

Identification and characterization of important cockroach allergens

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Allergens extracted from American and German cockroach species have been identified as significant sensitizing agents in the induction/exacerbation of asthma. In the present study, gel-filtration fractions of saline extracts of American cockroach (Periplaneta americana) whole bodies (AWBE fraction 2) and German cockroach (Blattella germanica) whole bodies (GWBE fraction 2) were used to identify and characterize important cockroach allergens by immunoprinting. In addition, allergens from AWBE and GWBE fractions 2 were additionally fractionated by chromatofocusing on polybuffer exchanger. Immunoprinting studies demonstrated several important acidic allergens in cockroach whole body extracts. All but one allergen had an acid isoelectric point (pH 2.80 to 5.20). Two allergens, one that focused at pH 3.50 and another allergen (or group of isoallergens) that focused between pH 4.15 to 4.55 were reactive with most sera obtained from cockroach-sensitive subjects. Chromatofocusing and subsequent skin test and RAST studies of AWBE and GWBE confirmed the presence of significant cockroach allergens with isoelectric points within the zone of pH 3.75 to 4.50. RAST-inhibition studies demonstrated the similarity of these allergens between AWBE and GWBE. Collectively, these observations identify the presence of several acidic cockroach allergens presumably shared between AWBE and GWBE. (J ALLERGY CLIN IMMUNOL 1990;86:781-6.)

Both the American and German cockroaches are present throughout most of the United States and have been demonstrated to be allergenic.¹⁻⁴ Previous studies have supported the clinical importance of cockroach allergens in the development of "inner city," allergic respiratory disease.^{1, 2, 5} Cockroach has even been demonstrated to sensitize laboratory workers.⁶ Most studies demonstrate a high prevalence of skin reactivity to cockroach allergens in atopic populations with asthma.⁷ Although major allergens have recently been identified in American cockroach with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotting techniques,⁸ these allergens are incompletely characterized. Data are also incomplete regarding al-

Abbreviations used

AWBE: American cockroach whole body extract
GWBE: German cockroach whole body extract
kd: Kilodalton
IEF: Isoelectric focusing
SPT: Skin prick test
PBS: Phosphate-buffered saline

lergens of other species and the distribution of common allergens among species.⁹ Therefore, cockroach allergens warrant additional study.

Earlier studies in our laboratory identified the presence of significant allergens in AWBE and GWBE that eluted from Sephadex G-75 in a molecular weight range of 12,500 to 75,000 daltons.¹⁰ This cockroach-allergen fraction is referred to as "fraction 2." The present studies were undertaken to further characterize fraction 2 from AWBE and GWBE by the methods of chromatofocusing and IEF, followed by immunoprinting. Additionally, chromatofocusing fractions were used for immediate SPT, direct RAST, and RAST-inhibition studies to analyze the occurrence of shared allergens between American and German cockroach species.

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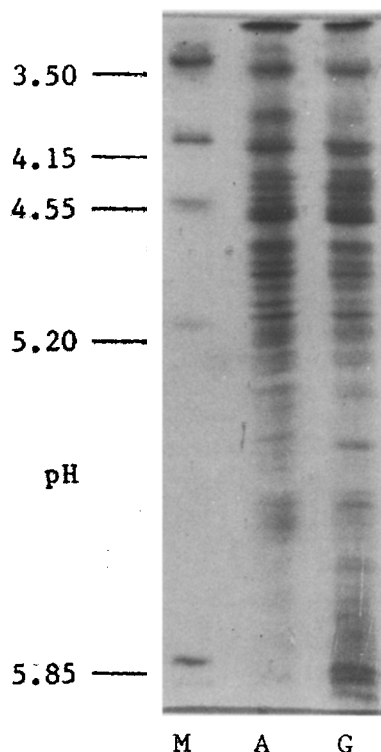


FIG. 1. IEF analysis of AWBE (A) and GWBE (G) fractions 2; 200 μ g (30 μ l) of protein of AWBE and GWBE were electrophoresed in 5% polyacrylamide gels with an ampholyte range of pH 4.0 to 6.5. The gels were stained with Coomassie brilliant blue dye; (M), pH markers.

MATERIAL AND METHODS

Cockroach whole body extracts and preparation of fraction 2

American cockroach (*Periplaneta americana*) and German cockroach (*Blattella germanica*) whole bodies were obtained from the Carolina Biological Supply Co., Burlington, N.C., and were of both sexes. Crude whole body extracts were prepared by freezing live roaches and weighing and suspending the whole bodies in physiologic saline (pH 7.4), followed by homogenization in a Waring (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) blender for 5 minutes at room temperature (five 1-minute pulses). The mixture was stirred overnight at 4° C and was then centrifuged at 18,000 g for 30 minutes at 4° C. Supernatants were concentrated by Amicon ultrafiltration (YM2, Amicon Corp., Danvers, Mass.) (molecular weight cutoff, 1000 daltons) and filtered through a 0.45 μ m Millipore filter (Millipore Corp., Bedford, Mass.). Glycerol was added in equal volumes with saline to allergen preparations for use in skin testing. Extracts were stored at 4° C. For allergen-fractionation studies, whole body extracts were applied to a Sephadex G-75 column. Fraction 2, the column eluate included in the column to the cytochrome c marker,

contained significant cockroach allergenic activity by RAST inhibition and was saved for additional study.

IEF and immunoprinting of AWBE and GWBE fractions 2

Polyacrylamide gel plates (5%) were obtained from Pharmacia LKB (Ampholine PAG plate, Pharmacia Fine Chemicals, Piscataway, N.J.) with an ampholyte range of pH 4.0 to 6.5 for IEF and immunoprinting. Allergen samples were dialyzed into 1% glycine solution for IEF. The amount of sample added to the gel varied between 30 to 40 μ l and contained 180 to 240 μ g of protein. Power settings and total focusing time were according to manufacturer's recommendations. Gels were fixed for 1 hour in a trichloroacetic acid and sulfosalicylic acid solution, stained with 0.1% Coomassie brilliant blue R-250, and destained with an ethanol/acetic acid solution. For immunoprinting studies, activated nitrocellulose was prepared by placing 10 by 13.2 cm sheets (membranes) of nitrocellulose (0.2 μ m pore size, Hoefer Scientific Instruments, San Francisco, Calif.) in a solution of 5% cyanogen bromide adjusted to a pH of 10 to 10.5 with 1 mol/L of NaOH and maintained at this pH for 20 to 30 minutes at room temperature. The activation was stopped by transferring membranes to cold (4° C) 0.005 mol/L of NaHCO₃. Activated membranes were air-dried and stored at 4° C. Isoelectric focused samples were blotted onto activated membranes by passive transfer. Unreacted sites on the membranes were quenched in buffer (0.4% Tween 20 and 1% bovine serum albumin in 0.04 mol/L of phosphate buffer, pH 7.5) for 1 hour at room temperature. Membranes were then laid on a glass plate and cut into 0.5 cm strips with a sharp scalpel. Strips were incubated overnight at room temperature with the subjects' sera diluted 1:3 with PBS-Tween 20 buffer. The next day the strips were washed with PBS-Tween 20 buffer, and 50,000 cpm of ¹²⁵I-labeled antihuman IgE (Kallestad Laboratories, Austin, Texas) was added to each strip. After overnight incubation at room temperature, the strips of nitrocellulose were washed with PBS-Tween 20 and air-dried. Strips were placed in an x-ray cassette with intensifying screens and exposed for 72 hours.

RAST/RAST inhibition

Direct RAST was performed in duplicate with the use of antigen-coated paper disk for the detection of circulating IgE antibodies specific for cockroach allergens.¹⁰ For evaluation of results, a RAST percent binding was obtained by dividing the mean counts per minute of test disks by total counts per minute of ¹²⁵I-labeled anti-IgE added. For RAST inhibition, inhibitor was incubated with pooled sera (10 subjects) in the presence of allergen-coated disks overnight on a rotator at room temperature. Disks were washed, and radiolabeled anti-IgE was added, followed by an overnight incubation. Disks were washed free of unbound label, and the level of remaining activity (percent bound) was determined in a gamma counter. Results are expressed as the percent inhibition of the RAST reaction by the test solution, relative to the RAST reaction inhibited with PBS.

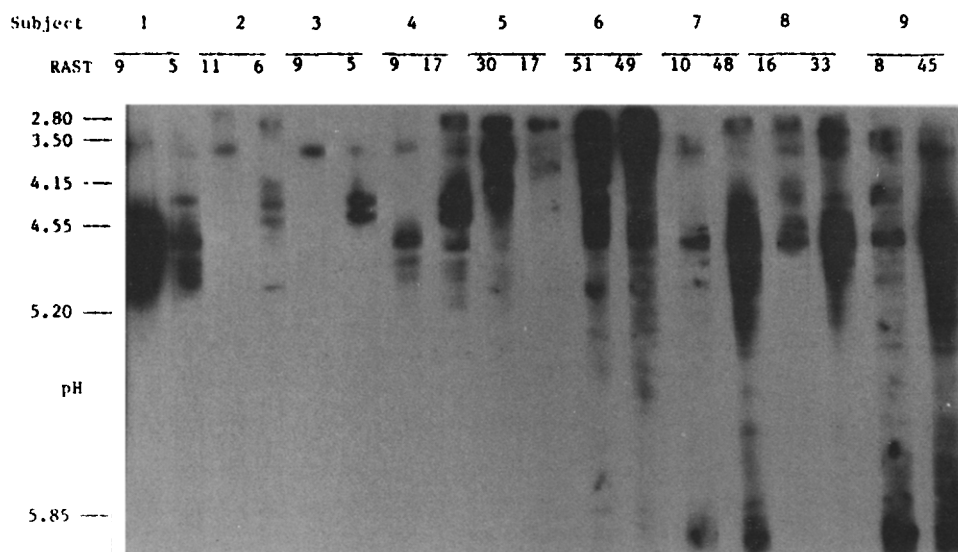


FIG. 2. Immunoprinting studies of AWBE and GWBE. Sera from nine subjects with skin test and RAST reactivity to AWBE and GWBE were individually printed against AWBE (*left side* of each paired pattern) and GWBE (*right side* of each paired pattern). RAST percent binding with AWBE and GWBE fractions 2, respectively, is illustrated above each immunoprint. Approximately 200 μ g of protein from AWBE and GWBE was first focused on 5% polyacrylamide gels with an ampholyte range of pH 4.0 to 6.5 and then transferred to activated nitrocellulose. The migration of pH markers is illustrated on the *left side* of the figure.

Chromatofocusing

AWBE and GWBE fractions 2 were chromatofocused on polybuffer exchanger (PBE-94, Pharmacia Fine Chemicals) through a 0.9 cm by 30 cm column with gel volume of 10 ml. Elution was at a rate of 50 cc/hr beginning with a histidine HCl buffer, pH 6.0, and followed with polybuffer (PBE-74) diluted 1:8 with distilled water. The total volume of the gradient was 240 cc. Twenty-five to fifty milligrams of protein was applied to each column, and 2 ml fractions were collected. Absorption of protein was read at 280 nm. For purposes of skin testing, fractions of AWBE and GWBE eluted from the columns were precipitated with saturated ammonium sulfate and then dialyzed against physiologic saline (pH 7.4).

RESULTS

IEF and immunoprinting of AWBE and GWBE fractions 2

Preliminary studies with a wide range pH gel (pH, 3.5 to 9.5) demonstrated that protein bands in AWBE and GWBE fractions 2 only focused below pH 6.5. Therefore, in subsequent IEF and immunoprinting studies, a gel with pH range 4.0 to 6.5 was used. The IEF profile of AWBE and GWBE fractions 2 is illustrated in Fig. 1. There was a marked similarity in the protein band patterns of both cockroach species. Most proteins in AWBE and GWBE fractions 2 focused below pH 5.2.

The immunoprinting analysis is depicted in Fig. 2 of sera obtained from nine subjects with immediate SPT and serum RAST reactivity to AWBE fraction 2 and GWBE fraction 2. Both AWBE and GWBE fractions 2 were individually printed against each subject's serum, and direct RAST percent binding of each serum is depicted in the figure above each nitrocellulose strip. Multiple allergens were detected by immunoprinting in both AWBE and GWBE fractions 2. Although individual sera varied in their patterns of reactivity with AWBE and GWBE fractions 2, there were no electrophoretically unique allergens apparent in either AWBE or GWBE fractions 2. One allergen (or group of isoallergens) that focused in the zone between pH 4.15 to 4.55 was reactive with sera from all nine subjects. Similarly, one allergen that focused at pH 3.50 was also reactive with sera from all nine subjects.

Chromatofocusing and RAST studies of AWBE and GWBE fractions 2

The column elution profile of AWBE fraction 2, chromatofocused on polybuffer exchanger, is illustrated in Fig. 3. Proteins were first eluted with a continuous pH gradient between 6.0 to 3.75, after which a second gradient between pH 3.75 to 3.0 was applied to the column. Eluted samples were pooled into four separate fractions (A, B, C, and D) according to pH

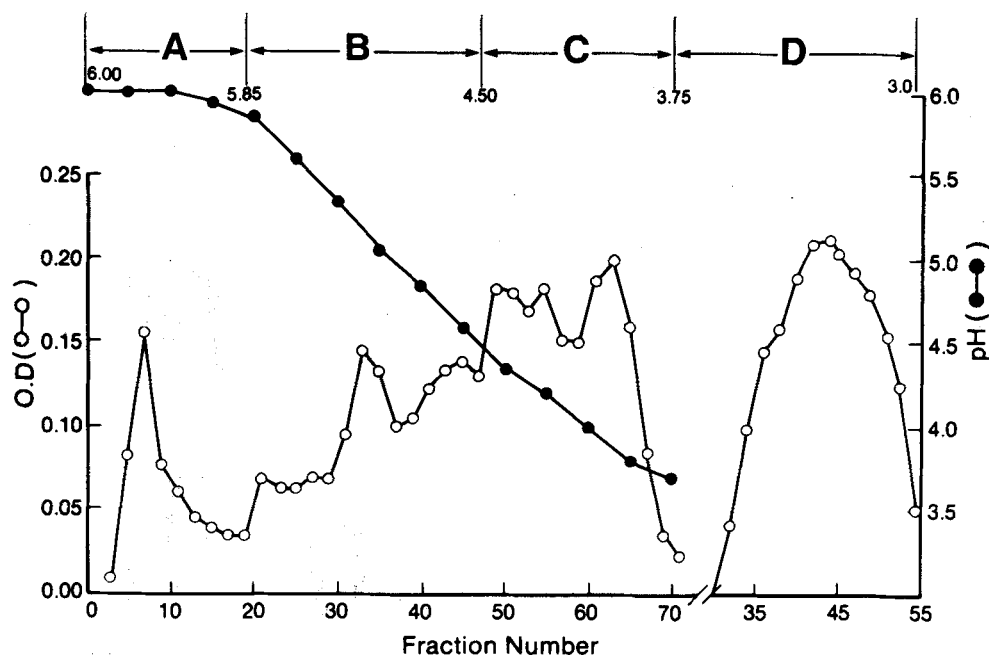


FIG. 3. Chromatofocusing profile of AWBE fraction 2. Fifty milligrams of AWBE fraction 2 was applied to polybuffer exchanger, and protein was eluted with a continuous pH gradient beginning with pH 6.0, histidine HCl buffer. Two milliliter fractions were collected, and UV absorption of protein was read at 280 nm. A second gradient from pH 3.75 to 3.0 was applied beginning with tube 71. Eluted protein was pooled into four separate fractions (A, B, C, and D) with the pH range of each fraction defined in the upper part of the figure.

TABLE I. RAST percent binding of chromatofocusing column-fractionated AWBE

		RAST % binding			
		Chromatofocusing fraction of AWBE 2			
Subject	AWBE 2*	A (pH 6.0-5.85)	B (pH 5.85-4.5)	C† (pH 4.5-3.75)	D (pH 3.75-3.0)
J. L.	23	2	4	27	2
F. B.	11	2	3	11	2
V. K.	20	1	17	18	4
J. J.	9	2	2	24	1
G. G.	15	2	12	15	3
D. V.	9	2	4	10	2
C. F.	6	1	3	21	2
S. S.	9	1	2	6	1
J. R.	30	2	4	48	25
W. T.	51	2	43	47	41
J. G.	10	2	3	18	5
S. H.	16	2	2	15	3
S. M.	7	2	3	5	2
M. Sm.	15	2	2	12	2
M. SI.	16	2	3	22	5
ANP‡	1	2	2	2	2

*Fraction 2 of AWBE prepared by column chromatography on Sephadex G-75.

†All patients demonstrated skin test reactivity to AWBE 2, C.

‡Atopic, cockroach skin test negative pooled sera.

TABLE II. RAST percent binding of column-fractionated GWBE

Subject	GWBE 2*	GWBE 2, C† (pH 4.5-3.75)
J. L.	37	57
F. B.	6	11
V. K.	30	35
J. J.	5	12
G. G.	4	2
D. V.	9	11
C. F.	4	4
S. S.	17	29
J. R.	17	16
W. T.	49	52
ANP‡	1	1

*Fractionated by Sephadex G-75.

†Fractionated by Sephadex G-75 and chromatofocusing.

‡Atopic, cockroach skin test-negative pooled sera.

and were then coupled to paper disks for use in the RAST. The direct RAST reactivity of each of the four pooled column fractions that reacted with sera from 15 cockroach-sensitive subjects is summarized in Table I. Chromatofocused column fraction C, which eluted between pH 3.75 to 4.5, was reactive by RAST and SPT with all 15 subjects. Several sera, however, also reacted with allergen(s) in fractions B and D. The IEF pattern of AWBE, fraction 2, C, is depicted in Fig. 4.

GWBE fraction 2 (obtained by Sephadex chromatography) was additionally fractionated by chromatofocusing. The results of direct RAST reactivity for GWBE fraction 2, C (the fraction that was subsequently eluted from polybuffer exchanger in the pH zone 3.75 to 4.5) is presented in Table II. As with AWBE, all sera that were reactive by RAST with GWBE fraction 2 also demonstrated significant RAST reactivity with GWBE fraction 2, C.

RAST-inhibition studies with chromatofocusing-column fractions

The results of inhibition of AWBE fraction 2 RAST are summarized in Table III. AWBE fractions 2 and 2, C were the only American cockroach fractions that produced significant RAST inhibition. Similarly, GWBE fraction 2, C produced significant inhibition of the AWBE fraction 2 RAST.

DISCUSSION

This study was undertaken to identify and characterize important cockroach allergens by the methods

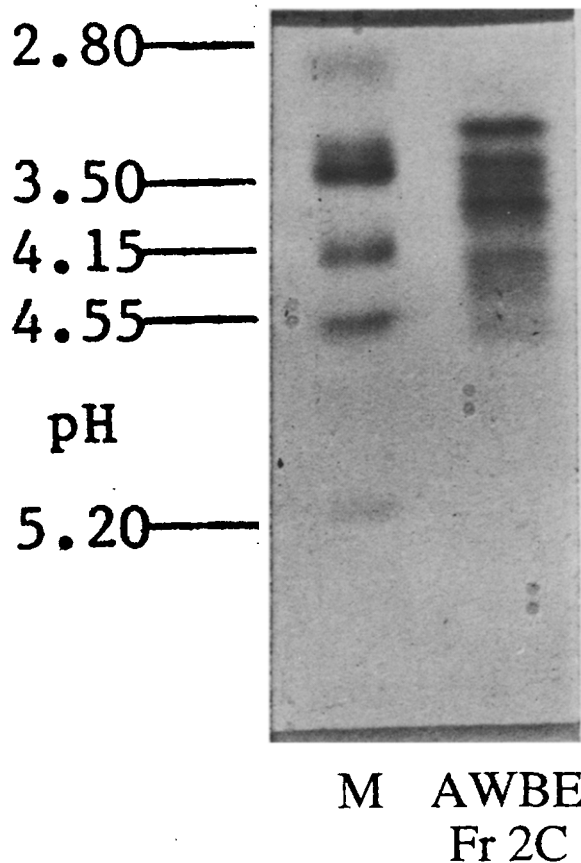


FIG. 4. IEF pattern of AWBE fraction 2, C (A 2, C). Approximately 50 μ g of AWBE fraction 2, C was electrophoresed in 5% polyacrylamide gel with an ampholyte range of pH 4.0 to 6.5; (M), pH markers.

of IEF, immunoprinting, RAST, RAST inhibition, and chromatofocusing. These procedures have proved useful in characterization of other clinically important aeroallergens. Accordingly, the analysis of cockroach AWBE and GWBE demonstrated strong similarities among allergens defined by immunoprinting and allergens isolated by chromatofocusing. Clearly, most cockroach allergens are acidic proteins with isoelectric points between pH 2.8 and 5.2. Two allergens, one that focused at pH 3.5 and the second allergen between pH 4.15 and 4.55, appear to be highly reactive allergens, as defined by immunoprinting studies. This second allergen may represent a group of isoallergens because at least two bands were consistently identified. Although the panel of nine sera used in this study was relatively small, immunoprinting analysis indicated that all sera reacted with both allergens. It remains to be determined whether these two allergens

TABLE III. RAST inhibition of AWBE fraction 2 disks with chromatofocusing-column fractions

Inhibitor (μ g)	% Inhibitor
AWBE	
Fraction 2	
0.1	9
1.0	25
10.0	58
100.0	80
Fraction 2, A	
0.1	6
1.0	6
10.0	5
100.0	9
Fraction 2, B	
0.1	6
1.0	5
10.0	5
100.0	10
Fraction 2, C	
0.1	5
1.0	10
10.0	45
100.0	55
Fraction 2, D	
0.1	4
1.0	5
10.0	7
100.0	7
GWBE	
Fraction 2, C	
0.1	5
1.0	7
10.0	32
100.0	54

are the 72 and 78 kd major allergens identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotting.⁸

Previous studies identified most cockroach allergens within the molecular weight range of 12.5 to 75 kd.⁸ This fraction is referred to as "fraction 2." The use of fraction 2 for chromatofocusing studies of AWBE and GWBE confirmed the presence of significant cockroach allergens that eluted from the polybuffer exchanger in the pH zone of 3.75 to 4.5. Direct RAST analyses of fractions 2, C from AWBE and GWBE demonstrated enhanced activity as compared to fraction 2. All sera reactive with fraction 2 from AWBE or GWBE were also reactive with fraction 2, C from AWBE and GWBE, respectively. RAST-inhibition studies confirmed the allergenic similarity between AWBE fraction 2, C and GWBE fraction 2, C.

Collectively, the above findings suggest close similarities among cockroach allergens in AWBE and GWBE. This finding agrees with earlier studies with SPTs and direct RAST analysis that demonstrated that >95% of cockroach-sensitive subjects were reactive to both AWBE and GWBE.¹⁰ Indeed, in the present study, neither AWBE nor GWBE had electrophoretically unique allergens. However, individual subject's sera did not always produce identical immunoprinting patterns when sera were reacted with AWBE and GWBE. This result could be due to some allergenic differences between AWBE and GWBE or may reflect a quantitative difference in individual allergen content within a defined amount (e.g., dry weight) of AWBE or GWBE.

The acid nature of most cockroach whole body allergens may have important clinical considerations from the standpoint of allergen exposure. Assuredly, intact cockroach whole body parts are not readily accessible to the human lower respiratory tract. However, earlier studies have suggested the importance of cockroach feces as a source of clinically relevant allergens.¹¹ The ability of these allergens to resist the acid environment of the gastrointestinal tract, together with their presumed desiccation in common household dust, would represent important characteristics that aid in host sensitization. Additional studies in our laboratory have confirmed the role of cockroach feces as an important source of clinically relevant allergens.¹²⁻¹⁴ These studies suggest that fecal allergens have similar physicochemical properties to those of cockroach whole body allergens and that fecal allergens reflect a restricted "acid extract" of whole body proteins.

In summary, this study has identified and characterized important cockroach allergens in AWBE and GWBE. These allergens appear to be similar by physicochemical and immunochemical analysis, and furthermore do not appear to be unique to either American or German cockroaches. At least two acid cockroach allergens are reactive with sera from most cockroach-sensitive subjects, and these may be major cockroach allergens.

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Effects of H₁-antihistamine drug regimen on histamine release by nonlesional skin mast cells of patients with chronic urticaria

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*Profiles of compound 48/80-induced histamine release (HR) from mast cells of uninvolved skin from patients with chronic urticaria (CU) and from a normal control (NC) group were compared, and the effects of anti-H₁ medications were assessed versus placebo. Then, patients with CU (15) and NC subjects (10) were randomly assigned to take either hydroxyzine (100 mg/day), terfenadine (120 mg/day), or placebo for 28 days. The effects of such treatment on the clinical response and on the profile of compound 48/80-induced HR during a 4-hour period were analyzed. Treatment with hydroxyzine in patients with CU improved the clinical symptoms and modified the profile of HR; more histamine was recovered at 1 hour ($p < 0.05$) and 2 hours ($p < 0.05$), as compared with baseline. Terfenadine and placebo had no effect on the clinical response or on the profiles of HR. In the NC group, the amounts of histamine recovered at 1 hour after challenge with compound 48/80 were lower than amounts of the pretherapy values ($p < 0.01$). It could be concluded that (1) the profile of HR in patients with CU is reproducible during a period of 28 days, (2) only hydroxyzine modifies both the clinical response and the profile of HR, and (3) anti-H₁ compounds decrease the HR in the NC group. (*J ALLERGY CLIN IMMUNOL* 1990;86:787-93.)*

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Histamine is released by skin mast cells on immunologic and nonimmunologic stimuli. A large body of evidence supporting its role in the pathophysiology of idiopathic CU has been demonstrated.¹⁻⁵ Indeed, clinical manifestations of CU are comparable to manifestations produced by intradermal injection of histamine, and both are suppressed by anti-H₁-antihistamine drugs. Furthermore, the amounts of histamine found in lesional skin or in skin blister fluids are more elevated in patients with active CU than in