foundation makeup. A positive P-K test to carmine with the patient's husband as recipient confirmed carmine allergy (11).

Patient 3, a 30-year-old woman, presented with a 2-year history of six episodes of angioedema and/or urticaria 4–5 h after ingestion of carmine-colored artificial crab (from several pieces to a half pound). The patient also experienced immediate pruritus and erythema after applying a blush, colored with carmine (Merle Norman®), directly to facial skin, but not over foundation makeup. Her past medical history was significant only for mild seasonal allergic rhinitis. Her food allergy to carmine was diagnosed by history, SPT, and double-blind, placebo-controlled food challenge (DBPCFC) to carmine. Open oral challenge to artificial crab without carmine was negative.

After diagnosis, all three patients avoided carmine-containing products, and did not have recurrence of anaphylaxis, urticaria, and/or angioedema.

Material and methods

Preparation of allergen extracts

In vivo tests. For SPTs, commercial carmine liquid containing not less than 3.5% carminic acid w/v (from Warner-Jenkinson, St Louis, MO, USA) was used without dilution. Histamine phosphate, 2.75 mg/ml, and 50% glycerin/50% buffered saline were used as positive and negative controls, respectively. For the oral challenge test, commercial carmine lake containing not less than 50% carminic acid was used. The US Food and Drug Administration (FDA) does not require a declaration of protein content of commercial carmine liquid or lake; consequently, this content was unknown.

In vitro tests. Pulverized dried cochineal insects (0.25 g) were washed once with methanol (10 ml) and three times with saturated ammonium sulfate (10 ml). PBS (10 ml) was added to the precipitate, and then the combination (0.75 ml) was mixed with 4× sample buffer (0.25 ml), boiled for 10 min, and centrifuged at 3200 g for 10 min. The supernatant was used as an antigen (4). Commercial carmine lake (0.5 g) containing not less than 50% carminic acid (Warner-Jenkinson, St Louis, MO, USA) was prepared as above. Both prepared antigens were used for SDS-PAGE and immunoblotting. Commercial carmine liquid containing not less than 3.5% carminic acid w/v was used for immunoblotting inhibition.

SPT

SPTs were performed with carmine and negative and positive controls. SPTs were performed on the three patients and 50 negative controls without a history of carmine allergy.

Oral challenge tests

Patient 1 underwent SBPCFC on a single day. Opaque capsules containing either lactose or commercial carmine lake (1, 5, 10 mg each) were used. The challenge dosages of carmine were determined by estimates of carmine ingested by the patient prior to reactions as well as the relative severity of her reactions. The patient was closely monitored by spirometry, arterial pressure, and pulse rates during the challenge. She was observed for 5 h after each dose because of her history of reactions developing 3–5 h after eating carmine-containing foods.

Patient 3 underwent DBPCFC on two separate days. Opaque capsules containing either lactose or carmine lake (25, 50, 100, and 200 mg each) were used. The challenge dosages of carmine were determined by estimates of carmine ingested by the patient before reactions. Lactose and carmine capsules were administered randomly on two separate days in increasing doses, until a positive reaction occurred. A normal control individual with negative SPT to carmine ingested the same dose of carmine ingested by the patient. The patient was observed for 5 h after each dose because of her history of reactions developing 4–5 h after eating carmine-containing foods. Open oral challenge to artificial crab from which the red coloring had been stripped (eliminating the carmine) was also performed 8 weeks after the DBPCFC to carmine.

SDS-PAGE of carmine and cochineal insects

SDS-PAGE was carried out on a 4% acrylamide stacking gel and 15% separating gel. Electrophoresis was performed at 20 mA until the bromophenol blue reached the bottom of the mini-gel. After electrophoresis, proteins were either stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane for immunoblotting.

Immunoblotting

Proteins in the SDS-PAGE gel (both cochineal extract and carmine) were blotted onto a nitrocellulose membrane (Biotrace® NT, Gelman Sciences) in transfer media (24 mM TRIS, 182 mM glycine, and 20% v/v methanol) by diffusion with the Schleicher & Schuell apparatus. After blocking in 10% w/v nonfat powdered dry milk in 5 mM Tris, 15 mM NaCl solution (pH 7.4) for 2 h at room temperature (RT), the nitrocellulose sheet was incubated overnight at RT with 1000 µl of 1:1 dilution and 1:9 of the patients' sera and 1000 µl of 1:1 dilution of control sera in incubation buffer (5 mM Tris, 15 mM NaCl, 10% w/v nonfat powdered dry milk, pH 7.4), with 1:500 dilution of a monoclonal, biotin-labeled mouse antihuman IgE for 3 h at RT, and finally with 1:500 dilution of streptavidine-alkaline phosphatase for 1 h at RT. Between each step, 5 mM Tris, 15 mM NaCl, and 0.05% Tween-20 solution (pH 7.4) were used for three washes. Bands were visualized by incubation with the BCIP-NBT substrate for 20 min.

Immunoblotting inhibition with carmine

To investigate whether carmine could inhibit patients' sera from recognizing cochineal insect proteins, we incubated the patients' sera (1000 µl of 1:1 and 1:9 dilution in incubation buffer) for 6 h at RT with 5 or 50 µl of undiluted commercial carmine liquid. The resultant mixtures achieved a 1:20 or 1:200 carmine concentration

for the inhibition test. These mixtures were then placed in the lane of blotting device, and the immunoblotting procedures described above were followed.

Results

SPT to carmine

SPTs were 20 × 16, 19 × 14, and 24 × 16 mm diameter wheals with pseudopods to carmine in all three patients, respectively. Fifty control subjects were negative in SPT to carmine.

Oral challenge test

In patient 1, 1 mg carmine induced itching sensation, chest tightness, and 19.2% fall in FEV₁ from 3.33 l (104% predicted) to 2.69 l (86% predicted) in 60 min. However, there was no urticaria/angioedema or hypotension. After albuterol nebulized mist treatment, the patient's symptoms and FEV₁ (2.91 l; 91% predicted) improved, and another 5 mg carmine was given 4 h later. FEV₁ dropped by 27.9% from first baseline 3.33 l to 2.4 l (75% predicted) 1 h after 5 mg carmine, and severe itching and urticaria developed 3 h later. The patient was treated with albuterol nebulized mist, 10 mg cetirizine, and 40 mg prednisone, with complete resolution within 12 h.

In patient 3, the DBPCFC with carmine elicited angioedema and generalized urticaria within 1 h at 25 mg carmine, but not with placebo. The spirometry and other vital signs remained stable. The patient was treated with 10 mg cetirizine; however, symptoms persisted intermittently for 36 h. Eight weeks later, patient 3 tolerated without sequelae open oral challenge to artificial crab without carmine.

Neither patient 1 nor 3 experienced a late reaction to carmine. The normal control individual with negative SPT to carmine experienced no symptoms at 25 mg carmine.

SDS-PAGE of carmine and cochineal

As shown in Fig. 1, lane 1, two main bands of 80 and 46 kDa and several minor bands (23–88 kDa) were detected in the cochineal insect extract. No bands were detected with commercial carmine lake (Fig. 1, lane 2).

Immunoblotting

As shown in Fig. 2, lane 1 (in patients 1, 2, and 3), several different cochineal insect extract bands (23–88 kDa), including those of 23 kDa (patient 2), 38 and 50 kDa (patient 1), and 88 kDa (patient 3), were recognized variably by the various patient sera. Patient 1's serum recognized two common protein bands (38 and 50 kDa) in both the cochineal insect extract (Fig. 2, I-lane 1) and carmine (Fig. 3, lane 1), although no bands on SDS-PAGE were detected with carmine. Patient 2's

serum diluted 1:1 reacted strongly to a common 23-kDa band in both cochineal insect extract (Fig. 2, II-lane 1) and carmine (Fig. 3, lane 2), and weakly recognized several other cochineal insect bands. This patient's serum diluted 1:9 still strongly recognized the 23-kDa band from the cochineal insect extract, but not the other bands recognized by 1:1 dilution serum (Fig. 2, II-lane 3). Patient 3's serum recognized an 88-kDa protein in cochineal insect extract (Fig. 2, III-lane 1), but did not recognize any protein in the carmine (Fig. 3, lane 3). Serum from an oral-challenge-negative individual failed to react to any protein bands from either extract (Fig. 2 C-lane 1; Fig. 3, lane C).

Immunoblotting inhibition with carmine

As shown in Fig. 2, all patient 1's bands with 1:1 dilution serum on the cochineal insect extract were inhibited with 1:20 liquid carmine (Fig. 2, I-lane 2). For patient 2, with 1:1 dilution serum, several bands, except for the strongly recognized 23-kDa band, were inhibited by 1:20 carmine (Fig. 2, II-lane 2). However, with 1:9 dilution serum, this strongly recognized 23-kDa band was inhibited by 1:20 carmine (Fig. 2, II-lane 3 and 4). For patient 3, with 1:1 dilution serum, the 88-kDa band was also inhibited by 1:20 carmine (Fig. 2, III-lane 1 and 2). For all patients, with 1:1 dilution sera, no immunoblot inhibition could be achieved with 1:200 carmine (not shown).

Discussion

Carmine (CAS Reg. no. 1390-65-4) and cochineal extract are biogenic dyes made from the dried females of the cochineal insect, *Dactylopius coccus costa* (*Coccus cacti* L), a parasite of the prickly pear cactus. The dried cochineal insects, which contain 17–24% carminic acid, are subjected to a carefully controlled extraction process in an acidic, aqueous, alcoholic solution. This solution of carminic acid is then precipitated (laked) on a substratum of aluminum hydrate with aluminum and

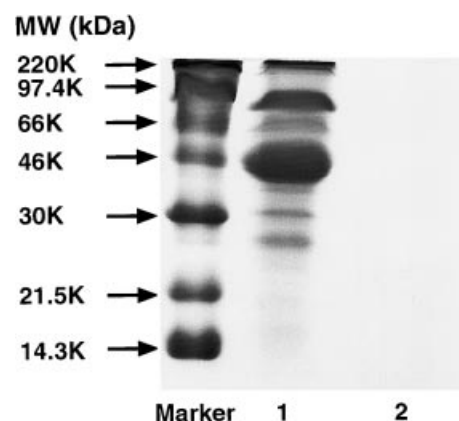


Figure 1. Coomassie brilliant blue stained-SDS-PAGE of molecular weight marker, cochineal insect extract (lane 1), and carmine lake (lane 2).

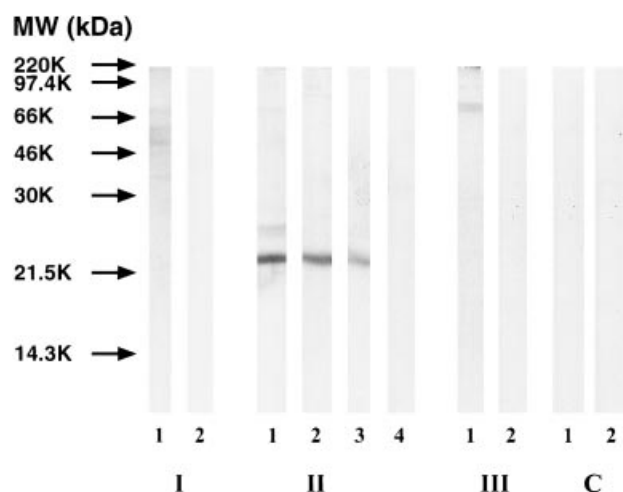


Figure 2. Specific IgE-immunoblot of three patients' sera to cochineal insect extract (I-lane 1 [1:1 dilution serum], patient 1; II-lane 1 [1:1 dilution serum], and 3 [1:9 dilution serum], patient 2; III-lane 1 [1:1 dilution serum], patient 3; C-lane 1 [1:1 dilution serum], control) and immunoblot inhibition with 1:20 carmine liquid (I-lane 2, patient 1; II-lanes 2 and 4, patient 2; III-lane 2, patient 3; C-lane 2, control).

calcium cations as precipitants. The resulting lake is called carmine. Carmine and cochineal extract share the same color index (no. 75470) and are also known in the world marketplace as CI natural red no. 4, and E120 in the European Economic Community (EEC) code. The coloring principle of both is believed to be carminic acid (CAS Reg. no. 1260-17-9), $C_{22}H_{20}O_{13}$ (MW 492.39).

The FDA specifies that cochineal extract should contain not more than 2.2% protein ($N \times 6.25$) (13). Although no corresponding guidelines for carmine exist, its protein levels are likely to be considerably higher because carmine is a more concentrated material. Water-soluble carmine is manufactured as powder (hydrosoluble carmine; not less than 50% carminic acid) or liquid (not less than 3.1–3.5% carminic acid). Neither carmine nor cochineal extracts are FDA-certifiable (synthetic) colorants. Foods and drugs containing these colorants (or any other FDA-approved dye exempt from certification) need not bear labels specifying these ingredients (14). For cosmetics, each ingredient (with the exception of flavors and fragrances) must be declared, in descending order of predominance, on the label (15, 16).

In this paper, Coomassie brilliant blue-stained SDS-PAGE of cochineal insect extract showed several definite protein bands in the range 23–88 kDa. No bands were detected with carmine lake. Despite this, immunoblotting using patient but not control sera recognized several protein bands from both electrophoresed pulverized cochineal insect extract and carmine. However, no two patients' sera recognized the same protein band. More proteins and stronger recognition were observed with cochineal insect extract than with carmine. These results suggest, as expected, that carmine contains fewer proteins from insects than the pulverized insect itself.

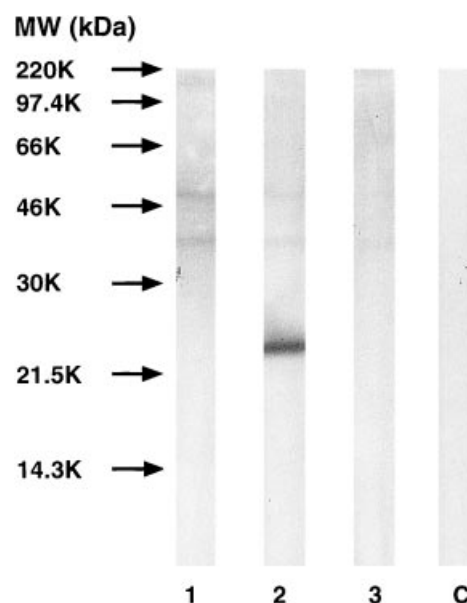


Figure 3. Specific IgE-immunoblot of three patients' sera to carmine lake (lane 1, patient 1; lane 2, patient 2; lane 3, patient 3; lane C, control).

To determine whether carmine liquid (which provoked positive skin reaction in carmine allergy patients) contains the same proteins as cochineal insects, we performed immunoblotting inhibition with carmine liquid. With appropriate concentrations of carmine and patient sera, all bands present in the above immunoblots with insect extract were inhibited. These results suggest that antigenically identical (or similar) proteins recognized by our patients' sera exist in carmine and the insect; however, the concentration may differ, and the proteins recognized may differ from patient to patient. It remains unclear whether the culpable allergens are simply proteins from cochineal insects or whether a protein-carminic acid complex is the recognized allergen in these patients. Furthermore, other lines of study are required to determine whether carminic acid is an essential antigenic determinant in carmine allergy in these patients.

In summary, SBPCFC or DBPCFC combined with skin testing in these three patients confirmed carmine as the allergen responsible for their food allergy. As indicated by immunoblotting and immunoblotting-inhibition experiments, several different cochineal insect proteins appear to be present in commercial carmine. Although all patients' sera recognized one or more of these proteins, no universal protein was recognized by all three carmine-allergic patients. Carmine avoidance remains problematic under current FDA labeling requirements for food. Future investigations to determine whether carminic acid is an essential antigenic determinant in carmine allergy, or whether pure carminic acid (free of any associated proteins) would be a safe alternative colorant are essential in improving the understanding of this condition.

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