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

Anna H. Nowak-Wegrzyn, MD,[a] Ramon Bencharitiwong, BS,[a] John Schwarz, PhD, MHSc,[b] Gloria David, PhD, MHSc,[b] Peyton Eggleston, MD,[c] Peter J. Gergen, MD, MPH,[d] Andrew H. Liu, MD,[e] Jacqueline A. Pongracic, MD,[f] Sampson Sarpong, MD,[g] and Hugh A. Sampson, MD[a] *New York, NY, Chapel Hill, NC, Baltimore and Bethesda, Md, Denver, Colo, Chicago, Ill, and Washington, DC*

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

From [a]Division of Pediatric Allergy and Immunology, Mount Sinai School of Medicine, New York; [b]Rho, Inc, Chapel Hill; [c]Division of Pediatric Allergy and Immunology, Johns Hopkins University School of Medicine, Baltimore; [d]the Division of Allergy, Immunology, and Transplantation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda; [e]Division of Pediatric Allergy and Immunology, National Jewish Medical and Research Center and the University of Colorado Health Science Center, Denver; [f]Division of Pediatric Allergy and Immunology, Children's Memorial Hospital, Chicago; and [g]Department of Pediatrics and Child Health, Howard University, Washington, DC.

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Reprint requests: Anna Nowak-Wegrzyn, MD, Mount Sinai School of Medicine, Department of Pediatrics, Box 1198, One Gustave L. Levy Place, New York, NY 10029. E-mail: anna.nowak-wegrzyn@mssm.edu.

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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

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