

Occupational Asthma to Carmine in a Butcher

Analysis of the Literature on Allergy to Carmine

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Key Words

Carmine allergy · E120 · Immunoblotting · Inhibition · Occupational asthma

Abstract

Hypersensitivity to carmine (E120) has been identified as a cause of food intolerance and occupational asthma. We present a case of occupational asthma following exposure to carmine in a manufacturer of sausages and review the literature. **Case Report:** A 42-year-old non-atopic male presented with a 5-year history of rhinoconjunctivitis and asthma on occupational exposure to food additive dusts. Symptoms increased after work. The patient had been exposed for more than 20 years. **Methods:** Skin prick tests were performed with a battery of common inhalant allergens and spices. Cochineal, carmine lake and additive mixes used by the patient were extracted and subsequently used for skin prick test, bronchial provocation and in vitro measurements (specific IgE, Western blot and chromatographic fractionation). **Results:** Prick tests were positive to carmine and carmine-containing additives; carmine-specific IgE and bronchial challenge tests were also positive ($PC_{20} = 0.0004$ mg/ml and 1.6 kU/l). Western blot showed IgE binding to bands of about 30 kDa on cochineal extract and a diffuse pattern at 40–97 kDa on carmine. This result was confirmed

by gel filtration chromatography and dot blot. Carmine completely inhibited IgE binding to cochineal extract.

Discussion: Carmine is a potential sensitizer in an occupational setting: 18 cases of occupational asthma have been described to date. Carmine allergens are poorly defined; in general, proteins from cochineal not removed by the extraction process are considered as the main allergens in carmine. Our results are consistent with this, but show that these proteins may be subject to chemical modification.

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Introduction

Carmine is a natural red dye prepared from cochineal, the dried bodies of gravid female cochineal insects (*Dactilopius coccus* or *Coccus cactus*). Cochineal extract (color index Natural Red 4, color index No. 75470; European Economic Community No. E120) is the concentrated solution obtained after removal of alcohol from an aqueous-alcoholic extract of cochineal. Carmine consists of an aluminum or calcium-aluminum chelate on an aluminum hydroxide substrate of carminic acid, the coloring principle of cochineal [1]. Production of carmine involves aqueous extraction of cochineal, salt precipitation of carmine, sterilization under steam and drying [2]. The final

Table 1. Composition of the mixtures and skin prick test (SPT) results

	Composition	SPT result	IgE Elisa OD/kU/l
Mix A	E451, E450, starch, potato flour, lactose, casein, nutmeg, clove, cinnamon, anise	–	– (0.047/0.2)
Mix B	salt, lactose, dextrose, sodium sulfite, sodium ascorbate, cochineal (E120), potassium meta-bisulfite, glutamate, sodium citrate	++ (7 mm)	++ (0.665/2.9)
Mix C	salt, paprika, sugars, color (E124), pepper, marjoram, garlic, clove	+	+
Mix D	sugars, preservatives E200 and E202, pepper, clove, cinnamon, nutmeg, coriander, anise	–	– (0.197/0.39)
Carmine	–	–	++ (0.05/0.2)
			++ (0.483/2.5)

powder is mixed with sucrose to adjust the color and canned. Typically, crude cochineal extract contains 2–4% carminic acid while commercial powdered carmine contains 20–50% [3].

Carmine is widely used in the manufacture of foods (drinks, meat products, dairy products, milk derivatives and confectionery), medicines, cosmetics and as a histological dye. Reactions to this natural dye were first described as a cause of cheilitis related to the use of lip salves colored with carmine [4]. Immediate hypersensitivity reactions among workers inhaling carmine were described by Burge et al. [2], although the involvement of IgE antibodies was later demonstrated by Quirce et al. [5]. Carmine hypersensitivity has also been identified as a cause of food allergy [6–8]. In addition, carmine inhalation has been linked to the development of allergic extrinsic alveolitis among workers in food and food additive factories [9]. In some of these workers precipitins to carmine have been found.

There are conflicting results on the nature of allergenic components of carmine. Protein remnants from the cochineal insect are thought to play an important role as carmine allergens, but no single allergen has been identified as a cause of sensitization [6, 10–12]. In addition, the role of carminic acid as a potential hapten has been suggested in several reports [13, 14]. We describe a case of occupational asthma following exposure to carmine in a butcher handling food additives. We report the clinical and immunological examinations and a study of carmine and the cochineal allergens involved. In contrast with previous reports, we found strong differences in carmine and cochineal extracts among the allergenic components recognized by the patient.

Case Report

A 42-year-old male had worked for 20 years as a blender of spices and food additives in a meat product factory. During the last 5 years, he had often noted shortness of breath and wheezing after work. The patient reported rhinorrhea, sneezing, nasal obstruction and watery eyes when exposed to mixes of spices containing carmine, which were used to color sausages. In the last year, the symptoms worsened, with angioedema of the larynx on exposure to ground spices, requiring strict avoidance measures and inhaled bronchodilators and steroids (salmeterol and fluticasone). An organic face mask was prescribed in an attempt to reduce work-related symptoms, with significant clinical improvement.

The patient has to prepare four different standard mixes (SM) of spices and food additives for the manufacture of sausages (table 1).

Methods

Allergen Extracts. SM used by the patient and water-soluble carmine (E120) containing not less than 50% of carminic acid (Sancolor Ref. P618; Sancolor, Barcelona, Spain) were extracted in phosphate-buffered saline (PBS) at 20 and 5%, respectively. Extracts were centrifuged at 10,000 g, dialyzed against purified water with 3,500-Da membranes (Pierce, Rockford, Ill., USA), filter sterilized (Millipore, Bedford, Mass., USA) and freeze dried. Total proteins in extracts were measured by the method of Bradford [15]. In addition, 5 mg/ml synthetic cochineal red (E124, Sancolor) solutions were prepared by dissolving in PBS and filter sterilization.

Skin Prick Test. A battery of common inhalant allergens (IPI, Madrid, Spain) was tested. Skin reactivity to SM, carmine and E124 extracts was assayed at 10 mg/ml with solutions containing 50% glycerin. Histamine dichlorhydrate at 10 mg/ml and normal saline were used as positive and negative controls, respectively. The response was read 15 min after puncture, and the results were expressed as the mean wheal diameter. A wheal diameter of at least 3 mm, compared with the saline control, was defined as a positive reaction [16].

Specific IgE. Specific IgE to spices contained in SM was determined by the Pharmacia CAP System RAST FEIA (Pharmacia, Uppsala, Sweden). For the determination of specific IgE to SM, we

adapted a modification of the basic RAST protocol on CNBr-activated paper discs as described [17, 18]. SM extracts were coupled to CNBr-activated paper discs at a protein concentration of 1 mg/ml (0.1 ml/disc). Serum samples were diluted 1:5 in PBS containing 1% bovine serum albumin and 0.1% Tween-20 (PBS-A-T), and incubated for 3 h at room temperature in a shaker device. After three washes in PBS-0.1% Tween-20, discs were incubated overnight in a 1:10,000 dilution of biotinylated rabbit antiserum to human IgE (Dako, Copenhagen, Denmark) supplemented with 5% normal rabbit serum. After further washing, biotinylated antibodies were detected with alkaline-phosphatase-labeled streptavidin (250 ng/ml) and a solution of *p*-nitrophenyl phosphate at 1 mg/ml (all from Sigma, St. Louis, Mo., USA) and read at 405 nm. IgE to SM was expressed in kU/l by comparison with an in-house reference standard, a serum pool from patients allergic to olive pollen titrated using the CAP System. Specific IgE levels in the calibration curve ranged from 0.2 to 26 kU/l.

IgE Inhibition Assays. The assays were performed with carmine (E120) extracts. In these experiments, 50- μ l aliquots of patient serum were mixed with 100 μ g carmine in a volume of 100 μ l. After a 2-hour incubation at 37°C, the remaining IgE was measured as above. Results of the inhibition experiments are expressed as percentage of inhibition relative to the serum incubated with PBS.

SDS-PAGE. Polyacrylamide gel electrophoresis in the presence of SDS was carried out as described previously [19]. Extracts were analyzed on 15% acrylamide discontinuous gels with 4% acrylamide stacking gels. After electrophoresis, protein bands were either detected by silver stain [20] or transferred to nitrocellulose membranes for immunoblotting.

Immunoblotting. Immunoblotting was carried out as described by Chung et al. [12] with minor modifications. Pulverized dried cochineal insects and commercial carmine powder were washed once in methanol and three times in saturated ammonium sulfate. The resulting precipitate was suspended in PBS, boiled for 10 min, centrifuged for 10 min at 10,000 g, and the supernatant retrieved for electrophoresis. After SDS-PAGE, proteins were blotted onto nitrocellulose membranes (Sigma) in Towbin buffer [21] with 20% methanol in a Hoeffer apparatus (Amersham Biosciences, Uppsala, Sweden). Blotted membranes were blocked for 1 h in TBS, pH 7.4 (Tris-HCl 50 mM, NaCl 150 mM), containing 0.3% gelatin and 0.5% bovine serum albumin. After blocking, 3-mm strips were cut and incubated overnight with serum samples, diluted 1:5 in incubation buffer (blocking solution supplemented with 0.1% Tween-20). The next day, membranes were washed, and bound IgE was detected with a biotinylated mouse monoclonal antibody to human IgE diluted 1:1,000 (Ingenasa, Madrid, Spain), followed by streptavidin-peroxidase (Sigma). Detection was performed with the ECL system (Amersham Biosciences). In a separate set of experiments, IgE binding to cochineal was inhibited with either carmine or cochineal. Briefly, 200 μ l of serum were preincubated with 50 μ g of carmine or cochineal extracts and with PBS, all in a volume of 100 μ l. After 2 h at 37°C, 700 μ l of incubation buffer were added, and strips were processed as above.

Chromatographic Fractionation of Carmine and Dot Blot. Carmine extract was fractionated by size exclusion chromatography on Superdex 75 (Amersham Biosciences). Elution was monitored at 280 nm, and absorbance peaks were pooled, concentrated by freeze drying and analyzed by SDS-PAGE as above. Pooled fractions were also analyzed in dot blot by spotting 1- μ l aliquots of each pool on nitrocellulose strips.

Specific Bronchial Challenge Test. Bronchial challenge was performed according to Cockcroft et al. [22] with some modifications [23]. Test material consisted of a lyophilized extract of carmine freshly reconstituted with buffer. The aerosolized particles were generated by a continuously pressurized nebulizer (Hudson 1720; Hudson Respiratory Care Inc., Temecula, Calif., USA) with an input of 7 liters/min and an output of 0.17 ml/min. The patient received a control challenge with buffer at tidal breathing for 2 min before antigen provocation and forced expiratory volume in 1 s (FEV₁) was recorded. Allergen dose was increased by tenfold increments at 60-min intervals, and FEV₁ was measured 20 min after inhalation of each concentration. The test was discontinued when there was a fall in FEV₁ of 20% or more compared to the buffer control value. The test result is expressed as the provocative concentration of carmine causing a 20% fall in FEV₁ (PC₂₀) estimated by interpolation of the last two concentrations. The same bronchial challenge test was used in 3 control subjects.

Results

Skin Prick Test

The standard inhalant allergen battery gave negative results. Positive skin response was found when carmine-containing SM was assayed (table 1). A skin test with carmine (E120) gave strong positive results (wheal 12 mm) while synthetic carmine (E124), both free and bound to human serum albumin, was negative.

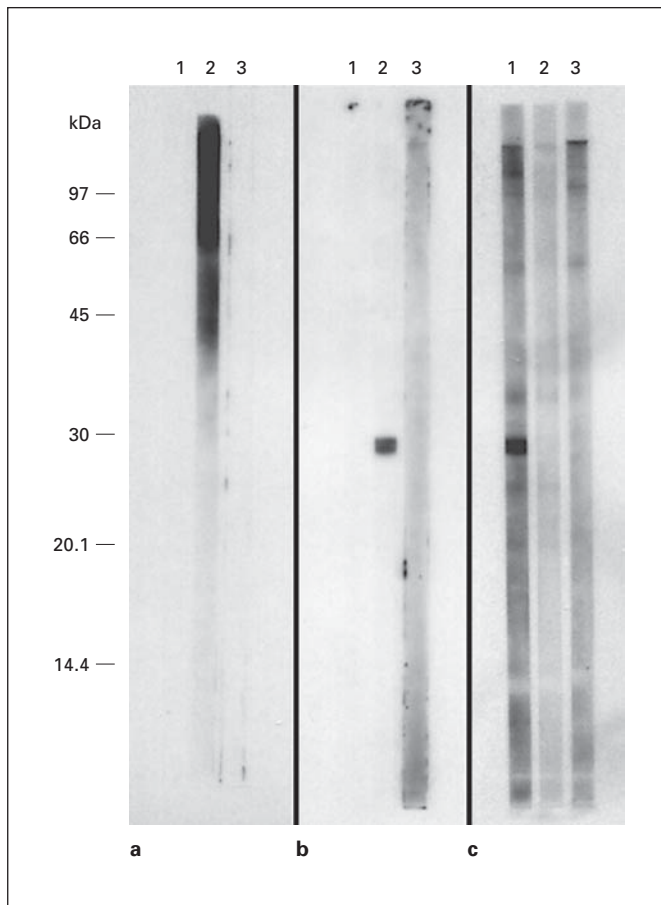
Prick tests were assayed in a group of 17 atopic and 10 non-atopic patients. All these controls displayed negative results except for 2 atopic patients in which positive results were found to mix C: both patients displayed sensitization to spices.

IgE Measurements

Total IgE was 70.7 IU/ml. Specific IgE to a wide range of spices was measured using the CAP System. A positive result was found to carmine and a blend of curry named Santa María (1.6 and 2.11 kU/l, respectively). Specific IgE to SM was assayed by in-house ELISA (table 1). A positive result was obtained with mix B (2.9 kU/l). Preincubation of serum with carmine decreased IgE binding to mix B by 59%, while no effect on IgE binding was found in a non-related allergen system (olive pollen). A series of specific IgE determinations did not detect sensitivity to the spice components in SM (cinnamon, garlic, anise, nutmeg, black pepper, clove and marjoram).

SDS-Page and Immunoblotting

As in previous reports, SDS-PAGE with carmine (E120) extract showed no distinctive pattern of protein bands. In contrast, cochineal showed several bands over



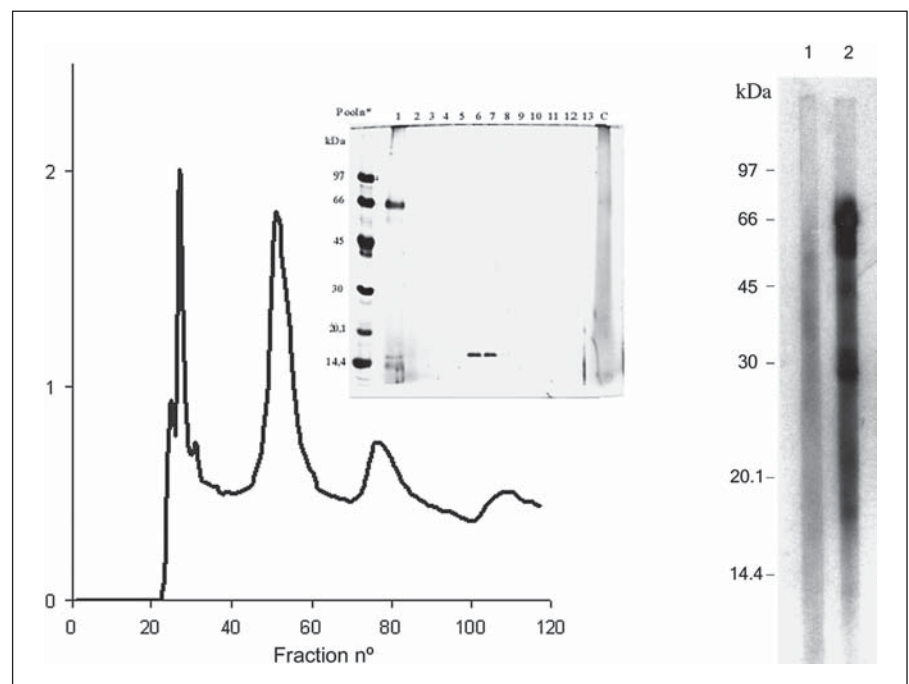
a wide molecular weight range. Immunoblotting with the patient's serum produced a different pattern of IgE binding on carmine and cochineal strips (fig. 1). In carmine, high-molecular-weight components ranging from about 40 to over 97 kDa were identified. In contrast, the patient's IgE clearly recognized a doublet of about 30 kDa in cochineal extract. In immunoblotting inhibition experiments, carmine produced complete inhibition of IgE binding to cochineal (fig. 1), while no effect was observed when carmine was used as inhibitor in a non-related allergen system (olive pollen Western blot, data not shown).

Chromatographic Fractionation of Carmine

After chromatography on Superdex 75 and SDS-PAGE analysis, protein bands were detected in only two of the pools, one containing the void volume (112.8- and 57-kDa bands) and the other with a single 14.6-kDa band (fig. 2). Dot blot experiments with the patient's serum showed specific IgE binding restricted to the higher-molecular-mass fractions, and no recognition of smaller pro-

Fig. 1. **a, b** IgE immunoblotting of carmine (**a**) and cochineal (**b**). Lane 1 = Medium control; lane 2 = patient; lane 3 = normal control. **c** Immunoblot inhibition of IgE binding to cochineal with PBS (lane 1), cochineal (lane 2) and carmine (lane 3).

Fig. 2. Immunoblotting of Superdex-75-fractionated cochineal extract. Fractions showing IgE binding on dot blot were pooled and probed with the patient's serum. Lane 1 = Non-reduced; lane 2 = reduced with 2-mercaptoethanol.



teins. Positive fractions were pooled and submitted to SDS-PAGE and immunoblotting (fig. 2). IgE binding was detected at 57 and 54 kDa. Treatment of sample with 2-mercaptoethanol produced an increase in IgE binding with bands over a wide molecular weight range.

Specific Bronchial Challenge Tests

A bronchial challenge test with carmine extract was positive with $PC_{20} = 0.0004$ mg/ml. The same test gave negative results in 3 non-atopic controls.

Discussion

We describe a patient with asthma and rhinoconjunctivitis related to professional exposure to mixtures of food additives and spices used in the manufacture of sausages. Among these additives, four SM were identified, extracted and tested on the patient. Positive SPT was found with two of the extracts, one of them containing cochineal carmine (E120) and the other synthetic carmine (E124). We carried out a search for a causative agent among the components of additive mixes by SPT and specific IgE. These tests gave only positive results with cochineal carmine, while synthetic carmine (E124, Ponceau 4R) and spices were negative. The involvement of carmine as a causative agent of occupational asthma in this patient was established by the presence of specific IgE to carmine both in serum and skin prick test, and by specific bronchial challenge, which was strongly positive ($PC_{20} = 0.004$ mg/ml). In addition, carmine inhibited the patient's IgE binding to the mix containing cochineal carmine (mix B), indicating that the reactivity to mix B was attributable to its carmine content.

To date, 31 cases of immediate hypersensitivity reactions to carmine have been described (table 2). According to the clinical features and origin of sensitization, these patients can be clustered in two groups. In 18 cases, hypersensitivity was caused by professional exposure in the manufacture of dyes or handling of spices and food additives [2, 5, 10, 11, 14, 24–26, present report]. These occupational cases were mainly men (12/18), and among them an atopic background did not seem to be a predisposing factor, since only 3 out of the 18 displayed additional sensitivities. In all these cases, inhalation of carmine was associated with respiratory symptoms (asthma and/or rhinoconjunctivitis). The case of occupational asthma described here shares several of the features commonly found in these patients with carmine-related occupational disease, including male predominance and

non-atopic background. The remaining 13 patients described presented hypersensitivity reactions to carmine-colored foods [6–8, 12, 13, 27]. All these patients were female, and about half of the cases were atopic. Clinical features of these cases included cutaneous (urticaria and angioedema), digestive (nausea, vomiting and diarrhea), and respiratory symptoms (dyspnea, wheezing, cough, sneezing, watery rhinorrhea and nasal congestion), triggered by oral intake of foods colored with carmine (Campari®, yogurt, artificial crab, fruit juice, and ice cream). In addition to these patients orally sensitized to carmine, at least 2 cases of inhalative occupational allergy to carmine also showed oral intolerance to this substance [2, 10] confirmed by positive oral challenge test. Nevertheless, workers with inhalant allergy to carmine can tolerate ingestion of carmine [5], like the patient described here, who regularly consumed carmine-containing foods with no adverse effect.

Cases of occupational disease induced by carmine inhalation have been described in factory workers producing, mixing or storing dyes. In those settings, high levels of exposure can be inferred from the generation of airborne cochineal dust, as has been described in the manufacture of carmine [5], and the occasional involvement of workers in non-production areas such as administrative departments. In a recent study [25] on workers from a natural-dye factory, the prevalence of sensitization to carmine and/or cochineal was 30% while respiratory allergy to carmine (occupational asthma) affected 8% of the workers. In addition, allergy to carmine has been linked to the manufacture of meat products [26, and present report], where it can be linked to activities such as mixing of additives in which carmine dusts are generated, increasing exposure and the risk of sensitization.

Carmine is a common constituent of food and cosmetic products. In a search performed in the PubMed database (www.ncbi.nlm.nih.gov), just 13 cases of non-occupational allergy to carmine were found (table 2), so the rate of sensitization among the general population seems quite low. Interestingly, most of these patients suffering from food allergy complained of cutaneous symptoms after use of cosmetics containing carmine, such as lipsticks, blushes or eye shadows. This could account for the observed predominance of females among orally sensitized individuals.

Allergens involved in sensitization to carmine have been poorly characterized. The first immunoblotting results published are from Acero et al. [10] using cochineal extracts. In that paper, IgE from a sensitized worker of a spice warehouse bound high-molecular-weight proteins,

Table 2. Studies on occupational asthma or patients allergic to carmine

References	Sex	Age years	Atopy	Exposure	Occupation	Duration of exposure years	Carmine prick test	Specific IgE	Immuno- blotting (Mw)	Challenge test
Burge et al. [2] 1979	M	36	no	inhalative	dye factory	0.4	–	ND	ND	BCT+
	M	54	no	inhalative	cosmetic factory (blender)	5	–	–	ND	BCT+ OCT+
Tenabene et al. [24] 1987	F	54	no	inhalative	spice manufacture (blender)	10	–	–	ND	BCT+
	M	36	no	inhalative	food dye handling	3	+	ND	ND	BCT+
	M	52	no	inhalative	food dye handling	0.15	+	ND	ND	BCT+
Quirce et al. [5] 1994	M	27	no	inhalative	dye manufacture	2	+	+	ND	BCT+ OCT–
	F	40	no	inhalative	dye factory (cleaner)	3	+	–	ND	ND
	M	23	no	inhalative	dye factory (ex-plant worker)	1	+	–	ND	ND
Kägi et al. [6] 1994	F	34	yes	oral	–	–	+	+	ND	ND
Beaudouin et al. [7] 1995	F	35	no	oral	–	–	+	ND	ND	ND
Stücker et al. [14] 1996	M	26	no	inhalative	food factory	5	+	+	ND	ND
Wütrich et al. [8] 1997	F ^a	34	yes	oral	–	–	+	+	ND	ND
	F	33	yes	oral	–	–	+	+	ND	ND
	F	46	no	oral	–	–	+	+	ND	ND
	F	25	yes	oral	–	–	+	+	ND	ND
	F	39	no	oral	–	–	+	+	ND	ND
Baldwin et al. [27] 1997	F	27	yes	oral	–	–	+	+	(P-K test)	ND
Acero et al. [10] 1998	M	37	no	inhalative+oral	spice warehouse (packer)	0.5	+	ND	+ (Mw not determined)	BCT+ OCT+
DiCello et al. [13] 1999	F	27	no	oral	–	–	+	ND	ND	ND
	F	42	no	oral	–	–	+	ND	ND	ND
Lizaso et al. [11] 2000	M ^b	32	no	inhalative	plant worker (food dye factory)	4	+	ND	pooled serum (28.50)	BCT+
	F	29	no	inhalative	office worker (food dye factory)	6	+	ND	pooled serum (28.50)	BCT+
	F	27	no	inhalative	chemist (food dye factory)	3	+	ND	pooled serum (28.50)	BCT+
Chung et al. [12] 2000	F	32	yes	oral	–	–	+	ND	+	OCT+
	F ^c	27	yes	oral	–	–	+	ND	(38.50) +	ND
	F	30	yes	oral	–	–	+	ND	(23.50) +	OCT+
Tabar et al. [25] 2003	F ^d	26	yes	inhalative	office worker (dye factory)	2.5	+	+	ND	BCT+
	F ^d	29	no	inhalative	laboratory (dye factory)	6	+	+	ND	BCT+
Anibarro et al. [26] 2003	M	47	yes	inhalative	butcher	35	+	ND	+	BCT+
	M	24	yes	inhalative	butcher	4	+	ND	(10) +	BCT+
Present report	M	42	no	inhalative	sausage factory (spice blender)	15	+	+	+	(28) BCT+

^a A patient already described [6].^b Previously described by Quirce et al. [5].^c Previously described by Baldwin et al. [28].^d Characteristics similar to those described previously [11].

Mw = Molecular weight; ND = not determined; BCT = bronchial challenge test; OCT = ocular challenge test.

although no accurate molecular weight estimations were available. Recently, Lizaso et al. [11] reported the results of immunoblotting in 4 cases of occupational sensitization to cochineal in a dye factory. In their study, three proteins were responsible for IgE binding to carmine (28 and 50 kDa) and to cochineal (17, 28 and 50 kDa). The authors claim that identical proteins from both cochineal and carmine are responsible for sensitization. In another study, immunoblotting was performed on 3 patients orally sensitized to carmine (table 2), and bands of 23, 38, 50 and 88 kDa were recognized in carmine, showing a different pattern in each patient, with 2 of them displaying a predominant pattern of high-molecular-weight bands.

The patient described here showed positive immunoblotting to carmine and cochineal, although the binding pattern to both allergens was different (fig. 2). IgE-binding components of cochineal displayed an apparent molecular weight of 28–30 kDa, while carmine showed high-molecular-weight allergens (from about 40 to over 97 kDa). The specificity of this result was further assessed by fractionation of carmine extract by size exclusion chromatography and dot blot. Dot blot experiments showed that the reactivity of the patient's IgE to these proteins was restricted to higher-molecular-weight fractions, confirming our previous findings on Western blot. It has been suggested that cochineal proteins are the main allergens in carmine. Since our results argue against this (i.e. allergens with different molecular weights were found in cochineal and carmine), we tried to assess the antigenic identity of allergens from these sources by inhibition. A close similarity in the antigenic nature of both allergens could be inferred from the strong inhibition of IgE binding to cochineal achieved by preincubation with carmine

(fig. 1). These results indicate that (proteinaceous) material from cochineal insects is responsible for the sensitivity to carmine and that chemical modification occurs during the process of carmine extraction. Several groups have suggested the possibility that chemical modification of cochineal proteins renders them allergenic. As an example, protein haptenization by carminic acid has been shown to be a relevant factor for the ability of cochineal proteins to bind IgE from some patients [14]; the persistence of a strong red color in high-molecular-weight (extensively dialyzed) preparations of carmine supports this possibility. Aggregation of proteins by disulfide bond scrambling or glycation induced by heating or storage may contribute to the allergenicity of some processed substances [reviewed in ref. 28]. Our results show that carmine allergens are highly sensitive to disulfide bond reduction (fig. 2), producing an increase in the number of IgE-binding components. This may reflect aggregation of allergenic proteins derived from cochineal via disulfide bonds, and could explain the apparent differences in allergen content of cochineal and carmine preparations observed by us.

We conclude that exposure to cochineal carmine (E120) is a significant risk factor for developing occupational disease even among non-atopic workers. A dual clinical and epidemiological profile is found in the literature: (1) non-atopic males with professional airway disease (as the present case) and (2) atopic or non-atopic females with food allergy to carmine-containing foods. Our results indicate that at least among patients occupationally sensitized to carmine, chemical modification of cochineal proteins may account for its allergenic potential.

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