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Isolation of the 36-kD German (*Blattella germanica*) Cockroach Allergen Using Fast Protein Liquid Chromatography

Key Words

Cockroach

Allergen

Abstract

Cockroach allergens have been established as important sensitizing agents in the induction of asthma. In the present investigation, we identified a 36-kD allergen from German cockroach (*Blattella germanica*) using fast protein liquid chromatography that reacted in the Bla g II monoclonal assay developed by Pollart et al. Defatted whole body German cockroaches were extracted in phosphate-buffered saline and fractionated using gel filtration (Superdex 75) and anion exchange (MonoQ) chromatography. The 36-kD allergen was isolated from this enriched allergen preparation by excision from modified preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, electroelution, dialysis and lyophilization. SDS-PAGE/Western immunoblot analysis using a serum pool of cockroach-sensitive individuals revealed a single band. The lyophilized 36-kD allergen was then analyzed for amino acid composition and digested for peptide amino acid sequencing.

Introduction

The role of the cockroach as an etiologic agent of respiratory allergic disorders is well documented [1-7]. Based on purification studies, Twarog et al. [8] suggested the occurrence of shared allergenic components between whole body extracts of German and American cockroaches. They further identified three major allergenic components: CR I (25.5 kD), CR II (63-65 kD) and CR III (10 kD). Richman et al. [9] reported that allergens in the extracts of these two species demonstrated little cross-reactivity, whereas Stankus and O'Neil [10] suggested the occurrence of shared, as well as specific, allergens between these two species. In earlier reports, we suggested species-

specific and possibly genus-specific as well as insect cross-reactive allergens among several cockroach species [11, 12].

In order to investigate the nature of cockroach allergens, we established a physicochemical/immunochemical scheme to isolate specific German cockroach allergens using serum containing IgE specific to cockroach. German whole body cockroach extracts and a 36-kD allergen isolated from German cockroach extracts reacted with the Bla g II monoclonal assay developed by Pollart et al. [13]. Our peptide analysis of the four CNBr/tryptic digests of this 36-kD allergen is the first demonstration of partial sequencing for this allergen.

Materials and Methods

Cockroach Extracts

Frozen whole body German cockroaches were supplied by the Medical and Veterinary Entomology Research Laboratory (USDA-ARS, Gainesville, Fla., USA). To facilitate extraction, adult male and female cockroaches were snap frozen in liquid nitrogen and lyophilized for storage at -20°C . Lyophilized cockroaches were then crushed in a mortar and pestle, defatted for 3–4 h with petroleum ether in a Soxhlet extractor and dried at room temperature. Defatted cockroaches were extracted (1:10 w/v) in phosphate-buffered saline (PBS) (pH 7.4). Protein was determined by the bicinchoninic acid (BCA) procedure (Pierce Chemical Co., Rockford, Ill., USA) using bovine serum albumin (BSA) as standard.

Human IgE Antibodies

A human serum pool containing elevated IgE to German cockroach allergens was prepared using sera from 4 urban patients from Chicago who were cockroach sensitive as determined by clinical history, skin test (wheal diameter > 10 mm, 1:20 w/v, prick test; Hollister-Stier Laboratories, Spokane, Wash., USA), and radioallergen sorbent testing ($> 200\%$ control serum) (sera provided by Dr. Bann Kang, University of Kentucky, Lexington, Ky., USA). All serum donors were urban asthmatics and none had received cockroach immunotherapy. Details for criteria of the donors can be found in references by Kang [3, 4]. Serum obtained from individuals in Little Rock (Ark., USA) was obtained from patients presenting to the Immunology/Allergy Clinic, Arkansas Children's Hospital (wheal diameter > 10 mm, 1:20 w/v, prick test, Greer Laboratories, Lenoir, N.C., USA). These sera and serum samples received from various other sources were cockroach positive as determined by both skin test and clinical history.

Allergen Fractionation

Whole body German cockroach extracts were sequentially fractionated using gel filtration (Superdex 75 column) and ion exchange chromatography (MonoQ column) (fast protein liquid chromatography (FPLC); Pharmacia, Piscataway, N.J., USA). Approximately 40 mg protein of crude German cockroach extract were applied to the Superdex 75 column. Each peak was dialyzed against 100 mM ammonium bicarbonate buffer (pH 7.6) at 10°C using Spectra/por 1 membranes (Baxter) with a molecular weight cut-off of 6,000–8,000 daltons and lyophilized. Protein and IgE-binding activity of material in each peak was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) immunoblot analysis. Lyophilized samples representing fraction No. 2 (visibly the highest concentration of the 36-kD allergen band) of the gel filtration column were reconstituted in 20 mM BisTris (pH 7.4) buffer and 1.0-ml aliquots applied to the MonoQ (anion exchange) column equilibrated with 20 mM BisTris. Samples applied in starting buffer (20 mM BisTris) were eluted using a step salt gradient (limit buffer = 20 mM BisTris/1.5 M NaCl, pH 7.4). Material eluted as peaks in each NaCl step was collected immediately following the run, pooled, dialyzed, lyophilized and stored at -20°C . Allergenic activity was assessed by SDS-PAGE/immunoblot analysis.

The eluate obtained from the 20% NaCl step of anion exchange fractionation (visibly the highest concentration of the 36-kD IgE-binding protein) was applied to 10% SDS-PAGE gels in Tris-glycine buffer (pH 7.2) and run at 20 mA/gel. Protein bands were identified by negative staining with Rapid Reversible Stain (Diversified Bio-

tech, Newton Center, Mass., USA) and the single band representing the 36-kD protein electroeluted in Tris-glycine-SDS buffer (BioRad Laboratories Richmond, Calif., USA). The electroeluted 36-kD protein was exhaustively dialyzed against 100 mM NH_4HCO_3 , pH 7.6, and lyophilized. Relative purity and IgE binding of the isolated 36-kD allergen were evaluated visually using the MiniBlot system.

Enzyme-Linked Immunosorbent Assay (ELISA)

Reagents for the two-site monoclonal based enzyme-linked immunoassay for Bla g II was provided by Drs. Pollart and Chapman (University of Virginia, Charlottesville, Va., USA) [13]. In the place of peroxidase-conjugated goat anti-rabbit IgG, we used alkaline phosphatase-conjugated goat anti-rabbit serum (BioRad) diluted 1:2,000 (v/v) and developed using the alkaline phosphatase substrate kit (BioRad). The absorbance was read at 409 nm (Titertek Multiscan; Flow Laboratories, McLean, Va., USA). Units of Bla g II were read from a standard curve of an in-house cockroach allergen extract standardized with the extract provided by Pollart and Chapman (UVA 89/01: 5,000 units/ml Bla g I and 3,000 units/ml Bla g II).

Direct ELISA was performed in duplicate using antigen-coated 96-well microtiter plates (Immunlon IV) to detect IgE antibodies specific for cockroach allergens. One hundred microfilters of whole body German cockroach extract (20 $\mu\text{g}/\text{ml}$) or 100 μl of the 36-kD allergen (5 $\mu\text{g}/\text{ml}$) in 100 mM carbonate buffer, pH 9.2, were added to individual wells at 4°C overnight. Wells were then washed with PBS/0.5% Tween 20 five times and 100 μl of patients' serum (1:10 v/v) in PBS/2% BSA was added for 90 min at 37°C . Wells were again washed and bound IgE determined by the addition of 100 μl peroxidase-conjugated goat anti-IgE (1:1000 v/v) for 60 min at 37°C . Wells were washed and a substrate buffer containing O-phenylenediamine (Sigma Chemical Co.) was added for 20–30 min, after which 100 μl of 2 N HCl was added to each well and the plates read in microtiter plate reader (BioRad) at 405 nm. For evaluation, an ELISA percent binding was obtained by dividing the OD of the test IgE sample minus blank control by the total OD of a negative serum (nonatopic) control minus blank control and multiplying by 100. Values which were equal to or greater than IgE (+) pool (1:10 v/v) were considered positive (whole body = 150%; 36 kD = 120%).

SDS-PAGE/Immunoblotting

SDS-PAGE was performed using a 12.5% resolving gel following the method of Laemmli [14] and stained with Coomassie brilliant blue (BioRad). For immunoblotting, protein bands were transferred to Immobilon nitrocellulose membranes (Millipore, Gaithersburg, Md., USA) [15] using the BioRad transblot apparatus at a constant voltage (100 V) for 30 min. Nitrocellulose membranes were either stained with amido black to ensure protein transfer or washed in PBS containing 0.5% Tween 20 for 60 min to block the remaining active sites. Immunoblots were performed by incubation with 12 ml of the human IgE pool (1:3 v/v in PBS/Tween 20) overnight at 10°C . Blots were then washed 3 times (PBS), incubated with 10 ml alkaline phosphatase-labeled goat anti-human IgE (1:2,000 v/v in PBS; Kirkegaard Perry Laboratories, Gaithersburg, Md., USA) for 2 h at room temperature, washed 3 times (PBS) and developed using BioRad alkaline phosphatase color developer (BioRad).

An enriched 36-kD allergen preparation was applied to 10% SDS-PAGE gels in Tris-glycine buffer (pH 7.2) and run at 20 mA/gel. Protein bands were negatively stained with Rapid Reversible Stain (Diversified Biotech) and the single band representing the 36-kD protein cut from the gel and electroeluted in Tris-glycine-SDS buffer

(BioRad). The eluted component was exhaustively dialyzed and lyophilized. Relative purity and IgE binding of isolated 36-kD protein was evaluated visually using the Miniblot system (BioRad).

Amino Acid Analysis and Sequencing

Individual aliquots of 50 μ g of lyophilized 36 kD protein were submitted to the Biotechnology Resource Laboratory (W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, Conn., USA) for amino acid analysis and sequencing [16]. A 10% sample in 70% formic acid was subjected to amino acid analysis using a Beckman Analyzer. Amino-terminal sequencing was performed on an Applied Biosystems sequencer with an on-line HPLC system with the resulting PTH amino acids being analyzed on a C18 column (220 $\times$ 2.1 mm) that was eluted with increasing concentrations of acetonitrile.

Direct NH_2 -terminal sequencing demonstrated that > 98% of the intact 36-kD protein had a blocked NH_2 terminus. Therefore, internal sequences were obtained from peptides that were isolated by reversed phase HPLC following cleavage of the 36-kD protein with cyanogen bromide (CNBr) and trypsin. Samples were applied and peptides were eluted from a 2.1 mm $\times$ 25 cm Vydac C-18 column that was equilibrated at 0.15 ml/min with 98% buffer A (0.06% TFA) and 2% buffer B (0.052% TFA, 80% acetonitrile). Peptides were eluted by a series of linear gradients utilizing increasing concentrations of buffer B [16]. A blank control and a transferrin control were treated in the same fashion.

Results

FPLC Fractionation/SDS-PAGE Immunoblot Analysis

The results of gel filtration with the Superdex 75 column are shown in figure 1. The crude whole body extract was resolved into three peaks ranging in apparent molecular weights of 6,500 to > 75,000 daltons and three additional peaks with apparent molecular weights of less than 1,500 daltons. Fraction 1 represents the void volume material; fraction 2 represents material eluting between 10,000 and 68,000 daltons; fraction 3 and fraction 4 the remainder of the column eluate with apparent molecular weights of

less than 6,500. Fraction 2 showed the highest visible concentration of the 36-kD protein.

Analysis of Superdex 75 fraction 2 by anion exchange (MonoQ) chromatography revealed the chromatogram shown in figure 2. The major peak eluting in the 20% NaCl gradient fraction showed the enriched 36-kD component (by visual comparison, approximately 80% of the applied material) as indicated by SDS-PAGE analysis. Analysis of the remaining Superdex 75 fractions revealed diminished

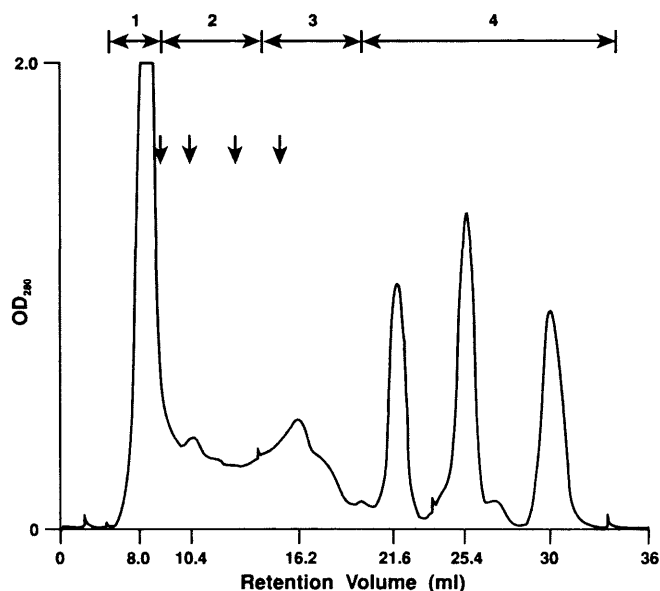
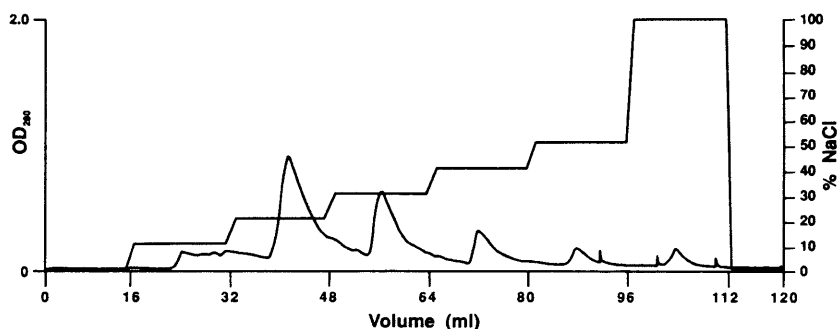


Fig. 1. Gel filtration of defatted whole body German cockroach extract on Superdex 75; 40 mg of extract was applied and eluted with PBS. The elution profile was monitored at 280 nm (OD) and peaks collected at the 10% absorbancy level in 5.0-ml fractions. Numbers across the top (between brackets) represent pooled fractions. Arrows indicate molecular weight standards (left to right): BSA = 68,000; chicken ovalbumin = 43,000; myoglobin = 17,600; apoferritin = 6,500.

Fig. 2. Anion exchange chromatography of fraction 2 isolated from Superdex 75 gel filtration (see fig. 1). Approximately 20 mg of fraction 2 were applied to a 1.0 ml 20 mM BisTris and eluted with a NaCl gradient (limit buffer = 20 mM BisTris/1.5 M NaCl). The elution profile was monitored at 280 nm (OD) and peaks collected at the 10% absorbancy level in 5.0-ml fractions.



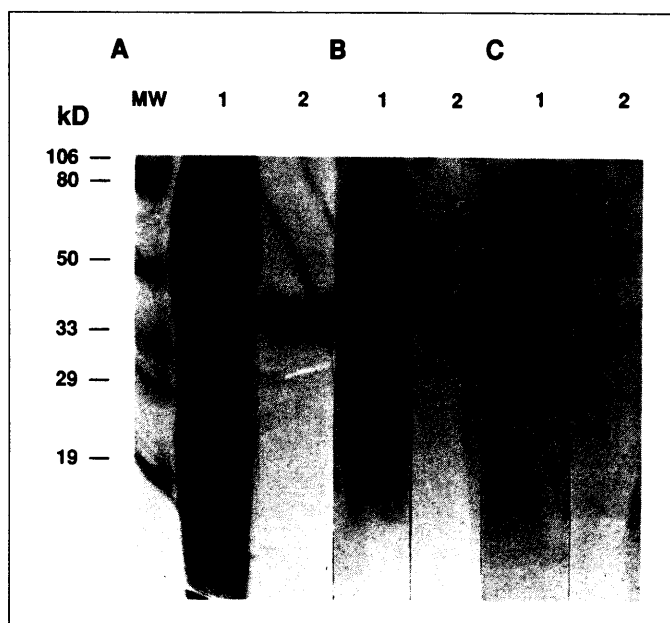


Fig. 3. SDS-PAGE (10%) and immunoblot analysis of the 36-kD electroeluted allergen. **A** Coomassie blue stained gel. **B** Immunoblot analysis using cockroach-sensitive sera. **C** Immunoblot analysis using nonatopic sera. Lane 1 = *B. germanica* whole body extract; lane 2 = 36-kD allergen.

Table 1. Concentrations (units) of Bla g II specific binding to whole body German extract and the 36-kD allergen band

Dilution	Units ¹	
	whole body extract	36 kD
10 ⁻¹	0.40	ND
10 ⁻²	0.23	ND
10 ⁻³	0.14	ND
10 ⁻⁴	0.07	0.89
10 ⁻⁵	0.01	0.19
10 ⁻⁶	0.00	0.01

ND = Not done.

¹ Bla g II units derived from standard curve using an in-house whole body German cockroach extract standardized with UVA 89/01.

yields of the 36-kD component. Multiple samples of the Superdex 75 fraction 2/20% NaCl MonoQ fraction were run on modified preparative SDS-PAGE gels to isolate the 36-kD allergen by electroelution.

A 50- μ g aliquot of the electroeluted 36-kD allergen preparation was reconstituted in Laemmli sample buffer

and analyzed for purity and IgE binding by SDS-PAGE/Western blot analysis. The results of the assessments are shown in figure 3. Lane 1 represents the crude whole body German cockroach extract and lane 2 represents the single purification of the 36-kD allergen. Immunoblotting of the purified 36-kD allergen with sera from cockroach-sensitive individuals and sera from a nonatopic control is shown in panels B and C respectively. Clearly, there is no binding with the negative control sera to any of the blotted bands in either the whole body German extract or the 36-kD allergen. Serum from the cockroach-sensitive individuals bound the 36-kD allergen with no indication of contaminating proteins.

Bla g II ELISA

The results of the Bla g II assay for German whole body cockroach extract and the 36-kD allergen are presented in table 1. The 36-kD allergen concentration was 0.89 U compared to the whole body extract starting material (0.07 U) at a 1:10,000 dilution. On a weight-to-weight basis, the 36-kD allergen represented approximately a 10,000 times more reactive preparation than the whole body extract.

Direct ELISA

The ELISA reactivity of each of 5 sera from cockroach skin test positive patients in Little Rock (AR); 8 sera from asthmatic patients in Chicago (IL), and 3 miscellaneous sera received from patients with skin test positive tests in New Jersey (NJ), Minnesota (MN), and Texas (TX) respectively is shown in table 2. All patient sera from the Chicago area reacted equally well to the whole body German extract as well as to the 36-kD allergen with the exception of patient CM, whose serum reacted poorly to both allergen preparations. The sera from patients in the Little Rock area reacted variably to the allergen preparations. KH reacted the best to both allergen preparations; however, NC and CR reacted far better to the whole body extract compared to the 36-kD preparation. CS and AD, although strongly skin test positive (personal communication from the donor) to mixed whole body cockroach extract reacted only marginally (CS) or not all (AD) to the allergen preparations. Similar reactivity was determined with individual serum from patients residing in NJ, MN or TX. IH (NJ) and VP (MN) reacted quite strongly to whole body German extract; while only VP (MN) reacted marginally to the 36-kD allergen preparation. Again, BD (TX) who was strongly skin test positive (personal communication from donor) failed to bind either allergen preparation. In summary, 10 of 15 cockroach-allergic individuals had IgE antibody to the 36-kD allergen.

Table 2. ELISA percent binding (negative control) of 36-kD allergen

Geographic area	Subject	ELISA % binding	
		German whole body cockroach extract	36-kD allergen
	(-) Control	100	100
	(+) IgE pool	157	122
	ANP	113	86
Little Rock, Ark.	KH	173	162 (+)
	NC	183	107 (-)
	CR	161	101 (-)
	CS	114	121 (+)
	AD	98	95 (-)
Chicago, Ill.	DP*	185	141 (+)
	MJ	167	138 (+)
	VW*	158	148 (+)
	LW*	182	176 (+)
	RM*	169	164 (+)
	MB	173	133 (+)
	GM	148	148 (+)
Miscellaneous	CM	119	106 (-)
	IH (NJ)	165	86 (-)
	VP (MN)	141	120 (+)
	BD (TX)	99	76 (-)

(-) Control = sera from nonatopic individual; * = sera used to prepare cockroach-sensitive IgE pool; ANP = atopic, cockroach skin test negative pooled sera; (+) = > 120%.

Amino Acid Analysis and Sequencing

The 36-kD band isolated from 10% SDS-PAGE preparative gels showed a total of 32.42 µg or 901 pmol of protein. The amino acid composition of this material is presented in table 3. Cysteine and tryptophan are destroyed to varying extents during hydrolysis and were not quantified.

Amino-terminal sequencing of the intact 36-kD protein failed. The N-terminally blocked 36-kD protein was digested with CNBr/trypsin and peptide fragments separated by HPLC. Recovery of the peptides ranged from 21 to 75%. Four peaks representing homogenous peptides (based on their HPLC profile) identified as 143-56, 143-76, 143-111 and 143-115 were sequenced. The amino acid sequences are shown in table 4. A search of the PIR, GENBANK or SWISS-PROTEIN databases revealed no homology with known protein amino acid sequences.

Table 3. Amino acid composition of the 36-kD allergen

Amino acid	Residues/100 residues
Asx	12.4
Thr	3.6
Ser	8.2
Glx	16.5
Pro	3.2
Gly	21.8
Ala	5.8
Val	3.8
Met	0.9
Ile	2.7
Leu	3.1
Tyr	1.8
Phe	2.4
His	1.6
Lys	4.0
Arg	8.3

Table 4. 36-kD cockroach allergen peptide sequence

<i>Peptide 143-56</i>												
*	Ile	Ala	Gly	Gly	Asn	Thr	Gln	Ala	Leu	Ile	(Asp)	
1	2	3	4	5	6	7	8	9	10	11	12	
Thr	Ser	Lys										
13	14	15										
<i>Peptide 143-76</i>												
*	Val	Glu	Val	Pro	(THr)	Ser	(Ile)	Asp	Glu			
1	2	4	4	5	6	7	8	9	10			
(Leu)	*	*	(Pro)									
11	12	13	14									
<i>Peptide 143-111</i>												
Ala	Pro	Pro	Gly	Cys	(Ser)	Asn	His	Phe	Asn	Leu		
1	2	3	4	5	6	7	8	9	10	11		
Glu	Thr	Val	Phe	Gly	Asn	Phe	Val	Lys				
12	13	14	15	16	17	18	19	20				
<i>Peptide 143-115</i>												
*	Gly	Leu	Asn	Ile	Cys	Gln	Ile	Asp	Cys	Asn	Lys	
1	2	3	4	5	6	7	8	9	10	11	12	
Ile	Gln	(Ser)	Leu	Pro	*	Leu	Val	Phe	Thr	Ile		
13	14	15	16	17	18	19	20	21	22	23		
Gly	(Gly)	(Asp)										
24	25	26										
* Undetermined amino acid.												

Discussion

The significance of the cockroach has been established as an important inhalant allergen in various parts of the world [1-13]. Following skin testing of adults and children in the Chicago area, Kang and Sulit [17] concluded that the prevalence of skin test reactivity to the cockroach was second only to the prevalence of skin test reactivity to house dust. Biological skin test reactivity has been demonstrated using both whole body extracts and fecal extracts [18].

From the extraction of German whole body extracts, we have identified several IgE-binding bands ranging from 12 to 100 kD and have emphasized the isolation of the 36-kD molecule earlier identified as a *Blattella* genus-specific cockroach allergen [12]. Our 36-kD IgE-binding molecule predominated at this relative molecular weight in both reducing (5% 2-mercaptoethanol) and nonreducing SDS-PAGE analysis. However, we still do not have clinical evidence of the species-specific nature of this allergen among common cockroach hypersensitivities.

In a previous publication we identified a chromatographic fraction as German cockroach fraction 3 (GCR3) of a Sephadex G-200 chromatographic isolation that was shown to contain an allergen with a pI of 6.7 and an apparent molecular weight of 36-kD [12]. SDS-PAGE/immunoblot analysis of GCR3 showed several components with molecular weights ranging from 12 to 75 kD. We sought to improve our isolation scheme to purify the 36-kD allergen using the improved chromatographic methods provided by the FPLC system. A 36-kD component isolated by combined gel filtration and anion exchange chromatography using the FPLC system was shown to be identical to the 36-kD component isolated using rabbit anti-GCR3 IgG [12]. Storage of pooled source material during the 2-week period did not show significant loss of protein bands as assessed by IgE-binding patterns. The FPLC system provided increased yield throughout and in purified allergen compared to the earlier Sephadex G-200 separation protocol.

To fully understand the nature of cockroach allergy and the specific allergens involved, investigators in the field

should standardize and share reagents for comparison. During the course of isolating our 36-kD component, Pollart et al. [19] described Bla g II as a 36-kD molecule using monoclonal antibodies. Through a gift from Drs. Pollart and Chapman we were able to analyze our 36-kD molecule in the same monoclonal assay. The results confirmed that the 36-kD component identified by our biochemical procedures was similar to that of the 36-kD molecule identified in their laboratory. Based on their ELISA, we found our 36-kD allergen preparation to be approximately 10,000 times more reactive on a weight-to-weight comparison than our whole body German cockroach extract starting material. One striking difference that remains to be resolved is the reported pIs. Pollart et al. [19] reported their 36-kD to have a pI of 5.2–5.4, whereas our pI was 6.7, suggesting the possibility of isoallergenic forms of Bla g II. Definitive proof of the identity of the two proteins depends on amino acid or gene sequencing [20–24].

We have, however, established a good clinical correlation of the immunoreactivity of the electroeluted 36-kD purified allergen preparation with a whole body German cockroach extract. Results from the Little Rock area and the serum obtained from individuals in other geographical areas of the United States suggest different allergens may be responsible for skin test reactivity. In this respect, our results are in agreement of the proposed explanations of Kang et al. [25]. In her discussion, Kang suggested that subjects from Lexington with asthma had a lower incidence of cockroach allergy than subjects from Chicago, or sera from the Lexington group demonstrated significantly lower allergy to one cockroach species as compared to others which may be present in the respective geographic area. Although she claims that all three cockroach species – American, German and Oriental – were probably pre-

sent in both cities, Lexington and Chicago, a discussion with a pest control organization in Little Rock revealed that the major species of cockroach in the area was the Smoky Brown (*Periplaneta fuliginosa*). Further study is necessary to confirm both the incidence of cockroach allergy and the species of cockroach responsible for sensitization in the Little Rock area.

In summary, our amino acid composition and partial sequencing of peptides can be used to compare allergen preparations characterized by various other investigators. Studies are underway to determine the full amino acid sequence of the 36-kD allergen as well as other cockroach allergens. A comparison of the amino acid sequences of the 36-kD allergen identified in the present investigation and the protein isolated by Pollart et al. [19] will be necessary to determine the identity of these two allergens isolated by independent protocols. The clinical usefulness of the purified allergen is uncertain. Large-scale clinical use will be needed to clarify the relative importance of the various cockroach allergens.

Acknowledgements

This work was supported by a University of Arkansas for Medical Sciences Biomedical Research Grant RR05350, the Arkansas Science & Technology Authority and NIH grant AI26629-04. The authors wish to thank Dr. Bann Kang for contribution of sera from cockroach-sensitive individuals; Drs. Pollart and Chapman for their contribution of monoclonal reagents for Bla g II identification, and the personnel at the W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, Conn., for their assistance in sequencing. The authors also wish to thank David E. Milne for technical assistance in Gainesville, Ms. Janette Breeding and Amy Brown for secretarial assistance and Ms. Majorie McMinn for her editorial comments.

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