

ORIGINAL ARTICLES

ANTIGENIC PROPERTIES OF CHLOROGENIC ACID

*Samuel O. Freedman, M.D., Robert Shulman, M.D., John Krupey, B.Sc., and
A. H. Schon, Ph.D., Montreal, Quebec, Canada*

PREVIOUS work in this laboratory has shown that chlorogenic acid, a simple phenolic compound of low molecular weight is an important allergenic constituent of green coffee bean, castor bean, and oranges.¹⁻⁴ It was found that coffee workers who developed occupational asthma, rhinitis, or dermatitis on exposure to finely dispersed coffee dust showed positive wheal-and-erythema responses to intradermal injections of chlorogenic acid, and positive passive transfer (P-K) reactions were obtained with their sera. Similar positive direct and passive transfer reactions to chlorogenic acid were elicited in patients allergic to castor bean and oranges. In addition, cross-neutralization experiments demonstrated that chlorogenic acid neutralized skin-sensitizing antibodies to crude extracts of green coffee, castor bean, and oranges. Chlorogenic acid has also been identified in a wide variety of other plant materials, such as apples, pears, carrots, beans, celery, lettuce, cucumber, cabbage, plums, cherries, sweet potatoes, and tobacco. Structurally, chlorogenic acid is the quinic acid ester of caffeic acid (Fig. 1.)

There have been relatively few reports of the production of immediate type skin reactions in humans by simple chemical substances. Large wheal-and-erythema responses with passive transfer of circulating antibodies were reported by Sherman and Cooke in a patient allergic to sulfadiazine.⁵ Similar positive direct skin tests and passive transfer responses to simple chemicals have been reported with toluene-sulfone chloramide (chloramine-T),⁶ gallic

From the McGill University Medical Clinic of the Montreal General Hospital and the Departments of Biochemistry and Chemistry, McGill University, Montreal, Canada.

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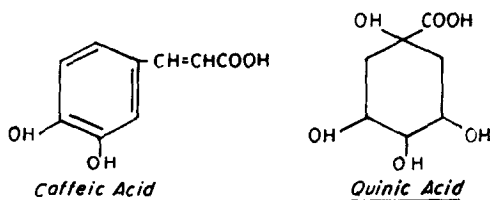
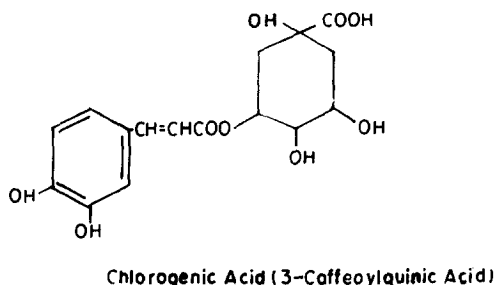


Fig. 1.

acid,⁷ penicillin,⁸ and several naphthoquinone and benzoquinone derivatives⁹ isolated from tropical woods.

On the other hand, delayed type contact hypersensitivity to low molecular weight compounds has been extensively studied in humans and in various animal species. During the course of these studies it has been observed in animals that simple chemical inducers of contact skin sensitivity are often capable of inducing serum antibody formation as well.¹⁰ Furthermore, it is generally agreed that induction of antibody formation by simple chemical substances depends on in vivo protein conjugation. Experiments concerned with proof of antibody formation in animals to simple chemicals have been based on results obtained by passive cutaneous anaphylaxis¹¹⁻¹² and more recently by a hemagglutination method utilizing antigen-erythrocyte conjugates.¹³

The purpose of the present investigation was to determine whether chlorogenic acid is antigenic in animal species. The demonstration that this low molecular weight compound is capable of inducing antibody formation in animals would lend strong support to the hypothesis that chlorogenic acid is an important naturally occurring sensitizer in human respiratory allergy to certain classes of plant substances.

METHODS AND MATERIALS

Chlorogenic Acid.—Chlorogenic acid was purchased from the Nutritional Biochemical Corporation, Cleveland, Ohio.

Aqueous Extract of Green Coffee.—Raw coffee beans were ground dry in a Waring Blendor and sieved through a 30 mesh screen. The ground material was defatted with ether in a Soxhlet apparatus and extracted in distilled water at room temperature for 30 minutes. The extract was filtered first through Whatman No. 5 filter paper and then through an "oxoid" membrane filter (British

Drug Houses). The filtered extract was lyophilized and stored at 4° C. The lyophilized preparation was reconstituted prior to use by the addition of physiologic saline.

Immunization of Animals.—Chlorogenic acid dissolved in physiologic saline was emulsified with Freund's complete adjuvant to yield a final concentration of 1 mg. of chlorogenic acid in 1 ml. of emulsion. Three male albino rabbits weighing 3 to 5 kilograms were given injections of this mixture 3 times weekly for 3 weeks. At each injection, 0.1 ml. was injected into the foot pad of the rabbit and 0.9 ml. intramuscularly in the thigh. The animals were bled 21 days after the final injection and were subsequently given weekly "booster" injections of 1 mg. of chlorogenic acid in Freund's adjuvant intramuscularly in order to maintain antibody levels. The rabbits immunized with chlorogenic acid in Freund's adjuvant were labeled F₁, F₂, and F₃. Three additional rabbits were immunized by the intravenous route with chlorogenic acid according to the same schedule of doses and were labeled I₁, I₂, and I₃. Another 3 rabbits were immunized with chlorogenic acid without Freund's adjuvant by the intramuscular route according to the same schedule of doses and were labeled C₁, C₂, and C₃.

Three male guinea pigs weighing 350 to 400 grams were injected with the same mixture of chlorogenic acid in Freund's adjuvant used to immunize the first series of rabbits. One milliliter of this mixture containing 1 mg. of chlorogenic acid was injected in the neck muscles once a week over a period of 3 weeks. The guinea pigs were labeled G₁, G₂, and G₃.

Skin Testing of Immunized Animals.—Skin testing was carried out by the intradermal injection of 0.1 ml. of chlorogenic acid at varying concentrations.

Systemic Anaphylaxis.—Attempts to induce acute systemic anaphylaxis in guinea pigs were made by the intracardiac injection of 1 mg. of chlorogenic acid in 1 ml. of physiologic saline.

Passive Cutaneous Anaphylaxis.—Intradermal injections of 0.1 ml. of rabbit antisera in varying dilutions were made in the shaved abdominal skin of male albino guinea pigs weighing approximately 350 grams. Six hours later the animals were given an intracardiac injection with 1 ml. of a mixture containing 1 mg. of chlorogenic acid and 1 mg. of Evans blue dye dissolved in 1 ml. of physiologic saline. The antiserum sites were examined for the characteristic extravasation of blue dye 15 minutes after the intracardiac injection and the reactions graded from 0 to 4⁺ according to the criteria recommended by Ovary.¹⁴

Agar Gel Precipitation.—Precipitation in agar gel was performed by the micro-Ouchterlony technique. A 1 per cent solution of Bacto-agar (Difco) in distilled water was spread in a very thin layer over a standard microscope slide. A second layer of 1.5 per cent Bacto-agar in physiologic saline was then poured over the first layer. As the second layer began to harden, wells were punched out with a 12 gauge hypodermic needle. The distance between the wells was approximately 10 mm. The wells were filled with undiluted antisera or with antigens at varying concentrations, and the slides were examined for precipitation bands in 18 hours.

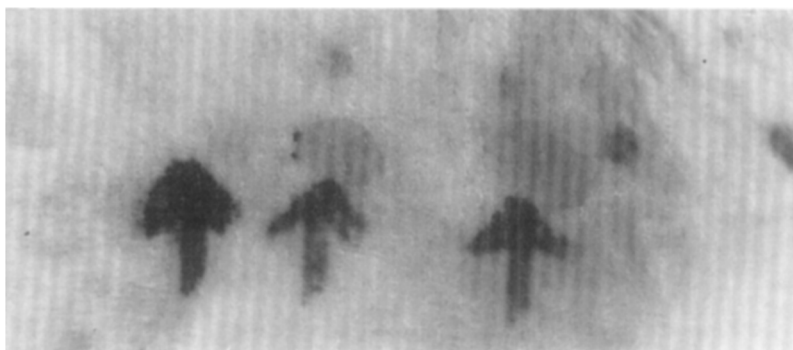


Fig. 2.—Arthus reactions to chlorogenic acid in a rabbit sensitized with chlorogenic acid, photographed at 24 hours. The site on the left was injected with saline, the middle with chlorogenic acid 100 μ g per milliliter, and the right with chlorogenic acid 1,000 μ g per milliliter.

Precipitation-inhibition experiments were carried out by placing the suspected inhibitor substance in the antiserum wells 2 hours before the addition of the antiserum.

Hemagglutination.—The bis-diazotized benzidine (BDB) technique was employed for the hemagglutination studies. The technique described by Schon¹⁵ was followed in all respects except that an aqueous extract of green coffee was used as the antigen instead of an aqueous extract of ragweed pollen. With this method the ratio of benzidine to antigen is critical for satisfactory sensitization of erythrocytes, and the optimum concentrations must be established for each batch of BDB. With the green coffee antigen, it was found that best results were obtained by adjusting the antigen concentration to 0.5 to 0.75 mg. per milliliter and by adding 1 ml. of BDB phosphate solution to each 3 ml. aliquot of erythrocytes suspended in antigen solution.

Hemagglutination-inhibition was undertaken by incubating the inhibitor with the antiserum dilutions at 37° C. for 30 minutes prior to the addition of sensitized cells. Fifty micrograms of inhibitor was added to each hemagglutination tube.

EXPERIMENTS AND RESULTS

Only the rabbits (F_1 , F_2 , and F_3) sensitized with chlorogenic acid emulsified with Freund's complete adjuvant showed evidence of antibody formation. The results reported in the remainder of this paper are for this group of rabbits only.

Intradermal Skin Testing.—Intradermal skin tests were performed on 3 sensitized rabbits by use of 0.1 ml. of a solution of chlorogenic acid in physiologic saline at a chlorogenic acid concentration of 100 μ g per milliliter. The test sites were inspected for evidence of Arthus or delayed type skin reactions at 6, 12, 24, and 48 hours. At 6 hours, there was evidence of erythema and induration at the test sites, and at 12 hours the reactions had reached their maximum intensity (Table I). There was no grossly visible central necrosis of the lesions (Fig. 2). Microscopic examination of punch biopsy sections ob-

TABLE I. INTRADERMAL REACTIONS TO CHLOROGENIC ACID IN RABBITS SENSITIZED TO CHLOROGENIC ACID

ANIMAL	DIAMETER OF SKIN REACTION		
	6 HOURS	12 HOURS	48 HOURS
F ₁	8 mm.	10 mm.	10 mm.
F ₂	12 mm.	15 mm.	15 mm.
F ₃	10 mm.	15 mm.	15 mm.

tained 24 hours after the injection of chlorogenic acid showed blockage of the small blood vessels with leukocyte and platelet thrombi, as well as massive infiltration of the section with polymorphonuclear cells and eosinophils. On the basis of the histologic appearance and the time sequence of the gross changes, the intradermal response to chlorogenic acid was considered to be of the Arthus type. Intradermal testing with green coffee extract produced large typical Arthus reactions with visible central necrosis and sloughing. Control intradermal tests with physiologic saline in sensitized rabbits and with chlorogenic acid in normal rabbits produced no reaction.

Active Systemic Anaphylaxis.—Three guinea pigs sensitized to chlorogenic acid were given intracardiac injections of 1 mg. of chlorogenic acid in 1 ml. of physiologic saline. Within 10 minutes the animals became restless, signs of intense dyspnea appeared in about 15 minutes, and the animals finally died of asphyxia 20 to 25 minutes after the intracardiac injection. This pattern was repeated in all 3 animals. Control intracardiac injections with physiologic saline in sensitized guinea pigs and with chlorogenic acid in normal guinea pigs failed to produce evidence of systemic anaphylaxis.

Passive Cutaneous Anaphylaxis.—Passive cutaneous anaphylaxis was performed in 4 guinea pigs with antiserum obtained from rabbit F₂ sensitized to chlorogenic acid. Chlorogenic acid was used as the intracardiac challenging agent. The results of these experiments are recorded in Table II. When phys-

TABLE II. PASSIVE CUTANEOUS ANAPHYLAXIS IN THE GUINEA PIG (RABBIT ANTI-CHLOROGENIC ACID SERUM F₂ CHALLENGED WITH CHLOROGENIC ACID)

GUINEA PIG	SERUM DILUTION				
	1:10	1:100	1:1000	1:5000	1:10,000
A	+++	++	+	—	—
B	+++	++	++	+	—
C	++	+	+	—	—
D	+++	++	+	—	—

TABLE III. IMMUNOLOGIC PROPERTIES OF RABBIT ANTI-CHLOROGENIC ACID SERA REACTED AGAINST GREEN-COFFEE EXTRACT

ANTISERUM	PRECIPITATION IN AGAR GEL	HEMAGGLUTINATION TITER
F ₁	Positive	102,400
F ₂	Positive	204,800
F ₃	Positive	204,800

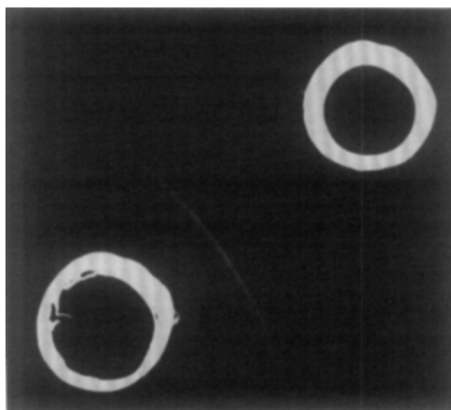


Fig. 3.—Agar gel precipitation between green coffee extract (lower well) and rabbit anti-chlorogenic acid serum (upper well).

iologic saline was substituted for antiserum or when the animals were challenged with an intracardiac injection of saline, no positive reactions were obtained.

Agar Gel Precipitation.—Attempts to obtain precipitation bands between solutions of chlorogenic acid and rabbit antisera to chlorogenic acid were unsuccessful. It was therefore decided to use an aqueous extract of green coffee (1 mg. per milliliter) as the antigen since this material contains large amounts of chlorogenic acid presumably complexed with larger molecules. Undiluted antisera from 3 rabbits sensitized to chlorogenic acid were tested against the green coffee extract in agar gel by the micro-Ouchterlony technique. Single precipitation bands were obtained with each of the 3 sera (Table III). A typical agar gel diffusion experiment between rabbit anti-chlorogenic acid serum and green coffee extract is illustrated in Fig. 3. No precipitation was obtained when physiologic saline was substituted for green coffee extract or when green coffee extract was tested against normal rabbit serum.

Precipitation-inhibition experiments were performed with chlorogenic acid (1 mg. per milliliter) and rabbit anti-chlorogenic acid serum F_2 . The chlorogenic acid produced complete inhibition of the expected precipitation band between green coffee extract (1 mg. per milliliter) and the undiluted rabbit antiserum. No inhibition was obtained with caffeic acid, with quinic acid, or with a mixture of caffeic and quinic acids.

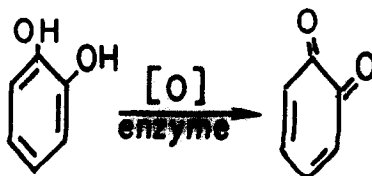


Fig. 4.

TABLE IV. HEMAGGLUTINATION INHIBITION EXPERIMENTS (RABBIT ANTI-CHLOROGENIC ACID SERUM F₀* AND ERYTHROCYTES SENSITIZED WITH GREEN COFFEE EXTRACT)

INHIBITOR SUBSTANCE	HEMAGGLUTINATION TITER, NO INHIBITOR ADDED	HEMAGGLUTINATION TITER, INHIBITOR ADDED
Chlorogenic acid	6400	0
Green coffee extract	6400	0
Caffeic acid	6400	6400
Quinic acid	6400	6400
Quinic and caffeic acids	6400	6400
Castor bean extract	6400	1600
Castor bean CB-SRI	6400	400

*Antiserum F₀ obtained at different bleeding from that used in the experiments recorded in Table III.

Hemagglutination.—Aqueous green coffee extract was used as the antigen in the BDB hemagglutination experiments because uniformly negative results were obtained when chlorogenic acid was used as the antigen. An attempt to adapt the allergen-erythrocyte conjugation technique of Johnson to the chlorogenic acid system was also unsuccessful. Hemagglutination titers to green coffee extract obtained with rabbit antiserum to chlorogenic acid are recorded in Table III. Control experiments with (*a*) nonsensitized cells and rabbit anti-chlorogenic acid serum and (*b*) sensitized cells and normal rabbit serum showed no evidence of hemagglutination.

Hemagglutination-inhibition experiments were undertaken using rabbit anti-chlorogenic acid serum F₀ and erythrocytes sensitized with green coffee extract as the hemagglutination system. The results of attempts at inhibition with chlorogenic acid, aqueous green coffee extract, caffeic acid, quinic acid, an equimolar mixture of caffeic and quinic acid, an isopropyl alcohol extract of castor bean, and castor bean fraction CB-SRI* are recorded in Table IV. From these results it is apparent that complete inhibition of hemagglutination was obtained with chlorogenic acid and with green coffee extract, partial inhibition with the castor bean preparations, and no inhibition with caffeic or quinic acids. None of the inhibitor substances tested produced inhibition of a ragweed hemagglutination system. This latter finding would suggest that the hemagglutination inhibition in these experiments was specific for chlorogenic acid.

DISCUSSION

The results obtained in this investigation have demonstrated (1) that chlorogenic acid is capable of inducing antibody formation in experimental animals and (2) that circulating antibodies induced by chlorogenic acid are also directed against aqueous extracts of green coffee. In addition, the findings are of considerable theoretical interest in relation to the role of simple chemical sensitizers in hypersensitivity reactions.

The induction of circulating antibodies in animal species by low molecular weight substances has been described previously,^{11, 16} but the elicitation of

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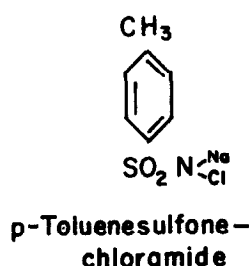
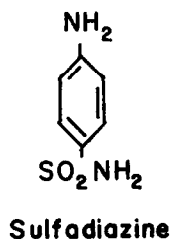
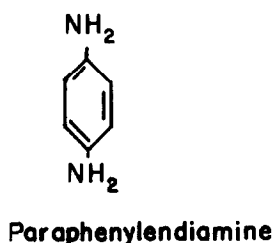
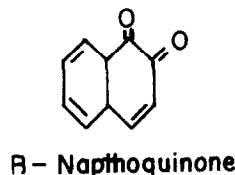
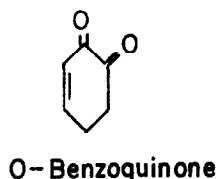
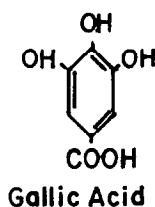


Fig. 5.

Arthus reactions, passive cutaneous anaphylaxis, and active systemic anaphylaxis by a simple chemical hapten, rather than by a hapten-protein conjugate, represents an unusual series of findings. However, comparable results were obtained by Chase¹¹ in a series of experiments with the sera of guinea pigs actively sensitized to simple chemical compounds such as acid anhydrides and acyl chlorides. Using a technique essentially similar to passive cutaneous anaphylaxis, he was able to demonstrate the passive transfer of immediate type skin sensitivity to normal guinea pigs. Skin reactions were elicited in the recipient animals both by simple haptens and by hapten-protein conjugates. This latter finding was interpreted as evidence in favor of *in vivo* conjugation between the simple chemical sensitizer and body proteins.

Chlorogenic acid is also one of the few simple chemical substances which is capable of evoking a wheal-and-erythema response in specifically hypersensitive human subjects. If current theories regarding the mechanisms of hypersensitivity to low molecular weight substances are accepted, it follows that chlorogenic acid must be capable of rapid *in vivo* combination with tissue proteins.

Although no detailed information regarding the metabolic fate of chlorogenic acid in the mammalian organism is available at the present time, it is possible on the basis of current knowledge to postulate a mechanism for the *in vivo* conjugation of an *o*-diphenol such as chlorogenic acid with tissue proteins. Phenol oxidases, which are widely distributed throughout human and animal tissues, are responsible for the dehydrogenation of *o*-diphenols to *o*-quinones¹⁷ (Fig. 4). The *o*-quinones and *p*-quinones produced as metabolic intermediates through the action of phenol oxidases are among the most reactive compounds occurring in living matter and have been shown to combine readily

with amino, imino, sulfhydryl, and free heterocyclic groupings. Inasmuch as proteins contain all of these groupings, they react readily with *o*-quinones and *p*-quinones. It is not without significance that several of the other simple chemical substances previously reported as being capable of evoking wheal-and-erythema reactions in humans are theoretically capable of metabolic transformation to quinones (Fig. 5). For instance, positive immediate type intradermal reactions to tannic acid in humans were demonstrated to be due to the gallic acid portion of the tannic acid molecule,⁷ and benzoquinones and naphthoquinones were reported as elicitors of immediate type skin reactions in patients sensitive to tropical woods. Paraphenylenediamine, a black dye, has long been known as a cause of immediate type respiratory allergy in the fur industry. It was suggested as early as 1928 that hypersensitivity to this compound was based on its oxidation to a quinone.¹⁸ For a detailed discussion of some of the theoretical aspects of sensitization to quinones, the reader is referred to the excellent review article by R. L. Mayer,¹⁹ in which it is postulated that aromatic amines and their nitro derivatives, certain azo dyes, sulfonamides, *p*-aminobenzoic acid and its derivatives, and certain polyphenols are antigenic primarily because of their ability to undergo metabolic oxidation to quinones.

During the course of the present investigation, it was noted that chlorogenic acid obtained from different suppliers and different lots of chlorogenic acid obtained from the same supplier varied considerably in their ability to induce antibody formation and in their ability to evoke allergic responses in sensitized animals. A possible explanation for this variability in antigenic activity is that commercially available chlorogenic acid is obtained by extraction from botanical sources. It is known that there are at least three naturally occurring isomers of chlorogenic acid.²⁰ Therefore, it is conceivable that different batches of chlorogenic acid contain isomers in different proportions and that not all of these isomers are allergenically active. The various chlorogenic acid samples tested all gave identical spots on paper chromatography and a single peak at 322 m μ on ultraviolet absorption spectrophotometry. However, no conclusions can be drawn from these results regarding the presence or absence of isomers because it was recently demonstrated that samples of chlorogenic acid which appeared to be homogeneous on paper chromatography gave more than one band on silica gel partition chromatography.²¹

Another possible explanation for the variation in allergenic activity between samples is the presence of protein or other contaminants. All of the samples tested gave negative ninhydrin reactions, but trace amounts of nitrogen (0.12 to 0.23 per cent) were demonstrated to be present in all samples when they were analyzed by a method using Nessler reagent.* However, it is unlikely that the nitrogen contaminant was immunologically significant because positive direct skin tests in humans and positive P-K reactions were obtained with synthetically prepared chlorogenic acid.† This latter observation

*Analyses done by the Schwartzkopf Microanalytical Laboratory, Woodside, N. Y.

†Kindly supplied by Dr. M. L. Scarpati, Florence, Italy.

would indicate that chlorogenic acid free of protein contamination is capable of eliciting an immediate type response. Animals were not sensitized with the synthetic compound because insufficient quantities for immunization are available at the present time.

It is therefore postulated that the presence of naturally occurring isomers in varying proportions in chlorogenic acid isolated from botanical sources best accounts for differences in allergenic activity between samples.

The finding that castor bean preparations only partially inhibited hemagglutination between rabbit anti-chlorogenic acid serum and green coffee extract is in keeping with previously reported experiments in which partial cross-neutralization was obtained between chlorogenic acid and castor bean.⁴ It would thus appear that chlorogenic acid is only one of the allergens in castor bean.

SUMMARY

Rabbits sensitized to chlorogenic acid by means of intramuscular and foot pad injections with chlorogenic acid emulsified in complete Freund's adjuvant showed positive Arthus reactions to chlorogenic acid and to green coffee extract. Sera obtained from the sensitized rabbits gave positive PCA reactions to chlorogenic acid and contained hemagglutinating and precipitating antibodies to green coffee extract. Guinea pigs sensitized to chlorogenic acid developed active systemic anaphylaxis when challenged with an intracardiac injection of chlorogenic acid. These findings lend further support to the hypothesis that chlorogenic acid is an important simple chemical sensitizer responsible for human respiratory allergy to certain types of plant materials.

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