

Environmental assay for cockroach allergens

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A sandwich ELISA was developed to measure the concentration of cockroach allergen in the environment. The assay was based on a monospecific rabbit antibody preparation reactive with determinants shared by the important allergens, Per a 1 and Bla g 1, from American and German cockroaches. The sensitivity was 0.2 ng Lowry protein of Per a 1 equivalents per milliliter, corresponding to 1 ng of Per a 1 equivalents per gram of dust (Per a 1 eq/gm). The assay did not react with noncockroach-allergen sources. Dust samples from 73 households in a cockroach-infested area were assayed. The concentration in these samples varied from below detection to 200,000 ng of Per a 1 eq/gm of dust. Three commercial cockroach-allergen extracts all contained the allergen. The assay will be valuable for studies of the clinically relevant cockroach-allergen exposure levels and for assessment of efficacy of allergen-avoidance measures. Furthermore, the assay could be used for sanitary documentation in bakeries, restaurants, etc. (J ALLERGY CLIN IMMUNOL 1991;87:828-34.)

It is well established that cockroaches cause problems for allergic subjects living in cockroach-infested environments.^{1, 2} These insects can cause inhalant allergy³ as well as direct cutaneous sensitivity.^{4, 5} It has been demonstrated that allergic subjects have a higher incidence of positive skin test reactions to cockroach extracts than do nonallergic subjects.² These reactions have been correlated tentatively to degree of exposure. Exposure, however, has not been easy to measure unequivocally and has not been expressed in absolute quantities but rather by terms reflecting the general impression of the individual patient. Cockroaches are not easily enumerated in the patients' environment, and this approach will, in any case, not account for the entire allergen load since feces and small fragments of the insects are likely to remain a long time after an eradication program has been performed. To provide a framework for future studies of the relationship between cockroach-allergen exposure and the development of allergic disease, it was considered necessary to design an assay that would directly measure the concentration of a marker allergen in extracts of environmental dust.

Abbreviations used

Bla g 1:	Major allergen from German cockroach
BSA:	Bovine serum albumin
CB:	Citrate buffer
CIE:	Crossed immunoelectrophoresis
Per a 1:	Major allergen from American cockroach
PNU:	Protein nitrogen unit
WBE:	Whole body extract
HRP:	Horseradish-peroxidase
PBS:	Phosphate-buffered saline

Recently, we identified and purified an important allergen from each of two cockroaches of major domiciliary importance, that is, the American cockroach, *Periplaneta americana*, and the German cockroach, *Blattella germanica*.^{6, 7} The allergens were tentatively named *Per a 1* and *Bla g 1*, respectively, and were demonstrated to be immunologically cross-reactive proteins with a molecular weight of approximately 25,000 in sodium dodecyl sulfate-polyacrylamide electrophoresis. The allergens had a tendency to break down to fragments with an apparent molecular weight of 6000 in sodium dodecyl sulfate-polyacrylamide electrophoresis. The clinical importance of the molecules was demonstrated by skin testing with purified material as well as by IgE blotting to cockroach WBEs.⁷ These allergens have been chosen as marker allergens and were used to develop an ELISA-based environmental assay for cockroach exposure.

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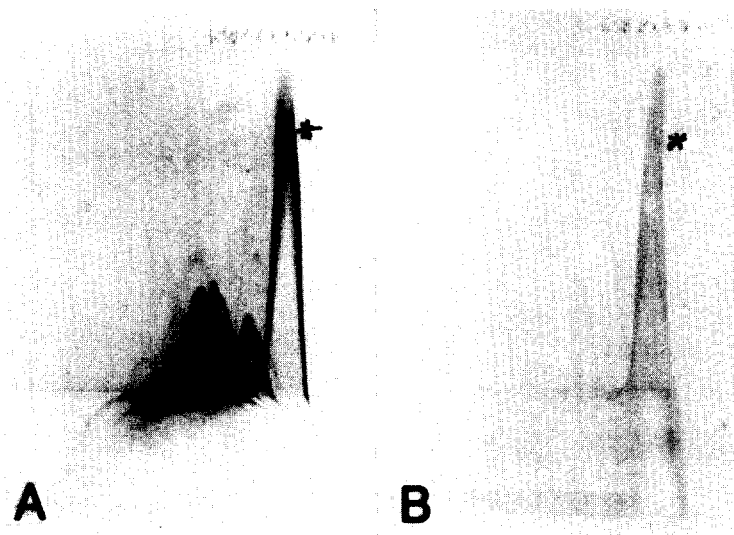


FIG. 1. CIE of WBE of American cockroach; 40 μ g of protein was separated in the first dimension. The second dimension gel contained 150 μ l of rabbit antibody to American cockroach (*Ra anti-Per a*). **A**, The gel was stained with Coomassie brilliant blue R-250 to reveal the full array of precipitated antigens. **B**, The gel was incubated (at 1:250 dilution) with overlay of HRP-labeled monospecific rabbit antibody to *Per a* I and *Bla g* I (*Ra anti-Per a* I and *Bla g* I); this antibody preparation reacts with only *Per a* I. The *Per a* I precipitate is marked with an asterisk.

MATERIAL AND METHODS

Antigen preparations and allergen extracts

The following cockroach species were used for this study: *Periplaneta americana*, *Blattella germanica*, *Blatta orientalis*, *Supella supellecillum*, *Pycnoscelus surinamensis*, and *Leucophaea maderae*. The insects were obtained from cultures at the State Pest Infestation Laboratory, Lyngby, Denmark, and the University of Copenhagen. Cultures contained all developmental stages. The dry-wood termite, *Cryptothermes brevis*, was a kind gift from Dr. M. Pallaske, Krefeld, Germany. Two species of crickets, that is, *Gryllus campestris* and *G. domesticus*, were from the University of Copenhagen. The house isopod, *Oniscus* sp., was collected outdoors. The cultures were killed by freezing. Cultures of house dust mite, *Dermatophagoides pteronyssinus*, and the three storage mites, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*, and *Acarus siro*, were obtained from ALK, Hørsholm, Denmark. Standardized extracts of cat and dog were Aquagen SQ from ALK. Extracts of fungi, that is, *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Mucor racemosus*, and *Penicillium expansum*, were from ALK. Three cockroach extracts commercially available in the United States were bought from the manufacturers.

Reference allergen

Purified cockroach-allergen *Per a* I from *P. americana* was used as a reference antigen in the quantitation of allergen in extracts of dust samples. Purified cockroach-

allergen, *Bla g* I, from *B. germanica* was used for affinity purification of reagent antibodies. The allergens were purified from whole cockroach bodies as previously described.^{6,7}

House dust samples

Vacuum cleaner bags containing dust from 73 homes were collected in the Tampa area, south Florida; two bags were obtained from 23 of the households. Cockroaches are not found as household pests in Greenland, which has an arctic climate, and six vacuum cleaner dust samples from there were included as negative controls. One gram of unsieved dust was weighed and used for extraction.

Extraction

All extractions were performed on shaker tables. Extraction of allergen sources was performed at 10% wt/vol for 20 hours at 5° C in 0.125 mol/L of NH_4HCO_3 . The extract was centrifuged at 4° C for 60 minutes at 27,000 g and stored at -20° C. Extraction of dust samples was performed at 20% wt/vol for 3 hours at room temperature in 0.125 mol/L of NH_4HCO_3 . The extract was centrifuged at room temperature for 10 minutes at 27,000 g; 15 mmol/L of NaN_3 was added as preservative and stored at -20° C.

Protein concentration

Determination of protein concentration was performed according to the method of Lowry et al.⁸ BSA was used as the reference protein.

TABLE I. Specificity of cockroach assay as determined by radioimmunoassay inhibition

50% Inhibition by*	<i>Per a</i> I (ng/ml)	<i>Bla g</i> I (ng/ml)
Cockroaches (WBE)		
<i>P. americana</i>	250	170
<i>B. germanica</i>	2,000	1,400
<i>B. orientalis</i>	350	235
<i>S. supellectilium</i>	4,000	770
<i>P. surinamensis</i>	400,000	15,000
<i>L. maderae</i>	>350,000	>350,000
Purified allergens		
<i>Per a</i> I	23	12
<i>Bla g</i> I	300	26
Noncockroach insects (WBE)		
<i>G. campestris</i>	>510,000	>510,000
<i>G. domesticus</i>	>570,000	>570,000
<i>C. brevis</i>	>140,000	58,000
Isopod (WBE)		
<i>Oniscus</i> sp	>420,000	>420,000
Arachnids (WCE)		
<i>D. pteronyssinus</i>	>80,000	>80,000
<i>L. destructor</i>	>80,000	>80,000
<i>T. putrescentiae</i>	>80,000	>80,000
<i>A. siro</i>	>80,000	>80,000
Mammals (pelt)		
Cat	>80,000	>80,000
Dog	>80,000	>80,000
Fungi		
<i>M. racemosus</i>	>80,000	>80,000
<i>P. expansum</i>	>80,000	>80,000
<i>A. fumigatus</i>	>80,000	>80,000
<i>A. alternata</i>	>80,000	>80,000
<i>C. herbarum</i>	>80,000	>80,000

WCE, whole culture extract; >, no inhibition occurred at the highest concentration tested as presented.

Inhibition concentrations are nanograms of Lowry protein per milliliter.

*The binding of 9.6 ng/ml of *Per a* I or 7.5 ng/ml of *Bla g* I was inhibited to 50%.

Radiolabeling

Iodination of the allergens with ^{125}I was performed by the chloramine-T method.⁹ Unbound iodine was separated from protein by Sephadex G-25 gel filtration.

Reagent antibodies

Rabbit serum to the *Per a* I allergen of *P. americana* was raised by immunizing three rabbits subcutaneously at monthly intervals with purified protein (dosage, 0.01 mg of protein in 0.05 ml plus 0.05 ml of Freund's incomplete adjuvant). The animals were bled monthly, and sera were pooled as *Ra anti-Per a* I. Rabbit antibodies to determinants shared by allergens *Per a* I and *Bla g* I were immunopurified as outlined: 15 mg of purified *Bla g* I protein was coupled

to 1.5 gm of CNBr-activated Sepharose 4B (Pharmacia Ltd., Uppsala, Sweden) as recommended by the manufacturer. Six milliliters of *Ra anti-Per a* I was applied to the gel at room temperature. The gel was subjected to consecutive washes of 0.05 mol/L of CB, pH 6.2, CB, pH 6.2 plus 0.5 mol/L of NaCl, and CB, pH 2.6, plus 1.0 mol/L of NaCl. The pH of eluted fractions was immediately brought to 7 with NaHCO_3 . The eluted antibodies were revealed by fused rocket immunoelectrophoresis¹⁰ with swine antirabbit immunoglobulin (code Z 196, DAKO AS, Glostrup, Denmark) and pooled accordingly. After extensive dialysis against 0.005 mol/L of NH_4HCO_3 and subsequently against distilled water, the antibodies were freeze-dried and weighed. (Typically, 4.5 mg of immunoglobulin was purified from 6 ml of serum.) The ensuing preparation was called *Ra anti-Per a* I and *Bla g* I. This antibody reacted with only *Per a* I and *Bla g* I when it was tested toward WBEs of the two cockroach species as peroxidase-labeled preparation in crossed immunoelectrophoresis overlay technique as described elsewhere.¹¹ Aliquots of 1 mg of the antibody preparation were conjugated with 1000 units of HRP (295 U/mg, Kem-En-Tek, Copenhagen, Denmark), and possible remaining reactive groups were blocked by the addition of 0.2 mol/L of lysine. The HRP-antibody preparation was stored at 4° C with 15 mmol/L of sodium azide.

ELISA assay procedure

Polystyrene microtiter trays (96-well Immuplate, NUNC, Roskilde, Denmark) were coated with 1 $\mu\text{g}/\text{ml}$ of *Ra anti-Per a* I and *Bla g* I (100 μl per well). Reaction occurred overnight at 20° C in 0.1 mol/L of sodium carbonate buffer, pH 9.6. After trays were thoroughly washed in PBS, pH 7.4, 0.05% vol/vol Tween 20, duplicate dilution series of samples were incubated 2 hours at room temperature in PBS, 0.5% wt/vol BSA. Each tray included dilution series of standard, that is, purified *Per a* I of defined protein concentration. After trays were thoroughly washed again, the bound allergen was revealed by incubation for 1 hour at room temperature with HRP-conjugated *Ra anti-per a* I and *Bla g* I diluted 1000 times in PBS, 0.5% wt/vol BSA. Substrate (3.0 mmol/L of hydrogen peroxide and 5.0 mmol/L of o-phenylenediamine in 0.1 mol/L of sodium-CB, pH 5.0) was prepared immediately before use, and reaction occurred for 15 minutes at room temperature in darkness, after which 1 mol/L of sulfuric acid was added. The absorbance at 490 nm was read on a Teknunc immunoreader NJ-2000, Nippon InterMed, Tokyo, Japan.

Specificity measurements

Polyvinylchloride microtiter plates (96-well Immuno Assay Plate, Titertek, Flow Laboratories, Denmark) were coated with 1 $\mu\text{g}/\text{ml}$ of *Ra anti-Per a* I and *Bla g* I (100 μl per well). Reaction occurred overnight at 20° C in 0.1 mol/L of sodium-carbonate buffer, pH 9.6. Washing was as described in "ELISA assay procedure." Initially, dilution series of iodinated *Per a* I and *Bla g* I (from 5400 cpm) were incubated overnight in PBS, 0.1% wt/vol BSA at room temperature. After wells were washed, the wells were cut and counted in a gamma counter. Binding capacity

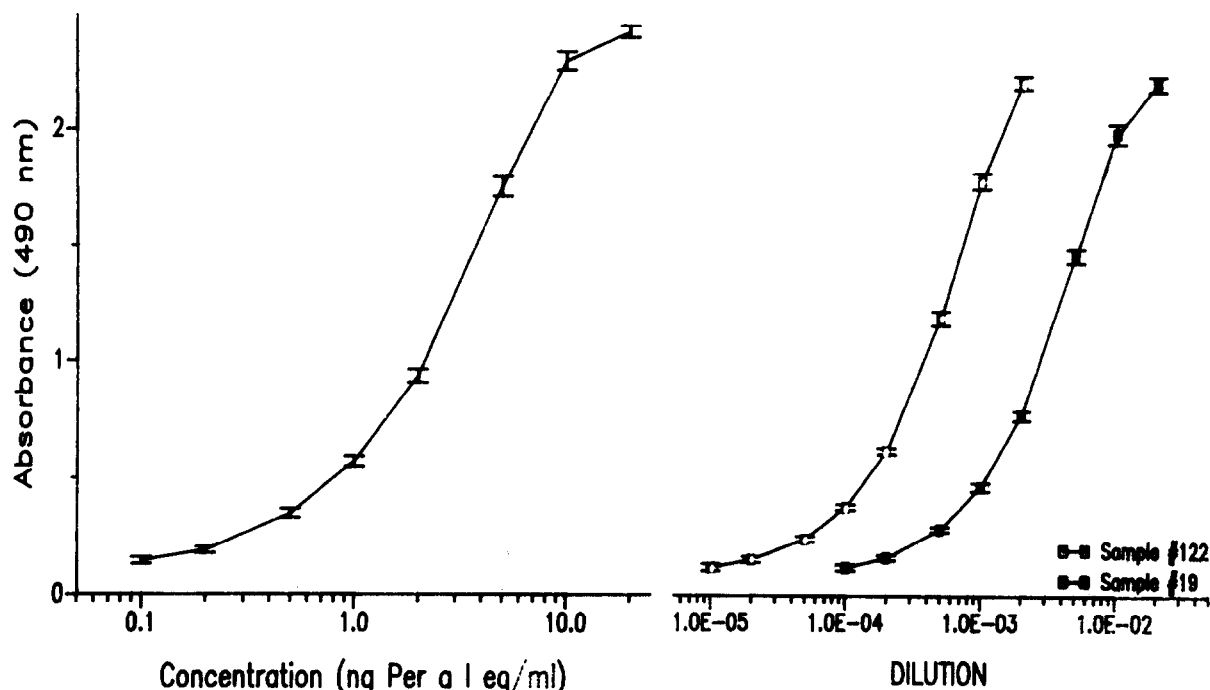


FIG. 2. Dose-response curves for purified *Per a I* standard from American cockroach (left curve) and two vacuum cleaner dust samples (right curves). Horizontal axis: nanograms of *Per a I* per milliliter (standard) and log of sample dilutions of 1:5 (wt/vol) extracts; vertical axis: absorption units; curves are means $\pm$ SD of five individual setups.

of the antibodies was 1600 cpm for *Bla g I* and 1100 cpm for *Per a I*. This saturation was obtained with 7.5 ng/ml for *Bla g I* and 9.6 ng/ml for *Per a I*, and these concentrations were chosen as working concentrations for the ensuing specificity analyses. The background was 25 cpm. For specificity measurements, 50 μ l of radiotagged allergen was mixed directly in the well with 50 μ l of the test sample at appropriate dilution. All dilutions were in PBS, 0.1% wt/vol BSA, and incubated overnight at room temperature. Washing and counting were performed as above. Inhibition curves were drawn, and the concentration of inhibitor needed to suppress allergen binding to 50% of maximal counts per minute was calculated.

RESULTS

Antibody preparation

The present ELISA system is based on rabbit antibodies directed to determinants that are shared by the marker allergens, *Per a I* and *Bla g I*, from American and German cockroaches, respectively. The antibody preparation was produced by immunizing rabbits with purified *Per a I* and subsequently immunopurifying the antibodies by reaction with solid-phase *Bla g I*. The antibodies eluted from the solid phase were used for the cockroach-allergen ELISA as capture antibody, as well as detection antibody in HRP-labeled form. The monospecificity with respect to re-

action with *Per a I* and *Bla g I* was tested with overlay technique in CIE. The monospecific reaction of the antibody preparation is illustrated in Fig. 1.

Specificity

The specificity of the assay was tested by measuring the displacement of radio-tagged purified *Per a I* and *Bla g I* by preparations containing antigens that could potentially be present in the same environment as cockroach allergen. Displacement resulting in lowering the bound radioactivity to 50% of maximal binding was used as a measure of interference by competing material. The working concentration of radiolabeled purified allergen was 9.6 ng/ml for *Per a I* and 7.5 ng/ml for *Bla g I*. The results of the specificity measurements are presented in Table I. Extracts of several mites and fungi, as well as cat and dog, were tested at competing concentrations up to 80,000 ng/ml, but none were able to displace the two cockroach allergens. The highest concentration possible to test for house isopod was 420,000 ng/ml, and no interference with the assay was observed. Among noncockroach insects, the two cricket extracts tested at concentrations above 500,000 ng/ml did not react. The drywood termite extract at 58,000 ng/ml displaced the binding of *Bla g I* to 50%, but no interference was observed with *Per a I*. The purified cockroach aller-

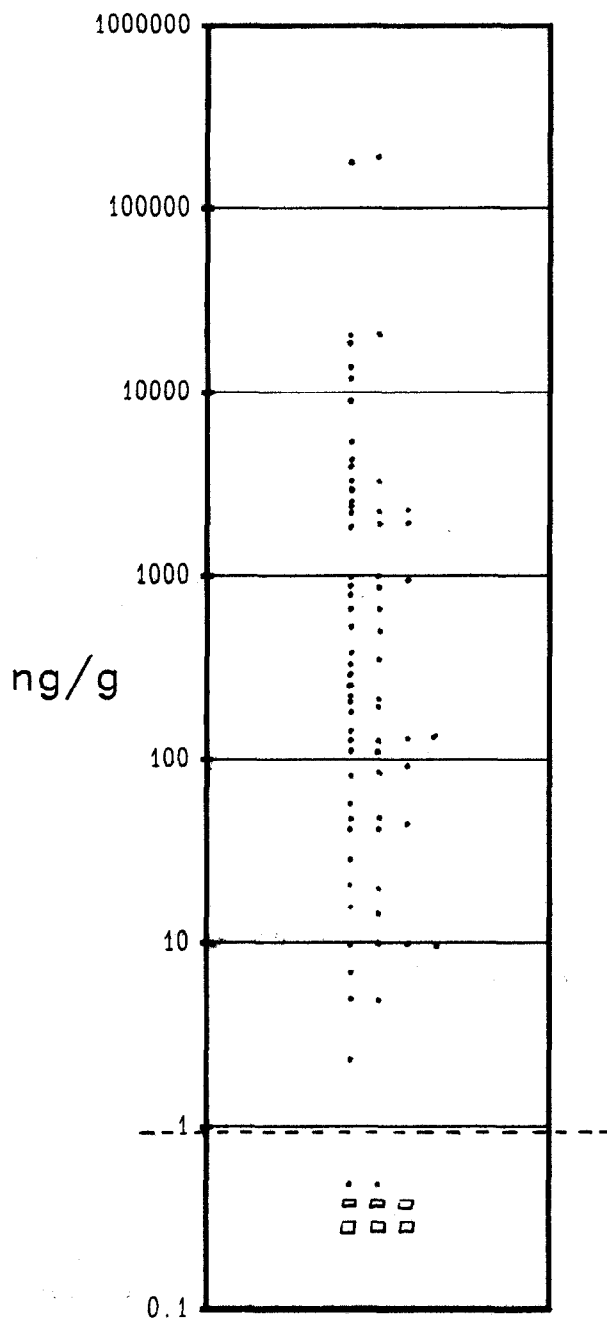


FIG. 3. Levels of cockroach allergen in vacuum cleaner dust from 73 households in Florida (●) and from six households in Greenland (□). In cases in which several bags were obtained from individual households, the values are means. Figures on vertical axis denote nanograms of *Per a I* eq/gm of dust. Observations positioned under the broken line were below the limit of detection.

gens, as well as extracts of several cockroach species, interfered with the assay to a varying degree, as will be discussed later. Since the assay cross-reacts with domiciliary cockroaches other than the American and German, it was decided to express the allergen concentrations measured in extracts of dust samples as

"nanogram equivalents of *Per a I* Lowry protein per gram of dust," abbreviated to ng *Per a I* eq/gm.

Measurements on house dust samples and commercial cockroach extracts

Titration of house dust extracts were compared to titrations of purified cockroach allergen *Per a I* that served as a reference preparation. Titration curve with standard error for five different runs of the reference preparation is illustrated in Fig. 2. The lower limit of detection was set at four times the absorption of blanks and corresponded to readings at 15% of maximal absorption. The upper limits of the range of measurements (corresponding to readings at 90% of maximum absorption) were approximately 50 times the lower limit. Extraction was performed on household vacuum cleaner bags containing dust from 73 homes in the Tampa area of south Florida. Because cockroaches are not likely to occur as domiciliary pests in the arctic climate of Greenland, extracts of six household vacuum cleaner dust samples from that area were included in the study as negative controls. Dose-response curves for samples containing cockroach allergen were approximately parallel to the reference curve (Fig. 2). The concentration of cockroach allergen in the 73 American and the six Greenland homes is illustrated in Fig. 3. All Greenland homes as well as two American homes contained cockroach-allergen concentrations below the lower detection limit, that is <1 ng *Per a I* eq/gm. The remaining 71 American household dust samples contained from 2 to 200,000 ng of *Per a I* eq/gm; most samples (59/71) contained between 10 and 10,000 ng of *Per a I* eq/gm. Two bags were obtained from 23 of the households, covering concentrations from 4.8 to 10,600 ng of *Per a I* eq/gm. There was a good correlation between the results of measurements on the two sets of bags (Pearson's $r = 0.931$; $p < 0.00001$). Three commercial cockroach extracts were investigated (Table II); all extracts contained *Per a I*, although the concentration per 100 PNU varied between the extracts.

DISCUSSION

The assay described here has been devised to measure the concentration of an allergen that relates to the total cockroach-allergen load in the environment of allergic patients. Although the density of cockroaches in houses may be lowered by intensive eradication measures, very little is currently known about the effect of such measures on the concentration of cockroach allergen in the environment.

Among the most common and most widespread domiciliary cockroaches are the American and the German. It was therefore decided to develop an assay based on immunologic cross-reactivity between

two recently^{6,7} purified marker allergens, *Per a* I and *Bla g* I, from the American and the German cockroach, respectively. It is not now known if the "sensitizing activity" of different species of domiciliary cockroaches is in the same range of concentrations. It is therefore acceptable to measure "overall" cockroach level rather than the concentration of each of several potentially important allergens from the individual species.

The antibody preparation used in the present study reacts with determinants shared by the allergens *Per a* I and *Bla g* I. The avidity for *Per a* I is, however, higher than for *Bla g* I, as demonstrated by the competition tests. Because of this cross-reactivity, the measured allergen concentrations are expressed as nanogram equivalents of *Per a* I Lowry protein per gram of dust, abbreviated as ng *Per a* I eq/gm. WBEs of American and German cockroaches were required in about 10 times the concentrations of the corresponding purified allergens to suppress the bound radioactivity to 50%. This observation is in accordance with earlier data^{6,7} indicating that the two allergens comprise about 10% of the protein in WBEs of the two species.

The Oriental cockroach (*Blatta orientalis*) cross-reacted strongly in the assay; therefore, it is conceivable that this insect, which is also a domiciliary pest, contains a molecule that is homologous to *Bla g* I and *Per a* I. Oriental and American cockroaches both belong to the subfamily Blattinae.¹² The brown-banded cockroach (*Supella supellectilium*), which is also known to occur in human dwellings, cross-reacted in the assay as well; brown-banded and German cockroach both belong to the subfamily Blattellinae. It is perhaps not unexpected that the Madeira cockroach (*L. maderae*) does not react in the assay and that only a slight reaction was observed with another tropical species, *P. surinamensis*; these latter species belong to the Blaberidae family and are hence distantly related to the American and German cockroaches.¹²

The allergen was present in three commercial cockroach extracts available in the United States. The concentration per protein nitrogen unit was lowest in "cockroach mix"; this could be due to the inclusion in the extract of cockroach species with a low concentration of *Per a* I or its equivalents.

Extracts of several other allergen sources likely to occur in the environment of allergic subjects were tested for cross-reaction in the assay. Only in one instance, that is, the dry-wood termite, was a low level of interference detected. Even if there is a slight cross-reactivity with termite, the assay is considered having adequate specificity for studies of cockroach exposure.

The assay sensitivity of 1 ng *Per a* I eq/gm

TABLE II. Allergen concentration in commercial cockroach extracts

Supplier	Source	Allergen concentration
A	"Cockroach"	0.9 µg <i>Per a</i> I eq/100 PNU
B	"American"	0.9 µg <i>Per a</i> I eq/100 PNU
C	"Cockroach mix"	0.5 µg <i>Per a</i> I eq/100 PNU

is considered ample for environmental surveys. It is comparable to the sensitivity obtained in radioimmunoassays, for example, for *Fel d* I¹³ and *Der p* I/*Der f* I¹⁴ and superior to that obtained in ELISA assays for *Der p* I/*Der f* I/*Der m* I.^{15,16} The clinically relevant levels of cockroach-allergen exposure have not been established, but levels may fall within the same concentration range as levels proposed for the house dust mite group I allergens, that is, 2 µg/gm of fine dust for sensitization and 10 µg/gm for the mite-allergic patients to develop acute asthma.¹⁷

The dust samples used in the present study have not been sieved, since it is unlikely that the marker allergens detected in the assay are primarily tied to fragments of any particular size. This finding is in contrast to *Der p* I/*Der f* I/*Der m* I, which is primarily contained in fecal particles.¹⁸ The concentrations of cockroach allergen measured here arise from vacuum cleaner dust samples from the whole house and therefore probably represent an overall allergen load in the houses in question. It is a well-known phenomenon that domiciliary cockroaches are likely to be more numerous in kitchen regions than elsewhere. Accordingly, we have detected very high levels of cockroach allergen in kitchen cabinet dust, that is, up to 10⁶ ng *Per a* I eq/gm (data not presented; samples kindly supplied by Dr. Susan Pollart, Charlottesville, Va.¹⁹). It is not established where the sensitization of patients takes place; therefore, probably overall allergen load in patients' homes will be the best framework for studies of clinical aspects of cockroach-allergen levels in the environment.

The concentration of cockroach allergen measured varied from below detection limit, that is, from two American homes and the six Greenland control homes, to approximately 200,000 ng *Per a* I eq/gm. The concentration range is comparable to that detected for *Der p* I/*Der f* I/*Der m* I in extracts of mattress dust.^{15,16} Subjects from several of the households tested were skin test positive for cockroach (data not presented), but no correlation between skin test positivity and concentration of cockroach allergen was found. This finding is perhaps not unexpected since the life histories of the subjects tested were not known in detail; therefore, it is likely that the skin test-

positive subjects were sensitized earlier in their lives in houses with different levels of cockroach-allergen exposure.

The assay presented in this study is a fast and sensitive tool for the measurement of the cockroach-allergen load in dust samples and should be valuable for controlling the effect on allergen load of eradication programs. The assay could be useful for establishing clinically relevant levels of cockroach-allergen exposure.

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