

Cloning of the American cockroach Cr-P-II allergens: Evidence for the existence of cross-reactive allergens between species

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Background: Previously, we have identified the 28 and 32 kd proteins as additional important allergens from the American cockroach (*Periplaneta americana*) Cr-P-II allergenic fraction.

Objective: The aim of this study was the cloning of *P. americana* Cr-P-II allergens.

Methods: A λ gt22A cDNA library constructed from *P. americana* mRNA was packaged into *Escherichia coli* Y1090 (r⁻), and clones recognized by murine anti-Cr-P-II monoclonal antibodies and human IgE antibodies were isolated, sequenced, and subcloned into pET 21 and expressed in *E. coli* BL21(DE3).

Results: Six Cr-P-II-positive clones recognized by human IgE antibodies were isolated. Two clones, C6 and C17, were sequenced, and we found encoding proteins of 228 and 274 amino acids with no cysteine or any potential N-glycosylation site, with predicted masses of 25.8 and 31.14 kd, respectively. Both molecules contain internal repeated sequences with a 94% identity between them. C6 and C17 showed 59% and 77.3% skin reactivities, respectively, on 22 cockroach-sensitive atopic patients. Both clones were found to have 28.9% to 31.8% identities to ANG12 protein, a precursor of the African malaria mosquito (*Anopheles gambiae*) and 82.7% to 85.1% identity to a nucleotide sequence of the German cockroach (*Blattella germanica*) Bla g Bd90K allergen. The anti-C6 and anti-C17 antibodies were able to recognize Cr-P-II, recombinant proteins, five commercial American extracts, and two German cockroach extracts. Moreover, the binding of anti-C6 and anti-C17 antibodies to recombinant protein can be inhibited by *B. germanica* crude extract. Furthermore, Northern blot analyses have shown that *B. germanica* mRNAs could be detected by both cDNA probes.

Conclusion: Our findings provide the first evidence of antigenic cross-reactivity between *P. americana* and *B. germanica*

allergens on molecular levels. The results will be a great aid in facilitating the epitope mapping and improving diagnostic and therapeutic reagents for both cockroach species.

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Key words: American cockroach, Cr-P-II allergen, cDNA cloning, cross-reactive allergen

Cockroach allergens are one of the major etiologic risk factors for IgE-mediated allergic respiratory illnesses throughout the world,¹⁻¹⁴ such as allergic rhinitis and asthma. Although cockroach skin reactivity is second only to house dust reactivity, molecular dissecting of cockroach allergens and immunobiologic understanding of cockroach-induced asthma are just beginning. The cloning of allergens for generating recombinant proteins offers the possibility of obtaining pure allergens that would be very difficult to produce by conventional fractionation methods. Recombinant allergens will provide significant quantities for detailed characterization. This will lead to a better understanding of the mechanisms of these allergens and the development of major improvements in the management of asthmatic reactions.

The significant *Periplaneta americana* allergens, with molecular weights ranging from 6 to 120 kd, have been identified by various immunochemical techniques.¹⁵⁻²⁴ Our previous studies have purified two allergenic fractions, Per a 3 (Cr-PI) and Cr-P-II, from crude American cockroach extract (CRa-A) by using Sephadex G-150 SF resin. Per a 3 and Cr-P-II allergens elicit skin reactivity in 73% and 93% of patients sensitive to CRa-A, respectively.¹⁶ The 78 and 72 kd major allergens of Per a 3 cause T-cell proliferation in patients allergic to cockroaches,²⁵ and monoclonal antibodies (mAbs) to Per a 3 have been generated.²⁶ Four Per a 3 cDNAs encoding 79.3, 75.5, 56.2, and 46.7 kd allergens have recently been sequenced, and it was found that they are isoallergens.^{27, 28}

Two IgE-binding components of 32 and 28 kd in Cr-P-II that bind at least 83% of the sera tested have recently been identified as additional important *P. americana* allergens, and mAbs recognizing these two allergens have also been generated.²⁴ In this article we report the nucleotide and deduced amino acid sequences of two partial cDNA clones, C6 and C17, encoding *P. americana* Cr-P-II allergens. Sequence

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Abbreviations used

bp:	Base pairs
CRa-A:	Crude American cockroach extract
CRa-G:	Crude German cockroach extract
EDTA:	Ethylenediamine tetraacetic acid
IPTG:	Isopropyl thiogalactose
kb:	Kilobase pairs
mAbs:	Monoclonal antibodies
PBS:	Phosphate-buffered saline
PMSF:	Phenylmethylsulfonyl fluoride
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

similarity is found between *P. americana* clones and Bla g Bd90K²⁹ and ANG 12 of *Anopheles gambiae*. No significant similarities to other known proteins were found.

METHODS

Molecular cloning of Cr-PII cDNAs

A λ gt22A cDNA library was constructed from *P. americana* mRNA³⁰ as previously described.³¹ The library was plated by using standard procedures³² on 100 mm Petri dishes, and it was screened on a 0.45 mm nitrocellulose membrane with polyclonal anti-Cr-PII antibodies (1.0 μ g/ml) preabsorbed with *Escherichia coli* lysate. After plaque purification of immunopositive clones, candidates were amplified and rescreened on dot blots by using 10 mmol/L isopropyl thiogalactose (IPTG)-saturated 0.45 mm nitrocellulose membrane and probed either with a pool-purified anti-Cr-PII mAb (0.5 μ g/ml of HW-4 and HW-8)²⁴ or human atopic serum pool (1:30). Preimmunized rabbit or BALB/c mouse serum (1.0 μ g/ml) was used as a control. Peroxidase-conjugated goat anti-rabbit IgG (1:1000; Sigma, St. Louis, Mo.), rabbit anti-mouse IgG (1:8000; Bio-Rad, Hercules, Calif.), or ¹²⁵I-labeled anti-human IgE (Pharmacia, Piscataway, N.J.) were used as secondary antibodies. Detection of bound secondary antibody was performed by the 4-chloro-1-naphthol substrate or by autoradiography.²⁶ Purified DNA was digested with enzymes *Sal*I and *Not*I, and the size of the insert was determined by electrophoretic analysis.³¹

Sequencing of cDNAs

C6 and C17 cDNA were isolated and purified from λ gt22A by a polyethylene-glycol precipitation method.³² The inserts were released by *Not*I and *Eco*RI digestion and then subcloned into pSPORT 1 (GIBCO BRL/Life Technologies, Grand Island, N.Y.). A set of nested deletions was created by using the "Erase-a-base" Exo III/S1 nuclease system (Promega, Madison, Wis.). DNAs were prepared from the transformed *E. coli* DH5 α colonies, and dideoxynucleotide sequencing³³ was performed with the T7-Sequencing kit (Pharmacia) according to the manufacturer's directions. ³⁵S-labeled products were separated on 6% polyacrylamide gels and autoradiographed.

Computer analysis

Sequence data were analyzed by using the PC/GENE software (IntelliGenetics, Inc., Mountain View, Calif.), and sequence homology searches were performed at NCBI by using the BLAST network service. The version of BLAST used was BLAST 1.4.9MP (26 March 1996).

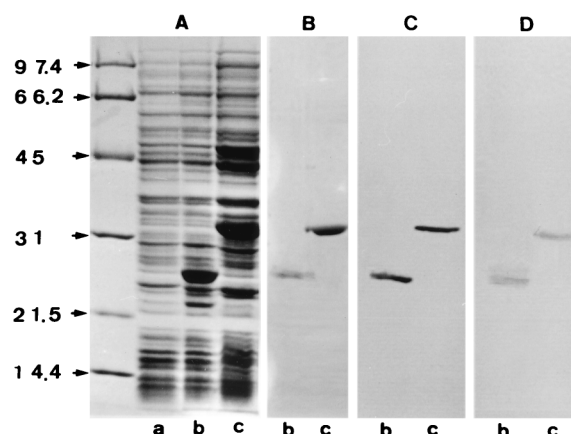


FIG. 1. SDS-PAGE analysis (A and B) and immunoblots (C and D) of recombinant allergens. IPTG-induced *E. coli* BL21(DE3), recombinant lysates (A), and purified recombinant allergens (B) were stained with Coomassie Blue. Purified C6 and C17 allergens were probed with murine anti-Cr-PII mAbs (C) or IgE of human allergic serum pool (D). Lane a, *E. coli* BL21(DE3); lane b, C6; and lane c, C17. Numbers at left indicate sizes of protein markers in kilodaltons.

Target gene expression

Purified DNA of C6 or C17 was ligated into the *Sal*I and *Not*I sites of pET 21b(+) (Novagen, Inc., Madison, Wis.), and recombinant protein was overexpressed in *E. coli* BL21(DE3). Briefly, transformed *E. coli* BL21(DE3) was grown for 1 hour at 37° C in Luria-Bertani medium containing 50 μ g/ml ampicillin. IPTG was added to a final concentration of 0.4 mmol/L and incubated for an additional 16 hours. Cells were harvested and washed with 20 mmol/L Tris-HCl buffer, pH 8.0, containing 200 mmol/L NaCl and 0.1 mmol/L ethylene diamine tetraacetic acid (EDTA) (sonication buffer). The pellets were resuspended in sodium dodecylsulfate (SDS) sample buffer or sonication buffer containing 1.0 mmol/L phenylmethylsulfonyl fluoride (PMSF) for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis or protein purification, respectively.

Detection of recombinant proteins by SDS-PAGE and immunoblotting

Proteins were separated on SDS-PAGE gels under denatured conditions with a discontinuous buffer according to the method of Laemmli.³⁴ After electrophoresis, proteins were transferred onto a 0.45 mm nitrocellulose membrane by the method of Towbin.³⁵ Immunologic detection was performed as described previously.³¹ The blot was either probed with the anti-Cr-PII mAbs (15 μ g/ml) or the human atopic serum pool (1:30). Horseradish peroxidase-labeled anti-mouse IgG at a dilution of 1:30,000 (Sigma) or alkaline phosphatase-conjugated mouse anti-human IgE at a dilution of 1:500 (Pharmingen, San Diego, Calif.) was used as a secondary antibody.

Purification of recombinant protein

Cells of recombinant proteins were resuspended in sonication buffer (20 mmol/L Tris-HCl buffer, pH 8.0, containing 200 mmol/L NaCl, 0.1 mmol/L EDTA, and 1.0 mmol/L PMSF) and sonicated. The lysate was centrifuged at 10,000 g for 30 minutes at 4° C, and the supernatant was mixed with 10 volumes of ice-cold buffer A (50 mmol/L sodium phosphate, pH 7.4; 0.5 mmol/L EDTA; and 1.0 mmol/L PMSF). The resulting mixture

[illegible]

FIG. 2. Nucleotide sequences of C6 and C17. Sequences are aligned with translated region, and gaps (*dashes*) are introduced to optimize alignment. Asterisks represent homology with C17. TGA stop codon and AATAAA polyadenylation signal sequences are underlined. Nucleotide sequences have been deposited in GenBank database with accession numbers U78970 (C6) and U69261 (C17).

was applied to a hydroxyapatite column equilibrated with buffer A. The column was washed with three column volumes of buffer A and then eluted with a 50 to 500 mmol/L sodium-phosphate gradient. Fractions containing C6 or C17, which migrate at 26 or 32 kd on SDS-PAGE gels, were pooled, dialyzed against phosphate-buffered saline (PBS), and concentrated and stored at -70°C . We were able to obtain about 18 mg purified recombinant protein from 1 L of culture by this method.

Cockroach extracts and preparation of CRa-A and Cr-P11

Commercial cockroach skin test reagents and CRA-G were obtained from Allergy Laboratories, Inc. (Oklahoma City, Okla.), Berkeley Biologicals (Berkeley, Calif.), Center Laboratories (Port Washington, N.Y.), Greer Laboratories (Lenoir, N.C.), and Hollister-Stier Laboratories (Spokane, Wash.). CRA-A was prepared by extracting the whole body of insects with Coca's solution in our laboratory.¹⁶ Per a 3 and Cr-PII was partially purified from the CRA-A by gel filtration¹⁶ and preparative isoelectric focusing, respectively.²⁴

Immunodetection of Cr-Pil allergens in cockroach extracts by ELISA with anti-C6 and anti-C17 antibodies

Antibodies to C6 and C17 recombinant proteins were generated from immunized BALB/c mice with purified protein by the method of Mahana and Paraf.³⁶ ELISA was performed as described previously.²⁷ Wells were coated either with 1.0 µg CRa-A, 0.5 µg Per a 3, 0.5 µg Cr-PII, 0.5 µg purified C6 and C17 recombinant proteins, or 100 µl of a 1:20 dilution of commercial skin test extracts in triplicate. The anti-C6 or anti-C17 antibody (500 ng) and peroxidase-labeled anti-mouse immunoglobulin (1:8000, Sigma) were used as primary and

secondary antibodies, respectively. The optical density was determined at 415 nm on an SLT SPECTRA (SLT-Labinstruments, Salzburg, Austria).

Inhibition of binding to anti-C6 and anti-C17 antibodies by cockroach allergens

Microtiter plates were either coated with 0.5 $\mu\text{g}/\text{well}$ of purified C6 or C17 recombinant proteins in triplicate. Different concentrations of CRA-A, CRA-G, Per a 3, and Cr-PII were preincubated with anti-C6 or anti-C17 antibodies (0.5 μg) at room temperature for 2 hours and added to the wells. Peroxidase-labeled anti-mouse immunoglobulin (1:8000, Sigma) was used as a secondary antibody, and the reaction was developed as described above.

Skin test and IgE antibodies to CRa-A

Commercial American cockroach extracts (1:20 wt/vol, Greer Laboratories), Cr-P_{II}, and recombinant proteins at concentrations of 100 µg/ml in PBS containing 50% glycerol were used for skin testing. Allergens were applied to the testheads with the Dip 'N Touch dispenser (Center Laboratories, Port Washington, N.Y.), and an epicutaneous skin test was performed with the sterile disposable Multi-Test applicator of eight testheads (Lincoln Diagnostics, Decatur, Ill.). PBS containing 50% (vol/vol) glycerol and 1.0 mg/ml histamine were included as negative and positive controls, respectively. The reaction was read at 15 minutes, and the largest and smallest diameter of the wheal and erythema were measured. The mean diameters of the wheal and erythema were graded against the controls. A reaction comparable to the negative control was considered as negative. A response with a wheal 2.0 mm larger or an erythema 5.0 mm greater than those produced by the negative control or a wheal 4.0 mm larger or an erythema 10 mm greater than those

A

C17	VGVDGLIDDI IAILPIDDLKALFQEKLETS LDFKAFYDAVRSPEFQSI VQTLNAMPEYQD	60
C6	-----*IS*****	14
C17	LLQKLRDKGVDVDHYIELIRALFGLTREARNLQDDLNDFLALIPTDQILAIAMDYLANDA	120
C6	***N*****A*****	74
C17	EVQELVAYLQSDDFHKIINTIEALPEFANFYNFLKGHGLDVANYINEIHSIIGLPPFVFP	180
C6	*****T**G*****DF*****	134
C17	SRRHARRGVINGLIDDVIAILPVDELKTLFQEKLETSDFKALYDAIRSPEFQSIISTL	240
C6	*****A**D*****	194
C17	NAMPEYQELLQNLNRDKGVDVDHFIELIRSWFGLP	274
C6	*****D*****L****	228

B

5	GLIDDI IAILPIDDLKALFQEKLETS LDFKAFYDAVRSPEFQSI VQTLNAMPEYQDLLQKLRDKGVDVDHYIELIRALFGL	85
193	*****V*****E**T*****P****L****I*****IS*****E***N*****F*****SW***	273

C

1	SIISTLNAMPEYQDLLQNLNRDKGVDVDHYIELIRALFGL	39
189	*****F*****S****	227

FIG. 3. Alignment of deduced amino acid sequences of C6 and C17. Single-letter amino acid code is used, and gaps (dashes) are introduced to optimize alignment. Asterisks represent homology with C17 (A). Internal repeat sequences of C17 and C6 are presented in B and C, respectively. Repeating sequences were aligned visually. Identities are denoted by the asterisks, and similar conserved residues are shown in bold.

produced by the negative control was graded as 1+ or 2+, respectively. A reaction compatible with the positive control, which usually had a wheal of 7 to 10, mm and an erythema greater than 20 mm was graded as 3+. A reaction greater than the positive control that was sometimes associated with pseudopodia and itching was graded as 4+. Sera of cockroach-sensitive atopic subjects were assayed for CRa-A-specific IgE antibodies by fluoro-allergosorbent test, and a class-scoring system based on specific IgE concentration was assigned according to the manufacturer's recommendation as previously described.²⁷

Northern analysis of *P. americana* and *B. germanica* mRNA

P. americana and *B. germanica* mRNA were isolated from total RNA as described.³⁰ Samples containing 5 µg mRNA were electrophoresed on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane. Filters were probed with digoxigenin-labeled C6 and C17 cDNA and hybridized at 42°C overnight in 5× standard sodium citrate containing 1% blocking reagent, 0.1% N-lauroylsarkosine, and 0.1% SDS solution as recommended (Boehringer Mannheim Biochemica, Mannheim, Germany). After hybridization, digoxigenin-labeled probes were detected by using alkaline phosphatase-labeled anti-digoxigenin antibody. Luminescence was performed by a 10-second exposure to Hyperfilm-MP (Amersham International plc, Amersham, U.K.).

Protein determination

Protein concentrations were determined by using the Bio-Rad Bradford assay (Bio-Rad) with either bovine serum albumin or bovine γ-globulin as standard.

RESULTS

Isolation of cDNA clones and immunodetection of recombinant proteins

Six anti-Cr-P11-positive clones were isolated from a λgt22A cDNA library derived from *P. americana* mRNA by screening with allergic human serum and anti-Cr-P11 mAbs. Clones C3, C6, C17, C18, C33, and C42 contain inserts of 1.5, 1.0, 1.1, 2.1, 1.6, and 1.7 kb, respectively, and were unable to recognize Per a 3 mAbs (data not shown). These fragments were isolated and cloned into the *Sal*I and *Not*I sites of pET 21, and proteins were overexpressed in *E. coli* BL21(DE3). Immunoblot analysis of clones C3, C6, C17, C18, C33, and C42 reveal that only recombinant proteins of 38, 26, 32, 62, 47, and 48 kd were recognized by anti-Cr-P11 mAbs and human IgE antibodies, respectively, and no protein from *E. coli* BL21(DE3) was recognized by either probe (data not shown). SDS-PAGE analysis and immunoblots of C6 and C17 are presented in Fig. 1.

Nucleotide and amino acid sequences of Cr-P11

Sequences of C6 and C17 were shown to be 890 and 1024 bases long (Fig. 2). The C6 insert contains a 1 bp 5'-proximal end sequence and a 684 bp open reading frame that starts with a serine codon (AGC) and ends at the nucleotide position 686-688 (TGA stop codon). The 3'-untranslated region, 202 bp long, includes an AATAAA polyadenylation signal and a 43 bp poly(A) tail. The C17 cDNA includes a 1 bp 5'-proximal end sequence followed by an 822 bp open reading frame that

Cr4H	RNLDDDLQDFLKLVPVDQIIAATDYIANDAENVQAAVAYLQSDDEFETIVV	50
C6	RNLQDDLNDFLALIPTDQILAIAMDYLANDAEVQELVAYLQSDDFHKIIT	93
C17	RNLQDDLNDFLALIPTDQILAIAMDYLANDAEVQELVAYLQSDDFHKIIN	140
	..*** *.***.*** **.*.***.***.***.***.***.***.	
Cr4H	TVDALPELQNFNLFLETNGLNAIDFLNGIHDLGIPHPVSGRKYHIRRG	100
C6	TIEGLPEFANFYNFLKEHGLDVADFLNEIHSIIGLPPFVPPSR-HARRG	142
C17	TIEALPEFANFYNFLKGHGLDVANYINEIHSIIGLPPFVPPSR-HARRG	188
	*...***.***.***.***.***.***.***.***.***.***.***.***.	
Cr4H	VGITGLIDDVLAAILPIDDLKALFNEKLETSPDFLALYNAIKSPEFQSIVQ	150
C6	VGINGLIDDVIAAILPVDELKALFDEKLETSPDFKALYDAIRSPEFQSIIS	192
C17	VGINGLIDDVIAAILPVDELKTLFQEKLETSPDFKALYDAIRSPEFQSIIS	238
	..***.***.***.***.***.***.***.***.***.***.***.	
Cr4H	TLNDMPEYQNLLQKLREKGVVDVKIIEILIRALFGLTLNAKAS	192
C6	TLNAMPEYQDLLQNLRDKGVDVDFHFIELIRSLFGL-----P	228
C17	TLNAMPEYQELLQNLRDKGVDVDFHFIELIRSWFGL-----P	274
	..***.***.***.***.***.***.***.***.***.***.***.	

FIG. 4. Comparison of deduced amino acid sequences of C6 and C17 with "repeat 1" sequence of Bla g Bd90K (Cr4H). Single-letter amino acid code is used, and gaps (dashes) were introduced to optimize alignment. Asterisks and periods represent positions in alignment that are perfectly and well conserved, respectively, and only region of homology is represented.

TABLE I. Detection of cockroach allergens by anti-recombinant protein antibody-based ELISA

Cockroach allergen	A _(415 nm)	
	Anti-C17	Anti-C6
CRa-A	0.630	0.734
Per a 3	0.015	0.013
Cr-PH	0.508	0.618
C17	1.425	1.037
C6	1.108	1.319
A (Cockroach mix)	0.103	0.158
B (American cockroach)	0.344	0.438
C (American cockroach)	0.249	0.351
D (American cockroach)	0.334	0.453
E (American cockroach)	0.219	0.264
F (German cockroach)	0.098	0.108
G (German cockroach)	0.109	0.136

begins with a valine codon (GTT) and terminated at the nucleotide position 824-826 (TGA stop codon) followed by a 198 bp 3'-end untranslated region with an AATAAA polyadenylation signal and a 29 bp poly(A) tail. Open reading frames of 228 and 274 amino acids are predicted from C6 and C17 nucleotide sequences, and they exhibit 91.8% identity. No initiator methionine, cysteine, or any potential N-glycosylation site could be found in either gene. The calculated molecular weights of C6 and C17 are 25.79 and 31.14 kd with predicted isoelectric point values of 4.29 and 4.29. Fig. 3, A presents the alignment of the deduced amino acid sequences of C6 and C17. The comparison of deduced amino acid sequences of C6 with C17 reveals 93.86% identities, and both molecules contain repeated sequences (Fig. 3, B and C). Database searches showed

that besides ANG12, protein precursor of *A. gambiae*, and Bla g Bd90K (an allergen of *B. germanica*), no significant sequence homology was found between Cr-PH clones and any other gene reported. Alignment of amino acid sequences of C6 and C17 revealed 31.75% and 28.91% identity with unpublished sequence of ANG12 (data not shown). Nucleotide alignment revealed sequence identity between C6 and Bla g Bd90K and C17 and Bla g Bd90K of 85.1% and 82.7%, respectively (data not shown). Bla g Bd90K contains seven tandem repeats, and the only translatable region, "repeat 1," shares a 66.2% identity with C6 and a 64.1% identity with protein sequence of C17 (Fig. 4).

Detection of Cr-PH by anti-C6 and anti-C17 antibodies

The sequence similarity among C6, C17, and Bla g Bd90K prompted us to detect potential cross-reactivity among them. Therefore polyclonal antibodies were produced against purified C6 and C17 (Fig. 1, B), and their capacity and specificities were examined by ELISA (Table I). The response of Cr-PH to both anti-C6 and anti-C17 antibodies in the ELISA was dose dependent, and no Cr-PH allergen was detected when preimmunized mouse serum was used in ELISA (data not shown). Polyclonal anti-C6 and anti-C17 antibodies were able to detect the CRa-A, Cr-PH, C6, and C17. All five commercial American cockroach extracts tested contain detectable Cr-PH allergens, although the reactivities were different among the quantity of allergens in the extracts. Interestingly, both anti-C6 and anti-C17 antibodies were able to recognize two commercial CRa-G extracts. No detectable allergen was found in Per a 3 allergens by using anti-C6 and anti-C17 antibodies.

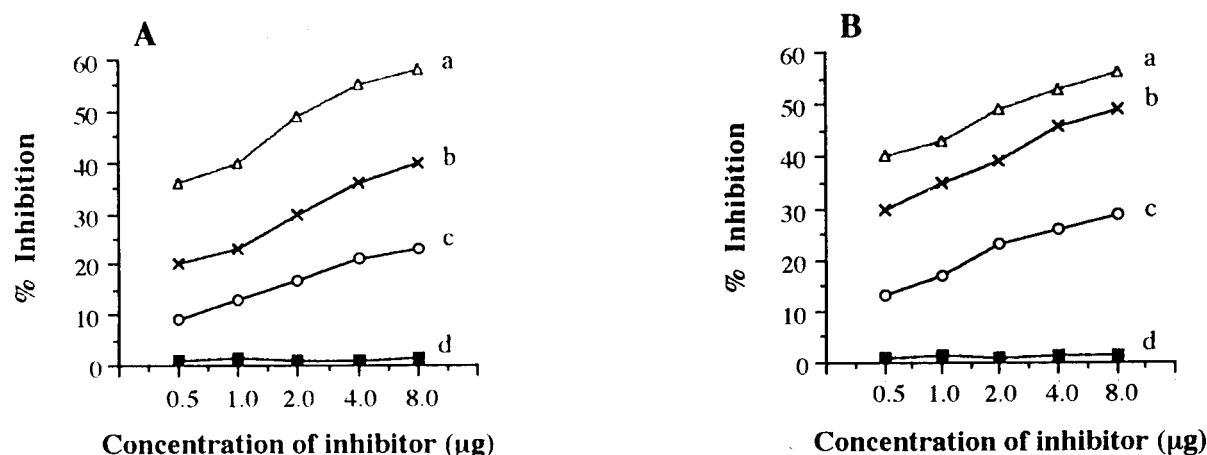


FIG. 5. Inhibition of Binding to anti-C6 and anti-C17 antibodies by Cr-Pil (a), CRa-A (b), CRa-G (c), and Per a 3 (d). Microtiter plates were either coated with purified C6 (A) or C17 (B) recombinant protein, and results are average of experiment performed in triplicate as described.

Inhibition of binding to anti-C6 and anti-C17 antibodies by CRa-G

To look for Cr-Pil cross-reactive German cockroach allergens, we performed inhibition of binding to anti-C6 and anti-C17 antibodies by cockroach allergens (Fig. 5). CRa-G inhibited 23% and 29% of the anti-C6 and anti-C17 binding to C6 and C17 at a concentration of 8.0 µg, respectively. On the other hand, 58% and 56% and 40% and 49% inhibition of binding to anti-C6 and anti-C17 were found at a concentration of 8.0 µg by Cr-Pil and CRa-A, respectively. Per a 3 was used as negative control, and no significant inhibition of Per a 3 was observed.

Allergy skin test and IgE antibodies

Thirty-five allergic patients (16 to 57 years of age) and 15 nonallergic adults were skin tested with the commercial American cockroach extracts and Cr-Pil allergens purified from the CRa-A extract.²⁴ CRa-A- and Cr-Pil-positive patients ($n = 22$) were further tested with the purified recombinant proteins. Table II shows the results of skin tests in which C6 and C17 showed 59% (13 of 22) and 77.3% (17 of 22) positive skin reactions, respectively. CRa-A-specific IgE of allergenic serum was also determined by using the fluoro-allergosorbent test, and all patients were found to carry CRa-A-specific IgE. On the other hand, 1 of 15 (6.7%) members of the nonallergic control group had positive reactions to CRa-A, and none showed any cockroach-specific IgE. Nonallergic control subjects were also tested with C6 and C17, and all had negative skin test responses.

Northern analysis

Northern blot analysis was used to compare expression of Cr-Pil mRNA in *P. americana* and *B. germanica*. The predicted 1.0 and 1.1 kb *P. americana* mRNA transcripts hybridized with digoxigenin-labeled C6 and C17 cDNA probe, respectively. In addition, *P. americana*

mRNAs at 4.3, 3.4, 2.5, 2.0, 1.7, and 1.5 kb (Fig. 6, A and B, lane a) were detected by C6 and C17 cDNA probes. On the other hand, 4.0, 2.2, 1.8, and 1.2 kb *B. germanica* mRNAs were detected by both *P. americana* cDNA probes (Fig. 6, A and B, lane g). Taken together, these results show that *B. germanica* mRNAs are detectable with C6 and C17 cDNA probes.

DISCUSSION

Several cockroach allergens have recently been cloned and sequenced from *B. germanica*, including Bla g 2 (36 kd, an aspartic protease),³⁷ Bla g 4 (21 kd, a calycin),³⁸ Bla g 5 (23 kd, a glutathione transferase),³⁹ and Bla g Bd90K,²⁹ and Per a 3 (79 kd, an insect hemolymph protein)^{30, 31} has been cloned from *P. americana*. Bla g 2, Bla g 4, and Per a 3 are species-specific allergens, and no homologies were found among them and Cr-Pil-cloned allergens presented in this article. Bla g Bd90K (clone Cr4H), a 90 kd *B. germanica* allergen binding 77% cockroach-allergic IgE antibody, carries 4125 bp nucleotides containing seven 576 bp tandem repeats (around 90% homology among them), with a short region at either end, and only 373 of 576 nucleotides were determined on "repeat 5."²⁹ Deduced amino acid sequence was untranslatable from the all seven repeats except "repeat 1" of the Bla g Bd90K cDNA sequence.

In this study we presented the sequences of two new cockroach allergens, C6 and C17. C6 and C17 share 94% identity and elicit 59% and 77.3% skin reactivity on CRa-A-positive patients. These results reconfirm our previous findings²⁴ that Cr-Pil is a group of prominent allergens in *P. americana*. Besides high identity between them, C6 and C17 share a common antigenic determinant or determinants. The anti-C6 and anti-C17 antibodies were able to recognize CRa-A, Cr-Pil, recombinant proteins, and five commercial American cockroach extracts but not Per a 3. Moreover, Northern analyses revealed that both predicted 1.0 and 1.1 kb *P. americana*

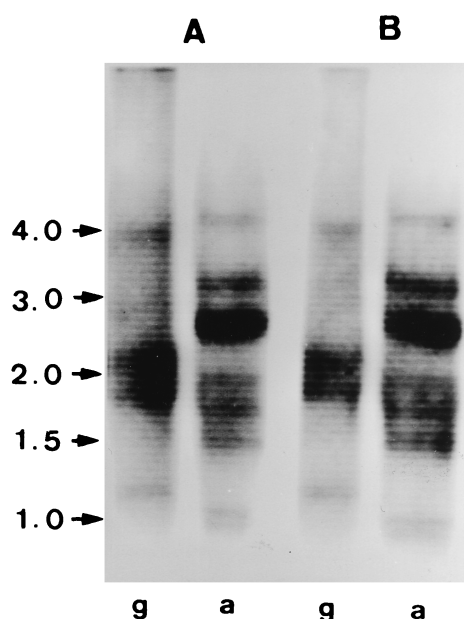


FIG. 6. Northern blot analysis of *P. americana* and *B. germanica* mRNA. Five micrograms of *P. americana* (a) or *B. germanica* (g) was hybridized with digoxigenin-labeled insert from C17 (A) and C6 (B) probes. Numbers at left indicate sizes of standard RNA fragments in kilobase pairs.

mRNAs were detected by C6 and C17 cDNA probes. Sequencing of other Cr-PII clones are currently in progress and will be published elsewhere in due course. C6 and C17 show 31.8% and 28.9% identities to ANG12 of *A. gambiae* ANG12 protein precursor (23.6 kd; GenBank database, accession number Z22925), a probable secretory protein with unknown function, which is induced in the midgut of the female *A. gambiae* after eating (unpublished data). The significance of sequence homology between them has yet to be determined.

P. americana and *B. germanica* are two major domiciliary cockroaches found commonly in households worldwide. It is known that most of the population who are allergic to cockroaches usually have positive skin reactions to the extracts of American and German cockroaches and have specific IgE to both species.^{17, 40} On the basis of physicochemical characteristics, it has been suggested that crude extracts from German, American, Oriental (*B. orientalis*) and Asian (*B. asahinai*) cockroaches contained common IgE binding components.^{17-20, 40, 41} These include Bla g 1 of *B. germanica* and Per a 1 of *P. americana*, which have been purified but not yet cloned from both species and show antigenic cross-reactivity.^{18, 23} Both Bla g 1 and Per a 1 had approximately 33 to 37 kd from Sephadex G-75 gel filtration, but purified allergens revealed a minor band at 25 kd and a major band at 6 kd on SDS-PAGE.¹⁸ Whether it was the result of proteolysis or the active molecules contained two subunits is not clear at this point. Nevertheless, molecular structure and biologic

TABLE II. Skin tests to recombinant allergens and serum IgE antibodies to CRa-A on atopic patients

Patient no.	Diagnosis	Skin test/FAST class			
		CRa-A	Cr-PII	C6	C17
1	AS	3+/I	1+	1+	1+
2	AS	2+/II	1+	—	1+
3	AS	3+/II	3+	1+	3+
4	AS/ AR/AC	3+/I	2+	—	1+
5	AC/ AR/UR	2+/I	2+	1+	1+
6	AC	3+/III	3+	2+	1+
7	AS	2+/I	2+	—	1+
8	AS/ AR/AC	3+/I	1+	1+	—
9	AS/AR	2+/II	4+	1+	2+
10	AS	2+/II	4+	2+	2+
11	AR	3+/III	2+	—	1+
12	AS/ AR/AC	1+/I	1+	1+	—
13	AS	1+/II	1+	1+	—
14	AS/AR	2+/II	1+	—	2+
15	AS/ AR/AC	2+/I	1+	1+	2+
16	AS	4+/III	3+	2+	3+
17	AS/AR	3+/III	3+	2+	2+
18	AS/ AR/AC	1+/I	1+	—	—
19	AS/AR/ AC/UR	1+/I	1+	—	—
20	AR	3+/II	3+	—	1+
21	AS/ AR/AC	2+/I	2+	2+	1+
22	AR	3+/II	2+	—	2+

FAST, Fluoro-allergosorbent test; AS, asthma; AR, allergic rhinitis; AC, allergic conjunctivitis; UR, urticaria.

functions of these allergens in both species are poorly understood, especially in *P. americana*.

Both Bla g Bd90K clones, C6 and C17, contain repeated sequences, and these unique structures do not appear in other cockroach clones reported to date. The importance and function of these repeats are not yet known. Interestingly, C6 and C17 were found to have 66.2% and 64.1% identity to the "repeat 1" peptide of the Bla g Bd90K. In addition, the predicted 4.0 kb mRNA of Bla g Bd90K was also detected in *B. germanica* by both *P. americana* cDNA probes. Moreover, in ELISA anti-C6 and anti-C17 antibodies were able to detect two commercial German cockroach skin test extracts. In agreement with this, the binding of anti-C6 and anti-C17 antibodies to C6 and C17 can be inhibited by CRa-G. We have demonstrated the presence of cross-reactivity between Cr-PII proteins of *P. americana* and crude *B. germanica* extract by ELISA and Northern

blotting. To our knowledge, this is the first evidence of antigenic cross-reactivity between *P. americana* and *B. germanica* on both immunologic and molecular levels, and this observation may have clinical significance.

Dr. Martin D. Chapman and his associates (University of Virginia Health Sciences Center, Charlottesville, Va.) have recently cloned and sequenced one cDNA from *P. americana* and three cDNA clones from *B. germanica* (personal communication, 1997). They have identified these allergens as Per a 1 and Bla g 1 on the basis of their reactivity with anti-Per a 1 and anti-Bla g 1 mAbs. These clones also have repeat sequences and show extensive homology with Bla g Bd90K, C6, and C17 (unpublished observation). They suggest that *P. americana* Cr-PII allergens be designated as Per a 1 and *B. germanica* Bla g Bd90K allergen as Bla g 1. These results further support that C6, C17, and Bla g Bd90K may share a similar antigenic determinant or determinants that will improve our understanding of the antigenic relationships between *B. germanica* and *P. americana*. A new group of *P. americana* allergens cloned and sequenced will be useful in defining the epitopes of allergen interacting with IgE on specific T and B cells. We are currently mapping the epitopes of a Cr-PII allergen that can be recognized by mAbs and IgE from allergic individuals. Moreover, these recombinant allergens will now facilitate the development of improved diagnostic reagents, and structurally modified allergens can be used to develop new forms of immunotherapy.

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