

Identification of important allergens in German cockroach extracts by sodium dodecylsulfate–polyacrylamide gel electrophoresis and Western blot analysis

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Background: Despite recent advances in the purification and characterization of cockroach allergens, identification of clinically important allergens and their source have not been completely elucidated. This study investigated the allergen content of German cockroach (*Blattella germanica*) whole body (GWBE) and fecal (GFE) extracts.

Methods: Sera from 37 subjects with asthma, with positive skin test results to cockroach, were used for RAST and Western blot (after sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS-PAGE] under reducing conditions); this serum panel is the largest used to date for cockroach allergen analysis.

Results: RAST reactivity to GWBE and GFE were highly correlated ($r = 0.882$, $p < 0.001$). SDS-PAGE and Western blotting showed that GWBE and GFE had similar patterns of IgE binding. Furthermore, Western blot inhibition investigations revealed that either GWBE or GFE could almost completely inhibit the reactivity of the other extract. SDS-PAGE and Western blotting demonstrated in both extracts numerous bands that displayed a high prevalence of IgE binding. Protein bands at 67, 50, 45, and 36 kd bound more than 50%, and the band at 60 kd bound approximately 80% of the sera tested.

Conclusions: In summary, this investigation identified German cockroach allergens, established their relative importance by prevalence of reactivity to a large serum panel, and demonstrated that cockroach feces possess significant allergenic activity. Five allergens identified demonstrated reactivity with up to 50% to 80% of the 37 subjects' sera tested. (*J ALLERGY CLIN IMMUNOL* 1995;95:877-85.)

Key words: German cockroach allergy, whole body allergens, fecal allergens

Cockroaches have been demonstrated to be an etiologic factor in allergic diseases, especially in large urban areas of the United States and throughout the world.¹⁻¹³ Significant quantities of cockroach allergens are found in house dust,¹⁴⁻¹⁸ and a high prevalence of positive skin test results (up to 70%) and IgE antibodies to cockroach exist

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Abbreviations used

CNBr:	Cyanogen bromide
GFE:	German cockroach fecal extract
GWBE:	German cockroach whole body extract
IEF:	Isoelectric focusing
MW:	Molecular weight
PBS:	Phosphate-buffered saline solution
SDS-PAGE:	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

among persons with atopic disease, especially those in lower socioeconomic classes.¹⁹⁻²⁴ Furthermore, sensitivity to cockroach places persons with asthma at significant risk for exacerbations requiring emergency medical care.²⁵

Several investigators have attempted to identify and isolate major cockroach allergens with different physicochemical and immunochemical techniques.^{19, 26-30} Recently, cockroach-reactive monoclonal antibodies³¹ have been used to isolate two allergens, *Bla g* I and *Bla g* II, from extracts of the German cockroach. The source of airborne cockroach allergens, however, has not been conclusively defined. On the basis of skin test studies, Bernton and Brown³² first suggested that cockroach feces may be the major source of relevant cockroach allergens. Skin testing, RAST, RAST inhibition, isoelectric focusing (IEF) with immunoprint, and immunoprint inhibition studies established that cockroach feces possess significant allergenic activity and therefore may be the source of clinically relevant allergens.^{11, 18, 33-35} In contrast, only one study³⁶ reported that cockroach feces did not have significant allergenic activity.

This investigation was undertaken to identify the important allergens in German cockroach (*Blattella germanica*) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting techniques under reducing conditions with sera from 37 cockroach-sensitive persons with asthma. This represents by far the largest number of sera used to date in immunoblot investigations of cockroach allergens. Additionally, the reactivity of fecal and whole body allergens was compared by Western blot inhibition studies. The German cockroach was chosen because it is the most prevalent indoor cockroach species in the United States, including the metropolitan New Orleans area (D. Nichol, United States Department of Agriculture, personal communication).

METHODS

Sera

Sera from 37 patients with atopy (two or more positive skin test results from a panel of 15 to 18 common aeroallergens, which included house dust mite, grass, tree, and weed pollens) and asthma, all from the metropolitan New Orleans area, were selected for study. All 37 subjects had positive reactions to German cockroach whole body extract (GWBE) (Center Laboratories, Port Washington, N.Y.) by skin test (wheal ≥ 3 mm) and demonstrated a positive RAST to GWBE as defined in the subsequent section. Equal volumes of each of the sera were pooled and used for the immunoprint inhibitions. Sera from five subjects with atopy and asthma with a negative skin test reaction to cockroach served as controls.

Cockroach allergen extracts

Preparation of the cockroach extracts has been previously described in detail.^{33, 34} Briefly, GWBE was pre-

pared by homogenizing the cockroaches in physiologic saline solution with three 1-minute pulses of a blender. The homogenate was mixed overnight at 4° C and then centrifuged (105,000g). The final supernatant was concentrated (Amicon YM 2; molecular cutoff, <1 kd; Danvers, Mass.) and recentrifuged (105,000g). The supernatant was aliquoted and stored at -20° C. German cockroach fecal extract (GFE) was similarly prepared, except for a final concentrating step of dialysis against Ficoll (Pharmacia, Piscataway, N.J.) to obtain a protein concentration approximately equal to the GWBE. Protein concentrations were determined by the Pierce protein assay (Rockford, Ill.).

RAST

Cellulose disks, activated with cyanogen bromide (CNBr), according to the method of Ceska and Lundkvist,³⁷ were coated with GWBE or GFE, 1 μ g protein per disk. Antigen-coated disks were incubated overnight with 100 μ l undiluted serum. After washing with physiologic saline solution, 100 μ l iodine 125-labeled anti-IgE antiserum (Sanofi, Chaska, Minn.), diluted with saline solution to 15,000 counts per minute, was added. After overnight incubation the disks were washed with saline solution and bound ¹²⁵I was determined in a gamma counter (Gamma 5500, Beckman Instruments, Irvine, Calif.). All RASTs were performed in duplicate, and mean results are expressed as the percentage of total added radioactivity bound to the disk. RAST results were considered positive if the percentage bound was more than 2 standard deviations greater than the mean of the controls. RAST reactivity to GWBE and GFE was compared by linear regression of the logarithmically transformed values for each subject.

SDS-PAGE and Western blotting

SDS-PAGE was performed with 15% T, 2.7% C separation gels and 4% T, 2.7% C stacking gels in a SE 600 electrophoresis chamber (Hoefer Scientific Instruments, San Francisco, Calif.) according to Laemmli's method.³⁸ Samples were boiled for 5 minutes in treatment buffer containing 125 mmol/L tris(hydroxymethyl)aminomethane HCl (pH 6.8), 1% SDS, 5% glycerol, and 2% 2-mercaptoethanol. Twenty micrograms protein of each extract per 5 mm of gel were electrophoresed at 60 mA (constant current) until the bromophenol tracking dye was approximately 1 cm from the bottom of the gel.

Portions of the gels were fixed in a solution of 3.5% sulfosalicylic acid and 11.5% trichloroacetic acid and then stained with 0.1% Coomassie Brilliant Blue R250. Proteins in the unstained portions of the gels were then transferred electrophoretically for 1 hour at 200 mA to CNBr-activated nitrocellulose³⁹ in 100 mmol/L tris(hydroxymethyl)aminomethane-glycine transfer buffer (pH 10.4) containing 20% methanol. Unreacted sites on the membrane were quenched with 100 mmol/L phosphate-

blocking buffer containing 0.05% polysorbate 20 and 1% bovine serum albumin. Blotted and quenched membranes were washed in phosphate-buffered saline solution (PBS) with 0.05% polysorbate 20 and cut into 5 mm wide strips. One strip from each transfer was incubated overnight in india ink (Pelikan AG, Hannover, Germany) at 1:1000 dilution with PBS-0.05% polysorbate 20 to stain proteins and to assess the adequacy of transfer.⁴⁰

Remaining strips of nitrocellulose were incubated overnight with 1 ml of each of the 37 sera diluted 1:4 with 100 mmol/L phosphate-blocking buffer containing 0.05% polysorbate 20 and 1% bovine serum albumin and then washed with PBS-0.05% polysorbate 20. They were then incubated overnight with 50,000 counts per minute of radiolabeled anti-IgE. After incubation the strips were washed again and allowed to dry. Autoradiography was performed on x-ray films for 12 hours to 3 days with intensifier screens (Dupont Cronex Lightning-plus DL) at -70° C. Different exposure lengths were used to best identify different bands on the blots. The number and molecular weight (MW) of IgE-binding bands was determined by visual inspection of two independent observers. The observers had generally excellent agreement; a third independent observer was used to resolve differences.

Western blot inhibitions

GWBE and GFE (20 µg protein of each extract per 5 mm of gel) were fractionated by SDS-PAGE and transferred onto CNBr-activated nitrocellulose membranes, which were then cut into 5 mm strips as outlined in the preceding section. A sera pool was created by mixing equal volumes of sera from the 37 subjects with positive RAST reactions. Decreasing concentrations of either GWBE or GFE (20, 2, 0.2, 0.02 µg) and the subjects' sera pool (1 ml, 1:10 final dilution) were incubated with each blot. The strips were washed, radiolabeled anti-IgE was added, and strips were incubated overnight, as outlined in the preceding section. A 1:10 final dilution, instead of 1:4 used in the previous Western blotting, was chosen to increase the sensitivity of the inhibition by diluting the amount of IgE present in the sera pool. These strips were washed again, and autoradiography was performed for 10 days to 2 weeks. Degree of inhibition was determined by visual inspection of two independent observers.

RESULTS

RASTs

Results of GWBE and GFE RASTs are presented in Table I. Percentage of total radioactivity bound to each disk for the 37 subjects ranged from 2.2% to 32.2% for GWBE and 2.2% to 38.3% for GFE. All control sera bound less than 0.1%; the mean percentage bound $\pm$ 2 SD of the five con-

TABLE I. RAST, expressed as percentage bound of total IgE added,* of GWBE and GFE with 37 study subjects and five control subjects

Subject No.	RAST (% bound)	
	GWBE	GFE
1	13.3	12.9
2	10.7	18.9
3	27.2	12.7
4	27.1	29.2
5	3.8	2.2
6	3.2	3.9
7	9.0	9.5
8	29.4	24.1
9	24.3	25.2
10	30.3	20.6
11	20.0	12.5
12	11.2	17.0
13	19.6	9.8
14	9.8	14.8
15	30.8	30.9
16	12.1	14.7
17	6.5	8.7
18	32.2	31.1
19	3.0	3.1
20	2.2	4.4
21	10.0	11.4
22	31.8	38.3
23	3.7	4.5
24	2.5	3.4
25	15.3	17.6
26	21.3	23.5
27	2.9	3.4
28	5.1	4.1
29	23.4	28.7
30	10.1	19.7
31	6.9	2.4
32	7.7	8.5
33	11.6	19.0
34	20.7	17.2
35	10.9	13.5
36	10.1	8.5
37	9.6	14.5

*Positive RAST was defined as percentage bound greater than the mean percentage bound of five controls $\pm$ 2 SD (GWBE, 0.12% $\pm$ 0.05%; GFE, 0.11% $\pm$ 0.06%).

trols for GWBE was 0.12% $\pm$ 0.05% and was 0.11% $\pm$ 0.06% for GFE. RAST values for the two extracts were logarithmically transformed and compared by least-squares linear regression analysis (Fig. 1). The regression between GFE and GWBE reactivity was highly and significantly correlated ($r = 0.882$, $p < 0.001$).

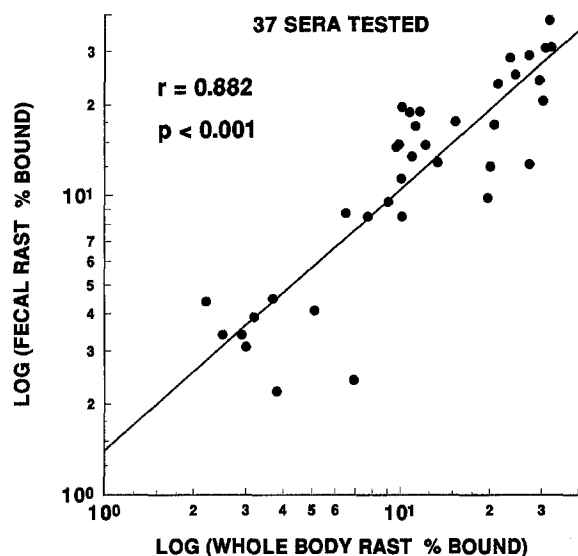


FIG. 1. Linear regression of logarithmically transformed GWBE and GFE RAST results. 37 sera from cockroach-sensitive persons were tested.

SDS-PAGE and Western blotting

SDS-PAGE of GWBE and GFE yielded well-defined banding of proteins by Coomassie staining, suggesting minimal degradation of protein in the extracts. Staining demonstrated at least 25 protein bands in the GWBE with a MW range of 16 to 85 kd and 17 proteins in GFE with a MW range of 13 to 85 kd (Fig. 2). A good transfer of proteins from the polyacrylamide gels was obtained, as indicated by india ink staining of the transferred proteins on the nitrocellulose (Fig. 3).

Western blot analysis revealed 20 protein bands in GWBE and 22 protein bands in GFE that bound IgE from skin test reactive-positive sera. Individual sera had significant reactivity to GWBE bands at 13 to 85 kd and GFE bands at 11 to greater than 100 kd. Western blots of the first 14 subjects, whose reactivity is representative of the 37 sera tested, are presented in Fig. 4. The 37 sera identified 20 bands in GWBE of which one (85 kd) was not present in GFE. Twenty-two bands were detected in GFE; three bands (11, 14.5, and >100 kd) were not present in GWBE. The total number of sera that displayed reactivity to a band was expressed as a percentage of the 37 RAST-positive sera tested in Fig. 5. The five control sera did not demonstrate any detectable IgE binding to GWBE or GFE by SDS-PAGE and Western blotting.

The allergen bands at 60 and 67 kd showed the most significant reactivity; in GWBE and GFE 81% and 76% of the sera bound to the 60 kd band,

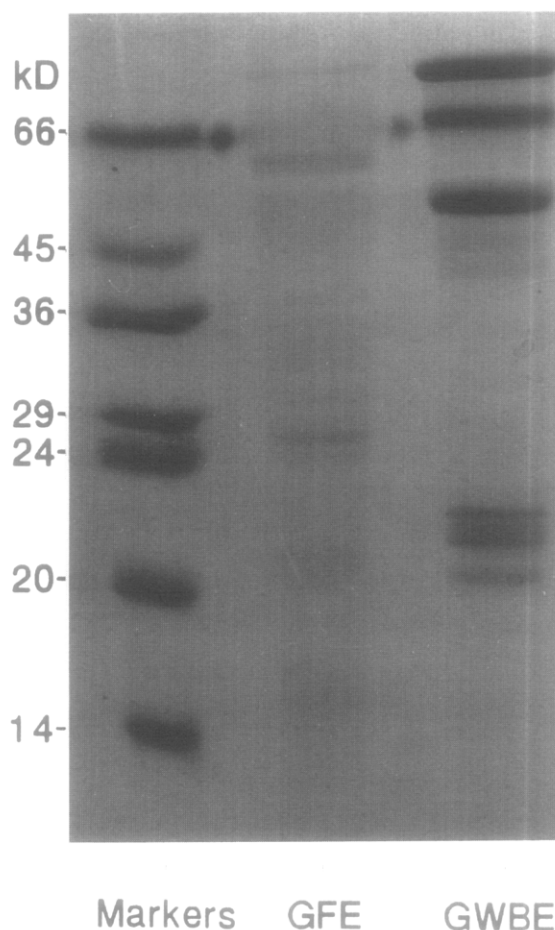


FIG. 2. SDS-PAGE of protein in GWBE and GFE. Approximately 20 μ g of protein per lane of GWBE and GFE were electrophoresed in 15% polyacrylamide gels and then stained with Coomassie Brilliant Blue.

and 60% and 54% to the 67 kd band, respectively. Other bands exhibiting significant activity were the 36 kd band with 43% and 62%, the 46 kd band with 65% and 41%, and the 50 kd with 51% and 51% binding of the subjects' sera to GWBE and GFE, respectively.

Western blot inhibition

As revealed by reduction in IgE-binding intensity, the GWBE immunoprint was inhibited by both the homologous (GWBE) and heterologous (GFE) inhibitors in an almost identical dose-dependent manner; however, GFE inhibited reactivity to a slightly greater degree. The GFE immunoprint inhibition demonstrated similar results, with almost equal inhibition by the homologous (GFE) and the heterologous (GWBE) inhibitors, and, again, slightly better inhibition by GFE. These

studies indicate that the allergens in GFE and GWBE contain shared IgE-binding sites (Fig. 6).

DISCUSSION

Previous studies have documented that cockroach allergens are present in house dust¹⁴⁻¹⁸ and that they are a significant etiologic factor in allergic asthma, particularly in large urban areas.^{1-13, 19-25} A successful environmental program of allergen reduction must be based on knowledge of the primary source(s) of allergens. Potential sources of relevant cockroach allergens in house dust include whole bodies, cast skins, secretions, egg castings, and/or feces.

The current study confirms that both cockroach whole body and feces have significant and similar allergenic activity. The direct RAST results show a high correlation of reactivity of individual sera to both GWBE and GFE. Similar reactivity to whole body and feces has been reported by other investigators.³²⁻³⁵ A similar prevalence of positive skin test results to GFE and GWBE has been previously demonstrated.^{32, 33, 35} Inhibition of GFE or GWBE RAST with GFE or GWBE suggested similar allergen content.³³ In contrast, only one study failed to demonstrate significant allergenic activity in cockroach feces on the basis of RAST and RAST inhibition with sera from 13 cockroach-sensitive atopic subjects.³⁶

SDS-PAGE–Western blot analysis in the present study demonstrates a similarity in GWBE and GFE IgE-binding patterns. Furthermore, almost complete inhibition of Western blot activity of either GWBE or GFE was obtained by both extracts. Findings of allergenic similarity between GWBE and GFE agree with a previous IEF-immunoprint inhibition investigation.³⁴ The SDS-PAGE–Western blot inhibition results in the present study, together with previous RAST and IEF-immunoprint inhibition studies clearly demonstrate that GFE and GWBE contain similar allergens.

Overall, reactivity to GWBE and GFE were similar, although certain subjects did display different degrees of reactivity or discordant reactivity between RAST and Western blotting. Differences in the degree of reactivity to the two extracts may be due to differences in the relative concentrations of allergens present in whole body and feces, the presence of unique allergens in whole body or feces, and/or cleaved fragments of larger GWBE allergens in GFE because of natural degradation caused by proteolytic enzymes present in the cockroach alimentary tract. Even when these subjects are considered, GWBE and GFE RAST results

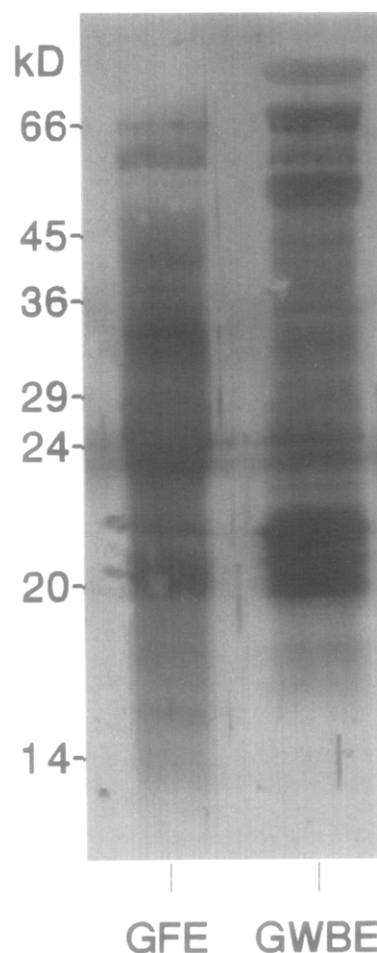


FIG. 3. India ink staining of nitrocellulose membrane after transfer of proteins from polyacrylamide gel. Approximately 20 μ g of protein per lane of GWBE and GFE were electrophoresed in 15% polyacrylamide gel. Proteins in gels were then electrophoretically transferred to CNBr-activated nitrocellulose and stained with india ink (1:1000 dilution with PBS–0.05% polysorbate 20).

were found to be highly and significantly correlated ($r = 0.882$, $p < 0.001$), and SDS-PAGE–Western blot analysis show small differences in percent of sera reactive to each band and only four IgE-binding bands reactive to either only GWBE (85 kd) or GFE (>100, 14.5, and 11 kd). The differences in reactivity between RAST and SDS-PAGE–Western blotting in certain subjects could be due to inherent differences in the two allergen analysis systems. The differences seen could be due to any of these factors; however, overall reactivity to GWBE and GFE as determined by RAST and Western blotting were similar for the sera panel.

An immunohistology investigation by Zwick et al.⁴¹ supports our finding that cockroach feces are

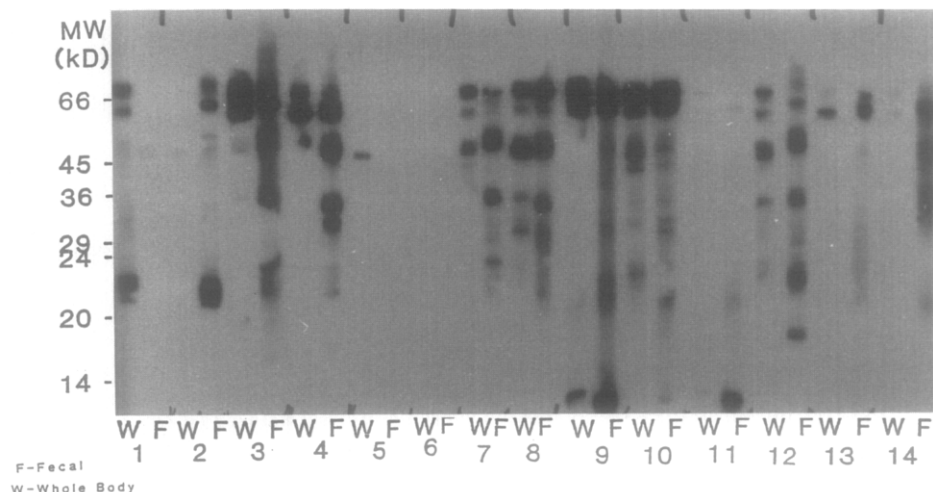


FIG. 4. SDS-PAGE-Western blot analysis of GWBE and GFE with sera from 14 cockroach-sensitive subjects representative of reactivity of 37 sera tested. Approximately 20 μ g of protein per lane of GWBE and GFE was electrophoresed by SDS-PAGE on 15% polyacrylamide gel and then transferred to CNBr-activated nitrocellulose.

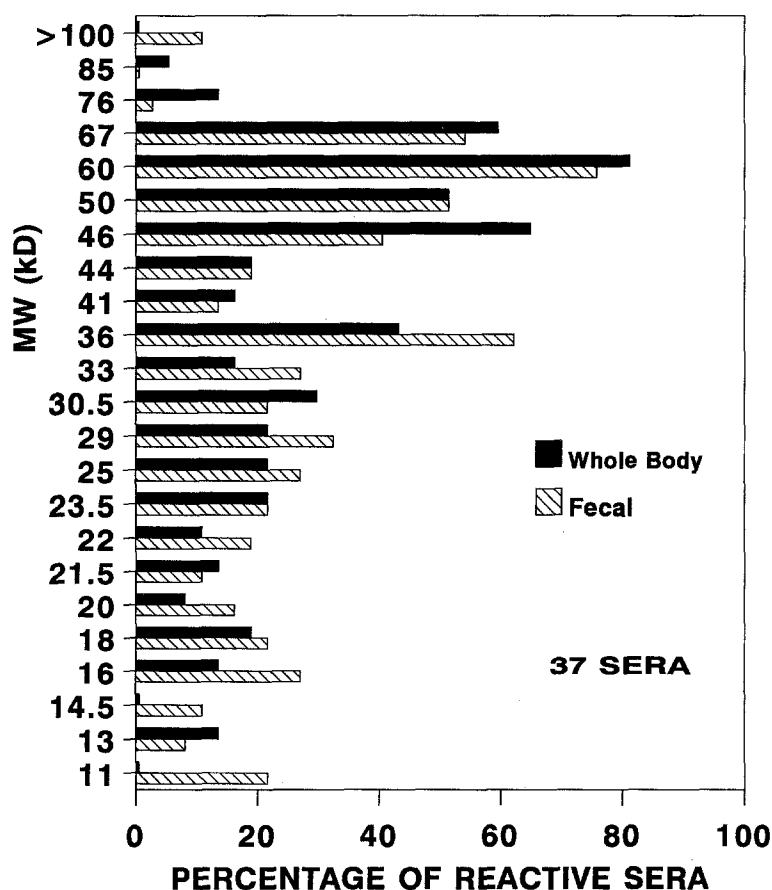


FIG. 5. Prevalence of IgE reactivity by SDS-PAGE-Western blotting among 37 sera from cockroach-sensitive persons tested determined by visual inspection. Total number of sera that displayed reactivity to band was expressed as percentage of 37 RAST-positive sera assessed.

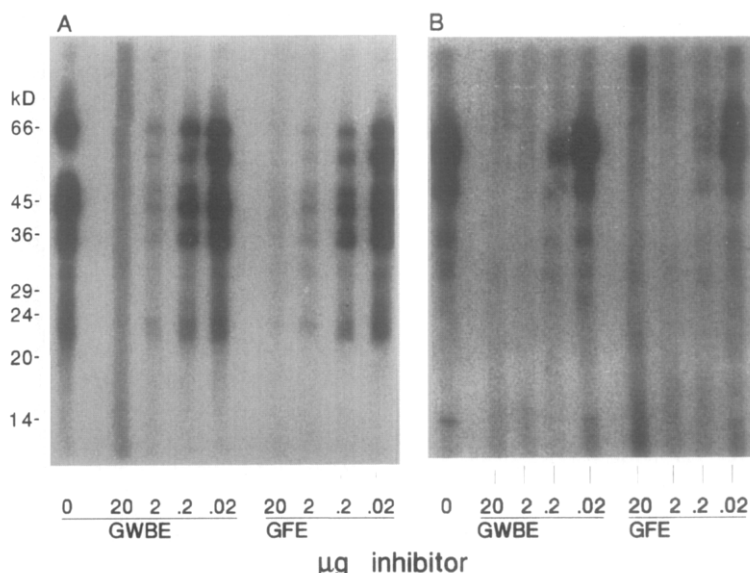


FIG. 6. SDS-PAGE–Western blot inhibition of (A) GWBE (solid phase) and (B) GFE (solid phase), performed separately with both GWBE and GFE. Nitrocellulose strips were reacted with 0.5 ml of pooled sera from cockroach-sensitive persons (final dilution, 1:10) mixed with 0.5 ml of inhibitor solution (either GWBE or GFE) containing indicated amount of inhibitor. A and B represent different SDS-PAGE runs.

probably an important allergen source. This study analyzed IgE-specific binding to different German cockroach body parts by immunofluorescence testing with sera from five cockroach-sensitive persons. The most significant IgE binding occurred to the gastrointestinal epithelium and the contents of the intestinal tract. The study of Zwick et al. demonstrates that a major source of cockroach allergens originates in the cockroach digestive tract and may be digestive enzymes.

Evidence presented in this study and that from previous studies clearly demonstrates that cockroach feces possess significant allergenic activity and suggests that fecal particles are a potential significant source of cockroach allergens in house dust. Cockroach feces are likely to be the source of relevant cockroach allergens because desiccated feces particles probably disintegrate to become incorporated into house dust more readily than chitinous body parts and encased internal organs. Cockroach infestations may produce allergenic material, although cockroach bodies and/or body parts are not noticed. One survey of house dust samples found that approximately 20% of homes tested had detectible levels of cockroach allergens, although there was no obvious evidence of infestation.¹⁶ Clearly, the possibility of occult infestations should be investigated in the management of cockroach allergy.

Regardless of the source of clinically relevant cockroach allergens, the major allergens, as determined by this study, are present in both whole body and feces. Of the 37 sera tested, the protein bands with the highest prevalence of IgE binding were found at 60, 67, 36, 46, and 50 kD. The band at 60 kD bound approximately 80% of sera tested in both GWBE and GFE.

Several other studies have attempted to identify important cockroach allergens by analysis of fractionated extracts. Twarog et al.,¹⁹ using sequential purification steps with a Sephadex column, diethylaminoethyl cellulose, and agarose gel electrophoresis, identified two allergens with MWs of 25.5 and 63 to 65 kD; the 63 to 65 kD allergen demonstrated positive skin test reactivity in 70% of 16 subjects who had positive skin test reactions to crude cockroach extract. Using a Sephadex column and SDS-PAGE–Western blotting with a sera pool from 12 persons sensitive to cockroach, Helm et al.²⁶ found significant binding of IgE at 36 and 55 kD. In a further investigation, Helm et al.²⁸ demonstrated significant IgE binding at 92, 67, 36, and 25 kD by SDS-PAGE–Western blotting with sera from four persons sensitive to cockroach. Kang et al.,²⁹ using SDS-PAGE and Western blotting with sera from two geographically distinct sera groups (10 sera from Chicago and six from Lexington, Ky.), found that two allergens with MWs of 85 to 95 and

30 to 35 kd had the highest prevalence of IgE binding. This study confirms the existence of several allergens identified in previous studies and establishes the importance of other relatively high-MW allergens. Further, this study provides strong evidence of the relative importance of these allergens by using a large serum panel and testing each serum individually in the Western blot analysis.

The disparity in identification of the most clinically relevant allergens in German cockroach between this study and previous investigations may be due to differences in extract preparation (source material and procedure), separation and isolation techniques, and serum source. Kang et al.²⁹ demonstrated that the locale of the subjects whose sera were tested influenced allergen-binding patterns. It is of note, however, that the present study used by far the largest number of sera in immunoblotting investigations of cockroach allergens to date. Additionally, the prevalence of reactivity to the allergens with MWs of 36 and 25 kd in the present study agrees closely with the RAST reactivity demonstrated by Pollart et al.³¹ to *Bla g* I and *Bla g* II, allergens of 36 and 25 kd, respectively, and thus provide further support for the allergen reactivity reported in the present study.

In summary, this investigation, which used RAST, SDS-PAGE and Western blotting, and Western blotting inhibition demonstrated that cockroach feces have significant allergenic activity and similar allergen content to GWBE. Important German cockroach allergens were identified by immunoblotting with sera from 37 persons sensitive to cockroach. These results confirm other reports of high-MW allergens and provide strong evidence of the importance of these larger allergens relative to previously characterized *Blattella* allergens. Progress in identification, purification, and subsequent standardization of cockroach extracts for clinically important allergens will lead to better diagnosis and management of patients allergic to cockroach.

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