

Identification of low molecular weight allergens of American cockroach and production of monoclonal antibodies

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Background: Previous studies have identified two allergenic fractions (Cr-PI and Cr-P II) from crude American cockroach extract, from which the 72- and 78-kilodalton (kD) components of Cr-PI have been identified as major allergens.

Objective: The objective of this study was to identify important allergens in Cr-P II and produce monoclonal antibodies.

Methods: Sera of cockroach-sensitive atopic patients were examined for IgE binding to Cr-P II proteins and monoclonal antibodies were generated by fusion between spleen cells of BALB/c mice immunized with Cr-P II and FO cells.

Results: Immunoblotting revealed that eight allergenic components in Cr-P II are able to bind specific IgE. Allergens of 18, 28, 32 and 45 kD bound 16.7% (2/12), 100% (12/12), 83.3% (11/12), and 100% (12/12) of the atopic sera tested, respectively. Cell fusion resulted in three stable subclones secreting monoclonal antibodies, and none of the monoclonal antibodies recognized any epitopes of German cockroach and Cr-PI of American cockroach. One monoclonal antibody reacted strongly with the 28- and 32-kD allergens of Cr-P II, and two monoclonal antibodies were able to bind the 18- and 12-kD components of Cr-P II with similar epitope specificities. All the epitopes defined by monoclonal antibodies are recognized by human IgE as demonstrated by a fluoroallergosorbent test (FAST) inhibition assay. Five commercial cockroach extracts were compared for Cr-P II levels by monoclonal antibody-based ELISA, and all extracts were found to contain detectable Cr-P II.

Conclusion: The 28-, 32-, and 45-kD components of Cr-P II may be considered as additional important allergens of American cockroach and monoclonal antibodies can be used to identify and define American cockroach allergens.

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INTRODUCTION

Cockroaches are recognized as one of the major causes of perennial rhinitis and asthma, and a high inci-

dence of cockroach hypersensitivity in atopic (20% to 55%) and asthmatic (49% to 60%) populations has been reported.¹⁻¹³ Early studies by Bernton et al¹⁻³ have clearly demonstrated that cockroach antigen can induce skin-sensitizing antibodies. The causative association of skin sensitivity to cockroach antigen with allergic bronchial asthma has been proved by the bronchial provocation test.^{4,9,10,13} Over 50 pestilent species of cockroaches have been described worldwide. The most common and major domiciliary cockroaches in the United States and Taiwan are the American (*Periplaneta americana*) and German (*Blattella germanica*) cockroaches, and allergenicities of both the American and German cock-

roaches have been demonstrated.^{1,7,10,12-21}

In recent years, allergenic components of the German cockroach have been extensively investigated by different workers in several studies.^{12,14,16,17-21} In contrast, the allergenic properties of the American cockroach are still under initial phases of investigation. The significant allergens of the American cockroach with molecular weights ranging from 6 to 120 kD have been identified by various immunochemical methods in different laboratories.^{11,15,16,18,19}

In earlier studies Wu and Lan reported that two allergenic fractions (Cr-PI and Cr-P II) from the crude extract of the American cockroach were eluted from Sephadex G-150 SF resin.¹⁵ Cr-PI contained high molecular weight (>50 kD) allergenic components of the American cockroach that were detected in 11 of 15 (73.0%) cockroach-sensitive patients using the skin prick test. Two prominent proteins of 78 and 72 kD in Cr-PI have been identified as the major allergens of the American cockroach¹⁵ and monoclonal antibodies to 78 and 72 kD have been generated.²² Recently, Wu and his associates^{23,24} reported that the cDNAs encoding Cr-PI allergens of the American cockroach were isolated and two clones were able to express recombinant proteins of 78 and 72 kD recognizing both human atopic IgE antibodies and anti-Cr-PI monoclonal antibodies. The Cr-P II fraction containing low molecular weight (12 to 45 kD) allergens elicited reactivity in 93% (14/15) of patients sensitive to the crude extract. Cr-P II appears to contain highly reactive allergens as defined by skin test; thus the identification of other important allergens warrants additional study. The present study

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describes the identification of allergens in Cr-PII and the production of monoclonal antibodies against the Cr-PII allergens of the American cockroach.

MATERIALS AND METHODS

Preparation of Crude American

Cockroach Extract, Cr-PI and Cr-PII

Crude American cockroach extracts were prepared by extraction of *P. americana* with Coca's solution, and Cr-PI and Cr-PII were prepared from crude extract of *P. americana* by gel filtration as previously described.¹⁵ Bioactive Cr-PI and Cr-PII fractions were pooled, concentrated, dialyzed against 0.02 M PBS, pH 7.5 and then stored at -70°C . Cr-PII was further fractionated twice by preparative isoelectric focusing (Rotofor cell, Bio-Rad, Richmond, CA) using Bio-Lyte 4/6 (Bio-Rad) and the test was performed according to the manufacturer's instruction. Fractions below pH 5.5 were pooled, concentrated, dialyzed against 0.02 M PBS, pH 7.5 and then stored at -70°C .

Commercial Extracts

Commercial cockroach extracts were obtained from Allergy Laboratories, Inc. (ALI, 1:20 wt/vol, American cockroach, Lot No. A5366; Oklahoma City, OK), Berkeley Biologicals (BB, 1:20 wt/vol, American cockroach, Lot No. 588953; Berkeley, CA), Center Laboratories (CL, 10000 PNU/mL, American cockroach, Lot No. 15411; Port Washington, NY), Greer Laboratories (GL, American cockroach, 1:20 wt/vol, Lot No. GB26-30B-1A5 and German cockroach, 1:20 wt/vol, Lot No. GB 46-3B-1A3, Lenoir, NC), and Hollister-Stier Laboratories (Cockroach mix, 1:10 wt/vol, Lot No. 6581ED; Spokane, WA). All other insect extracts and allergens, ant mix (1:10 wt/vol, Lot No. A4IE2174), Kentax fly mix (1:10 wt/vol, Lot No. K2IJ2954), mosquito mix (1:10 wt/vol, Lot No. D41G0106), house dust mix (20,000 PNU/mL, Lot No. 4795ED), mixed vespid venom protein (300 $\mu\text{g/mL}$, Lot No. 3823901), standardized *D. farinae* (10,000 AU/mL, Lot No.

K2IH3597) and *D. pteronyssinus* (10,000 AU/mL, Lot No. E2IC4801) were obtained from Hollister-Stier Laboratories (HSL).

Isoelectric Focusing

Polyacrylamide gel plate (5%) was obtained from Pharmacia LKB (Ampholine PAG plate, Piscataway, NJ) for isoelectric focusing. Isoelectric focusing standard (PI 2.8 to 6.5, Pharmacia LKB) was used as protein markers. Power setting and total focusing time were according to manufacturer's recommendation. Gel was stained for 1 hour in a 0.1% Coomassie brilliant blue R-250/crocein scarlet solution containing isopropanol, acetic acid and copper sulfate, and destained with an isopropanol/acetic acid/copper sulfate solution.

SDS-PAGE and Immunodetection of Allergens

Proteins were separated by SDS-PAGE (12% running gel, 4% stacking gel) according to the method of Laemmli.²⁵ Electrophoresis was carried out in a vertical slab gel apparatus (Bio-Rad) at 15 mA for one to two hours and then at 20 mA for three to four hours. After SDS-PAGE, protein bands were transferred onto 0.45 μm polyvinylidene fluoride membrane (Immobilon-P, Pharmacia) by the methods of Towbin et al²⁶ or stained in 0.2% Coomassie brilliant blue. After electrophoretic transfer, polyvinylidene fluoride membranes were washed in 10% methanol in Tris-buffered saline (TBS, 20 mM Tris, 0.5 N NaCl, pH 7.5) and reacted with individual sera from atopic and control subjects (diluted 1:15 in TBS containing 0.05% Tween and 0.5% gelatin). Immunodetection was performed with ¹²⁵I-labeled anti-human IgE (Pharmacia) and autoradiographed on Hyperfilm-Mp (Amersham International plc, Amersham, UK) for five days at -70°C with intensifying screen.

Production of Polyclonal Antibodies Against Cr-PII

Young adult New Zealand white rabbits were immunized with multiple injections of 400 μg Cr-PII in complete

Freund's adjuvant (Difco, Detroit, MI) and were boosted at least four times with 200 μg Cr-PII in incomplete Freund's adjuvant at 2-week intervals. Serial bleedings were performed, tested, pooled, and stored at -70°C .

Immunization and Production of Monoclonal Antibodies to Cr-PII

BALB/c mice were immunized intraperitoneally with 30 μg of Cr-PII in complete Freund's adjuvant and were boosted at least three times with 20 μg Cr-PII in incomplete Freund's adjuvant at intervals of ten days. Four days before fusion, the mice were injected intravenously with 20 μg of Cr-PII. Cell fusion experiments between immunized spleen cells and murine plasmacytoma FO cells were performed by the method of Galfre and Milstein²⁷ as described.²² Hybrid cells were screened for antibody production by ELISA.

Screening and Determination of Antigen Specificity of the Monoclonal Antibodies by ELISA

An ELISA was used to screen culture supernatants and determine antigen specificity of the monoclonal antibodies. Microtiter plates (Immunolon II, Dynatech, Alexandria, VA) were either coated with 100 μL of American cockroach extract (40 $\mu\text{g/mL}$). German cockroach extract (40 $\mu\text{g/mL}$) Cr-PI (5 $\mu\text{g/mL}$) or Cr-PII (5 $\mu\text{g/mL}$) in 0.1 M sodium carbonate buffer, pH 9.6 for two hours at 37°C . Coated wells were washed with PBS containing 0.05% Tween, pH 7.4 and blocked with 2% bovine serum albumin (BSA) and washed. Wells were then incubated with 100 μL of culture supernatants and reacted with peroxidase-labeled anti-mouse immunoglobulin (100 μL of 1:2000, Sigma, St. Louis, MO) and developed using 100 μL of 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (55 mg/mL in 0.1 M sodium citrate buffer, pH 4.2 containing 0.03% of H_2O_2). The reaction was stopped with 50 μL of 5% sodium dodecyl sulfate, and the optical density was determined at 415 nm on an

EAR 400 AT Easy Reader (SLT-Lab Instruments, Salzburg, Austria). The isotype of each monoclonal antibody was determined by ELISA with goat anti-mouse class and subclass-specific antisera (Zymed Laboratories, So. San Francisco, CA).

Immunodetection of Cr-P II Components by Monoclonal Antibodies

Electrophoretic transferred polyvinylidene fluoride membranes were washed in 10% methanol in TBS and blocked with 3% gelatin in TBS. After washing, the blots were probed with purified monoclonal antibody (15 $\mu\text{g}/\text{mL}$) diluted in TBS containing 0.05% Tween, 0.5% gelatin and alkaline phosphatase-labeled goat anti-mouse IgG (Sigma, 1:30,000). Following extensive washing, strips were immersed in a substrate solution of 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium in 100 mM Tris-HCl, pH 9.5 containing 100 mM sodium chloride and 5.0 mM magnesium chloride²⁸ and rinsed in distilled water and air dried.

Purification of Monoclonal Antibodies

Hybridomas were cloned in hypoxanthine-thymidine medium at least twice by limiting dilution and then expanded further. Hybrid cells (5×10^6 cells/mouse) were propagated in the peritoneal cavity of female BALB/c mice after prior injection of pristane (0.5 mL/mouse). The ascitic fluids of the mice were harvested 10 to 14 days later, and monoclonal antibodies were purified by Protein A-agarose affinity chromatography using Affi-Gel Protein A MAPS II kit (Bio-Rad) according to the method recommended by the manufacturer as described.²² The eluted IgG fractions were collected and dialyzed against 0.05 M PBS, pH 7.5, overnight, at 4 °C and finally lyophilized. Concentration of immunoglobulin was determined by immunodiffusion with anti-mouse IgG antisera (The Binding Site Ltd, Birmingham, UK).

Assaying for Epitope Specificity of the Monoclonal Antibodies by ELISA
Microtiter plates were coated with 0.5 $\mu\text{g}/\text{well}$ of purified monoclonal antibodies diluted in sodium carbonate buffer, and then blocked with BSA overnight at 4 °C. After washing, wells were incubated with Cr-P II (0.5 $\mu\text{g}/\text{well}$) at room temperature for two hours in triplicate, and serial dilutions of anti-Cr-P II monoclonal antibodies (diluted from 20 μg of purified monoclonal antibodies) were then added to the wells. After a two-hour incubation at room temperature, the remaining Cr-P II activity was determined by purified rabbit anti-Cr-P II antibodies (1.0 mg/mL, 1:2000) followed by goat anti-rabbit IgG-peroxidase (100 uL of 1:4000, Bio-Rad). The reaction was developed and determined as previously described.

Binding Inhibition of Human IgE to Cr-P II by Monoclonal Antibodies

Cockroach-specific IgE antibody of atopic subjects was determined by an enzyme immunoassay kit (IgE FAST-Plus Test, 3M Diagnostic System Inc), and test was performed according to the manufacturer's instruction. Optimal concentrations of Cr-P II (0.25 $\mu\text{g}/\text{well}$) were determined by checkerboard titration and coating onto a microtiter plate (Immunolon II, Dynatech). Wells were then incubated with 50 uL of serial dilutions of inhibitor (80 $\mu\text{g}/\text{mL}$ of monoclonal antibodies) at room temperature for two hours in triplicate. A nonspecific monoclonal antibody F-10 generated during the screening of anti-Cr-P II monoclonal antibodies was used as negative control. After incubation and washing, a mixture of sera of the same 12 cockroach-sensitive patients that were used in the above experiments was added and incubated at room temperature for two hours. Enzyme-labeled anti-human IgE was added and incubated for two hours at room temperature. Fluorescence substrate (4-methylumbelliferyl phosphate in 2-amino-2-methyl propanol buffer, pH 9.5) was then added into the well and incubated at room temperature for 30 minutes. Af-

ter incubation, fluorescence signal units were determined by the CytroFluor 2350 multiwell fluorescence plate reader (Millipore Corporation, Bedford, MA) with excitation at 360 nm and emission at 450 nm. Inhibition was expressed as:

$$[1 - (b-c)/a-c] \times 100$$

where a = fluorescence signal units in the absence of inhibitor; b = fluorescence signal units in the presence of inhibitor; and c = fluorescence signal units in the absence of a reaginic serum.

Detection of Cr-P II Allergens by ELISA Using Monoclonal Antibodies
Microtiter plates (Immunolon II, Dynatech) were coated with 100 μL of 1/10 dilution of commercial extracts or 5.0 $\mu\text{g}/\text{mL}$ of Cr-P II (diluted in 0.1 M sodium carbonate buffer, pH 9.6) per well for two hours at 37 °C. After incubation, coated wells were washed with PBS/Tween and blocked with 2% BSA overnight at 4 °C. After washing, wells were incubated with 100 μL of culture supernatant of monoclonal antibodies (antibody concentration that gave an absorbance of about 1.0 was used) at room temperature for two hours in duplicate and washed as before. Then 100 μL of peroxidase labeled rabbit anti-mouse IgG conjugate (Sigma, diluted 1/2500 in PBS containing 0.05% Tween) was added to each well. After two hours incubation at room temperature, wells were washed, and the reaction was developed and determined as previously described.

Protein Determination

Protein concentration was assayed by Coomassie brilliant blue G-250 protein-dye binding method of Bradford²⁹ using BSA as a standard.

RESULTS

Identification of Cr-P II Allergens

Preliminary studies with a wide range pH gel (3.5 to 9.5) revealed that proteins in crude American cockroach extract only focused below pH 6.5. Subsequently, isoelectric focusing was

performed in a gel with pH 4.0 to 6.5, and a profile of crude American cockroach extract, Cr-PI and Cr-P-II is presented in Figure 1. Cr-PI was mainly focused at pH 6.2 (Fig 1, lane c), and Cr-P-II was focused at pH 2.8 to 5.3 (Fig 1, lane d). The molecular weights of the isolated proteins estimated by SDS-PAGE under dissociating conditions are demonstrated in Fig 2A. At least 28 protein bands, molecular weights ranging from 12 to 100 kD, representing crude American cockroach extract were visible after Coomassie brilliant blue staining (Fig 2A, lane b). Cr-PI reveals six bands (Fig 2A, lane c) and Cr-P-II revealed as many as 15 clear bands (Fig 2A, lane d). Two significant bands, 45 and 43 kD, and two major bands at 28 and 18 kD, were prominent in Cr-P-II. Sera of 12 cockroach-sensitive atopic patients were examined for IgE binding to Cr-PI and Cr-P-II proteins after electroblotting. All 12 sera (100%) demonstrated binding to the 78- and 72-kD components of Cr-PI (Fig 2B). As for

Cr-P-II, as many as eight proteins were able to bind specific cockroach IgE (Fig 2C). Sera from two subjects, nonatopic and cockroach-negative atopic, did not bind specific human IgE to any proteins as demonstrated by negative autoradiographs (Fig 2B and 2C, lanes m and n). Three major proteins—28, 32, and 45 kD—bound IgE from 100% (12/12), 83.3% (10/12) and 100% (12/12) of the sera from atopic subjects tested, respectively. Results are summarized in Table 1.

Production of Monoclonal Antibodies to Cr-P-II

Cell fusion between spleen cells of BALB/c mice immunized with Cr-P-II and FO cells resulted in a series of hybridomas specific for Cr-P-II. These cell cultures were subcloned, and three stable cell lines secreting monoclonal antibodies against Cr-P-II were obtained and expanded in vitro as determined by ELISA with high absorbance values (Table 2). These monoclonal antibodies, designated as HW-4,

HW-8, and HW-19 were chosen for further studies. All three monoclonal antibodies were characterized as IgG2b, kappa-type subclass by ELISA and were unable to bind any components of German cockroach and Cr-PI (Table 2). Immunoblot analyses were performed with crude American cockroach extract, Cr-PI and Cr-P-II to determine the specificity of these monoclonal antibodies. Culture supernatant of FO was included as negative control and showed no reactivity with any components of Cr-P-II, crude American cockroach extract and Cr-PI (Fig 3, lanes b, f and j). HW-4 reacted moderate to strongly with 16-, 18-, 20-, 22-, 24-, 26-, 28-, and 32-kD components of Cr-P-II (Fig 3, lane c) and crude American cockroach extract (Fig 3, lane g). HW-8 and HW-19 bound only weakly to the 12- and 18-kD components of crude American cockroach extract (Fig 3, lanes h and i). Only the 12-kD protein of Cr-P-II however, was detected weakly by HW-8 and HW-19 (Fig 3, lanes d and e). All monoclonal antibodies did not react with any components of Cr-PI (Fig 3, lanes k, l, and m).

Epitope Specificity and IgE Binding capacity of Monoclonal Antibodies

Epitope specificity of the monoclonal antibodies was analyzed by ELISA, and results are presented in Fig 4. The results show that the binding of HW-4 to Cr-P-II was inhibited by HW-4 (Fig 4A) and only slightly inhibited by HW-8 (Fig 4B) and HW-19 (Fig 4C). On the other hand, the binding of HW-8 and HW-19 to Cr-P-II was only slightly inhibited by HW-4 (Fig 4A). HW-8 inhibited Cr-P-II binding of HW-8 and HW-19 and vice versa (Fig 4B and 4C). Collectively, these results indicate that HW-8 and HW-19 recognized the same binding site, and HW-4 did not recognize the same epitope as HW-8 and HW-19. Figure 5 shows the results of inhibition of Cr-P-II by monoclonal antibodies. Sera of the same 12 cockroach allergic patients used in autoradiography studies (Fig 2) were pooled for the inhibition studies. HW-4 inhibited 20% and 13% of the

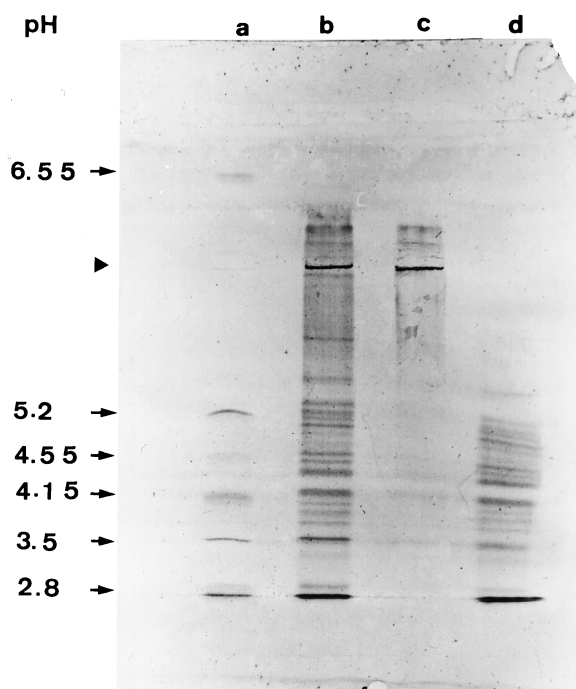


Figure 1. Coomassie brilliant blue stained isoelectric focusing gel (pH 4.0 to 6.5). a, PI markers (PI 2.8 to 6.55); b, crude American cockroach extract (40 μ g); c, Cr-PI (15 μ g); d, Cr-P-II (15 μ g). Sample application indicated (▲).

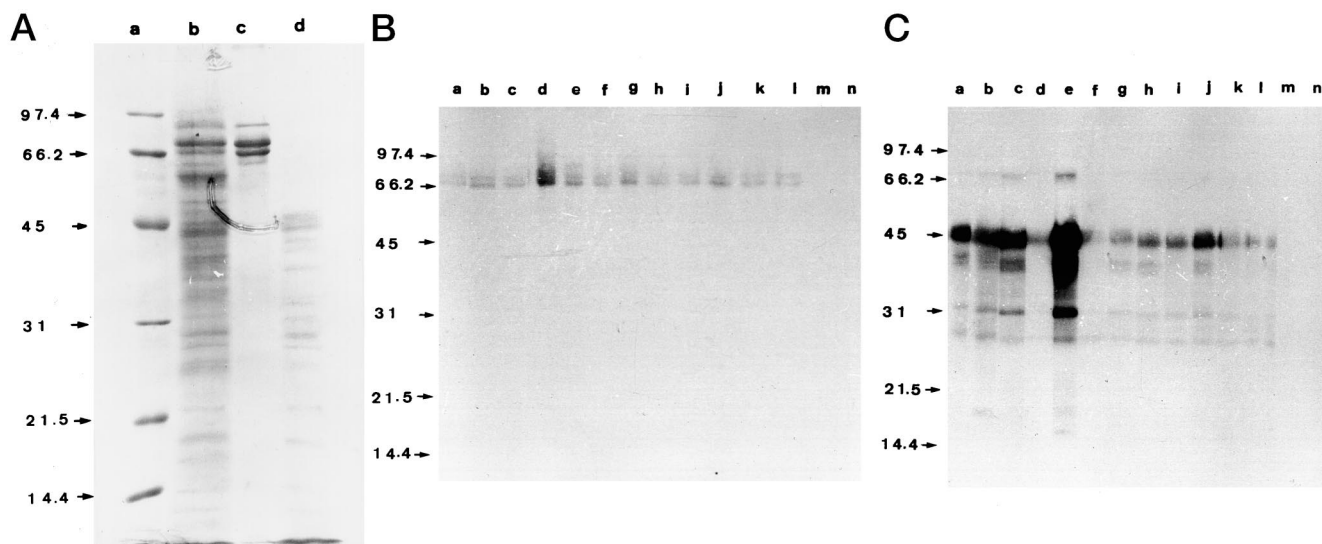


Figure 2. SDS-PAGE patterns of the crude American cockroach extract, Cr-PI and Cr-P-II (A), and autoradiographs of SDS-PAGE separated components of Cr-PI (B) and Cr-P-II (C) probed with human IgE antibodies. Concentration of proteins loaded for SDS-PAGE were 30 μ g of crude American cockroach extract and 10 μ g of Cr-PI or Cr-P-II. (A): Lane a, molecular weight markers; lane b, crude American cockroach extract; lane c, Cr-PI; lane d, Cr-P-II. (B) and (C): Lanes a-l: cockroach-positive atopics; lane m: cockroach-negative atopic; lane n: nonatopic.

Table 1. Components of Cr-PI and Cr-P-II that were Recognized by Cockroach-Specific IgE Antibodies in Sera of 12 Atopic Subjects

Serum	Components of Cr-P-II, kD								Cr-PI, kD	
	16	18	28	32	34	38	40	45	72	78
a			x	x		x	x	x	x	x
b		x	x	x	x	x	x	x	x	x
c			x	x	x	x	x	x	x	x
d			x					x	x	x
e	x	x	x	x	x	x	x	x	x	x
f			x					x	x	x
g			x	x		x	x	x	x	x
h			x	x		x	x	x	x	x
i			x	x				x	x	x
j			x	x		x	x	x	x	x
k			x	x				x	x	x
l			x	x				x	x	x
m*										
n†										
Y‡	8.3%	16.7%	100%	83.3%	25%	58.3%	58.3%	100%	100%	100%

Sera of CRa-A-negative atopic* and nonatopic† subjects.

‡ Y = Frequency recognized by specific IgE.

IgE binding to Cr-P-II at a concentration of 2.0 μ g and 1.0 μ g, respectively. A 13% inhibition of IgE binding was found at a concentration of 2.0 μ g by HW-8, and a similar result was found with HW-19. In a separate experiment, when a mixture of HW-4, HW-8, and HW-19 (2.0 μ g) was used, an inhibition rate of 39.5% was observed. These

results indicate that all epitopes defined by monoclonal antibodies are recognized by human IgE.

Detection of Cr-P-II in Commercial Extracts

A monoclonal antibody-based ELISA using HW-4 and HW-8 was performed for the detection of Cr-P-II allergens in

cockroach extracts. Five commercial cockroach skin test reagents that are currently marketed in the United States were compared for Cr-P-II levels by the ELISA and the results are shown in Table 3. All extracts contained detectable Cr-P-II, although there was a difference between the quantity of allergens in the extracts. In addition, seven

Table 2. ELISA Optical Densities of Culture Supernatants of FO and anti-Cr-P II Hybridoma Against the Crude Extracts of American and German Cockroaches, Cr-PI, and Cr-P II*

Hybridoma	Isotype of Monoclonal Antibodies	American Cockroach	German Cockroach	Cr-PI	Cr-P II
FO	—	0.005	0.001	0.002	0.001
HW 4	IgG2b, kappa	0.507	0.008	0.012	2.075
HW 8	IgG2b, kappa	0.480	0.002	0.004	2.431
HW 19	IgG2b, kappa	1.148	0.007	0.063	2.240

* Results are average of the experiment performed in triplicate as described.

common insect extracts (ant mix, Ken-tex fly mix, mosquito mix, house dust mix, mixed vespid venom protein, *Dermatophagoides farinae* and *D. pteronyssinus*) were also tested for Cr-P II allergens. Except for the house dust mix, none of those insects' extracts were found to contain any detectable Cr-P II as determined by the ELISA (Table 3).

DISCUSSION

Antigenic and allergenic components of both American and German cockroaches have been found to be solely acidic (PI between 2.8 to 6.5) by different workers.^{17-18,20,30} In this study, similar results were obtained. Isoelectric focusing revealed that components of crude American cockroach extract focused only below pH 6.5, and Cr-PI (pH 6.2) and Cr-P II (below pH 5.3) had an acidic isoelectric point (Fig 1).

Among 13 proteins of Cr-P II (Fig 2C) separated by SDS-PAGE, eight proteins in the range of 16 to 45 kD were able to bind specific IgE. Components of 45 and 28 kD bound IgE from 100% of the sera tested from atopic subjects. The next most frequently recognized components of 32, 38, and 40 kD bound 83.3%, 58.3% and 58.3%, respectively (Table 1).

Kang et al¹⁹ have reported allergenic components in the range of 40 to 45 kD that bound IgE of most of the sera tested. Twarog et al¹¹ and Schou et al¹⁸ isolated allergens of 25.5 kD and 28 kD (*Per a I*) showing significant IgE binding capacity. Stankus et al^{16,30} reported two components of allergenic "fraction 2" of crude American cockroach extract focusing between pH 3.5

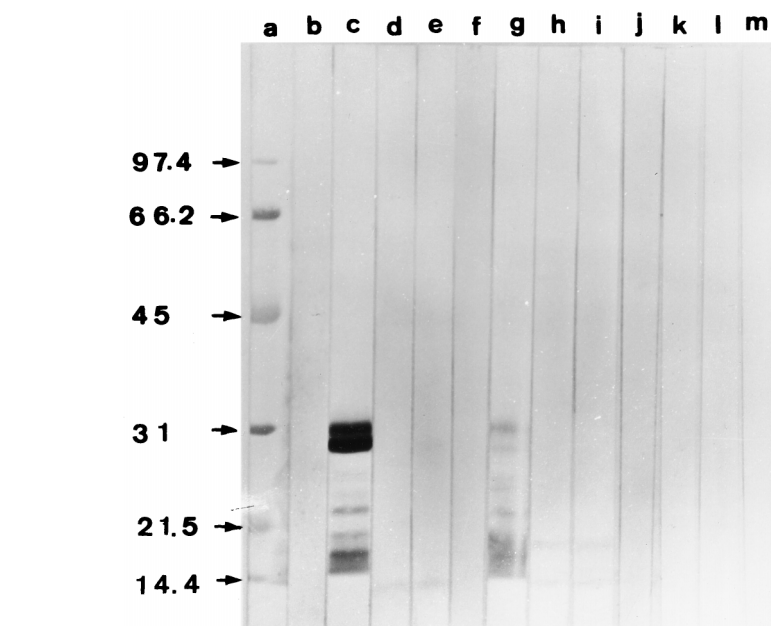


Figure 3. Immunoblot analysis of Cr-P II, crude American cockroach extract and Cr-PI. SDS-PAGE separated components of Cr-P II (10 µg, lanes b–e), crude American cockroach extract (30 µg, lanes f–i) and Cr-PI (10 µg, lanes j–m) were electrotransferred onto polyvinylidene fluoride membranes and followed by immunodetection with FO (lanes b, f and j), HW-4 (lanes c, g and k), HW-8 (lanes d, h and l) and HW-19 (lanes e, i and m). Lane a: amido black stained protein markers.

to 4.55 that reacted with most cockroach-sensitive atopic sera tested.

It remains to be determined whether allergens identified in Cr-P II are the same American cockroach allergens identified by other groups. The preliminary data presenting in this study suggests strongly that the 45-, 40-, 38-, 32-, and 28-kD components of Cr-P II are significant allergens of American cockroach alongside the 72- and 78-kD components of Cr-PI.

Three stable subclones secreting monoclonal antibodies against Cr-P II as defined by high ELISA optical densities were generated (Table 2). HW-4

bound moderate to strongly with seven components ranging from 16 to 40 kD, and HW-8 and HW-19 bound only weakly to the 12- and 18-kD proteins of crude American cockroach extract as demonstrated by immunoblotting (Fig 3). Changes in conformational configuration of Cr-P II allergens induced by denaturation in SDS-PAGE may explain the weak binding of HW-8 and HW-19 by immunoblotting.

Specific monoclonal antibodies reacting with multiple components of allergenic extracts have been reported.^{22,31-34} The high activities of major allergenic components may be due to

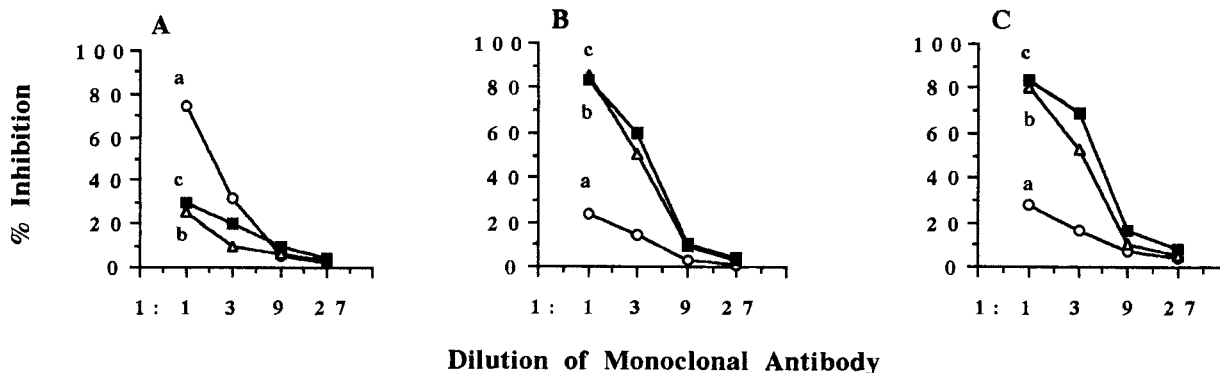


Figure 4. Determination of the epitope specificities of monoclonal antibodies (a, HW-4; b, HW-8; and c, HW-19) by ELISA. Microtiter plates were coated with purified HW-4 (A), HW-8 (B) or HW-19 (C). Results are average of the experiment performed in triplicate as described.

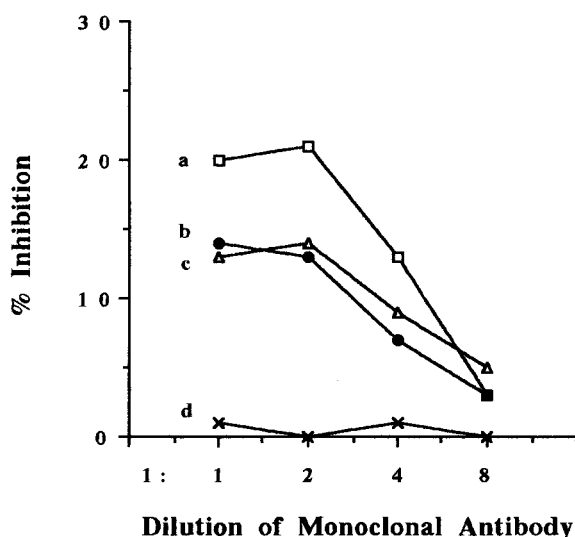


Figure 5. Inhibition of binding of human IgE to Cr-P-II by monoclonal antibodies (a, HW-4; b, HW-8; c, HW-19 and d, F-10). Results are average of the experiment performed in triplicate as described.

the possession of determinant groups common to other proteins as postulated.³⁵ It seems reasonable that multiple components sharing a certain epitope is a common feature in some allergenic extracts as well as in the American cockroach. Both species-specific and shared interspecies allergens in cockroaches have been demonstrated.^{11,16,18-20} All monoclonal antibodies failed to react with the crude extract of the German cockroach as demonstrated by ELISA (Table 2).

Using an ELISA based on the consideration that there is inhibition of antigen binding to the monoclonal antibody 1 by the monoclonal antibody 2,

HW-8 and HW-19 were found to have similar epitope specificities. These two monoclonal antibodies and HW-4 have different epitope specificities (Fig 4). All monoclonal antibodies to Cr-P-II did not recognize any components of Cr-PI (Table 2 and Fig 3). Thus, it appears that the 72-kD and 78-kD major allergens in Cr-PI and the low molecular weight allergens of Cr-P-II are different groups of allergens since they do not share the common determinants. In an inhibition assay (Fig 5), it was found that HW-4 can inhibit the binding of IgE to Cr-P-II by 20%, whereas HW-8 and HW-19 were less efficient (13%). One reason for the low

inhibition by monoclonal antibodies may be the mixture of several allergens present in the partially purified Cr-P-II fraction. Nevertheless, all three monoclonal antibodies' epitopes were shown to be human IgE binding epitopes.

We used an ELISA to compare allergen levels in commercial cockroach skin test extracts. Three extracts prepared from the American cockroach contained similar detectable levels of Cr-P-II. One extract (HSL) contained lower Cr-P-II levels defined by HW-8; however, this extract was prepared from a mixture of American and German cockroaches and would be expected to contain lower levels of Cr-P-II. The remaining extract (ALI) contained significantly lower Cr-P-II levels as compared with the other extracts. The results demonstrate significant differences of detectable Cr-P-II levels defined by monoclonal antibodies in the commercial extracts.

When monoclonal antibodies were used to examine the possibility of crossreactive components in some common insects' extracts, none of those insects' extracts were found to contain any detectable Cr-P-II except for house dust mix (Table 3). Detectable Cr-P-II found in house dust mix extract may be due to aerosolized allergens becoming airborne over cockroach-infested environments.³⁶

In conclusion, at least eight IgE-binding components have been identified in Cr-P-II, and components of 45,

Table 3. Detection of Cockroach and Insect Extracts by Anti-Cr-P II Monoclonal Antibodies ELISA

Extracts	Optical Density at 415 nm	
	HW-4	HW-8
Cr-P II (5 μ g/mL)	0.983	1.021
ALI (1:20 wt/vol, American cockroach)	0.430	0.490
BB (1:20 wt/vol, American cockroach)	1.037	0.712
CL (10,000 PNU/mL, American cockroach)	0.641	1.804
GL (1:20 wt/vol, American cockroach)	0.990	0.988
HSL (1:10 wt/vol American and German cockroach mix)	1.435	0.077
HSL (1:10 wt/vol Ant mix)	0.003	0.004
HSL (1:10 wt/vol Kentex fly mix)	0.002	0.015
HSL (1:10 wt/vol Mosquito mix)	0.001	0.007
HSL (300 μ g/mL, Mixed vespid venom protein)	0.002	0.003
HSL (20,000 PNU/mL, House dust mix)	0.172	0.343
HSL (10,000 AU/mL, standardized <i>D. farinae</i>)	0.022	0.004
HSL (10,000 AU/mL, standardized <i>D. pteronyssinus</i>)	0.012	0.003

Well was coated with 100 μ L of commercial crude extracts (1/10 dilution) or Cr-P II (5 μ g/mL). Culture supernatants (100 μ L) were added and immunodetected with peroxidase labeled rabbit anti-mouse IgG as described.

32, and 28 kD may be considered important allergens of the American cockroach besides the 78 and 72 kD of Cr-PI. Hybridomas were produced after immunization with Cr-P II. Two stable subclones with different epitope specificities were obtained and shown to bind human IgE epitopes. One of the monoclonal antibodies reacted strongly with 28- and 32-kD important allergens of the American cockroach. None of the monoclonal antibodies recognized any common epitopes of the German cockroach and Cr-PI.

Monoclonal antibodies can be used for the standardization, isolation, and purification of allergens from crude extracts and the cloning of cDNA encoding American cockroach allergens. Identification of additional important allergens and production of monoclonal antibodies will be useful in dissecting and defining cockroach allergens, and have implications for clinical applications and treatment of hypersensitive patients.

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ANTIBODIES TO FUNGI AND RESPONSE TO KETOCONAZOLE IN PATIENTS WITH ATOPIC DERMATITIS

To investigate possible relationships between immune responses to fungi and atopic dermatitis the authors determined the presence of IgE antibodies to *Pityrosporum orbiculare* (ovale) and *Candida albicans* in patients with atopic dermatitis and healthy controls by radioallergosorbent test (RAST), and evaluated responses to treatment with ketoconazole. Twenty-eight (44%) of 63 adults with atopic dermatitis had IgE antibodies to *P. orbiculare* and 21 (33%) to *C. albicans*. The RASTs disclosed neither antibody in sera of 11 patients with seborrheic dermatitis and 39 controls with no cutaneous disorders.

Atopic dermatitis improved significantly in 18 patients with IgE antibodies to *P. orbiculare* (and *C. albicans* in most) during treatment with ketoconazole as determined by changes in clinical scores. The patients received ketoconazole, 200 mg daily for 2 months and then 200 mg twice each week on consecutive days for 3 more months. Some patients were able to stop treatment with topical steroids while receiving ketoconazole. Both total serum IgE and *P. orbiculare*-specific IgE decreased significantly. *C. albicans*-specific IgE correlated significantly with global clinical scores.

The reason for improvement in these patients is not known because ketoconazole has both antiinflammatory and antifungal effects. There is need for further study with placebo controls.

—RMS

Bäck O, Scheynius A, Johansson SGO. Ketoconazole in atopic dermatitis: therapeutic response is correlated with decrease in serum IgE. *Arch Dermatol Res* 1995;287:448-51.