Mediator release assay for assessment of biological potency of German cockroach allergen extracts

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Background: Cockroach is an important allergen in inner-city asthma. The diagnosis and treatment of cockroach allergy has been impeded by the lack of standardized cockroach extracts.
Objective: We investigated the utility of a mediator release assay based on rat basophil leukemia (RBL) cells for comparing the potency of German cockroach extracts.
Methods: RBL cells (line 2H3) transfected with human FcεRI were passively sensitized with sera from subjects with cockroach allergy and stimulated with serial dilutions of 3 commercial cockroach extracts (1:10 weight/volume). In addition, the in-house prepared extract was tested in separate experiments with pooled sera that produced optimal performance in the RBL assay. N-hexosaminidase release (NHR) was used as a marker of RBL cell degranulation and was examined in relation to the intradermal skin test (ID_{50}EAL) and serum cockroach-specific and total IgE levels.
Results: The median cockroach-specific IgE concentration in 60 subjects was 0.72 kU_A/L (interquartile range, 0.35-2.97 kU_A/L); 19 sera (responders) produced a minimum 10% NHR to more than 1 extract. Responders had higher median cockroach-specific IgE (7.4 vs 1.0 kU_A/L) and total IgE (429 vs 300 kU/L) levels than nonresponders. Ranking of extract potency was consistent between the mediator release assay and the ID_{50}EAL. For the in-house prepared cockroach extract, the dose-response curves were shifted according to the concentration of the extract. NHR was reproducible between different experiments by using pooled sera.
Conclusion: The mediator release assay measures biologic potency and correlates with the ID_{50}EAL. It should be further evaluated to determine whether it could be used to replace intradermal skin test titration for assessing the potency of cockroach extract. (J Allergy Clin Immunol 2009;123:949-55.)

Key words: *Cockroach, cockroach allergy, cockroach extract, rat basophil leukemia cells, passive sensitization, mediator release, mediator release assay, extract potency, ID_{50}EAL, biologic potency, cockroach extract standardization*

German cockroach *(Blatella germanica)* is an important allergen for asthmatic subjects in urban areas of the United States.[1-3] Exposure to high levels of the major cockroach allergen Bla g 1 is associated with asthma morbidity in cockroach-sensitized children.[1] Cockroach mitigation is difficult; sensitization has been detected in the setting of low household cockroach allergen levels.[4-6] Specific immunotherapy is a proved treatment for environmental allergens.[7] Immunotherapy with cockroach allergen is an attractive option for cockroach-associated respiratory disease, but it requires well-characterized, potent allergenic extracts. The current US Food and Drug Administration–approved method of standardization of allergenic extract potency is based on *in vivo* skin test titration (the ID_{50}EAL).[8] This methodology is uncomfortable, is time and labor intensive, and carries the risk of systemic reaction.

A previous publication from our group assessed the biologic potency of cockroach extracts by using 3 methods: the ID_{50}EAL, *in vitro* competition ELISA with human and rabbit sera, and specific allergen content (Bla g 1, Bla g 2, and Bla g 5).[9] The purpose of this study was to determine the utility of a functional *in vitro* mediator release assay based on rat basophil leukemia (RBL) cells transfected with the human high-affinity IgE receptor type 1 and passively sensitized with human IgE for assessment of German cockroach extract's biologic potency and to compare this assay with the ID_{50}EAL.

METHODS

Serum samples

Sera were obtained from participants with cockroach allergy (age, 18-65 years) in the Cockroach Allergen Standardization Evaluation study. Subjects self-reported perennial respiratory symptoms (rhinitis or asthma) and had a positive skin prick test response with a commercial German cockroach extract

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Abbreviations used
ED_{50}: Extract concentration that induced half maximal response
$ID_{50}EAL$: Intradermal skin test method of allergen potency determination
NHR: N-hexosaminidase release
RBL: Rat basophil leukemia

at 1:10 wt/vol (extract C).[9] All enrolled subjects underwent evaluation with intradermal skin test titration (the $ID_{50}EAL$).

Cockroach allergen

Three cockroach extracts, A, B (1:20 wt/vol), and C (1:10 wt/vol), were purchased from major manufacturers in the United States.[9] Cockroach powder for extract E was purchased from the manufacturer of extract C and was mixed in house. *In vitro* testing of the allergen extracts was performed as previously published.[9]

Cockroach-specific IgE antibody concentration measurement

Serum cockroach-specific IgE levels were measured with UniCAP (Phadia, Portage, Mich); the lower limit of detection is 0.35 kU_A/L, and the upper limit is 100 kU_A/L. The allergen extract used to produce the UniCAP sorbent was not one of those tested in this study. Specific IgE levels to recombinant cockroach allergens (ie, rBla g 1, rBla g 2, rBla g 4, and rBla g 5) in sera with detectable cockroach-specific IgE were measured with the streptavidin CAP assay (Indoor Biotechnologies Ltd, Charlottesville, Va).[9]

Mediator release assay

The RBL-2H3 cell line transfected with human FcεRI and the protocol for the assay were kindly provided by Dr S. Vieths.[10] RBL cells were cultured in Eagle minimal essential medium, 15% RPMI with 10% FCS, and G418 sulfate (pH 7.4, in 20 mmol/L HEPES; ACROS, Morris Plains, NJ). RBL cells were incubated with serum at a final dilution of 1:40 at 37°C in 5% CO_2 for 18 to 20 hours in 96-well tissue-culture plates (BD Falcon; BD, Bedford, Mass). Sensitized cells were stimulated with 100 μL per well of the dilutions of cockroach extracts in a release buffer with 50% D_2O (ACROS) at 37°C in 5% CO_2 for 1 hour. Rabbit IgG anti-human polyclonal IgE (Bethyl Laboratories, Inc, Montgomery, Tex) was used as a positive control for IgE-mediated degranulation. Thirty microliters of supernatant was gently mixed with 50 μL of P-nitrophenyl-N-acetyl-β-D-glucosaminide solution (pH 4.5; Sigma-Aldrich, St Louis, Mo) to determine N-hexosaminidase release (NHR). After 1 hour at 37°C in 5% CO_2, 100 μL of 0.2 mol/L glycine solution (pH 10.7) was added, and absorbance was measured at 405 nm. RBL cells were lysed with 1% Triton X-100 (Sigma Chemical Co, St Louis, Mo) for total release. Results were expressed as the percentage of release from cells sensitized with individual serum minus spontaneous release (with buffer), which was then divided by total release. Responders were arbitrarily defined as those sera that produced greater than 10% NHR to at least 1 cockroach extract.

$ID_{50}EAL$ method

Intradermal skin test titration in 60 subjects was performed according to the protocol described by Turkeltaub et al.[8,9] In this test biologic potency is estimated by determining the extract dilution at which the sum of perpendicular erythema diameters is 50 mm. Briefly, serial 3-fold dilutions of cockroach extracts A, B, and C (starting from the lowest concentration) were injected intradermally on the back. Erythema was measured at 15 minutes, and the sum of erythema diameters was calculated by adding the longest possible diameter across the area of erythema and the shorter diameter perpendicular to and through the midpoint of the longest diameter. The objective was to establish a dose-response curve in each subject, with the sum of erythema diameters ranging from 0 to 125 mm and containing at least 4 valid data points that bracketed 50 mm.

Statistical analysis

The results of the RBL assay and skin test data were analyzed with the drc package[11] with R software.[12] The data were fit by using 4-parameter logistic models.[11] The parameters in the model estimate the minimum and maximum responses, the extract concentration that induced half maximal response (ED_{50}), and the relative slope of ED_{50}. Results from the fitted model were used to compare the maximum and minimum responses, as well as to compare the relative fit of different extracts. Each individual's data were fit with a single model, producing a different curve for each extract to account for subject-specific variation across the extracts. Models were fit separately for skin test data and RBL assay data. Additionally, models were fit by combining 6 subjects' responses, producing 1 set of curves for the skin test results and a second set based on the RBL data. Interpolation on the fitted models from the skin test data was used to determine $ID_{50}EAL$ values. The potency of the extracts using the skin test data was calculated as bioequivalent allergy units[13] with the following formula: $BAU/mL = 10^5 \times 3^{(D50-14)}$.

Potency measures evaluated with the RBL assay data were calculated by using ED_{50} values. ED_{50} values were obtained from the parameter estimates of the models, and the potency measured was defined as $1/ED_{50}$. Plotting the curves produced by the modeling allowed for comparison of the different extracts.

The study was approved by the institutional review boards of the participating institutions, and informed consent was obtained before subject enrollment.

RESULTS

Serum cockroach-specific IgE antibody concentrations

Sixty serum samples from subjects evaluated with the $ID_{50}EAL$ method were screened[9]; of those, 40 had cockroach-specific IgE antibody levels of greater than 0.35 kU_A/L, with a median level of 0.72 kU_A/L (interquartile range, 0.35-2.97 kU_A/L). Comparisons between responders and nonresponders are shown in Table I.

Mediator release assay

In our initial experiments, we noted a significant non–IgE-mediated release with cockroach extract alone (without the presence of human IgE) when RBL cells were stimulated with higher concentrations (first through third 3-fold dilutions and 10^{-2} dilution) of cockroach extracts. In subsequent experiments we considered the fourth 3-fold dilution and 10^{-3} dilution to be representative of the highest IgE-mediated release. Of note, we have not observed this non–IgE-mediated (presumably pharmacologic) effect with high concentrations of other allergens, such as birch pollen, dog dander, cow's milk, egg white, and shrimp extracts (not shown).

Serum was considered to be responsive if NHR to at least 1 extract (A, B, or C) was greater than 10% at the fourth 3-fold dilution. There were 19 responders and 41 nonresponders. Cockroach-specific IgE was necessary but not sufficient for good performance in the mediator release assay. None of the sera with cockroach IgE levels of less than 0.35 kU_A/L produced greater than 10% NHR; among the sera with detectable cockroach IgE, only 19 (46%) of 40 sera produced greater than 10% NHR. Responders had significantly higher levels of cockroach–specific IgE, total serum IgE, and specific/total IgE ratio (Table I). Among the 18 responders with detectable IgE levels to recombinant cockroach allergens, 3 (16.7%), 9 (50%), 5 (27.8%), and 11 (61%) had

TABLE I. Comparison between responders and nonresponders with detectable serum cockroach-specific IgE levels (>0.35 kUA/L)*

	Responders† (n = 19)	Nonresponders (n = 21)	*P* value
Cockroach-specific IgE (kU_A/L)	7.40 (2.12-27.91)	1.00 (0.5-1.9)	.00017
Total IgE (KU/L)	429.0 (237.46-877.49)	300.0 (107-504)	.031
Cockroach-specific/total IgE	0.017 (0.01-0.04)	0.005 (0-0.01)	.01

*Data are presented as medians (interquartile ranges).
†Responders were defined as those whose sera produced greater than 10% NHR.

TABLE II. Spearman rank order correlation coefficient between NHR at the fourth 3-fold dilution and IgE levels

	Extract A	*P* value	Extract B	*P* value	Extract C	*P* value
Cockroach-specific IgE	0.61	.0068	0.56	.015	0.79	.0001
Total IgE	0.13	.6004	0.38	.1145	0.35	.1562
Cockroach-specific/total IgE	0.65	.0034	0.46	.0056	0.73	<.0001
rBla g 1 IgE*	0.43	.0745	0.44	.0677	0.52	.0272
rBla g 2 IgE*	0.26	.2992	0.44	.0662	0.36	.1416
rBlag 4 IgE*	0.33	.1879	0.5	.0334	0.47	.0511
rBla g 5 IgE*	0.45	.0639	0.49	.0399	0.46	.0571

*Although we found a positive correlation between specific IgE directed against the recombinant cockroach allergens rBla g 4 and rBla g 5 for extract B and rBla g 1 for extract C, the significance of these observations is unclear because there was very little detectable Bla g 5 in extract C, and the content of Bla g 4 was not measured. There were only 3 subjects with detectable rBla g 1 IgE.[9]

detectable IgE to rBla g 1, r Bla g 2, rBla g 4, and rBla g 5, respectively, versus 0 (0%), 2 (15.4%), 3 (23%), and 3 (23%) among the 13 nonresponders. There was a significant positive correlation between NHR and cockroach-specific IgE level and the ratio of cockroach-specific/total IgE for all extracts (Table II).

Comparison between NHR and sum of erythema diameters in responders

The mediator release assay and ID_{50}EAL dose-response curves for cockroach extracts A, B, and C are shown side by side for 3 representative subjects (Fig 1). For 16 (84%) of the 19 responders, the 3 extracts were ranked in identical order in both assays (see Fig E1 in this article's Online Repository at www.jacionline.org).

We chose sera from 6 RBL responders with optimal performance in the RBL assay (defined as the highest NHR at the fourth 3-fold dilution for all 3 extracts) to calculate the biologic potency of cockroach extracts and for comparison with the biologic potency based on the ID_{50}EAL (Fig 2 and Table III). Extract B was the most potent and extract A was the least potent in both assays. Extract potency ranking with these 2 methods was also consistent with the ranking by means of competition ELISA with rabbit and human sera and specific allergen content (Bla g 1, Bla g 2, and Bla g 5).[9]

Mediator release assay measures cockroach allergen potency

In-house prepared cockroach extract E was mixed at 1:2.5, 1:10, 1:25, and 1:250 wt/vol. Serial 10-fold dilutions were tested with a serum pool (cockroach-specific IgE level, 28.3 kU_A/L) made of equal parts of sera from 7 responders, as well as serum from an individual subject (cockroach-specific IgE level, 19.6 kU_A/L). The dose-response curves had the same slopes, but ED_{50} differed by about a factor of 5. Half-maximal release occurred at the higher dilution of the 1:2.5 extract than the 1:25 and 1:250 extracts, demonstrating that the performance of the cockroach extract in the mediator release assay reflects the concentration of cockroach source material (Fig 3).

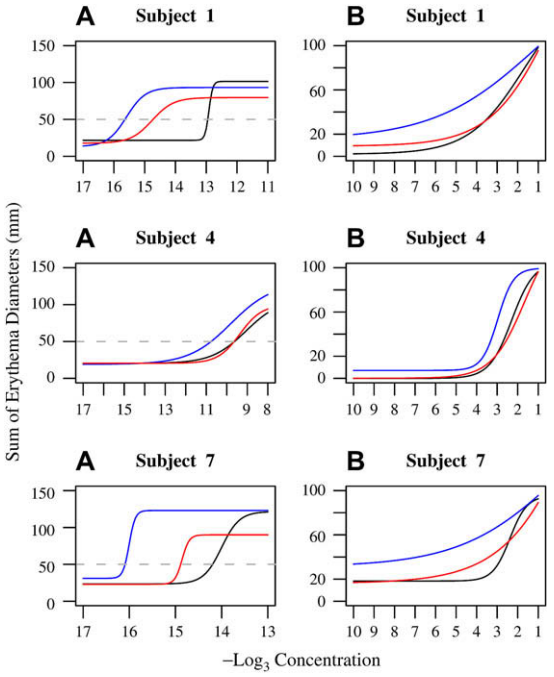

FIG 1. Side-by-side comparison of 3 cockroach extracts in skin tests **(A)** and mediator release assays **(B)**. Data are shown for 3 representative subjects (subjects 1, 4, and 7). Dose-response curves were generated with serial dilutions of cockroach extract A *(black)*, extract B *(blue)*, and extract C *(red)*. Data for all 19 responders are included in Fig E1 in this article's Online Repository at www.jacionline.org.

Mediator release assay is reproducible

Identical experiments were performed on 4 different days. RBL cells were sensitized with sera from 6 individual subjects and

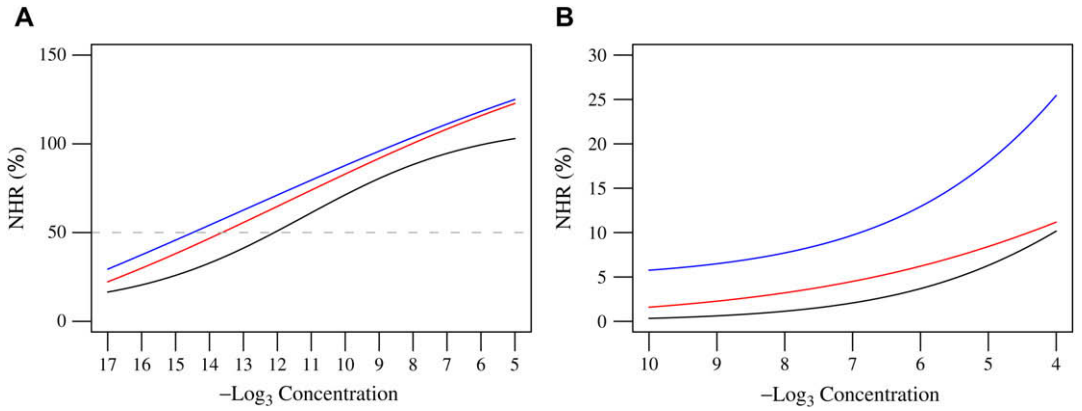

FIG 2. Comparison of dose-response curves generated with serial dilutions of 3 cockroach extracts in 6 representative subjects (responders in the RBL assay) by skin test **(A)** and mediator release assay **(B)**. *Black lines* represent extract A, *blue lines* represent extract B, and *red lines* represent extract C; *symbols* represent average values for 6 subjects.

TABLE III. Comparison of biologic potency of cockroach extracts calculated based on NHR* and ID_{50}EAL in 6 responders†

	Extract A	Extract B	Extract C
NHR			
ED_{50} raw	0.008723	0.040477	0.032529
ED_{50} on $\log_3$ scale	2.589	1.192	1.391
Biologic potency units	0.386	0.839	0.719
ID_{50}EAL			
D_{50} raw	1.70E-06	1.21E-07	3.13E-07
D_{50} on $\log_3$ scale	12.091	14.497	13.632
Biologic potency units	12282.98	172559.1	66754.09

*Maximum release was measured at the fourth 3-fold dilution.
†Six responders in the RBL assay were subjects with the greatest NHR to the fourth 3-fold dilution to all 3 cockroach extracts.

stimulated with serial 10-fold dilutions of extract E at 1:2.5, 1:10, and 1:25 wt/vol (Fig 4). Individual variances were stable within each extract dilution. In each case 88.9% to 90.1% of the variance was attributed to differences between individuals, and the remaining portion represented the within-individual variance.

DISCUSSION

We report, for the first time, the comparison between the ID_{50}EAL methods of allergen extract standardization and an *in vitro* mediator release assay based on the RBL cell line transfected with human IgE receptor type 1 and passively sensitized with human IgE. We demonstrate that although an *in vitro* mediator release assay measures the potency of cockroach extract with accuracy comparable with that of the ID_{50}EAL, it has significant advantages over the ID_{50}EAL for allergen standardization. The *in vitro* mediator release assay is less laborious and less expensive than the ID_{50}EAL, and it has no risk for a systemic allergic reaction. Furthermore, it can be performed with sera selected for optimal performance and stored frozen for prolonged periods of time, resulting in less variability compared with the ID_{50}EAL.

Accurate assessment of the cockroach extract's biologic potency is crucial for diagnosis and immunotherapy. Allergen extracts have inherent biovariability because of collection methods, storage, processing of raw materials, and extraction and manufacturing techniques.[14] Standardization based on the concentration of the source material (expressed as weight per volume) or the protein content in the source material (expressed as protein nitrogen units) is not reliable because extracts from natural sources have varying protein patterns over time.[15] In addition, there is little correlation between these designations and biologic measures of allergen potency.[16,17] In the United States cockroach extracts vary in protein content, electrophoretic banding patterns, relative potency, and Bla g 2 levels.[18]

Immunochemical assays, such as IgE inhibition tests and allergen detection by means of Western blotting, are subject to epitope alteration/destruction caused by adsorption of allergens to solid matrices, are susceptible to the competition from IgG of the same specificity, and often do not correlate well with data obtained by means of skin testing.[19-22] Measurement of major allergens depends on the use of polyclonal antibodies or mAbs and does not include measurement of biologic activity.[23,24] Although this approach is favored in Europe, standardization based on a selected major allergen might neglect the minor allergens of clinical importance.[24] Furthermore, it requires identification of clearly immunodominant allergens, which has not been accomplished in the case of cockroach allergy.[9,25]

In the United States standardization of extracts is based on a functional *in vivo* ID_{50}EAL assay.[8] This method requires serial intradermal skin testing (titration) and is labor intensive, expensive, and unpleasant to the tested individuals. In addition to the potential variability associated with the skin test (eg, different batch of extract, amount of allergen injected, depth of the injection, and location of the test on the back), it is usually not feasible to bring back the original subjects for repeat testing year after year, which further limits the reproducibility of the ID_{50}EAL.

Functional assays based on histamine release from human donors' basophils and basophil activation (expression of CD63 and CD203c) detected by means of flow cytometry have been evaluated in research studies.[26-28] Practical application of basophil activation assays has similar limitations as skin testing, namely the availability of basophil donors and time constraints (basophil assays have to be processed promptly after blood collection).

We chose to evaluate an *in vitro* mediator release assay using the RBL cell line RBL-2H3 transfected with human high-affinity

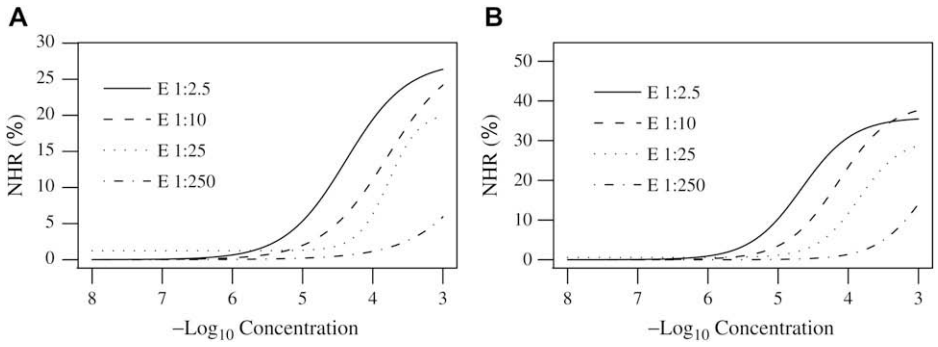

FIG 3. Dose-response curves generated with serial dilutions of 4 starting concentrations of extract E (1:2.5, 1:10, 1:25, and 1:250 wt/vol) and a 7-subject serum pool **(A)** and an individual subject's serum **(B)**. NHR to cockroach extract at 10^{-2} dilution without serum reached a similar value to the NHR in the presence of serum; therefore this data point was not displayed.

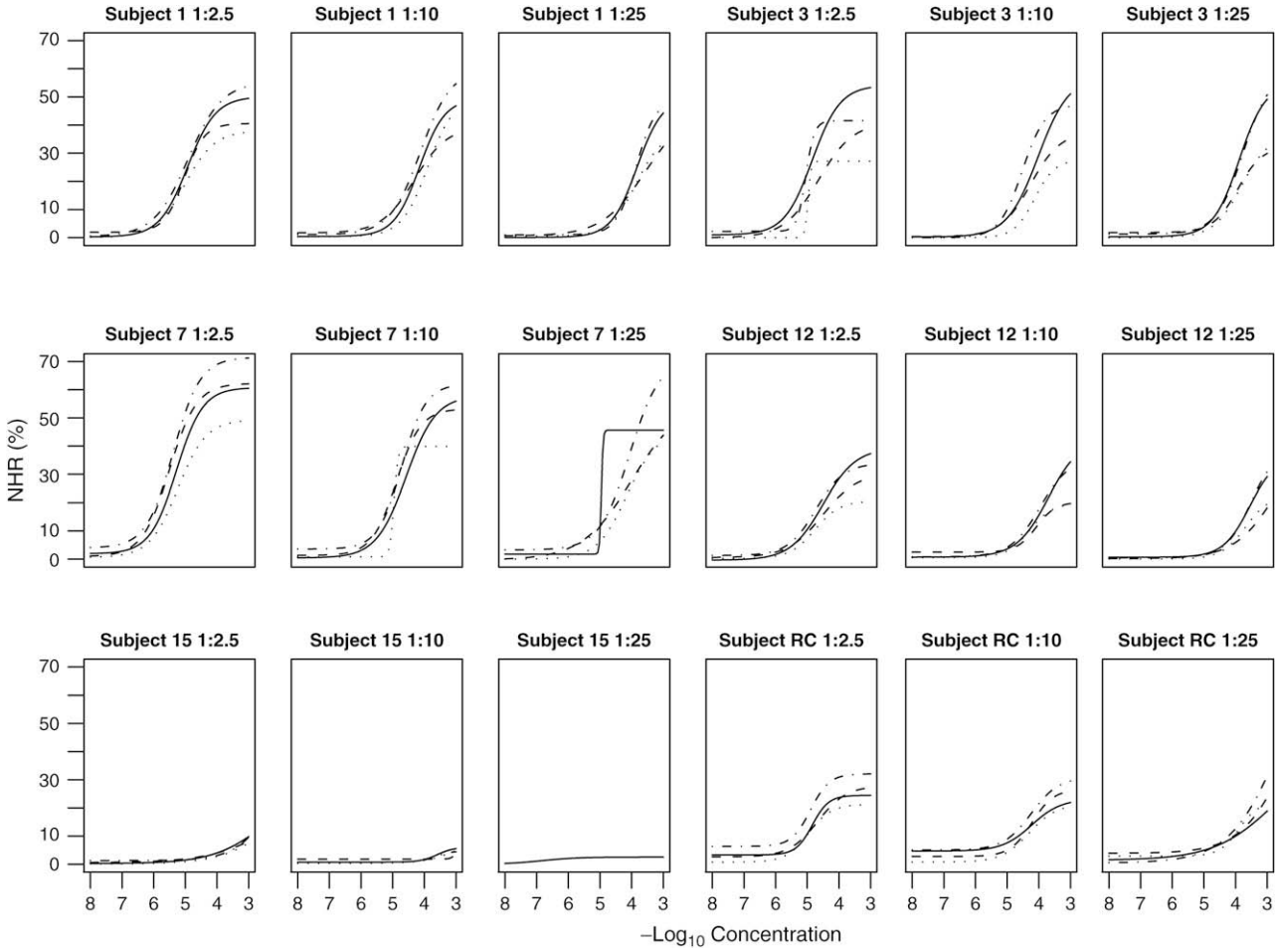

FIG 4. Results of 4 identical experiments repeated on 4 different days. Dose-response curves were generated with serial 10-fold dilutions of 3 starting concentrations of extract E (1:2.5, 1:10, and 1:25, wt/vol) and sera from 6 subjects. For subject 15 at 1:25 extract concentration, we were not able to fit the results of 3 of the experiments with the 4-parameter logistic model.

FcεRI and passively sensitized with human IgE. RBL-2H3 cells are stable transfectants that can be maintained in culture for long periods of time.[10,29] RBL cells have the functional characteristics of mast cells in regard to IgE-induced mediator release, but in contrast to wild-type mast cells, cross-linking of IgG through the Fc receptor on RBL-2H3 cells does not induce any detectable mediator release. The RBL cell–based mediator release assay was reported to detect very low levels of allergen; for example, the cat allergen Fel d 1 produced detectable release at the level of 10 pg/mL.[30] The RBL cell–based assay was previously reported to

estimate the potency of tree and grass pollen, house dust mite, cat dander, and peanut extracts.[10,19,30] The assay was sensitive and had a low coefficient of variation, 15% for NHR and 25% for ED_{50}, which made it suitable for detecting differences between batches of allergen extracts.[19]

In the RBL model the effect of interaction between the allergen and allergen-specific IgE is demonstrated through mediator release. The mediator release assay was dependent on the presence of cockroach-specific IgE and on the concentration of cockroach allergen. We found a significant positive correlation between NHR and (1) cockroach-specific IgE levels and (2) the ratio of cockroach-specific IgE to total IgE. These observations suggest that both parameters might be important in preselecting sera for evaluation in the mediator release assay. Finally, we demonstrated that the mediator release assay was reproducible on different days and thus might be used for standardization of different batches of extract over periods of time.

Our group has recently reported that the ID_{50}EAL method provided a valid estimate of cockroach extract biologic potency that was in agreement with the relative potency determination with an *in vitro* competition ELISA by using rabbit and human sera and specific content of Bla g 1, Bla g 2, and Bla g 5.[9] We demonstrated that the *in vitro* mediator release assay paralleled the results of ID_{50}EAL skin testing for the potency of individual German cockroach extracts in adult subjects sensitized to cockroach. We chose to express biologic potency as $1/ED_{50}$ rather than to establish a more precise unit because of the small number of serum samples with optimal performance in the mediator release assay. This limitation reflected the selection strategy, which was based on the results of skin prick test reactivity to cockroach extract and resulted in a cohort that included many subjects with undetectable or low levels of cockroach-specific IgE. In future studies selection of serum samples should be based on detection of moderate-to-high levels of cockroach-specific IgE and prescreening of serum samples to identify "high responders."

As in many bioassays, there are subjects (and sera) with detectable levels of cockroach-specific IgE who do not induce good mediator release in the mediator release assay (nonresponders). For instance, about 10% of atopic donors have nonresponsive basophils in the histamine release assay.[31] Discordance between the magnitude of mediator release in the *in vitro* assay and serum allergen-specific IgE levels has been reported previously.[10,32,33] Lack of responsiveness in the mediator release assay despite detectable allergen-specific IgE might be due to factors that are not accounted for in the immunochemical tests. These factors potentially include (1) low affinity and avidity of IgE,[34] (2) effect of the steric site of allergen recognition,[35,36] (3) availability of free (not bound in complexes) IgE,[37,38] and (4) low ratio of cockroach-specific IgE to total serum IgE (dilution effect).[33,39] However, once "high-performing" sera are identified, they can be stored for long periods of time (estimated stability of IgE immunoreactivity in frozen serum samples stored at temperatures $<-20°C$ is about 5 years; R. G. Hamilton, personal communication) and used to standardize different batches of allergenic extracts. This would minimize the variability from performing skin test titration in different allergic individuals over time and would also be less laborious and less expensive.

We observed a consistent non–IgE-mediated release on stimulation of nonsensitized RBL cells (without added human serum) at high concentrations of cockroach extract (present in the experiments performed on different days and with different extracts), which has not been reported with other allergen extracts (pollen, cat dander, peanut, and dust mite). Although none of the known cockroach allergens are proteolytically active, protease activity has been observed in the whole-body extract of German cockroach and might potentially contribute to the non–IgE-mediated effect.[24,40-42] We were able to reduce this non–IgE-mediated effect by incubating cockroach extract with protease inhibitors before exposing RBL cells to cockroach extract without human serum (data not shown). Additional reasons for the non–IgE-induced mediator release might be the presence of endotoxin, (1,3)-β-d-glucans, or both.[43] The mechanism of the non–IgE-mediated effect of cockroach extract will need to be further explored.

In conclusion, the *in vitro* mediator release assay using RBL cells transfected with the human high-affinity IgE receptor type 1 and passively sensitized with human IgE estimates the biologic potency of cockroach extract with similar accuracy as the ID_{50}EAL method. Considering a number of significant advantages over the laborious ID_{50}EAL, the *in vitro* mediator release assay should be further evaluated for application in the standardization of commercial cockroach allergen extracts for diagnosis and immunotherapy.

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Clinical Implications: Mediator release assay with RBL cells and human serum measures cockroach extract potency and deserves evaluation as a safer, more efficient method of cockroach extract standardization for diagnosis and immunotherapy.

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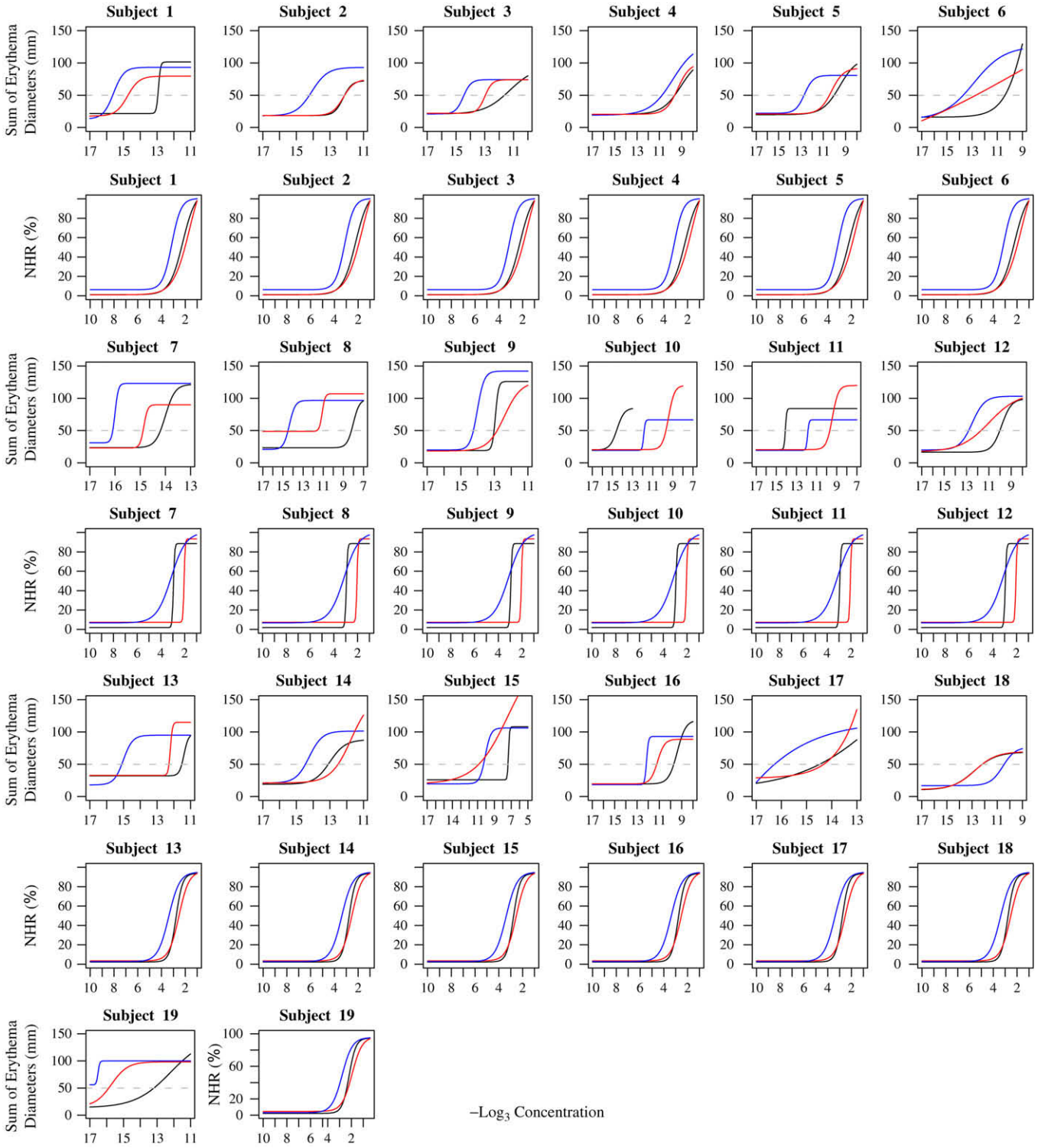

FIG E1. Side-by-side comparison of 3 cockroach extracts in skin tests **(A)** and mediator release assays **(B)**. Data are shown for 19 responders. Dose-response curves were generated with serial 3-fold dilutions of 3 cockroach extracts. *Black lines* represent extract A, *blue lines* represent extract B, and *red lines* represent extract C.