Published in final edited form as:

J Allergy Clin Immunol. 2009 April ; 123(4): 949–955.e1. doi:10.1016/j.jaci.2009.01.070.

Mediator Release Assay for Assessment of Biological Potency of German Cockroach Allergen Extracts

A. Nowak-Wegrzyn, MD^a, R. Bencharitiwong, BS^a, J. Schwarz, PhD, MHSc^b, G. David, PhD, MHSc^b, P. Eggleston, MD^c, P. J. Gergen, MD, MPH^d, A. H. Liu, MD^e, J. A. Pongratic, MD^f, S. Sarpong, MD^g, and H. A. Sampson, MD^a

^a Mount Sinai School of Medicine, New York, NY, USA

^b Rho, Inc., Chapel Hill, NC, USA

^c Johns Hopkins University School of Medicine, Baltimore, MD, USA

^d DAIT, NIAID, NIH, Bethesda MD, USA

^e National Jewish Medical and Research Center, and University of Colorado Health Science Center, Denver, CO, USA

^f Children's Memorial Hospital, Chicago, IL, USA

^g Howard University, Washington DC, USA

Abstract

Background—Cockroach is an important allergen in inner-city asthma. 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 potency of German cockroach extracts.

Methods—RBL cells (line 2H3) transfected with human Fcε receptor-1 were passively sensitized with sera from cockroach-allergic subjects 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 gave optimal performance in the RBL assay. N-hexosaminidase release (NHR) was used as a marker of RBL degranulation and was examined in relation to intradermal skin test (ID50EAL), serum cockroach-specific and total IgE levels.

Results—The median cockroach-specific IgE concentration in 60 subjects was 0.72 kUA/L (inter-quartile range; 0.35, 2.97); 19 sera (“responders”) produced a minimum 10% NHR to > 1 extract. Responders had higher median cockroach-specific IgE (7.4 vs. 1.0 kUA/L) and total IgE (429 vs. 300 kU/L) than non-responders. Ranking of extract potency was consistent between mediator release assay and the ID50EAL. 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 using pooled sera.

Corresponding author: Anna Nowak-Wegrzyn, M.D. Mount Sinai School of Medicine, Department of Pediatrics, Box 1198, One Gustave L. Levy Place, New York, NY 10029, Phone: 212-241-5548, Fax: 212-426-1902, E-mail: anna.nowak-wegrzyn@mssm.edu.

Conflict of Interest Statement

All authors report no conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conclusion—The mediator release assay measures biological potency and correlates with ID₅₀EAL. 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.

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

Keywords

cockroach; cockroach allergy; cockroach extract; RBL; passive sensitization; mediator release; mediator release assay; extract potency; ID₅₀EAL; biological potency; cockroach extract standardization

INTRODUCTION

German cockroach (*Blattella germanica*) is an important allergen for asthmatics in urban areas in the US. (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; 5) (6) Specific immunotherapy is a proven 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 Food and Drug Administration-approved method of standardization of allergenic extract potency is based on the *in vivo* skin test titration (ID₅₀EAL). (8) This methodology is uncomfortable, time and labor intensive, and carries the risk of systemic reaction.

A previous publication from our group assessed biological potency of cockroach extracts by three methods: ID₅₀EAL, *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 cells (RBLs) transfected with human high affinity IgE receptor type 1 and passively sensitized with human IgE for assessment of German cockroach extract biological potency and to compare this assay with ID₅₀EAL.

METHODS

Serum samples

Sera were obtained from cockroach-allergic participants (age 18–65 years) in the Cockroach Allergen Standardization Evaluation study. Subjects self-reported perennial respiratory symptoms (rhinitis or asthma) and had a positive prick skin test with a commercial German cockroach extract at 1:10 weight/volume (extract C). (9) All enrolled subjects underwent evaluation with intradermal skin test titration (ID₅₀EAL).

Cockroach allergen

Three cockroach extracts A, B (1:20 weight/volume), and C (1:10 weight/volume) were purchased from major manufacturers in the USA. (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 was measured with UniCAP (Phadia, USA); lower limit of detection is 0.35 kU_A/L, 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 to recombinant cockroach allergens: rBla g 1, rBla g 2, rBla g 4, and rBla g 5 in sera with detectable cockroach-specific IgE was measured with treptavidin CAP assay (INDOOR Biotechnologies Ltd., Charlottesville, VA).(9)

Mediator release assay

RBL-2H3 cell line transfected with human Fcε receptor type-1 and the protocol for the assay were kindly provided by Dr. S. Vieths. (10) RBL cells were cultured in Eagle's MEM, 15% RPMI with 10% FCS and G418 sulfate (pH 7.4, in 20 mM Hepes; ACROS, New Jersey, USA). RBLs were incubated with serum at a final dilution of 1:40 at 37°C in 5% CO₂ for 18–20 hours in 96-well tissue-culture plates (BD Falcon™, Bedford, MA, USA). Sensitized cells were stimulated with 100 µl/well of the dilutions of cockroach extracts in a release buffer with 50% D₂O (ACROS, New Jersey, USA) at 37°C in 5% CO₂ for 1 hour. Rabbit IgG anti-human polyclonal IgE (Bethyl Laboratories, Inc., Montgomery, TX, USA) was used as a positive control for IgE-mediated degranulation. To determine N-hexosaminidase release (NHR), 30 µl 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, USA). After 1 hour at 37°C in 5% CO₂, 100 µl of 0.2 M glycine solution (pH 10.7) was added and absorbance at 405 nm was measured. RBLs were lysed with 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA) 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 ≥ 10% NHR to at least 1 cockroach extract.

ID₅₀EAL method

Intradermal skin test titration in 60 subjects was performed according to the protocol described by Turkeltaub et al. (8;9) In this test, the biological potency is estimated by determining the extract dilution at which the sum of perpendicular erythema diameters is 50 mm. Briefly, serial three-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 four valid data points that bracketed 50 mm.

Statistical analysis

The results of the RBL assay and skin test data were analyzed using the *drc* package(11) in R. (12) The data were fit by four parameter logistic models. (11) The parameters in the model estimate the minimum and maximum responses, ED₅₀ (extract concentration that induced half maximal response), and relative slope of the ED₅₀. 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 combining 6 subjects' responses producing one 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

ID₅₀EAL values. Potency of the extracts using the skin test data was calculated as bioequivalent allergy units (BAU)(13) using the formula

$$\text{BAU/mL} = 10^5 \times 3^{(\text{ID}_{50} - 14)}$$

Potency measures evaluated using the RBL assay data were calculated using ED₅₀ values. ED₅₀ values were obtained from the parameter estimates of the models, and the potency measured was defined as 1/ED₅₀. 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 the informed consent was obtained prior to subject enrollment.

RESULTS

Serum cockroach-specific IgE antibody concentrations

Sixty serum samples from subjects evaluated with ID₅₀EAL method were screened;(9) of those, 40 had cockroach-specific IgE antibody ≥ 0.35 kU_A/L with a median level (IQR) of 0.72 kU_A/L (0.35, 2.97). Comparisons between responders and non-responders 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 presence of human IgE) when RBL cells were stimulated with higher concentrations (1st-3rd 3-fold dilutions and 10⁻² dilution) of cockroach extracts. In subsequent experiments we considered the 4th 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 one extract (A, B, or C) was $\geq 10\%$ at the 4th 3-fold dilution. There were 19 responders and 41 non-responders. Cockroach-specific IgE was necessary but not sufficient for good performance in the mediator release assay. None of the sera with cockroach-IgE < 0.35 kU_A/L gave $\geq 10\%$ NHR; among the sera with detectable cockroach-IgE, only nineteen (19/40; 46%) sera gave $\geq 10\%$ 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 detectable IgE to rBla g 1, rBla g 2, rBla g 4 and rBla g 5 respectively, versus 0 (0%), 2 (15.4%), 3 (23%), and 3 (23%) among the 13 non-responders. There was a significant positive correlation between the NHR and cockroach-specific IgE level, and 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 ID₅₀EAL dose response curves for cockroach extracts A, B, and C are shown side by side for three representative subjects (Fig 1). For 16 of the 19 responders (84%), the three extracts were ranked in identical order in both assays (Fig E1).

We chose sera from six RBL responders with optimal performance in the RBL assay (defined as highest NHR at 4th 3-fold dilution for all three extracts) to calculate biological potency of cockroach extracts and to compare with the biological potency based on ID₅₀EAL (Fig 2, Table

III). Extract B was the most potent and extract A was the least potent in both assays. Extract potency ranking by these two methods was also consistent with the ranking by competition ELISA using 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 weight/volume. Serial 10-fold dilutions were tested with a serum pool (cockroach-specific IgE was 28.3 kU_A/L) made of equal parts of sera from 7 responders as well as with serum from an individual subject (cockroach-specific IgE was 19.6 kU_A/L). The dose response curves had the same slopes but ED₅₀ differed by about a factor of 5. Half maximal release occurred at the higher dilution of the 1:2.5 extract than 1:25 and than 1:250, demonstrating that the performance of the cockroach extract in the mediator release assay reflects the concentration of cockroach source material. (Fig 3)

Mediator release assay is reproducible

Identical experiments were performed on 4 different days. RBL cells were sensitized with serum from 6 individual subjects and stimulated with serial 10-fold dilutions of extract E at 1:2.5, 1:10, and 1:25 weight/volume. (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 intradermal skin test titration method (ID₅₀EAL) 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 while an *in vitro* mediator release assay measures potency of cockroach extract with comparable accuracy as ID₅₀EAL, it has significant advantages over ID₅₀EAL for allergen standardization. The *in vitro* mediator release assay is less laborious and less expensive than ID₅₀EAL, 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 as compared to ID₅₀EAL.

Accurate assessment of cockroach extract biological potency is crucial for diagnosis and immunotherapy. Allergen extracts have inherent bio-variability due to 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, wt/vol) or the protein content in the source material (expressed as protein nitrogen units, PNU) 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 biological measures of allergen potency. (16;17) In the US, 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 Western blotting are subject to epitope alteration/destruction due to 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 skin testing. (19–22) Measurement of major allergens depends on the use of polyclonal or monoclonal antibodies and does not measure biological activity. (23) (24) Although this approach is favored in Europe; standardization based on a selected major allergen may neglect the minor allergens of clinical importance. (24) Furthermore, it requires

identification of clearly immuno-dominant allergens, which has not been accomplished in the case of cockroach allergy. (9;25)

In the US, standardization of extracts is based on a functional *in vivo* ID₅₀EAL assay. (8) This method requires serial intradermal skin test (titration), is labor-intensive, expensive, and unpleasant to the tested individuals. In addition to the potential variability associated with the skin test (e.g., different batch of extract, amount of allergen injected, depth of the injection, 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 ID₅₀EAL.

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

We chose to evaluate an *in vitro* mediator release assay utilizing the RBL cell line RBL-2H3 transfected with human high affinity Fcε receptor type 1 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 *via* Fc-receptor on RBL-2H3 cells fails to induce any detectable mediator release. RBL-based mediator release assay was reported to detect very low levels of allergen, e.g, cat allergen Fel d 1 gave detectable release at the level of 10 pg/ml. (30) The RBL-based assay was previously reported to estimate 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 ED₅₀, 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 via mediator release. 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) ratio of cockroach-specific IgE to total IgE. These observations suggest that both parameters may be important in pre-selecting 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 utilized for standardization of different batches of extract over periods of time.

Our group has recently reported that the ID₅₀EAL method provided a valid estimate of cockroach extract biological potency that was in agreement with the relative potency determination with *in vitro* competition ELISA 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 ID₅₀EAL skin testing for the potency of individual German cockroach extracts in adult subjects sensitized to cockroach. We chose to express biological potency as 1/ED₅₀ rather than to establish a more precise unit of biological potency due to 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 the prick skin 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 upon detection of moderate to high levels of cockroach-specific IgE and pre-screening of serum samples to identify “high responders”.

As in many bioassays, there are subjects (sera) with detectable levels of cockroach-specific IgE that do not induce good mediator release in the mediator release assay (non-responders).

For instance, about 10% of atopic donors have non-responsive basophils in the histamine release assay.(31) Discordance between the magnitude of mediator release in the *in vitro* assay and serum allergen-specific IgE 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 lower than -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 upon stimulation of non-sensitized RBL cells (without added human serum) at high concentrations of cockroach extracts (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, 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 prior to exposing RBL cells to cockroach extract without human serum (not shown). Additional reasons for the non-IgE-induced mediator release might be the presence of endotoxin and/ or (1,3)- β -d-glucans. (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 utilizing RBL cells transfected with human high affinity IgE receptor type-1 and passively sensitized with human IgE estimates biological potency of cockroach extract with similar accuracy as intradermal skin test titration ID₅₀EAL. Considering a number of significant advantages over the laborious ID₅₀EAL, the *in vitro* mediator release assay should be further evaluated for application in the standardization of commercial cockroach allergen extracts for diagnosis and immunotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This project has been funded in whole or in part with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, under Contracts number N01-AI-25496 and N01-AI-25482. A. Nowak-Wegrzyn is supported in part by NIH NIAID AI 059318.

We thank Dr. Wayne Shreffler for helpful discussions and Russell Castro for excellent technical assistance.

Abbreviations used

ED ₅₀	Extract concentration that induced half maximal response
FCS	Fetal calf serum

ID ₅₀ EAL	Intradermal skin test method of allergen potency determination
IQR	Inter-quartile range
NHR	N-hexosaminidase release
RBL	Rat basophil leukemia cells

References

1. Rosenstreich DL, Eggleston P, Kattan M, Baker D, Slavin RG, Gergen P, et al. The role of cockroach allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma. *N Engl J Med* 1997;336(19):1356–63. [PubMed: 9134876]
2. Chew GL, Perzanowski MS, Canfield SM, Goldstein IF, Mellins RB, Hoepner LA, et al. Cockroach allergen levels and associations with cockroach-specific IgE. *J Allergy Clin Immunol* 2008;121(1):240–5. [PubMed: 17936887]
3. Wisnivesky JP, Sampson H, Berns S, Kattan M, Halm EA. Lack of association between indoor allergen sensitization and asthma morbidity in inner-city adults. *J Allergy Clin Immunol* 2007;120(1):113–20. [PubMed: 17531297]
4. Eggleston PA, Rosenstreich D, Lynn H, Gergen P, Baker D, Kattan M, et al. Relationship of indoor allergen exposure to skin test sensitivity in inner-city children with asthma. *J Allergy Clin Immunol* 1998;102(4 Pt 1):563–70. [PubMed: 9802363]
5. Eggleston PA, Wood RA, Rand C, Nixon WJ, Chen PH, Lusk P. Removal of cockroach allergen from inner-city homes. *J Allergy Clin Immunol* 1999;104(4 Pt 1):842–6. [PubMed: 10518830]
6. Gergen PJ, Mortimer KM, Eggleston PA, Rosenstreich D, Mitchell H, Ownby D, et al. Results of the National Cooperative Inner-City Asthma Study (NCICAS) environmental intervention to reduce cockroach allergen exposure in inner-city homes. *J Allergy Clin Immunol* 1999;103(3 Pt 1):501–6. [PubMed: 10069886]
7. Nelson HS. Allergen immunotherapy: where is it now? *J Allergy Clin Immunol* 2007;119(4):769–79. [PubMed: 17337297]
8. Turkeltaub PC, Rastogi SC, Baer H, Anderson MC, Norman PS. A standardized quantitative skin-test assay of allergen potency and stability: studies on the allergen dose-response curve and effect of wheal, erythema, and patient selection on assay results. *J Allergy Clin Immunol* 1982;70(5):343–52. [PubMed: 7130550]
9. Slater JE, James R, Pongracic JA, Liu AH, Sarpong S, Sampson HA, et al. Biological potency of German cockroach allergen extracts determined in an inner city population. *Clin Exp Allergy* 2007;37(7):1033–9. [PubMed: 17581196]
10. Vogel L, Luttkopf D, Hatahet L, Hausteine D, Vieths S. Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* 2005;60(8):1021–8. [PubMed: 15969682]
11. Ritz C, Streibig JC. Bioassay analysis using R. *J Statist Software* 2005;12(5):1–22.
12. R Development Core team. R Foundation for Statistical Computing. Vienna, Austria: 2007. R: A language and environment for statistical computing. URL <http://www.R-project.org>
13. James R, Mitchell H, Gergen PJ, Eggleston PA, Slater JE. Analyzing of ID50EAL data for the standardization of German cockroach allergen extracts in the U.S. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M* 2006;(95):117–27. [PubMed: 17393731]
14. Morrow KS, Slater JE. Regulatory aspects of allergen vaccines in the US. *Clin Rev Allergy Immunol* 2001;21(2–3):141–52. [PubMed: 11725602]
15. AAAAI. The use of standardized allergen extracts. *J Allergy Clin Immunol* 1997;99:583–6. [PubMed: 9155821]
16. Baer H, Godfrey H, Maloney CJ, Norman PS, Lichtenstein LM. The potency and antigen E content of commercially prepared ragweed extracts. *J Allergy* 1970;45(6):347–54. [PubMed: 4192485]

17. Baer H, Maloney CJ, Norman PS, Marsh DG. The potency and Group I antigen content of six commercially prepared grass pollen extracts. *J Allergy Clin Immunol* 1974;54(3):157–64. [PubMed: 4854999]
18. Patterson ML, Slater JE. Characterization and comparison of commercially available German and American cockroach allergen extracts. *Clin Exp Allergy* 2002;32(5):721–7. [PubMed: 11994096]
19. Kaul S, Luttkopf D, Kastner B, Vogel L, Holtz G, Vieths S, et al. Mediator release assays based on human or murine immunoglobulin E in allergen standardization. *Clin Exp Allergy* 2007;37(1):141–50. [PubMed: 17210052]
20. Kadooka Y, Idota T, Gunji H, Shimatani M, Kawakami H, Dosako S, et al. A method for measuring specific IgE in sera by direct ELISA without interference by IgG competition or IgG autoantibodies to IgE. *Int Arch Allergy Immunol* 2000;122(4):264–9. [PubMed: 10971117]
21. Gruber C, Buck D, Wahn U, Niggemann B. Is there a role for immunoblots in the diagnosis of latex allergy? Intermethod comparison of in vitro and in vivo IgE assays in spina bifida patients. *Allergy* 2000;55(5):476–83. [PubMed: 10843429]
22. Ricci G, Capelli M, Miniero R, Menna G, Zannarini L, Dillon P, et al. A comparison of different allergometric tests, skin prick test, Pharmacia UniCAP and ADVIA Centaur, for diagnosis of allergic diseases in children. *Allergy* 2003;58(1):38–45. [PubMed: 12580805]
23. van RR. Indoor allergens: relevance of major allergen measurements and standardization. *J Allergy Clin Immunol* 2007;119(2):270–7. [PubMed: 17166574]
24. van RR, Chapman MD, Ferreira F, Vieths S, Bryan D, Cromwell O, et al. The CREATE project: development of certified reference materials for allergenic products and validation of methods for their quantification. *Allergy* 2008;63(3):310–26. [PubMed: 18269676]
25. Satinover SM, Reefer AJ, Pomes A, Chapman MD, Platts-Mills TA, Woodfolk JA. Specific IgE and IgG antibody-binding patterns to recombinant cockroach allergens. *J Allergy Clin Immunol* 2005;115(4):803–9. [PubMed: 15806002]
26. Ostergaard PA, Ebbsen F, Nolte H, Skov PS. Basophil histamine release in the diagnosis of house dust mite and dander allergy of asthmatic children. Comparison between prick test, RAST, basophil histamine release and bronchial provocation. *Allergy* 1990;45(3):231–5. [PubMed: 2183646]
27. Ebo DG, Hagendorens MM, Bridts CH, Schuerwegh AJ, De Clerck LS, Stevens WJ. Flow cytometric analysis of in vitro activated basophils, specific IgE and skin tests in the diagnosis of pollen-associated food allergy. *Cytometry B Clin Cytom* 2005;64(1):28–33. [PubMed: 15668987]
28. Shreffler WG. Evaluation of basophil activation in food allergy: present and future applications. *Curr Opin Allergy Clin Immunol* 2006;6(3):226–33. [PubMed: 16670519]
29. Hoffmann A, Jamin A, Foetisch K, May S, Aulepp H, Haustein D, et al. Determination of the allergenic activity of birch pollen and apple prick test solutions by measurement of beta-hexosaminidase release from RBL-2H3 cells. Comparison with classical methods in allergen standardization. *Allergy* 1999;54(5):446–54. [PubMed: 10380775]
30. Hoffmann A, Vogel L, Scheurer S, Vieths S, Haustein D. Potency determination of allergenic extracts using mediator release of rat basophil leukemia cells. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M* 1997;(91):203–8. [PubMed: 9383911]
31. Bernstein IL, Vijay HM, Perelmutter L. Non-responder basophils in highly ragweed-sensitive subjects. *Int Arch Allergy Appl Immunol* 1977;55(1–6):215–6. [PubMed: 73510]
32. Marchand F, Mecheri S, Guilloux L, Iannascoli B, Weyer A, Blank U. Human serum IgE-mediated mast cell degranulation shows poor correlation to allergen-specific IgE content. *Allergy* 2003;58(10):1037–43. [PubMed: 14510723]
33. Palmer GW, Dibbern DA Jr, Burks AW, Bannon GA, Bock SA, Porterfield HS, et al. Comparative potency of Ara h 1 and Ara h 2 in immunochemical and functional assays of allergenicity. *Clin Immunol* 2005;115(3):302–12. [PubMed: 15893698]
34. Mita H, Yasueda H, Akiyama K. Affinity of IgE antibody to antigen influences allergen-induced histamine release. *Clin Exp Allergy* 2000;30(11):1583–9. [PubMed: 11069567]
35. Torigoe C, Inman JK, Metzger H. An unusual mechanism for ligand antagonism. *Science* 1998;281(5376):568–72. [PubMed: 9677201]

36. Ortega E, Schweitzer-Stenner R, Pecht I. Possible orientational constraints determine secretory signals induced by aggregation of IgE receptors on mast cells. *EMBO J* 1988;7(13):4101–9. [PubMed: 2977332]
37. Johansson SG. Anti-IgE antibodies in human serum. *J Allergy Clin Immunol* 1986;77(4):555–7. [PubMed: 2420851]
38. Jarzab J, Gawlik R. Immune complexes IgE/IgG in airborne allergy: increase during pollen season. *J Investig Allergol Clin Immunol* 2000;10(1):24–9.
39. Dibbern DA Jr, Palmer GW, Williams PB, Bock SA, Dreskin SC. RBL cells expressing human Fc epsilon RI are a sensitive tool for exploring functional IgE-allergen interactions: studies with sera from peanut-sensitive patients. *J Immunol Methods* 2003;274(1–2):37–45. [PubMed: 12609531]
40. Arruda LK, Vailes LD, Ferriani VP, Santos AB, Pomes A, Chapman MD. Cockroach allergens and asthma. *J Allergy Clin Immunol* 2001;107(3):419–28. [PubMed: 11240940]
41. Pomes A, Chapman MD, Vailes LD, Blundell TL, Dhanaraj V. Cockroach allergen Bla g 2: structure, function, and implications for allergic sensitization. *Am J Respir Crit Care Med* 2002;165(3):391–7. [PubMed: 11818327]
42. Page K, Hughes VS, Bennett GW, Wong HR. German cockroach proteases regulate matrix metalloproteinase-9 in human bronchial epithelial cells. *Allergy* 2006;61(8):988–95. [PubMed: 16867053]
43. Trivedi B, Valerio C, Slater JE. Endotoxin content of standardized allergen vaccines. *J Allergy Clin Immunol* 2003;111(4):777–83. [PubMed: 12704357]

FIG 1.

Side-by-side comparison of three cockroach extracts in skin test (panel A) and mediator release assays (panel B). Data are shown for three representative 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 the online repository (FIG. E1).

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

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 weight/volume) and a 7-subject serum pool (panel A), and an individual subject's serum (panel 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, weight/volume) and sera from 6 individual subjects. For subject 15 at 1:25 extract concentration, we were not able to fit the results of three of the experiments with the four parameter logistic model.

Table I

Comparison between responders and non-responders with detectable serum cockroach-specific IgE levels (≥ 0.35 kU_A/L)*

	Responders** N=19	Non-responders N=21	P
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 presented are medians (inter-quartile range).

** Responders were defined as those whose sera produced $\geq 10\%$ NHR.

Spearman rank order correlation coefficient between N-hexosaminidase release (NHR) at 4th 3-fold dilution and IgE levels

Table II

	Extract A	P	Extract B	P	Extract C	P
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
rBla g 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 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 since there was very little detectable Bla g 4 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)

Table III

Comparison of biological potency of cockroach extracts calculated based on N-hexosaminidase release* and ID₅₀EAL in 6 responders**

	Extract A	Extract B	Extract C
NHR			
ED ₅₀ raw	0.008723	0.040477	0.032529
ED ₅₀ on log3 scale	2.589	1.192	1.391
Biological potency	0.386	0.839	0.719
ID₅₀EAL			
D ₅₀ raw	1.70E-06	1.21E-07	3.13E-07
D ₅₀ on log3 scale	12.091	14.497	13.632
Biological potency units	12282.98	172559.1	66754.09

* Maximum release was measured at 4th 3-fold dilution.

** Six responders in the RBL assay were subjects with highest NHR to 4th 3-fold dilution to all three cockroach extracts.