Allergen content in German cockroach extracts and sensitization profiles to a new expanded set of cockroach allergens determine *in vitro* extract potency for IgE reactivity

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Background: Cockroach allergens are an important cause of IgE-mediated sensitization in inner-city asthmatic patients. However, cockroach extracts used for diagnosis and immunotherapy are not standardized.
Objective: We sought to determine the allergen content of nonstandardized German cockroach extracts and the levels of sensitization to an expanded set of cockroach allergens as determinants of *in vitro* extract potency for IgE reactivity.
Methods: Twelve German cockroach extracts were compared for allergen content and potency of IgE reactivity. Bla g 1, Bla g 2, and Bla g 5 were measured by using immunoassays. IgE antibody levels to 8 purified recombinant allergens from groups 1, 2, 4, 5, 6, 7, 9, and 11 were measured by using ImmunoCAP. IgE antibody binding inhibition assays were performed to assess extract *in vitro* potencies (concentration inhibiting 30% of the total IgE antibody-binding inhibition) relative to an arbitrarily selected reference extract in 5 patients with cockroach allergy.
Results: Allergen levels were highly variable. Three new major allergens (groups 6, 9, and 11), were identified among highly cockroach-sensitized subjects (CAP class ≥ 3). Sensitization profiles were unique per subject without immunodominant allergens. The sum of IgE to 8 allergen components showed a good correlation with cockroach-specific IgE levels ($r = 0.88$, $P < .001$). *In vitro* potencies varied among different extracts per subject and among subjects for each extract.
Conclusions: The *in vitro* potency of German cockroach extracts for IgE reactivity depends on allergen content and allergen-specific IgE titers of patients with cockroach allergy. These factors are relevant for selection of potent extracts to be used for immunotherapy and for the design and interpretation of data from immunotherapy trials. (J Allergy Clin Immunol 2019;143:1474-81.)

Key words: *Cockroach allergy, non-standardized extracts, cockroach allergen components, immunotherapy, diagnosis*

Cockroach allergy is an important health problem in the United States, especially in inner-cities, and is associated with chronic exposure and IgE sensitization to multiple allergens, which often results in the development of asthma.[1] Cockroach extracts for immunotherapy are currently not standardized. The doses of extract used for cockroach immunotherapy by the Inner-City Asthma Consortium were calculated based on content of the cockroach allergens Bla g 1 and Bla g 2.[2]

The maintenance dose in a trial for cockroach subcutaneous immunotherapy was established as 120 μg of Bla g 1 and 6 μg of Bla g 2 based on the relevance of these 2 allergens.[2] Bla g 2 is one of the most important major allergens from cockroach, with a prevalence of sensitization of 54% to 72%.[3,4] Although the IgE prevalence for Bla g 1 (26% to 40%) was lower than that for Bla g 2, both Bla g 1 and Bla g 2 have consistently been used as markers of environmental exposure to cockroach.[4,5] However, these 2 allergens do not account for all IgE reactivity against cockroach extracts.[4] Until recently, 5 cockroach allergens were known: Bla g 1, a gut microvilli-associated protein; Bla g 2, a gut inactive aspartic protease; Bla g 4, a lipocalin produced only in male cockroaches and excreted in the spermatophore during copulation; Bla g 5, a glutathione-S-transferase; and Bla g 7, a tropomyosin.[3,6-14] Satinover et al[4] showed that none of these 5 cockroach allergens were immunodominant in a US population, and cumulatively, they did not account for all the IgE reactivity against cockroach extracts.[4]

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Abbreviations used
APA: Advanced Protein Assay
IC30: Concentration inhibiting 30% of the total IgE antibody-binding inhibition

The current study extends the analysis of IgE reactivity to 3 additional cockroach allergens (from groups 6, 9, and 11). Bla g 6 is a troponin C involved in muscle contraction.[15] An arginine kinase was identified by means of proteomic approaches with a 34% IgE prevalence in a Taiwanese population.[16] However, this allergen, a putative Bla g 9, had not been listed as an allergen in the World Health Organization/International Union of Immunological Societies Allergen Nomenclature database (www.allergen.org). α-Amylases from both *Blattella germanica* and *Periplaneta americana* were recently described in Korea and China as group 11, with an IgE prevalence of 41% and 83%, respectively.[17,18]

This study addresses the variability in content among German cockroach extracts prepared from different sources and using different protocols. This variability poses a challenge in terms of extract standardization, which is the difficulty of producing batches of extracts with consistent relative amounts of allergen for preparation of consistent doses for clinical use.

The main goal of the study is to compare the *in vitro* potencies of a group of cockroach extracts for IgE reactivity in individual patients with cockroach allergy. To achieve this goal, 12 German cockroach extracts were compared for allergen content and for potency of IgE reactivity. The extract *in vitro* potency for IgE reactivity was investigated by using IgE antibody inhibition assays in 5 individual patients with cockroach allergy. Overall, this study analyzes the importance of 2 factors, extract content and IgE sensitization profiles of patients with cockroach allergy, on the extract *in vitro* potencies and implications of the results for the design and interpretation of the outcomes of cockroach immunotherapy.

METHODS

Study population

A cohort of 23 subjects sensitized to cockroach (IgE titer, >0.35 kU_A/L) were recruited from San Diego, California; St Louis, Missouri; and New York, New York, according to institutional review board approval (protocols VD-112-0217, 201305110, and GCO 13-0691). All had a history of allergy symptoms to cockroach, and most had asthma, rhinitis, or both. All subjects enrolled in this study provided written consent. Donor information is summarized in Table E1 in this article's Online Repository at www.jacionline.org. IgE antibody titers were determined from plasma by using the ImmunoCAP system (Thermo Fisher Scientific, Uppsala, Sweden). Seventy percent of subjects were female, mean age was 39 ± 10 years, and cockroach-specific IgE titers were 16.5 ± 22.8 kU_A/L (range, 0.9-76.2 kU_A/L) on average.

Cockroach extracts

Twelve German cockroach extracts were acquired or prepared in-house for this study (Table I). Nine commercial extracts were purchased from Greer Laboratories (Lenoir, NC). Batches from Greer were made from whole cockroach bodies and included 4 extracts for clinical use in human subjects (extracts 1-4), 2 extracts for veterinarian use (extracts 5 and 6), and 3 extracts for research use (extracts 7-9). All extracts were formulated in 50% glycerin, except for extracts for research use, which were aqueous. Extracts 1 and 7 to 9 used defatted cockroaches. In addition, aqueous German cockroach extracts were made in-house by different research laboratories.

Extract 10 was made from cockroach fecal matter at Yonsei University (Seoul, Korea), and extract 11 was made at the La Jolla Institute (La Jolla, Calif) from fecal matter collected at North Carolina State University (Raleigh, NC). The protocol for the preparation of these 2 fecal extracts is described elsewhere.[17]

Extract 12 was made from cockroach frass at Indoor Biotechnologies (Charlottesville, Va), as previously described, with few modifications.[19] The extract was prepared by stirring German cockroach frass (cockroach debris containing body parts, fecal material, and egg cases) for 24 to 48 hours at 4°C in PBS (pH 7.4; 0.19 g of frass/mL) and was not ether extracted.

Extract 13 is a negative control made from food chow for cockroaches. Protein levels in the extracts were measured by using the Advanced Protein Assay (APA; Cytoskeleton, Denver, Colo). The extracts were diluted 1:5 before performing the APA to reduce the effect of glycerin on protein determination.

Expression, purification, and quantification of 8 recombinant cockroach allergens

The German cockroach allergens Bla g 1, Bla g 2, Bla g 4, Bla g 6, Bla g 9, and Bla g 11 and the American cockroach allergen Per a 7 were expressed in *Pichia pastoris*, and Bla g 5 was expressed in *Escherichia coli*. All allergens were expressed and purified as described in the Methods section in this article's Online Repository at www.jacionline.org.

Measurement of Bla g 1, Bla g 2, Bla g 5, and endotoxin levels in cockroach extracts

Bla g 1, Bla g 2, and Bla g 5 levels were measured by ELISA. Endotoxin levels were measured by using the chromogenic *Limulus* Amoebocyte Lysate assay (Lonza, Walkersville, Md). Methods used are described in the Methods section in this article's Online Repository.

Measurement of IgE antibody levels by using ImmunoCAP

Cockroach-specific IgE antibody binding was measured with commercially available i6 ImmunoCAPs. Allergen-specific IgE antibody levels were measured by using streptavidin CAPs optimally loaded with biotinylated purified recombinant cockroach allergens, as described in the Methods section in this article's Online Repository. Measurements of IgE antibody binding were performed in a Thermo Fisher Scientific ImmunoCAP system (Phadia 250 Immunoassay Analyzer), according to the manufacturer's instructions.

IgE antibody binding inhibition assays

In vitro inhibition assays were performed to compare the capacity of each extract to inhibit binding of IgE antibodies from individual subjects with an extract chosen as a reference. Commercial extract 9 was selected as reference because it contained the highest concentrations of Bla g 1 and Bla g 2 among the commercial extracts (44.60 and 19.01 μg/mL, respectively). Extract 9 also contained a relatively high amount of Bla g 1, Bla g 2, and Bla g 5 per milligram of protein (10.27 μg/mg; Table I).

The window of IgE antibody binding inhibition was determined *a priori* with the reference extract only. Assays were performed to compare all the extracts for each subject at one time. Five subjects were selected for the inhibition assays because in a prescreening of 12 plasma samples, they showed the largest windows of IgE antibody binding inhibition at 1:4 or 1:2 dilutions compared to the other subjects. Most had high IgE titers (average, 45.68 kU_A/L; range, 4.82-76.20 kU_A/L; see Table E1).

An individual assay per each of the 5 subjects was performed to compare all the extracts. Microplates were coated with extract 9 at 10 μg/mL and incubated at 4°C overnight. After the plates were washed and blocked with PBS-0.05% Tween 20-1% BSA, each extract was preincubated with the

TABLE I. Content of cockroach allergen extracts

German cockroach extract		Bla g 1 (μg/mL)	Bla g 2 (μg/mL)	Bla g 5 (μg/mL)	Protein concentration, APA (mg/mL)	Bla g 1/ protein concentration (μg/mg)	Bla g 2/ protein concentration (μg/mg)	Bla g 5/ protein concentration (μg/mg)	Three allergens/ protein concentration (μg/mg)	Endotoxin Limulus Amoebocyte Lysate (EU/mL)
1	Commercial, WB, Hum	41.81	30.30	0.130	7.00	5.97	4.33	0.02	10.32	1,617
2	Commercial, WB, Hum	10.32	2.86	**0.020**	3.14	3.28	0.91	0.01	4.20	10,892
3	Commercial, WB, Hum	**7.59**	3.13	0.081	2.22	3.42	1.41	0.04	4.87	3,175
4	Commercial, WB, Hum	8.06	3.13	0.023	2.46	3.28	1.27	0.01	4.56	13,418
5	Commercial, WB, Vet	21.44	**0.10**	<0.005	1.75	12.27	0.06	0.00	12.33	**467**
6	Commercial, WB, Vet	9.01	4.88	0.244	2.96	3.04	1.65	0.08	4.77	3,069
7	Commercial, WB, Res	13.68	17.52	0.178	3.04	4.50	5.76	0.06	10.32	1,889
8	Commercial, WB, Res	22.79	12.68	0.030	4.35	5.24	2.91	0.01	8.16	1,546
9*	Commercial, WB, Res	44.60	19.01	**0.248**	6.22	7.17	3.06	0.04	10.27	13,782
10	In-house, fecal, Res	127.82	**72.80**	<0.005	**7.71**	16.58	9.44	0.00	26.02	**342,622**
11	In-house, fecal, Res	**150.13**	26.70	<0.005	6.03	24.90	4.43	0.00	29.33	152,713
12	In-house, frass, Res	49.75	22.96	<0.005	**1.35**	36.85	17.01	0.00	53.86	39,934
13†	Negative control	<0.008	<0.01	<0.005	5.39	0.00	0.00	0.00	0.00	1,412
Average‡		42.25	18.01	0.08	4.02	10.54	4.35	0.02	14.92	48,760
SD‡		47.81	20.11	0.10	2.18	10.65	4.74	0.03	14.74	101,932
		20×	728×	12×	6×					733×
		Averages								
	WB	19.92	10.40	0.11	3.68					
	Hum	16.95	9.86	0.06	3.71					
	Vet	15.23	2.49	0.12	2.36					
	Res	27.02	16.40	0.15	4.54					
	Fecal-frass	109.23	40.82	0.01	5.03					

The lowest and highest values of allergen and endotoxin concentrations are shown in boldface, and the fold difference between them is indicated in the bottom row.
Hum, Human use; *Res*, research use; *Vet*, veterinary use; *WB*, whole body.
*Reference extract.
†Negative control (allergen concentrations were lower than the indicated lower limit of detection).
‡Negative control was excluded from the calculation of average and SD.

plasma in a different mixing polypropylene plate. Extracts were prepared in microtubes at a predetermined optimal concentration, and 80 μL were added to the first wells of the mixing plate and diluted 1:4 in consecutive wells. Plasma was then added to each well to a final dilution of 1:2 to 1:5, mixed, and incubated for 1 hour. One hundred microliters from each well of the mixing plate were then transferred to the corresponding well of the ELISA plate and incubated for 3 hours. Affinity-purified peroxidase–labeled goat anti-human IgE antibody (KPL, Gaithersburg, Md) was added to the plate at a dilution of 1:1000 and incubated for 1 hour. The plate was washed and developed with ABTS in 70 mmol/L citrate phosphate buffer, pH 4.2, and a 1:1000 dilution of H_2O_2. Absorbance was read at 405 nm on a Bio-Tek EL800 Microplate Reader (Bio-Tek Instruments, Winooski, Vt) when the top standard concentration reached an OD of approximately 2.0.

Extract potencies were expressed as the concentration inhibiting 30% of the total IgE antibody-binding inhibition (IC30) when the same reference extract was used as an inhibitor. IC30 values were normalized versus reference extract values, which had an IC30 of 1. An IC30 of 1000 was assigned to extracts that did not reach 30% of inhibition.

RESULTS

Content of cockroach allergens

Allergen and endotoxin levels were highly variable in the cockroach extracts analyzed (Fig 1 and Table I).

The average amount of Bla g 1 and Bla g 2 was 5.5- and 3.9-fold greater in fecal and frass extracts (extracts 10-12) than in whole-body extracts (extracts 1-9; 109.2 ± 52.7 vs 19.9 ± 14.3 μg/mL for Bla g 1 and 40.8 ± 27.8 vs 10.4 ± 10.2 μg/mL for Bla g 2; Table I). However, Bla g 5 levels were 21.3-fold greater in whole-body extracts (0.11 ± 0.1 vs 0.01 ± 0.0 μg/mL). The variability in Bla g 1, Bla g 2, and Bla g 5 levels was 5.5-, 10.6-, and 6.5-fold in commercial extracts for human use versus 2.4-, 48.8-, and 48.8-fold in extracts for veterinary use, respectively. On average, Bla g 1, Bla g 2, and Bla g 5 content was 70%, 30%, and 0.3%, respectively, of the sum of the amount of the 3 allergens in the extracts. In extracts for human use, Bla g 1 content was on average 2.5-fold greater than Bla g 2 content, and Bla g 2 content was on average 138-fold greater than Bla g 5. The in-house extracts contained the largest amount of allergen per milligram of protein (26.0, 29.3, and 53.9 μg/mg for extracts 10, 11, and 12, respectively). All extracts had low amounts of Bla g 5.

IgE recognition of 8 cockroach allergens

The pattern of IgE recognition of 8 cockroach allergens was variable (Fig 2). The 8 allergens included (1) the "traditional" cockroach allergens Bla g 1, Bla g 2, Bla g 4, Bla g 5, and Per a 7 analyzed by Satinover et al[4] and (2) 3 additional allergens: Bla g 6, an arginine kinase homologous to Per a 9, and Bla g 11. Based on the data presented here, the arginine kinase was proved to be an allergen and was submitted to the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Sub-Committee, which approved the assignment of the name Bla g 9 to this new allergen.

Different allergens were dominant for different donors. The tendency was for subjects with greater cockroach-specific IgE

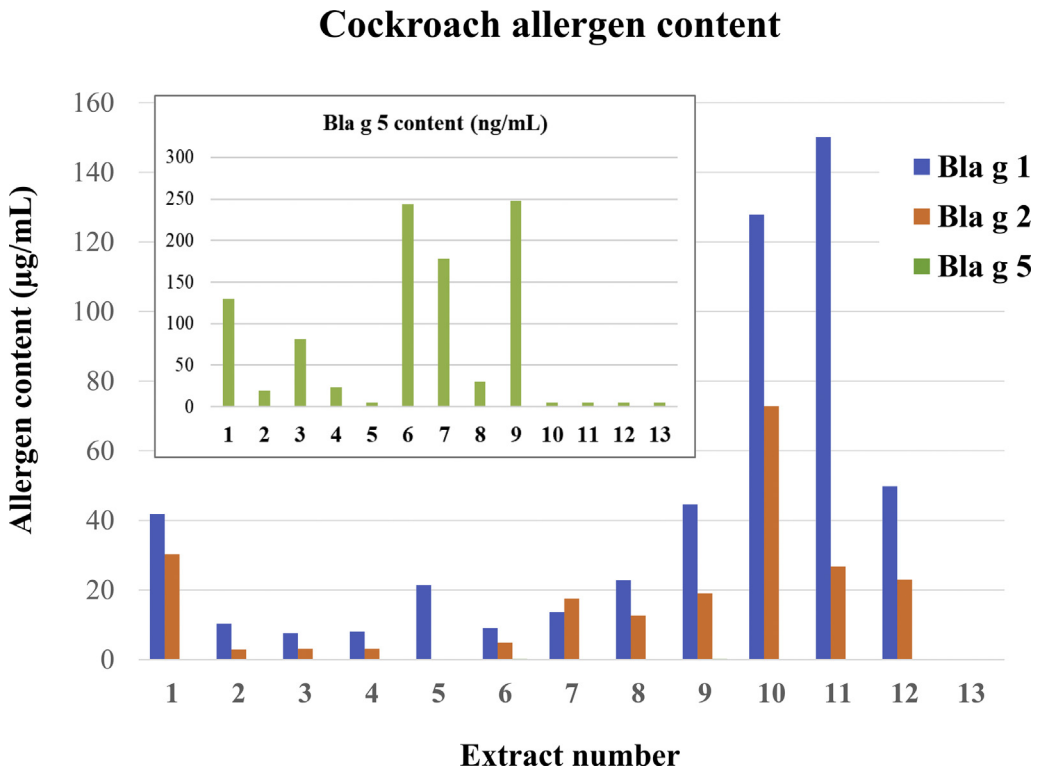

FIG 1. Allergen levels in cockroach extracts. Concentrations of Bla g 1, Bla g 2, and Bla g 5 in the extracts were measured with immunoassays (extract 13 is a negative control). The *inset* shows Bla g 5 concentrations in nanograms per milliliter.

titers to have IgE recognizing more allergens and at greater levels. For subjects with cockroach-specific IgE titers of less than 3.5 kU_A/L (CAP classes 0-2), 1 subject recognized 4 allergens, 4 recognized 1 to 2 allergens, and 3 did not recognize any allergen. For CAP classes 3 and greater (cockroach-specific IgE ≥ 3.5 kU_A/L), 3 subjects recognized 8 allergens, 2 recognized 7 allergens, 9 recognized 1 to 6 allergens, and 1 did not recognize any allergen. Four subjects with cockroach-specific IgE values of 1.23 to 4.47 kU_A/L did not recognize any of the 8 allergens tested.

The prevalences of IgE sensitization to the 8 allergens for patients with cockroach allergy (n = 23) were as follows: Bla g 1, 30%; Bla g 2, 57%; Bla g 4, 35%; Bla g 5, 39%; Bla g 6, 44%; Per a 7, 22%; Bla g 9, 44%; and Bla g 11, 57%. For a subgroup of only 15 subjects with CAP class 3 or greater, prevalences were as follows: Bla g 1, 47%; Bla g 2, 73%; Bla g 4, 47%; Bla g 5, 47%; Bla g 6, 60%; Per a 7, 33%; Bla g 9, 53%; and Bla g 11, 73%. These results show that the number of major cockroach allergens increases with the cockroach-specific IgE levels in a population. In this case there were 2 major allergens for 23 (Bla g 2 and Bla g 11) and 4 for a subgroup of 15 patients with high-level cockroach allergy (Bla g 2, Bla g 6, Bla g 9, and Bla g 11). The allergen with the highest geometric mean of allergen-specific IgE for 23 subjects was Bla g 2 (1.36 kU_A/L; Fig 3). The average of the percentages of allergen-specific IgE was greatest for Bla g 2 (21.3% ± 20.5%), followed by Bla g 9 (15.5% ± 13.2%), Bla g 5 (14.0% ± 14.1%), Bla g 4 (11.0% ± 10.2%), Per a 7 (10.7% ± 12.8%), Bla g 11 (10.1% ± 10.3%), Bla g 1 (8.8% ± 6.1%), and Bla g 6 (8.6% ± 6.4%; see Fig E1 in this article's Online Repository at www.jacionline.org).

There was a highly significant correlation between cockroach-specific IgE levels and the sum of allergen-specific IgE levels to 8 allergens (for $\log_{10}$ transformed data: $r = 0.88$, $P < .001$; n = 23; Fig 4). This correlation was an improvement versus that obtained when only 4 cockroach allergens (Bla g 1, Bla g 2, Bla g 4, and Bla g 5; $r = 0.78$, $P < .001$) or only the 3 allergens that were measured in the extracts ($r = 0.78$, $P < .001$) were considered. A weak correlation was observed between cockroach-specific IgE levels and skin prick test wheal sizes (n = 18; $r = 0.56$, $P = .015$).

Comparison of *in vitro* extract potencies using IgE inhibition assays

Cockroach extracts exhibited highly variable *in vitro* potency with respect to IgE recognition per subject. Differences in relative extract potencies for each subject varied up to more than 3 orders of magnitude (up to 2800-fold). Table II shows the relative potencies (IC30) estimated from the inhibition curves displayed in Fig E2 in this article's Online Repository at www.jacionline.org.

Commercial extracts 2, 3, 4, 6, and 9 showed the greatest relative potencies (IC30 value, 0.4-2.9). However, there was not a good correlation between the sum of 3 allergen levels in the extracts and the extract potencies. From the 5 subjects tested for extract potencies, 2 groups of subjects were identified because good correlations of the IC30 values between paired subjects were observed within these groups ($r > 0.9$, $P < .001$; see Table E2 in this article's Online Repository at www.jacionline.org). The pairs of subjects with a better *in vitro* potency correlation also tended to have a better correlation of specific IgE levels to 8 allergens (not significant; see Table E3 in this article's Online Repository at www.jacionline.org).

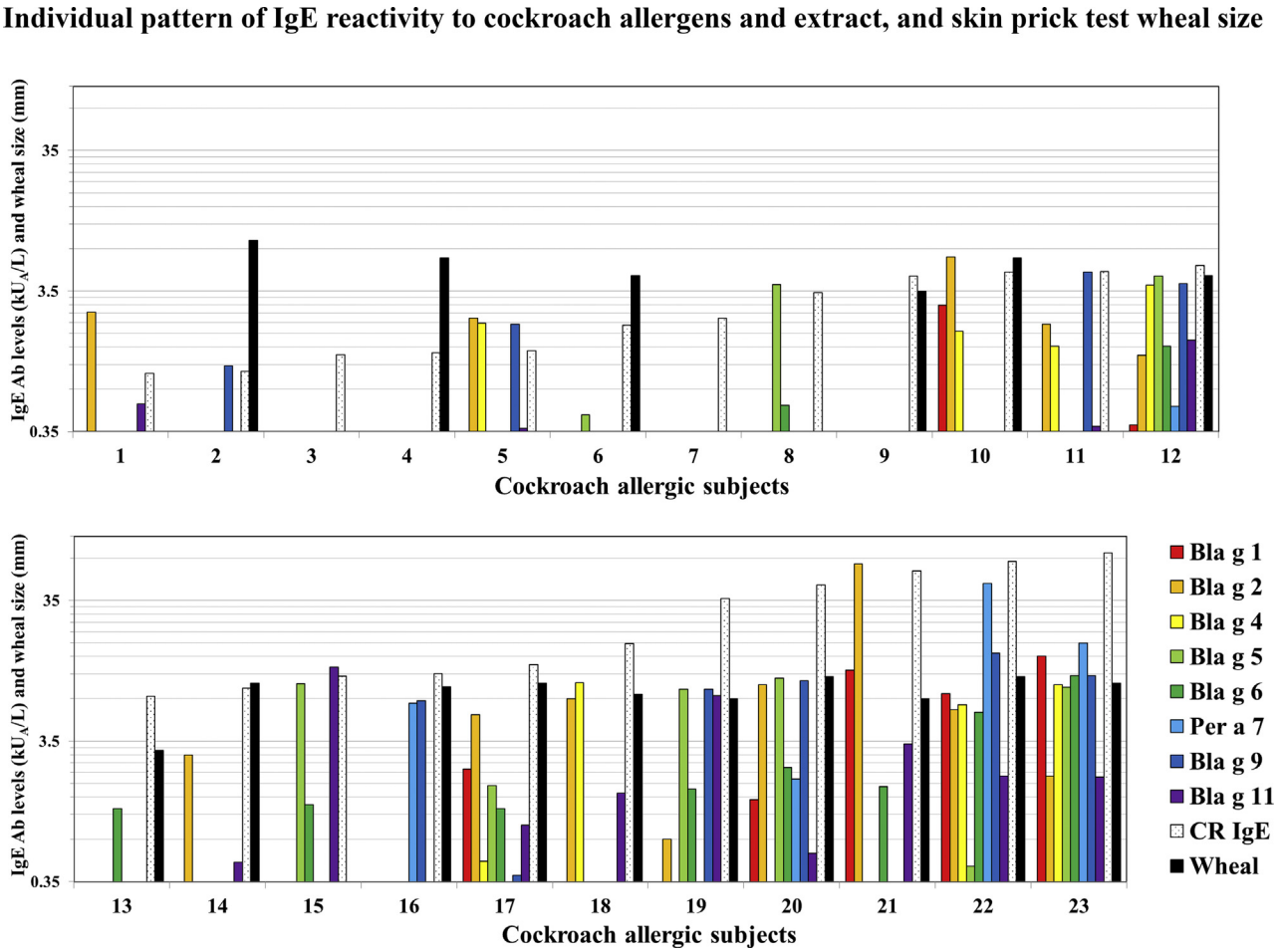

FIG 2. Allergen-specific IgE patterns of sensitization. Patterns of IgE sensitization to 8 purified cockroach allergens in a population of 23 patients with cockroach allergy. The *last 2 columns* show cockroach-specific IgE antibody levels and skin prick test wheal sizes (skin prick tests were not performed for patients 3, 7, 8, 11, and 15). Subjects are ordered by lowest to highest cockroach-specific IgE levels.

Analyses of German cockroach extract potencies versus allergen content and versus levels of IgE sensitization to cockroach allergens

Two additional systematic analyses of extract potency data were performed. First, for each of the 5 patients tested for extract potencies using inhibition assays, correlations between extract potencies and allergen content of the extracts were analyzed. Only patients 1445 and 1227 showed significant correlations between both variables ($r = 0.779$, $P = .0028$ for patient 1445 and $r = 0.773$, $P = .0032$ for patients 1277 when considering the 3 allergens measured; see Table E4 in this article's Online Repository at www.jacionline.org).

Interestingly, the correlations were greater when Bla g 1 alone was considered (0.871 [$P = .0002$] for patient 1445 and 0.838 [$P = .0007$] for patient 1227), whereas there were no significant correlations when considering only Bla g 2 (see Table E4).

These results might be associated with the fact that these 2 patients also had the highest levels of Bla g 1–specific IgE (14.06 and 7.56 kU$_A$/L for patients 1445 and 1277, respectively), whereas these levels were either low or undetectable for the 3 other patients (see Table E4). These results also agree with the fact that the allergen content of the 12 extracts was much greater for Bla g 1 than Bla g 2 (20.1-fold in average) and very low for Bla g 5. Therefore sensitization to Bla g 1 was shown to be relevant for the positive correlations observed between potencies and allergen content (high on Bla g 1) for those 2 patients.

Second, for each extract, the correlations between extract potencies for 5 patients and the sum of allergen-specific IgE levels of these patients were analyzed. The allergen-specific IgE considered for analysis was either the sum of IgE levels to Bla g 1, Bla g 2, and Bla g 5 (the 3 allergens measured in the extracts) or the sum of IgE levels to each of the 8 allergens. Correlations were not significant for the sum of specific IgE levels to only 3 allergens, and Pearson correlation coefficients increased significantly when 8 allergen-specific IgEs were considered (63.2-fold in average for the 12 extracts; see Table E5 in this article's Online Repository at www.jacionline.org). Most extracts showed significant correlations of potencies versus the sum of IgE levels to 8 allergens ($P < .05$; shown in boldface in Table E5), except 3 extracts (2, 4, and 6) that had the lowest levels of allergen content.

DISCUSSION

This study addresses 2 factors that determine the *in vitro* potency of German cockroach extracts for IgE reactivity. One is

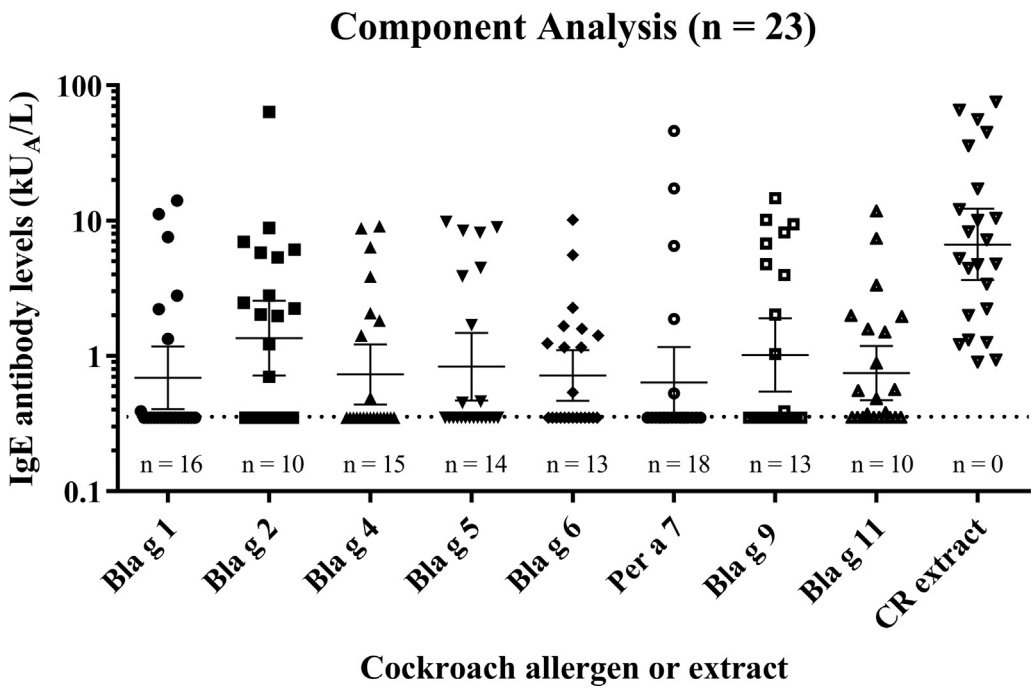

FIG 3. Component analysis of IgE reactivity to 8 cockroach allergens in a US population of cockroach-allergic subjects. Allergen- and cockroach-specific IgE antibody levels from 23 subjects are shown. *Long* and *short horizontal lines* indicate geometric means and 95% CIs, respectively. The cutoff level for IgE quantification (0.35 kU$_A$/L) is indicated by the *horizontal dotted line*. The number of negative results (<0.35 kU$_A$/L) is provided for each allergen under the corresponding cluster of symbols.

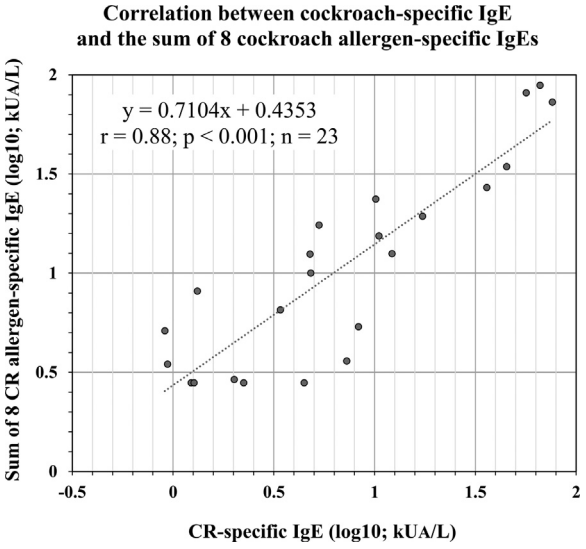

FIG 4. Correlation between cockroach *(CR)*–specific IgE levels and the sum of specific IgE levels to each of the 8 cockroach allergens for 23 subjects. Plasma cockroach-specific IgE levels and sum of allergen-specific IgE levels were plotted after $\log_{10}$ transformation for normalization of these variables.

the allergen content of the extract, and the other is the subject's sensitization profile to cockroach allergen components. Cockroach extracts are not standardized, and the variability of their allergen content makes it difficult to select a dose for clinical applications, such as diagnostics and immunotherapy. The 3 allergens measured in cockroach extracts (Bla g 1, Bla g 2, and Bla g 5), which are the only ones for which immunoassays are currently available, showed variability in allergen content ranging from 5.5- to 10.6-fold among commercial extracts for human use.

Highly variable content of protein, Bla g 1, and Bla g 2 in cockroach extracts has been described previously.[20] The large variations in allergen contents of the 12 cockroach extracts are likely related to the source of the extracts and the process of extract preparation.[21] For example, fecal extracts (extracts 10 and 11) contained the greatest amounts of Bla g 1 and Bla g 2, allergens that are known to be excreted in feces.[3,22] Bla g 5, an enzyme that is likely expressed in the cockroach fat body (analogous to liver), was poorly represented in fecal extracts. Other extracts made of cockroach whole body contained less Bla g 1 and Bla g 2 but greater relative amounts of Bla g 5 than fecal extracts. Bla g 1 and Bla g 2 were present in extracts at levels up to 3 orders of magnitude greater than Bla g 5. Consequently, for diagnostic and immunotherapy purposes, this might suggest a severe underrepresentation of Bla g 5 in the extracts, especially considering that the IgE prevalence to Bla g 5 (39% to 47%) is equivalent to that of Bla g 1 (30% to 47%) in this study. Most importantly, these 3 allergens do not cover the full cockroach-specific IgE reactivity.

Early studies by Satinover et al[4] reported that the reactivity profile of patients with cockroach allergy to 5 cockroach allergens was unique, without common immunodominant allergens. The 5 allergens tested from groups 1, 2, 4, 5, and 7 were not recognized by 36% of cockroach-sensitized subjects, which indicated that additional cockroach allergens existed. In the current study 3 more cockroach proteins were included: Bla g 6, Bla g 9, and Bla g 11. Bla g 6 showed a greater IgE prevalence (up to 60% for n = 15 subjects with CAP class ≥ 3) than that reported in previous cloning studies.[15] Interestingly, the 3 molecules turned

TABLE II. *In vitro* IgE potencies of cockroach extracts for 5 subjects with cockroach allergy

		Subjects with cockroach allergy				
		1445	1277	1424	1425	1864
	Cockroach extracts	Relative extract potencies (normalized IC30 data)*				
1	Commercial, WB, Hum	3.7	4.3	1.7	2.0	1.9
2	Commercial, WB, Hum	0.8	1.2	1.9	1.3	1.1
3	Commercial, WB, Hum	0.4	0.6	1.3	1.6	1.3
4	Commercial, WB, Hum	0.7	0.9	1.9	1.7	1.1
5	Commercial, WB, Vet	944.4	1000.0	350.0	25.0	1.8
6	Commercial, WB, Vet	0.8	0.4	1.6	2.8	1.5
7	Commercial, WB, Res	9.4	12.5	2.7	2.9	2.1
8	Commercial, WB, Res	15.0	187.5	1.0	1.0	1.0
9	Commercial, WB, Res	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
10	In-house, fecal, Res	444.4	1000.0	45.8	153.9	4.3
11	In-house, fecal, Res	1055.6	1000.0	3.0	10.0	15.5
12	In-house, frass, Res	1000.0	1000.0	22.5	12.2	1.6
13	Negative control	1000.0	1000.0	1000.0	1000.0	1000.0
		×2375	×2720	×1000	×1000	×1000

Hum, Human use; *Res*, research use; *Vet*, veterinary use; *WB*, whole body.
*Absolute *in vitro* potencies expressed in micrograms per milliliter were transformed into *in vitro* potencies (without units) relative to the values for extract 9 used as reference (potency of 1 *[boldface]*). One thousand was added for curves that did not reach the IC30 value.

out to be major allergens in the current study in a subpopulation of highly allergic subjects (CAP class ≥ 3). Four patients with cockroach allergy did not recognize the 8 allergens tested, which indicates that additional cockroach allergens still exist. Proteomic studies have reported new cockroach allergens in Asia.[16,18] The relevance of these potential allergens in a US population is currently being investigated.

In this study the expansion to a set of 8 cockroach allergens significantly improved the correlation between cockroach-specific IgE levels and the sum of allergen-specific IgE levels from an r value of 0.78 ($P < .001$) when calculated by using only 3 allergens to an r value of 0.88 ($P < .001$, n = 23) for 8 allergens. These results indicate that the 8 allergens account for a large proportion of cockroach sensitization.

Endotoxin, which has been reported to influence sensitization,[23] was also found in variable amounts in the extracts, but the effect of endotoxin in extracts during immunotherapy remains to be investigated. The current study is part of a larger one that analyzed potencies of the same German cockroach extracts at the T-cell level. Endotoxin levels were measured because they could be relevant for the T-cell *in vitro* potency of the extracts, but it is not relevant for the B-cell potency reported here. Levels of flagellin, a Toll-like receptor 5 ligand from bacterial flagella that is used as adjuvant in various vaccines,[24] were also measured and found to be undetectable in the extracts (data not shown).

The *in vitro* potency of 12 extracts was measured by using IgE antibody-binding inhibition assays in 5 subjects with an arbitrarily selected commercial extract as a reference. This approach is different from the one used by the US Food and Drug Administration, which measures biological potencies based on skin prick test responses, as previously reported for cockroach.[20,25] In one of these studies, highly variable extract biological potencies (up to 78-fold) were described using pooled allergic sera (n = 16).[20] In the other study, relative potencies were measured in a competitive ELISA by using a reference standard and pooled sera, which were found to parallel the biological potency of three extracts analyzed.[25] Here the goal was to investigate whether relative *in vitro* potencies would be different among different subjects. Therefore, relative extract potencies were obtained from experiments performed with individual instead of pooled plasma from 5 different subjects.

Inhibition assays have one limitation based on the fact that potencies are dependent on a reference extract, and it is not possible to know what proteins (assuming most of the allergens) are adsorbed from the crude allergen extract onto the wells. Nevertheless, measuring relative potencies was an advantage because it allowed to consistently and easily compare the 12 extracts for each subject. In general, there were large differences per subject in extract potencies of up to more than 3 orders of magnitude, with commercial extracts being the most potent for each subject. The differences observed here are presumably caused by differences in content of the extracts. However, it was not surprising to find a lack of correlation for 3 of the 5 patients tested between extract potency and the content of the only 3 allergens that were measured, presumably because these 3 allergens do not account for the total IgE reactivity to cockroach. In contrast, 2 of the patients with high IgE levels to Bla g 1 showed the best significant correlations between extract potencies and Bla g 1 content. These results indicate that potency depends on levels of sensitization to allergens that are present in the extracts.

Inhibition assays were performed with individual subjects to assess the importance of the unique subject's sensitization profile on extract potency. It was difficult to find a high number of subjects with plasma that showed a large enough window of IgE antibody binding inhibition to perform the assays. Nevertheless, the 5 subjects tested were sufficient to see that variability of extract potency is also dependent on the subject. Each extract showed different relative extract potencies per subject, presumably because of different subject sensitization profiles. In fact, 2 groups of subjects were identified, each containing individuals who showed the best correlations of extract potencies by pairs ($r > 0.9$, $P < .001$). The pairs of subjects showing the best potency correlations had a tendency to have a better correlation of allergen-specific IgE. For the 5 patients tested for extract potencies, most extracts (the ones with higher allergen content) showed significant correlations between extract potencies and the sum of specific IgE levels to 8 (but not to 3) allergens. These results indicate that the subject's levels of sensitization to a large

panel of cockroach allergens is also a determinant of extract potency.

Overall, these results show that cockroach extract potency depends on a combination of 2 factors: (1) extract allergen composition and (2) allergen-specific IgE sensitization profile. Both are relevant for the selection of potent extracts to be used for immunotherapy and the design and interpretation of data from immunotherapy trials. For example, if a subject is only allergic to Bla g 1 and Bla g 4, it would be preferable that these 2 allergens were present in the extract used to treat this subject. Cockroach allergy differs from cat allergy in that most patients with cat allergy are sensitized to Fel d 1, which covers most IgE reactivity to cat. Identification of new major allergens in a population with cockroach allergy, as shown here, also needs to be taken into consideration for B-cell component analysis (allergen-specific antibody analysis) and for design and data interpretation in immunotherapy trials. The unique IgE reactivity profile per patient and lack of immunodominant allergens in a population with cockroach allergy makes it difficult to select appropriate extracts for immunotherapy that contain and cover the allergens relevant to each subject. This variability in allergen-specific reactivity profiles within a cohort with cockroach allergy has also been observed at the T-cell level in a parallel study using the same extracts.[26]

The current study underscores the need for evaluating cockroach extracts to be used in clinical trials and avoiding the current limitation of measuring only Bla g 1 and Bla g 2 levels in the doses administered. Future approaches might include the use of standardized mixes of purified natural or recombinant allergens with known allergen concentrations to which the patients are sensitized. Alternatively, crude cockroach extracts should carefully balance the source material to include nymphs, adults of both sexes, egg cases, and feces because different proteins are expressed at different life stages.

The conclusions in this study set the stage for the imminent cockroach allergy trials that will be conducted by the Inner-City Asthma Consortium. The main recommendation is for the design of immunotherapy using extracts that are optimized for the presence of allergens relevant to the subject's sensitization profile.

Clinical implications: Allergen content, which is variable in nonstandardized German cockroach extracts, and IgE sensitization profiles to a new expanded set of cockroach allergens determine *in vitro* extract potency for IgE reactivity.

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METHODS

Measurement of Bla g 1, Bla g 2, and Bla g 5 levels in cockroach extracts determined by using ELISA

Levels of Bla g 1, Bla g 2 and Bla g 5 in cockroach extracts were measured by means of ELISA. Purified recombinant Bla g 1, purified natural Bla g 2, and purified recombinant Bla g 5, all prepared in 1% BSA/50% glycerol/PBS, pH 7.4, were used as standards. Concentrations of the 3 purified allergen standards were determined by using amino acid analysis. Antibody pairs used in each assay are specified as follows: Bla g 1, 10A6/pAb; Bla g 2, 7C11/pAb; and Bla g 5, 17B12/pAb. Each extract was analyzed at 2 starting concentrations (1:10 and 1:1000) with 11 doubling dilutions across the plate and tested in triplicate for each dilution. Assay development was performed as for IgE antibody binding inhibition assays.

Endotoxin measurement

Each cockroach extract was analyzed for endotoxin content by using the chromogenic *Limulus* Amoebocyte Lysate assay (Lonza). The extract was analyzed at a starting concentration of 1:100 with three 1:5 dilutions up to 1:12,500, and results were reported in endotoxin units per milliliter of extract.

Expression, purification, and quantification of 8 recombinant cockroach allergens

The German cockroach allergens Bla g 2 (GenBank accession code U28863), Bla g 4 (U40767), and Bla g 6 (DQ279092) were constitutively expressed in *P pastoris* by using pGAPZα vectors, whereas Bla g 1 (AF072219), Bla g 9 (DQ358231), and Bla g 11 (DQ355516) were expressed using pPICZ/pPICZα vectors by means of methanol induction for 48 to 96 hours. Per a 7 (isoform Per a 7.0102; AF106961) was expressed by using methanol induction with the *Pichia* pPIC9 vector. Per a 7.0102 is highly cross-reactive and shares 98.6% identity with Bla g 7.0101. Bla g 5 (U92412) was expressed in *E coli* by using the pET-21a vector.

Bla g 1, Bla g 2, and Per a 7 were purified by means of specific antibody affinity chromatography. Bla g 4 was purified by using phenol-sepharose chromatography, Bla g 5 by using glutathione-S-transferase affinity chromatography, and Bla g 6 by using ion exchange and size exclusion chromatography. Bla g 9 and Bla g 11 were purified by means of metal affinity chromatography. Bla g 1, Per a 7, Bla g 9, and Bla g 11 were quantified by using the Advanced Protein Assay (Cytoskeleton), whereas Bla g 2, Bla g 4, Bla g 5, and Bla g 6 were quantified by using OD_{280}.

Biotinylation and optimization of biotinylation

EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific, Rockford, Ill) was added to 2 mg of each allergen at a 10- to 20-fold molar excess, depending on the number of lysine residues in the sequence, and incubated for 30 minutes. The biotinylated mix was put over a prewashed Zeba Desalt Spin Column (Thermo Scientific) 2 times, and the concentration was determined after biotinylation with APA.

Quantification of biotinylation was carried out with a Quant Tag Biotin Kit (Vector Laboratories, Burlingame, Calif). Samples were tested in triplicate against a known biotin standard curve to determine the number of biotins per allergen molecule. The optimal number of biotins per molecule was considered between 2 and 6.

Optimization of biotinylated allergen loaded to the streptavidin ImmunoCAP

Streptavidin ImmunoCAPs (Thermo Fisher Scientific, Portage, Mich) were loaded and incubated on a Phadia 100 with biotinylated allergen at the following amounts: 0.5, 1, 2, 5, and 10 μg/CAP. Two different human plasma samples from subjects allergic to the allergen (that had been originally tested for IgE binding to 3 μg/CAP) were selected for optimization experiments. IgE binding to the allergen-loaded CAPs by the 2 selected plasma was measured in a Phadia 250, according to the manufacturer's instructions (Thermo Fisher Scientific). Results were plotted to select optimal amount of biotinylated allergen to be loaded to the streptavidin ImmunoCAPs.

Measurement of IgE antibody levels by using ImmunoCAP

Biotinylated allergen was loaded and incubated on streptavidin ImmunoCAPs with the Phadia 100. The ImmunoCAPs were transferred to the Phadia 250, where measurements of IgE antibody binding were performed according to the manufacturer's instructions. Cockroach-specific IgE antibody binding was measured by using commercially available CAPs loaded with cockroach extract (i6 ImmunoCAPs supplied by Thermo Fisher Scientific). Most subjects (except 3) did not have IgE antibodies against at least 1 of the 8 allergens. These negative IgE values served as negative controls and indicated that positive values were allergen specific. Also, sera from patients without cockroach allergy ($n = 10$) were used as negative controls. These sera were negative at a cutoff of 0.1 kU_A/L in in-house streptavidin ImmunoCAPs not loaded with allergen, and mostly negative (except 3 low values out of 70) in ImmunoCAPs loaded with each of 7 cockroach allergens (data not shown). Regardless, a conservative cutoff of 0.35 kU_A/L was chosen to make sure that IgE prevalences would not be overestimated because of low values between 0.1 and 0.35 kU_A/L. In addition, and to assess possible nonspecific IgE binding, all plasma samples were run in streptavidin CAPs not loaded with allergen. Crossreactive carbohydrate determinants (CCD) binding to the allergens was not expected for most allergens because only 3 had N-glycosylation sites (2 in Bla g 2 and 1 in Bla g 4 and Bla g 11). For the 3 plasma samples that reacted to all 8 allergens, a test was run with a CCD inhibitor for these 3 allergens to assess possible IgE binding to the carbohydrates (as explained below).

IgE binding to CCD present in allergens that contain N-glycosylation was assessed by adding a CCD inhibitor to the plasma before measuring IgE binding to rBla g 2 and rBla g 4 (rBla g 1 was used as a negative control because it lacks N-glycosylation sites). The lyophilized RIDA CCD-Inhibitor (R-Biopharm AG, Darmstadt, Germany) was dissolved in sterile H_2O, with vortexing. The CCD-Inhibitor (or sterile H_2O for corresponding sample without inhibitor) was added at a dilution of 1:41 to sample plasma and incubated on an orbital shaker for 1 hour at room temperature. Samples were run on the Phadia 250 immediately after incubation.

Specificity of the allergen-specific IgE measurements by using ImmunoCAP

IgE measurements for the component analysis were allergen specific. This was proved as follows: (1) all plasma had IgE antibody levels to streptavidin CAPs (not loaded with allergen) that were under the cutoff of 0.35 kU_A/L (except one with a low value of 0.59 kU_A/L that was used to correct the allergen-specific levels by subtracting 0.24 kU_A/L, the difference between 0.59 and the cutoff); (2) most plasma did not bind 1 or more of the 8 allergens (except 3 plasma that bound the 8 allergens), and these measurements acted as negative controls; and (3) the only 3 plasma with positive IgE values to all 8 allergens tested showed no difference in IgE levels in the presence versus absence of CCD inhibitor for 2 allergens with N-glycosylation (Bla g 2 and Bla g 4) and without as a control (Bla g 1).

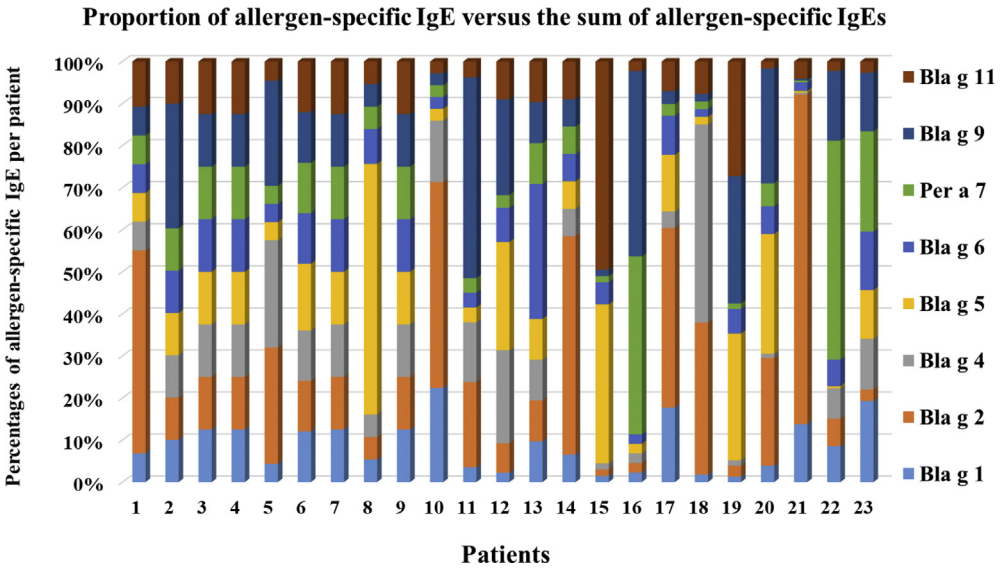

FIG E1. Proportion of allergen-specific IgE levels versus the sum of 8 allergen-specific IgE levels in subjects with cockroach allergy (n = 23). This figure represents a normalization to percentages of data from Fig 2, including data of less than the 0.35 kU_A/L threshold (as 0.35 values).

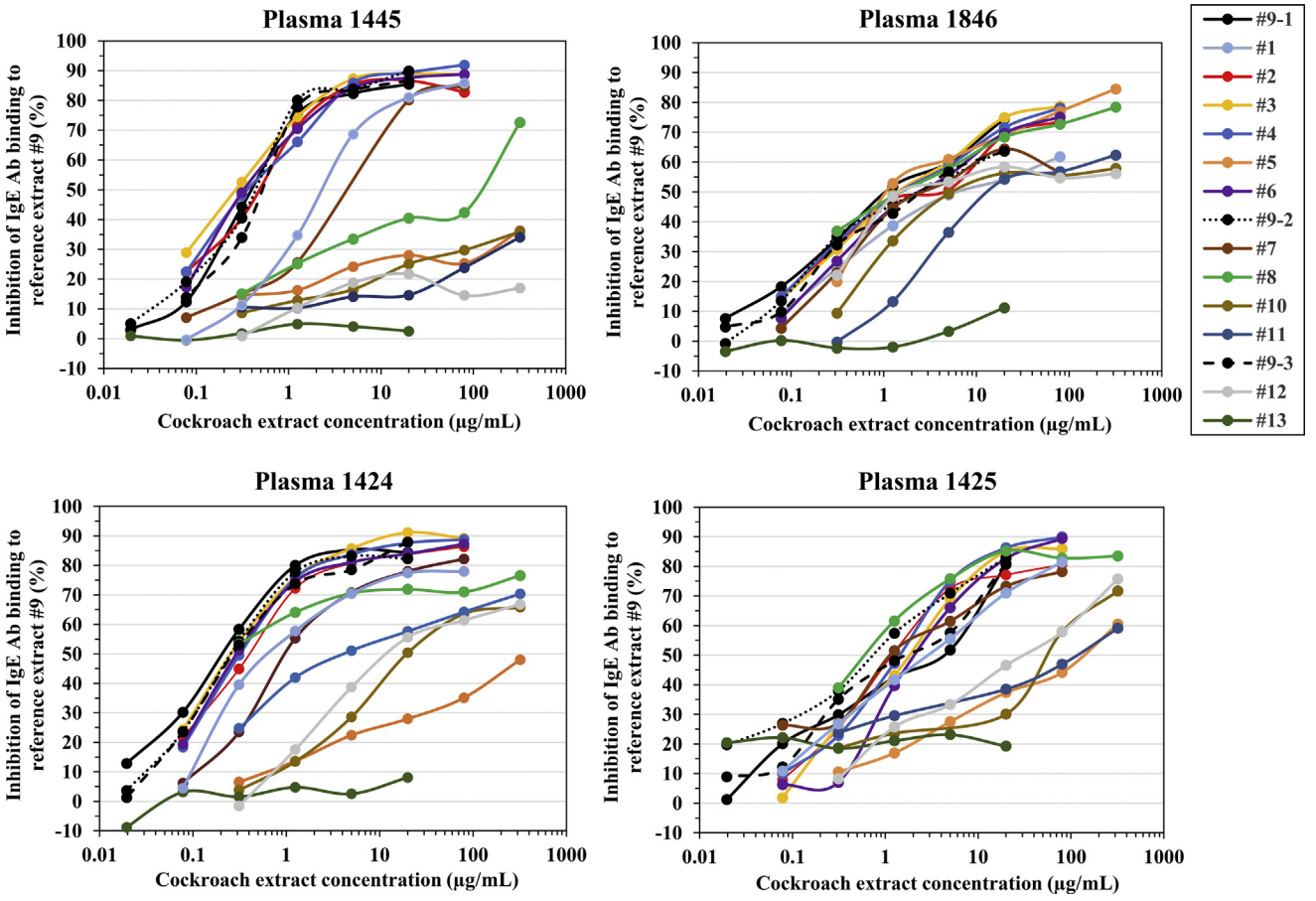

FIG E2. Inhibition assays to determine *in vitro* potencies for IgE reactivity of extracts in 5 patients with cockroach allergy. Results are from 4 representative subjects out of 5 analyzed. Plots show means with SDs of duplicates. Reference curves for each of the 3 plates used in the experiment are 9-1, 9-2, and 9-3. *Ab*, Antibody.

TABLE E1. Cockroach-specific IgE, skin prick test wheal size, age, and sex of the study cohort of 23 subjects sensitized to cockroach

	Information on donors with cockroach allergy				
Subject no.	**Donor ID**	**Cockroach-specific IgE (kU_A/L)**	**SPT wheal size (mm)**	**Age (y)**	**Sex**
1	1441	0.91	0	47	M
2	1439	0.94	8	32	M
3	2196	1.23	ND	53	M
4	1367	1.27	6	37	F
5	1006	1.32	0	44	M
6	1365	2.01	4.5	49	F
7	1665	2.24	ND	26	F
8	2083	3.41	ND	23	F
9	1231	4.47	3.5	23	F
10	1257	4.78	6	37	F
11	1864	4.82	ND	37	F
12	1406	5.30	4.5	41	F
13	1175	7.27	3	43	F
14	1437	8.32	9	38	F
15	2210	10.13	ND	28	F
16	1398	10.50	8.5	30	F
17	1229	12.20	9	49	M
18	1446	17.30	7.5	50	M
19	1425	36.00	7	39	F
20	1424	45.20	10	30	F
21	1228	56.50	7	54	F
22	1277	66.20	10	53	M
23	1445	76.20	9	32	F
	Average	16.46		38.9	69.6% F
	SD	22.76		9.8	30.4% M

F, Female; *M*, male; *ND*, not determined.

TABLE E2. Correlations between 13 extract potencies (IC30) for IgE reactivity from paired subjects among the 5 individuals tested

	1445†	**1277†**	**1424‡**	**1425‡**	**1864‡**
1445†	1				
1277†	**0.947**	1			
1424‡	0.550	0.502	1		
1425‡	0.447	0.436	**0.940**	1	
1864‡	0.426	0.372	**0.941**	**0.989**	1

Dagger (†) or double dagger (‡) of the donor ID denotes the 2 groups of subjects identified. Within each group, the correlations between pairs of subjects were significant ($P < .001$ *[boldface]*).

TABLE E3. Correlations between 8 allergen-specific IgE from paired subjects among the 5 individuals tested

	1445†	**1277†**	**1424‡**	**1425‡**	**1864‡**
1445†	1				
1277†	0.693	1			
1424‡	0.249	0.170	1		
1425‡	0.346	0.325	0.499	1	
1864‡	0.136	0.010	0.559	0.331	1

Dagger (†) or double dagger (‡) of the donor ID denotes the 2 groups of subjects identified according to results in Table E2. The best correlation was between subjects 1445 and 1277 ($r = 0.693$, $P = .057$).

TABLE E4. Correlations between extract potencies for IgE reactivity and allergen content of the 12 extracts for 5 patients with cockroach allergy

	Correlations between extract potencies (IC30) and extract allergen content (μg/mL)						Allergen-specific IgE (kU_A/L)		
	Bla g 1 + Bla g 2 + Bla g 5		Bla g 1		Bla g 2				
Patients	*r*	*P* value	*r*	*P* value	*r*	*P* value	Bla g 1	Bla g 2	Bla g 5
1445	0.779	.0028*	0.871	.0002*	0.469	.1245	14.06	1.98	8.45
1277	0.773	.0032*	0.838	.0007*	0.527	.0784	7.56	5.82	0.45
1424	0.026	.9358	0.123	.7035	0.193	.5481	1.34	8.83	9.81
1425	0.306	.3337	0.265	.4052	0.357	.2540	<0.35	0.7	8.14
1864	0.375	.2302	0.476	.1181	0.098	.7613	<0.35	2.03	<0.35

*$P < .05$ indicates significance.

TABLE E5. Correlations for each extract between German cockroach extract potencies and the sum of allergen-specific IgE levels of the 5 subjects analyzed

	Correlations between extract potencies (IC30) and:				
	Sum of IgE levels to 3 allergens* (kU_A/L)		Sum of IgE levels to 8 allergens (kU_A/L)		Three allergens/protein concentration per extract (μg/mg)
Extract	*R*	*P* value	*r*	*P* value	
1	0.395	.5108	**0.943**	**.0162**	**10.32**
2	0.024	.9678	0.382	.5254	4.20
3	0.621	.2635	**0.896**	**.0397**	**4.87**
4	0.139	.8241	0.608	.2764	4.56
5	0.696	.1921	**0.977**	**.0041**	**12.33**
6	0.407	.4969	0.765	.1319	4.77
7	0.452	.4449	**0.972**	**.0056**	**10.32**
8	0.037	.9521	**0.746**	**.0466**	**8.16**
9	NA	NA	NA	NA	10.27
10	0.275	.6540	**0.914**	**.0297**	**26.02**
11	0.552	.3343	**0.945**	**.0154**	**29.33**
12	0.553	.3341	**0.956**	**.0109**	**53.86**
13	NA	NA	NA	NA	0

P values of less than .05 indicate significance. Data associated with a significant correlation are shown in boldface.
NA, Not applicable (extract 9 is the reference extract, and extract 13 is the negative control).
*Bla g 1, Bla g 2, and Bla g 5.