

Identification of allergens involved in occupational asthma due to carmine dye

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Background: Carmine has been implicated as an etiologic agent of occupational asthma, but the allergens involved have not been yet identified.

Objective: To identify the allergens involved in occupational asthma due to carmine dye.

Methods: An in vitro study based in SDS-PAGE and IgE immunoblotting with carmine and cochineal extracts was performed. Sera from three carmine dye workers diagnosed with occupational asthma induced by carmine dye and from seven nonatopic subjects were used.

Results: Three proteins of around 30, 28, and 17 kD in raw cochineal extract and another protein of 50 kD in the boiled one were demonstrated by SDS-PAGE. Two proteins of around 50 and 28 kD were observed in the carmine extract by the same technique. Specific IgE binding bands at 17 kD in cochineal raw extract, at 50 kD in the boiled one, and at 28 kD in carmine extract were demonstrated by IgE immunoblotting.

Conclusions: We have identified three allergens of around 17, 28, and 50 kD implicated in occupational asthma of three carmine workers.

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INTRODUCTION

Carmine [Color Index No. [CI] 75470, E-120 in the European Community] is a natural red dye extracted from the dried bodies of the female insect *Dactylopius coccus* var. Costa (family Dactylopiidae, superfamily Coccoidea) (Fig 1). This insect lives as a parasite on some species of cacti, particularly *Nopalea cochenillifera*, mainly in Central and South America. In Europe, cochineal raising is restricted to the Canary Islands in Spain. Carmine (hydrated aluminum chelate of carminic acid) contains about 50% carminic acid [molecular weight (MW) 492.4 D] (Fig 2) and 20% proteinaceous material from cochineal. The process of

obtaining carmine comprises aqueous extraction of cochineal, precipitation of carmine with sodium sulfate and calcium carbonate, filtration, sterilization, and drying. It is then milled, mixed (usually with sucrose to adjust color by spectrophotometry on the basis of carminic acid content), and finally bagged (Fig 3). Today carmine is used in the food (mainly butchery and confectionery), cosmetic and pharmaceutical industries, and also as an histologic dye.¹

Carmine has been implicated as etiologic agent of occupational asthma^{2,3} and extrinsic allergic alveolitis.⁴ Our group reported a clinical and immunologic study carried out on 10 workers at a carmine dye factory.⁵ Three of these workers had work-related symptoms of rhinitis and/or asthma. In this study it was concluded that carmine might induce immunologic responses, likely IgE mediate, in workers with symptoms of occupational asthma.

The present in vitro study was designed to identify the allergens involved in occupational asthma in workers at the same carmine dye factory.

MATERIAL AND METHODS

Subjects

Frozen (–20°C) sera from three carmine dye workers diagnosed with occupational asthma induced by carmine dye were used for “in vitro” study. One of the workers was an asthmatic patient described in the previous study,⁵ and the other two were new affected patients. The clinical characteristics of the patients are presented in Table 1. The diagnosis of occupational asthma by carmine was based on a compatible clinical history; skin prick test with carminic acid, carmine, and cochineal extracts; baseline pulmonary function tests; and bronchial provocation test with methacholine, cochineal, and carmine. Table 2 shows the results of them in the three patients. All tests were performed according it was described at the previous study.⁵

Sera from seven nonatopic, unexposed subjects were used as controls. They were chosen at random among our Department patients and all of them had negative skin prick tests with pneumoallergens and foods. All had total IgE within normal ranges.

Dye Extracts

Untreated cochineal (dried female insects) was ground to a powder. Then a 10% wt/vol extract was prepared by dissolving this material in phosphate-buffered saline solution (PBS) (pH 7.3) at room temperature.

A 10% wt/vol carmine extract was prepared by dissolving water-soluble carmine in PBS.

SDS-PAGE

Sixteen percent acrylamide minigels were run according to Laemmli.⁶

The cochineal extract was mixed 1:1 with a sample buffer containing 0.5 M

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Figure 1. Dried cochineal.

Tris-HCl pH 6.8, glycerol, 10% SDS, 2 betamercaptoethanol, and 0.05% bromophenol blue. Sometimes, this mixture was boiled for 10 minutes and it was used without heating in other ones. The carmine extract was mixed 1:2 with the same sample buffer and it was used nonheated.

Twenty microliters of the mixture of every extract with sample buffer were electrophoresed at 150 V for approximately 45 minutes on a Mighty Small II, SE 250 cell (Hoeffer Scientific Instruments, San Francisco, CA). Molecular weight protein controls (Bio-Rad, Richmond, CA) provided seven calibration points between 6.5 and 204 kD.

After electrophoresis, the allergens were fixed with a 36% methanol, 6% trichloroacetic, and 3.6% sulfosalicylic acid solution for 30 minutes. Then the minigels were washed two times for 10 minutes with a 40% methanol and 10% acetic acid solution.

Thereafter, the minigels were stained for 45 minutes with a 0.125% Coomassie blue, 50% methanol, and 10% acetic acid solution. They were then destained with a 10% acetic acid and 7.5% methanol solution.

IgE Immunoblotting

After separation of allergen extracts by electrophoresis as described above, the minigels were transferred to nitrocellulose (0.45 μ m Hoefer Scientific, San Francisco, CA) by a diffusion method, as previously described.⁷ After blocking in 10 mM/L Tris, 0.15 mol/L NaCl, and 3% bovine serum albumin (BSA) (pH 7.4), some of the nitrocellulose sheets were placed in a Multi-Screen (Bio-Rad, Richmond, CA) and incubated overnight with a 1:6 dilution of the individual sera from symptomatic patients or from control subjects and other nitrocellulose sheets were incubated with a 1:20 dilution of pooled

sera from the same patients. The incubation buffer for sera dilution was composed of 10 mM Tris-HCl, 0.15 M NaCl, 3% BSA, and 1% Tween 20 at pH 7.4. Later the membranes were taken out of the device and after three washes, incubated for 3 hours with a 1:5000 dilution of a biotin-labeled monoclonal anti-IgE (Bio-Rad, Richmond, CA). After new washes, streptavidine alkaline phosphatase-labeled was added at a 1:2000 dilution for 1 hour and finally incubated with the substrate.⁸

RESULTS (Fig 4)

SDS-PAGE

Three protein-staining bands of around 30, 28, and 17 kD were demonstrated in the raw cochineal extract. Another protein of 50 kD was identified when the cochineal extract was boiled.

In the carmine extract, two protein-staining bands of around 50 and 28 kD were observed.

IgE Immunoblotting

Immunoblots with individual and pooled sera from the three workers diagnosed with occupational asthma dye demonstrated a specific IgE-binding band at 17 kD in the cochineal raw extract, at 50 kD in the cochineal boiled extract, and at 28 kD in the carmine extract. Only the immunoblots with pooled sera are shown because they are clearer than immunoblots with individual sera.

These allergens were not recognized by pooled sera from the seven nonatopic control subjects.

DISCUSSION

Carmine is a natural red dye extracted from the dried bodies of the female insect *Dactylopius coccus* var. Costa. It contains about 50% carminic acid and 20% of proteinaceous material from cochineal.¹ Carmine has been reported as etiologic agent of occupational asthma^{2,3} and extrinsic allergic alveolitis.⁴

Burge et al² in 1979 were the first to report work-related asthma caused by carmine inhalation, in whom dual responses to the bronchial provocation test with carmine and cochineal ex-

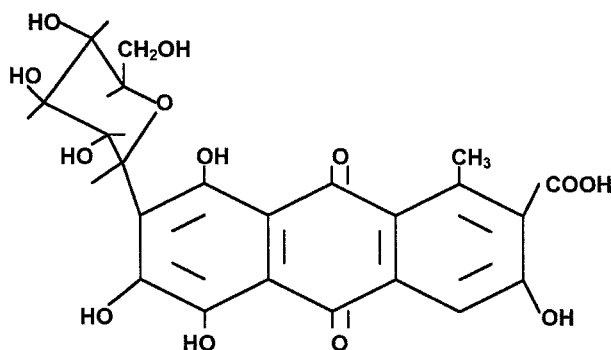


Figure 2. Structural formula of carminic acid.

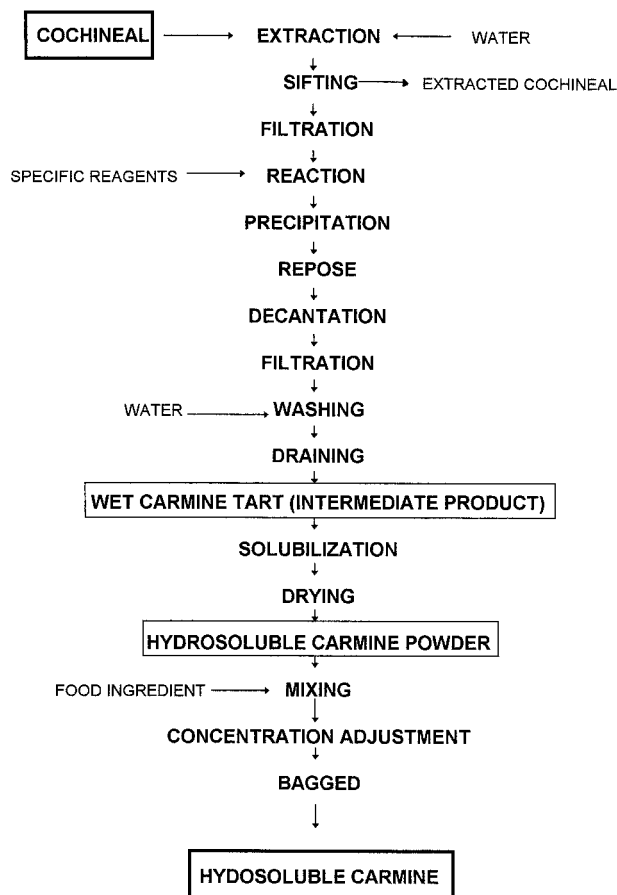


Figure 3. Process used to obtain carmine.

tract were observed. The two individuals in the Burge study had positive oral challenges with cochineal extract or a carmine-containing beverage. Specific IgE antibodies were not found in either patient by skin testing or RAST.

Tenabene et al³ described three workers with carmine-induced asthma confirmed by cabin exposure test. Two of them had positive reactions by skin test, although specific IgE was not demonstrated by RAST.

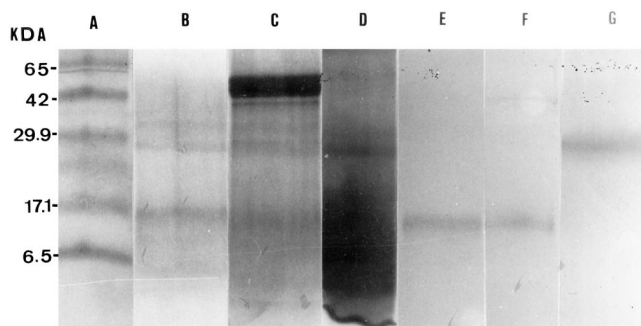


Figure 4. A = molecular weight protein controls. B = SDS-PAGE: raw cochineal extract. C = SDS-PAGE: boiled cochineal extract. D = SDS-PAGE: carmine extract. E = IgE immunoblotting: raw cochineal extract. F = IgE immunoblotting: boiled cochineal extract. G = IgE immunoblotting: carmine extract.

Christiansen et al^{8a} and Dietemann-Mo-
lard et al⁴ described several workers in
food and food additive factories with ex-
trinsic allergic alveolitis caused by car-
mine inhalation with demonstration of pre-
cipitins to carmine in some of these cases.

Initially our group described a case
report about a worker in a factory mak-
ing natural dyes who suffered from
occupational asthma due to carmine
inhalation.^{8b} The skin prick test with
carmine extract was positive and a dual
response to the bronchial provocation
test with carmine was obtained. In this
case, however, the presence of specific
IgE antibodies by serologic methods
was not tested.

Later, we studied all 10 workers in
this factory.⁵ We found a worker with
rhinitis and asthma due to carmine and
another worker with work-related rhi-
nitis, possibly caused by carmine inha-
lation. This could not be demonstrated,
however because the worker refused
provocation studies. In the first patient,
the prick test with carmine and cochineal
extract was positive. We also demon-
strated specific IgE antibodies by RAST
technique to carmine and cochineal. In
addition, we performed a RAST-inhibi-
tion study and we concluded that the
main allergen of carmine had a molecu-
lar weight between 10 and 30 kD.

Beaudouin et al⁹ reported a case
about an anaphylactic reaction follow-
ing the ingestion of carmine and in this
case the prick skin test and the leuko-
cyte histamine release test with car-
mine were positive, confirming the
IgE-dependent mechanism.

Baldwin et al¹⁰ reported another case of
anaphylaxis after ingestion of a popsicle
colored with carmine. In this case the skin
prick tests to the popsicle and carmine
were positive and were successfully trans-
ferred to a control in P-K format. This case
also supports the notion of a carmine-spe-
cific, IgE-mediated mechanism.

SDS-PAGE or IgE immunoblotting
studies have not been yet performed in
order to characterize the allergens in-
volved in this occupational asthma.
Quirce et al⁵ pointed out that the impli-
cated allergens of carmine are probably
cochineal proteins probably present in
the final product, the carmine. But, as

Table 1. Clinical Characteristics of Study Subjects

Subject No	Age (yr)	Sex	Occupation (yr employed)	Symptoms at Work (duration in mo)	Patient/Family Atopy	Smoking (pack yr)	Total IgE (kU/L)
1	32	M	Plant worker (4)	RC/A (44)	No/No	9	433
2	29	F	Office worker (6)	A (42)	No/No	0	171
3	27	F	Chemist (3)	A (27)	No/No	6	147

A = asthma and RC = rhinoconjunctivitis.

Table 2. Results of Skin Prick and Bronchial Provocation Test of Study Subjects

Subject No	SPT*				BPT		
	Carmine (10 mg/mL)	Cochineal (1:10 wt/vol)	Carmine Acid (10 mg/mL)	Histamine (10 mg/mL)	Metacholine† (PD20 in CBU)	Cochineal (1:10 wt/vol)	Carmine (10 mg/mL)
1	15 × 7	10 × 8	0	5 × 5	+(20)	+(Immed)‡	+(Immed)
2	10 × 4	11 × 6	10 × 6	8 × 5	+(40)	+(Immed)	+(Immed)
3	3 × 3	3 × 3	0	6 × 6	+(200)	+(Immed)	+(Immed)

* Perpendicular diameters of the wheal in mm;

† According method described by Chai et al¹² (PD20 in cumulative breath units (CBU); 1 unit = one inhalation of aerosol from 1 mg/mL solution of metacholine).

‡ Immed, immediate response.

pointed out Beaudouin et al,⁹ only IgE immunoblotting could help resolve the question about the nature of reactogenic antigen, and whether it is a hapten-induced or a protein-induced reaction.

So, we have performed a *in vitro* study based in SDS-PAGE and IgE immunoblotting with carmine and cochineal extracts. The sera used to perform this study were from three workers of a carmine dye factory who had been diagnosed with occupational asthma by a clinical history, positive skin prick test to carmine, and cochineal and bronchial provocations, with cochineal and carmine extracts.

We have identified three allergens implicated in causing occupational asthma in carmine workers. Their molecular weights are 17, 28, and 50 kD.

The coloring principle of carmine is chiefly carminic acid, but commercial carmine also contains about 20% proteinaceous material derived from the source insect during the extraction of the pigment.¹ These coccoid-derived proteins are potential aeroallergens, similar to other arthropod-derived proteinaceous material.¹¹ Our study confirm that all of the allergens implicated in occupational asthma due to carmine inhalation come from cochineal because all of them, the 17, 28, and 50 kD allergens,

are present in it. Only the 50 and 28 kD allergens, but not the 17 kD one, are presented in carmine extract.

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