

Isolation and characterization of a clone encoding a major allergen (*Bla g* Bd90K) involved in IgE-mediated cockroach hypersensitivity

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Previous studies have established that atopic individuals living in cockroach-infested housing become sensitized to cockroach aeroallergens and produce IgE antibodies to a variety of proteins. We describe the isolation of a complementary DNA clone from an expression library, constructed with messenger RNA from German (Blattella germanica) cockroaches, which encodes a major allergen involved in mediating cockroach hypersensitivity. Approximately 0.2% of the clones from a lambda ZAP XR cDNA library bound IgE from a patient with cockroach sensitivity. A randomly selected subset of these clones revealed that they were either different isolates of the same gene or members of a closely related gene family. One of the largest clones (a 4 kb insert) from this subset, Bla g Bd90K hybridized to a single mRNA of approximately the same size. DNA sequence analysis showed that this gene consisted of seven 576 bp tandem repeats with a short unique region at either end. No significant sequence homologies were found between the cockroach clone and any other gene reported in the GenBank database. Serum from 17 of 22 (77%) patients with cockroach hypersensitivity identified IgE-binding recombinant protein expressed from clone Bla g Bd90K in Escherichia coli XL-Blue cells as determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis/immunoblot analysis. This recombinant protein migrates with a molecular weight (90 kd) apparently similar to one identified in whole body extracts. We have identified and isolated a cDNA that encodes a major cockroach allergen (Bla g Bd90K) present in German cockroaches. (J Allergy Clin Immunol 1996;98:172-80.)

Key words: Cockroach allergens, cockroach sensitivity, cDNA library, mRNA

The prevalence of asthma in most human populations has been steadily increasing. However, triggers of an asthmatic episode are often unrecognized by the susceptible individual. A number of indoor allergens, which stimulate IgE production and cause IgE-mediated disease, have been identified.¹ Elevated levels of allergen-specific IgE and characteristics of inhalant allergens such as grass pollen, dust mites, animal danders, insects, and

Abbreviation used

SDS-PAGE: Sodium dodecylsulfate-polyacrylamide gel electrophoresis

fungi clearly increase an individual's risk of developing asthma.²⁻⁵

As an indoor aeroallergen component of house dust, the role of the cockroach as an etiologic agent of respiratory allergic disorders is well documented.⁶⁻⁸ Cockroach extracts including cast skins, egg shells, and fecal material⁹⁻¹¹ have been shown to contain several major and minor allergens¹²⁻¹⁴ involved in triggering an asthma attack. Despite the fact that positive skin test reactivity to cockroach is second only to house dust, the identification of the clinically relevant antigens and an

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understanding of the immunobiology of asthmatic reactions is just beginning.¹⁵ Numerous attempts have been made to isolate and characterize the major allergens of cockroaches¹⁶⁻²⁴; however, a lack of detailed information is at least partly due to the difficulty involved in purifying cockroach allergens from extracts in significant quantities for detailed characterization.

The cloning of allergens provides an efficient means of producing pure polypeptides, which in their native source form complex mixtures and are often represented in very small amounts. Because of the prevalence of asthma, coupled with the identification of major cockroach allergens ranging in molecular weight from 6000 to 100,000 d²⁰⁻²⁴ as etiologic agents in this process, we set out to clone a major allergen involved in IgE-mediated asthmatic reactions and to determine whether such a recombinant allergen would be recognized by serum IgE of patients with cockroach hypersensitivity. In this report we describe the isolation of a complementary DNA encoding a major cockroach allergen designated *Bla g* Bd90K. This cDNA encodes a protein with an apparent molecular weight of 90 kd and hybridizes to a 4 kb messenger RNA isolated from German cockroaches. Furthermore, we demonstrate that bacterially expressed *Bla g* Bd90K binds to IgE in 17 of 22 serum samples from cockroach-sensitive individuals, which bind to a similar allergen in whole body extracts.

METHODS

Insect extract

Frozen cockroaches (*Blattella germanica*) were supplied by Agriculture Research Services, Gainesville, Florida. Whole insects (male and female) were snap-frozen in liquid nitrogen, lyophilized, ground to a powder with a mortar and pestle, defatted for 4 hours with ether in a Soxhlet extractor (Baxter Diagnostics, Inc., Scientific Products Div., McGraw Park, Ill.), and dried at room temperature. Defatted cockroaches were extracted (1:10 wt/vol) in Dulbecco's phosphate-buffered saline (2 mmol/L polymethylsulfonate, 20 mmol/L ethylenediaminetetraacetic acid, pH 7.0) overnight at 4° C. The crude whole body extract was then clarified by centrifugation, and 1.0 ml aliquots of the supernatant were stored in 1.5 ml Eppendorf test tubes (Baxter Diagnostics, Inc., Scientific Products Div.) at -30° C.

Patients

Serum samples from 22 patients with documented cockroach sensitivity (clinical history, skin test, and RAST) were used to identify cockroach allergens. One individual with elevated serum IgE levels (but no cock-

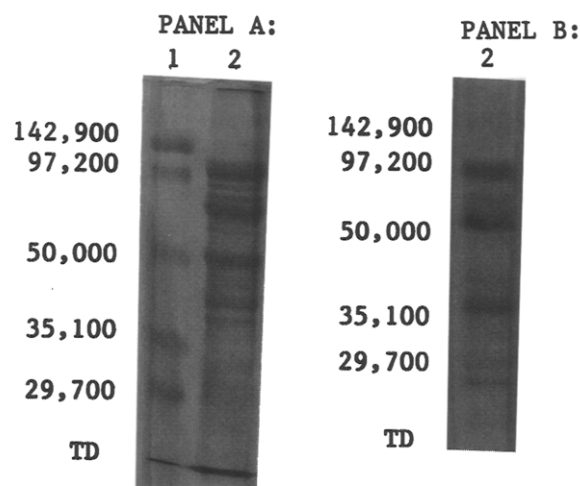


FIG. 1. SDS-PAGE (10%) and Western blot analysis of whole body German cockroach extract. **Panel A**, Coomassie blue-stained gel of whole body German cockroach extract. **Panel B**, Autoradiograph of IgE immunoblot of whole body German cockroach extract with a serum pool from eight cockroach-sensitive individuals, followed by radiolabeled anti-IgE. *Lane 1*, Molecular weight markers; *lane 2*, whole body extract; *TD*, tracking dye front.

roach-specific IgE or cockroach skin test reactivity) was used as a control subject in these studies. A serum pool, derived from sera of eight *Alternaria*-sensitive subjects, and a separate serum pool derived from sera of mite-sensitive subjects (generous gifts from Dr. John Yunginger, Mayo Clinic, Rochester, Minn.) were used as noncockroach, atopic control sera to define specificity. At least 5 ml of venous blood was drawn from each patient and allowed to clot, and the serum was collected.

Cockroach RNA isolation and Northern (RNA) gels

Total cellular RNA was isolated from 4 gm of frozen whole body cockroaches as previously described.²⁵ Poly A⁺ RNA, isolated by using a purification kit supplied by Collaborative Research (Bedford, Mass.) according to the manufacturer's instructions was subjected to electrophoresis in 1.2% formaldehyde agarose gels, transferred to nitrocellulose, and hybridized with phosphorous 32-labeled probes according to the methods of Bannon et al.²⁶

Complementary DNA expression library construction and screening

Cockroach poly A⁺ RNA and oligo dT primer were used to synthesize a lambda ZAP XR cDNA library with an estimated amplified titer of 1.6×10^{10} pfu/ml (Stratagene, La Jolla, Calif.). The library was 95% recombinants carrying inserts greater than 400 bp, as determined by sizing of randomly selected clones. The library was screened according to the methods of Scheiner et al.²⁷ by using IgE antibody from a patient with cockroach sen-

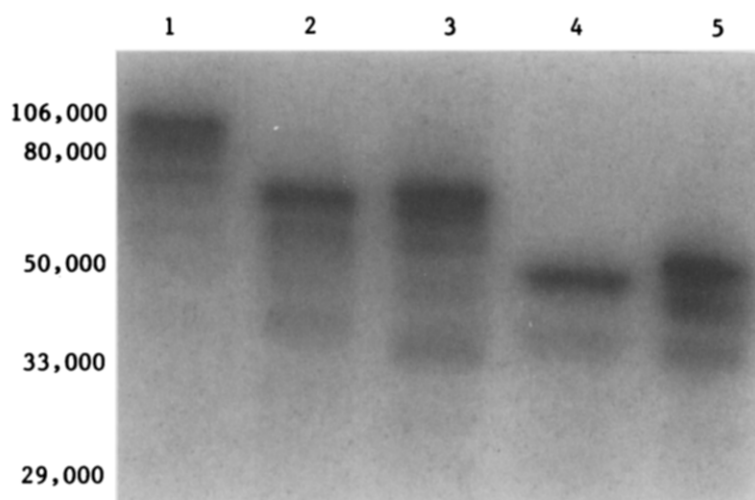


FIG. 2. Selected cockroach cDNA clones encode a variety of different molecular weight IgE-binding proteins. Representative autoradiograph of a 10% SDS-PAGE/immunoblot of five cDNA clones expressing IgE-binding proteins detected by radiolabeled anti-IgE. Lanes 1-5 represent separately isolated cockroach cDNA clones.

sitivity. The primary antibody was detected with either alkaline phosphatase-labeled anti-IgE (KPL, Gaithersburg, Md.) or iodine 125-labeled anti-IgE antibody (Sanofi Diagnostics, Chaska, Minn.) performed according to manufacturers' instructions. Positive cDNA clones were plaque-purified to homogeneity and subsequently rescreened with serum from a patient with elevated IgE but no cockroach sensitivity to control for nonspecific IgE binding.

IgE immunoblot analysis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli.²⁸ All gels were composed of a 10% acrylamide resolving gel and 4% acrylamide stacking gel. Electrophoretic transfer and immunoblotting on nitrocellulose paper were performed according to the procedures of Towbin et al.²⁹ The blots were incubated with antibodies diluted in Tris-buffered saline (100 mmol/L Tris/1 mol/L NaCl) and 1% bovine serum albumin for at least 12 hours at 4° C for 4 hours at room temperature. The primary antibody was detected with ¹²⁵I-labeled anti-IgE antibody as outlined previously.

Plasmid isolation

Plasmids were isolated from each of the IgE-selected cockroach clones by *in vivo* excision of pBluescript sk from the UniZAP XR vector (Stratagene). XL1-Blue cells were combined with a portion of each of the cockroach clone stocks and R408 helper phage. This mixture was incubated at 37° C for 15 minutes and then added to 5 ml of yeast-tryptone media and incubated for 3 hours at 37° C. This culture was heated to 70° C for 20 minutes, and the cells were pelleted by centrifugation at 4000 g for 5 minutes. The supernatant containing the packaged phagemid was then used to infect host XL1-

Blue cells. XL1-Blue cells containing the phagemid were selected on Luria-Bertani. Plasmids were isolated from these colonies by standard methods.³⁰

DNA sequencing and analysis

Sequencing was done according to the methods of Sanger et al.³¹ by using a series of clones constructed by *Exo III* digestion of the original DNA isolate. With the exception of repeat 5 of clone 4H, both strands of DNA were sequenced in most cases. Because of the highly repeated nature of this portion of the gene, we were only able to determine 373 of 576 nucleotides contained in this repeat. Sequence analysis was done on the University of Arkansas for Medical Science's Vax computer with the Wisconsin DNA analysis software package (Genetics Computer Group, Madison, Wis.).

RESULTS

Characterization of whole body German cockroach extract

SDS-PAGE/Western blot analysis was performed with IgE from patients who were sensitive to cockroach under conditions listed in Methods to inhibit proteolytic degradation. Fig. 1 shows that the whole body extract has Coomassie blue-stained proteins ranging in molecular weight from 30,000 to more than 100,000 d. Major staining bands can be identified at approximately 36,000, 50,000, 80,000, 90,000, and 97,000 d (Fig. 1, *Panel A*, lane 2). Fig. 1, *Panel B*, shows the IgE immunoblot analysis of the crude extract with major IgE-binding proteins at approximately 21,000, 36,000, 50,000, 80,000, and 90,000 d.

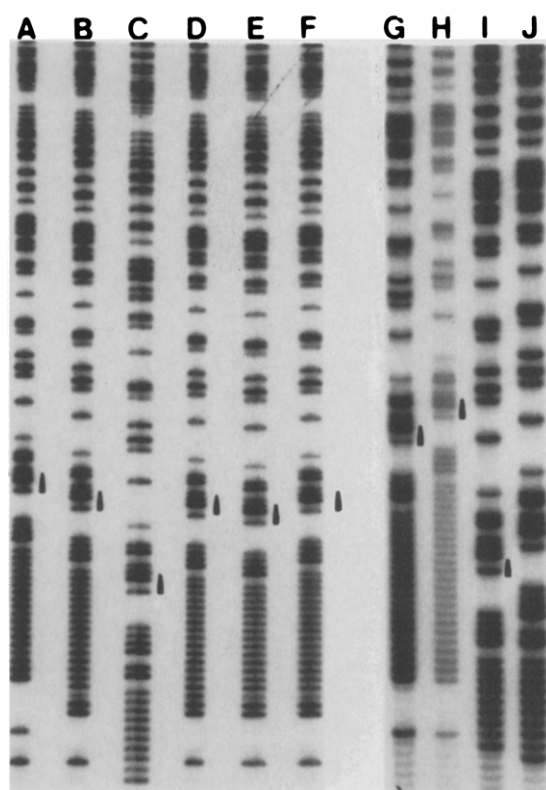


FIG. 3. The IgE-selected cockroach clones have highly similar nucleotide sequences. Dideoxythymidine DNA sequencing reactions were performed on the 3' end of each clone with the M13 forward primer. Each lane (A-J) represents a different IgE-selected cDNA clone. Arrowheads are reference points after the poly A tail, which indicate where each of the clones have identical patterns.

Isolation of clones that produce antigens recognized by serum IgE from a patient with cockroach sensitivity

RNA isolated from the German cockroach, *B. germanica*, was used to construct an expression library for antibody screening. Use of serum from a single cockroach-sensitive patient as a probe for screening 10^6 plaques resulted in the identification of 2000 recombinant cDNAs. Because the number of positive clones was so large in the primary screening, 10 primary positive plaques were randomly selected for further purification. These clones were plaque-purified to homogeneity and subsequently tested for their ability to react with serum IgE collected from a patient who was not cockroach-sensitive. All of the plaque-positive clones were intensely positive when incubated with serum IgE from a cockroach-sensitive patient and nonreactive with serum IgE from a control subject (data not shown).

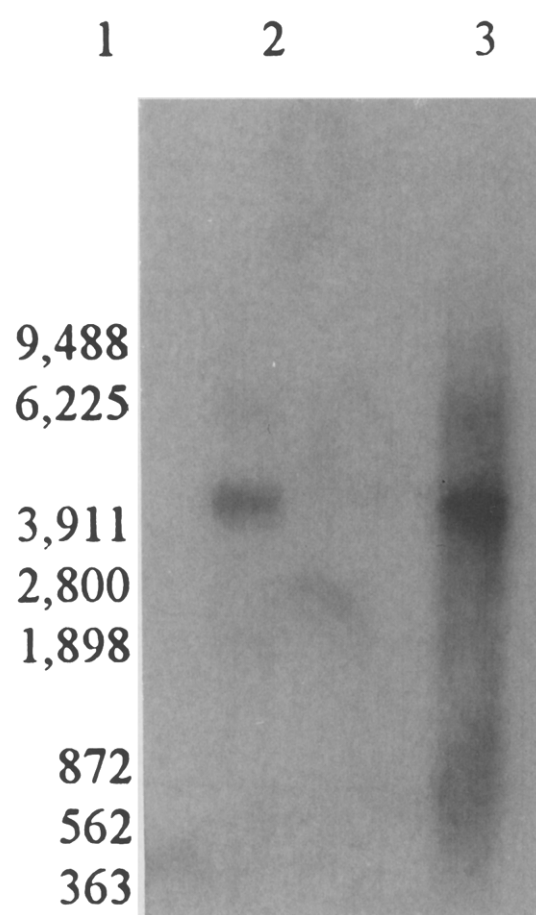


FIG. 4. Northern blot analysis of Bla g 90 kd. Cockroach poly A+ RNA was isolated from *B. germanica* species, and 3 μ g was electrophoresed on denaturing formaldehyde agarose gels. A purified, α - ^{32} P-deoxycytidine triphosphate-labeled 4 kb insert from clone Bla g 90 kd was used as hybridization probe of a Northern blot of this gel. Lane 1, RNA ladder expressed in kilobases; lane 2, total RNA; lane 3, poly A+ RNA.

Characterization of clones that encode cockroach allergens

IgE immunoblot analysis was performed with IgE from patients who were cockroach-sensitive as probes to detect immunoreactivity of recombinant cockroach proteins expressed from a variety of selected cDNA clones and to determine the approximate molecular weight of the allergen produced by each clone. Fig. 2 shows that the clones produce allergens varying in size from 90 to 50 kd. As a control, a duplicate of this blot was incubated with serum from an individual with elevated IgE but no cockroach hypersensitivity. The control blot had no detectable IgE binding (data not shown). These results show that we

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1  CAGAGATGAA GTTGGCACTC ATCTTTCTT CCTTCTGGG CTTGACATGT
51  GGCAAGTCCA TACCATCAAC ACGCAACCTA GATGATGACC TCCAAGACTT
101 CTTGAAACTT GTTCCAGTAG ATCAAATTAT TGCCATTGCC ACAGACTACA
151 TTGCTAACGA CGCTGAAGTA CAGGCTGCCG TTGCATACCT CCAATCAGAT
201 GAATTTGAGA CTATTGTTGT TACCGTTGAT GCACTCCCAG AATTGCAGAA
251 TTTCTCAAT TTCTGGAGA CCAATGGAAT CAATGCAATT GACTTCCTCA
301 ATGGAATCCA TGATCTTCTT GGAATTCCTC ACATTCCAGT CTCAGGACGC
351 AAATATCACA TCCGCAGAGG AGTTGGAATC ACTGGTCTCA TTGATGATGT
401 CCTCGCCATC CTTCCAATTG ATGACCTGAA GGCCCTCTTT AATGAGAAAT
451 TGGAAGTAG CCCAGATTTT CTTGCTCTGT ACAATGCCAT CAAATCCCCT
501 GAATTCCAGA GCATTGTGCA AACATTGAAT GACATGCCAG AATATCAGAA
551 TCTTCTGCAA AAACGCGTG AAAAGGGAGT TGATGTGGAC AAAATCATTG
601 AACTCATCAG AGCTCTTTT GGATTGACCC TAAATGCCAA AGCCTCC 7
3971 TCACTAGTGA GAACTCAAGA CTGATACGTT TAAGTGCCAA ACTTCATTTC
4011 TGATAATAAT CAGAATGTTA ACATTGTTCA TTTTGAAC ACTTGATTA
4071 TATTTTGATT GTTCCACGAA TAAATACTTC AAAATAAAAA AAAAAAAAAA
4121 AAAAA

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FIG. 5. Nucleotide sequence of a cockroach allergen. The nucleotide sequence of *Bla g* 90 kd is shown. **Boxed and bold nucleotides** represent one tandem repeat present in this gene. A total of seven, highly homologous (~90%) tandem repeats are contained within this cDNA clone. Numbers on the left indicate the position of the nucleotide sequence relative to the 5' end (nucleotide position 1) and 3' end (nucleotide position 4125) of the gene. The entire sequence has been deposited in the GenBank database, accession no. L47595.

have isolated numerous clones that encode IgE-recognizable antigens specific to patients with cockroach hypersensitivity.

To determine whether the isolated clones represented different allergens or were truncated versions of the same mRNA, dideoxythymidine DNA sequencing reactions were performed on the 3' end of each of the clones with the M13 forward primer. If the clones had similar nucleotide sequences, then the pattern of T residues would be the same when clones were compared with each other. Fig. 3 shows the results from these experiments, revealing that with the exception of variation in poly A tail length, all of the selected clones that were tested had identical or very similar T patterns in their 3' ends. This suggests that the allergens we identified from these isolated clones were encoded by a highly abundant mRNA from the amplified library or by a family of closely related mRNAs.

We selected one of these clones with the largest insert (*Bla g* Bd90K, ~4.0 kb; data not shown) for further characterization. To determine whether this clone represented the entire cockroach aller-

gen, an α -³²P-deoxycytidine triphosphate-labeled insert was used as a hybridization probe of a Northern blot containing cockroach poly A+ RNA (Fig. 4). This insert hybridized to a single-size mRNA of approximately 4 kb. Because the size of the insert was similar to the size of the mRNA to which it hybridized, it is likely that this clone represents the entire protein coding portion of the mRNA, although we do not have conclusive data at this time.

DNA sequence analysis

The primary DNA sequence of this clone was determined by Sanger dideoxy sequencing by using a series of subclones constructed by *Exo III* digestion of the insert. *Bla g* Bd90K carries a 4125 base insert that contains seven 576 bp long tandem repeats. Homology between the tandem repeats was ~90% (Fig. 5). A search of the GenBank database revealed no significant homology between *Bla g* Bd90K and any other sequence in this database, indicating that this clone encodes a previously unreported allergen.

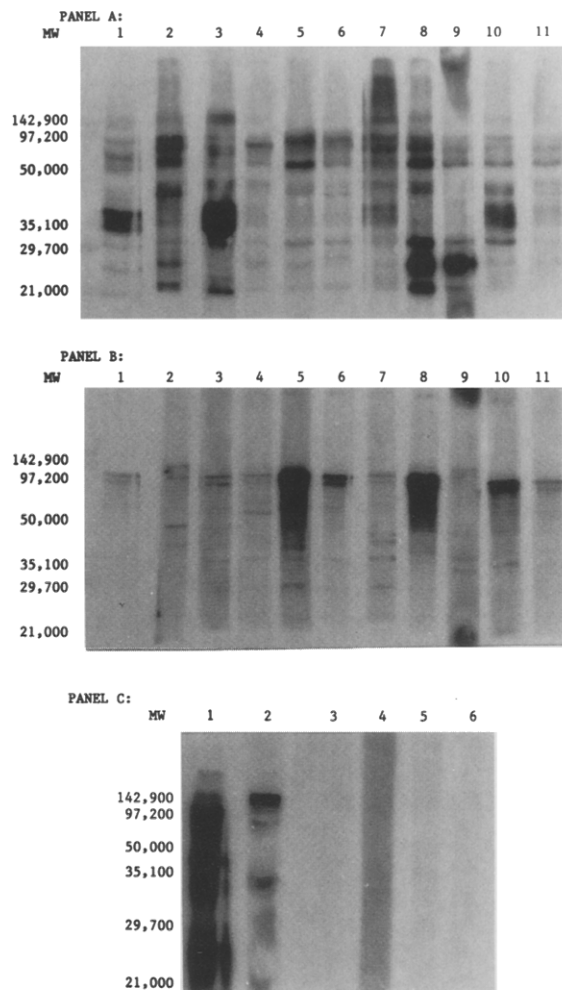


FIG. 6. Autoradiographic photographs of 10% SDS-PAGE/immunoblot analysis of German cockroach proteins incubated with serum from cockroach-sensitive, atopic, and nonatopic individuals, followed by radiolabeled anti-IgE. **Panel A**, Representative IgE immunoblot of a crude whole body German cockroach extract. **Panel B**, Representative IgE immunoblot of Bla g 90 kd expressed protein. **Panels A and B:** Lane 1, Pooled serum from eight cockroach-sensitive individuals; lanes 2-11, individual serum samples from known cockroach-sensitive individuals. Exposure time = 24 hours. **Panel C**, Representative IgE immunoblot of Bla g 90 kd expressed protein with control serum. Lane 1, Whole body extract incubated with pooled cockroach-sensitive serum; lanes 2-6, Bla g 90 kd expressed in *E. coli* cells; lane 2, serum from a known cockroach-sensitive individual; lane 3, nonatopic serum; lane 4, pooled serum from eight mite-sensitive individuals; lane 5, pooled serum from *Alternaria*-sensitive individuals; lane 6, atopic individual with high IgE titer, not cockroach-sensitive. Exposure time = 5 days.

Recognition of a recombinant cockroach allergen by individual patient sera in an IgE immunoblot assay

IgE immunoblot analysis was performed with serum IgE from patients with cockroach hypersensitivity and a recombinant cockroach protein expressed from Bla g 90 kd clone in *Escherichia coli* XL-Blue cells to address the question of how frequently this allergen was recognized by IgE from this pool of individuals. Fig. 6 shows 11 (lanes 2 to 11, Panel B; lane 2, Panel C) representative

immunoblot strips, which had been incubated with sera collected from individual patients. All of the patients showed strong IgE binding to the recombinant cockroach allergen. Of the 22 patient sera tested in this manner 17 (77%) had IgE that recognized this recombinant cockroach allergen (Table I). Failure of individual serum IgE to recognize this recombinant protein could be due to the requirement for a conformational epitope that is not conserved in the recombinant protein or the absence of a linear epitope in this clone. A serum

TABLE I. Summary of patient IgE binding to cockroach clone Bla g 90 kd protein product

Serum sample	Diagnosis	Laboratory test result	IgE blot	
			Whole extract	Recombinant protein
Patient sera				
1. KH	Asthma	+SPT	+	+
2. PA	Asthma	+SPT	+	+
3. SK	Asthma	+SPT	+	+
4. DM	Rhinitis	+SPT	+	+
5. EMa	Asthma	+SPT	+	+
6. KJ	AD	+RAST	+	+
7. JHor	Asthma/AD	+SPT	+	+
8. JP	Asthma/AD/AR	+SPT	+	+
9. GY	Asthma/AD	+SPT	+	+
10. Eme	Asthma	+SPT	+	+
11. MT	Asthma	+SPT	+	+
12. QM	Asthma	+SPT	+	+
13. VP	Asthma	+SPT	+	+
14. SS*	Asthma	+SPT/RAST	+	+
15. LS*	Asthma	+SPT/RAST	+	+
16. RL*	Asthma	+SPT	+	+
17. JD*	Asthma	Not done	+	+
18. CB	AD	+RAST	+	—
19. PT	Asthma	+RAST	+	—
20. BJ	AR	+SPT	+	—
21. MT	AR	+SPT	+	—
22. JHer	Asthma	+RAST	+	—
Control sera				
1. CC	Nonatopic		—	—
2. VS	Atopic, high IgE		—	—
3. Mite†	Atopic pool		—	—
4. Alt‡	Atopic pool		—	—

SPT, Skin prick test; AD, atopic dermatitis; AR, allergic rhinitis.

*Serum provided by Drs. Manuel Lopez, Samuel Lehrer, and Elliott Horner (Tulane Medical School, New Orleans, La.).

†Serum pool from mite-sensitive individuals provided by Dr. John Yunginger (Mayo Clinic, Rochester, Minn.).

‡Eight-person serum pool from *Alternaria*-sensitive individuals provided by Dr. John Yunginger (Mayo Clinic).

pool derived from sera of eight *Alternaria*-sensitive subjects and a separate serum pool derived from sera of mite-sensitive subjects were used as non-cockroach, atopic control sera to define specificity (Fig. 6, Panel C, lanes 3-6, respectively). These results demonstrate that no IgE bound to the recombinant cockroach protein, indicating that the clone we have isolated is specific to cockroach-sensitive individuals.

DISCUSSION

Cockroach allergens have been established as important sensitizing agents in the induction or exacerbation of respiratory diseases.³² A number of studies have been undertaken to investigate the nature of cockroach allergens and the process by which they elicit an asthmatic reaction.^{5, 8, 18} Two significant allergens, Bla g 1 and Bla g 2, have been

prepared by using monoclonal antibodies and protein purification techniques, and immunoassays have been widely used to detect these allergens in the environment.^{5, 22} However, the molecular nature of cockroach allergens has not been clearly defined, primarily because of the difficulty in obtaining purified allergens, although several abstracts have reported preliminary characterization.³³⁻³⁵

In this study a cDNA expression library was constructed by using mRNA isolated from German cockroaches. This RNA source had previously been shown by in vitro translation studies to encode many of the allergens recognized by serum IgE from patients with cockroach hypersensitivity.²⁵ After screening this library with serum IgE from a patient with known cockroach sensitivity, it was noted that a significant number of clones,

approximately 0.2% of the total, were positive for antibody binding. In contrast to *Bla g 1* and *Bla g 2*, they appeared to be allergens with apparent molecular weights greater than 40 kd. Ten cDNA clones were randomly selected to determine whether the high molecular weight IgE-binding proteins were encoded by these clones and to characterize the expressed protein products with naturally occurring allergens identified in whole body extracts. Southern blot analysis (data not shown) and DNA sequencing reactions strongly suggest that these clones are either different isolates of the same gene or members of a closely related gene family.

By definition, a major allergen is an antigen against which more than 50% of a group of patients allergic to the allergen source in question react.^{36, 37} The cockroach gene we have isolated is capable of producing a protein product in prokaryotic cells, which is recognized by serum IgE from 77% (17 of 22) of the patients tested, and therefore can be classified as a major allergen. This is within the range (30% to 70%) of other cockroach allergens identified to date.¹⁵ In addition, these results are significant in that they indicate that some of the allergenic epitopes responsible for this reaction are linear amino acid sequences that do not include a carbohydrate component because the bacteria are unable to posttranslationally add carbohydrates to the protein. Clearly, we have identified a recombinant allergen that can be produced in bacteria with immunologic reactivity to the natural protein extract (Table I). This is significant in that other indoor allergens, including mite Group I and II allergens produced in bacteria or yeast, show variable immunoreactivity compared with native allergens.³⁸

Allergen immunotherapy has been shown to be an effective treatment for patients with insect sting hypersensitivity when they have experienced significant systemic symptoms.³⁹ Because allergen immunotherapy can down-regulate the specific IgE response and the cellular response to allergens, treatment of patients with cockroach immunotherapy can now be studied as a possible option. The use of recombinant allergens that retain IgE-recognizable epitopes has been envisioned to provide a basis for improving treatment of patients with immediate hypersensitivity. Immunotherapy with specific recombinant allergen epitopes, rather than the crude allergen mixture, could prove to be a more effective treatment. The use of recombinant allergens in standard allergen immunotherapy would have several advantages over natural antigens, including better control of the batch-to-batch

variability of the specific allergens and the assurance of the representation of minor allergens in standard amounts.

Our results suggest that clone *Bla g Bd90K* represents a cockroach mRNA that can be used to produce significant quantities of this protein. We propose to use this protein to design experiments for the advancement of immunochemical and molecular strategies required for the identification and sequencing of cockroach T- and B-cell epitopes of this allergen. Our finding that the recombinant cockroach allergen we have identified is recognized by serum IgE of a large proportion of patients with cockroach hypersensitivity will allow further application of this technology to improve our understanding of the basic mechanisms and management of allergic disease.

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