

## ORIGINAL PAPER

# Biological potency of German cockroach allergen extracts determined in an inner city population

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## Clinical and Experimental Allergy

### Summary

**Background** Cockroach allergy is an important cause of inner city asthma. To perform valid studies on the diagnosis and treatment of cockroach allergy, biological potencies of test extracts need to be established, and a surrogate *in vitro* test for biological potency should be chosen.

**Methods** Sixty-two cockroach-allergic adult subjects were recruited for quantitative skin testing with three commercial German cockroach extracts. The intradermal D50 values were determined using linear interpolation, and the biologic potencies were determined from D50 data. The extracts were also analysed for relative potency, using a competition ELISA, and for specific allergen content, using a two-site ELISA.

**Results** Estimates of each extract's D50 were analysable in 48–55 subjects, with D50s between 10.3 and 11.8. All three extracts were bioequivalent using pre-set criteria. The biological potencies of the extracts were 1738–8570 bioequivalent allergy units (BAU)/mL (geometric mean = 3300), and these relative potencies were similar to those estimated by competition ELISA and specific allergen content. IgE against cockroach allergens were detected in sera from 34 subjects with analysable D50s, and 17 subjects had IgE directed against specific cockroach allergens. Although the presence of anti-Bla g 5 correlated with the subjects' skin test responses for 2/3 extracts, no single allergen was immunodominant. Antibody responses among the subjects were heterogeneous.

**Conclusions** Although commercial cockroach extracts are relatively low in potency, immunotherapeutic doses should be achievable. Biological potency may be estimated using D50 testing, a combination of specific allergen determinations, or by an overall potency assay such as the competition ELISA.

**Capsule Summary** The biological potency of three German cockroach allergen extracts, determined in an inner city population, was 1738–8570 BAU/mL. No one allergen was immunodominant, and surrogate *in vitro* testing methods were examined.

**Keywords** allergen, asthma, cockroach, immunotherapy, standardization

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### Introduction

Asthma is a multifactorial disease, and the role of IgE-mediated hypersensitivity to environmental allergens in the pathogenesis of asthma in certain populations is well established. Several recent publications have highlighted the importance of cockroach allergen exposure in the development of asthma in residents of US inner cities. In

a survey of over 400 children with asthma from eight US urban areas, Rosenstreich et al. [1] found that high bedroom levels of the cockroach allergen Bla g 1 were associated with asthma morbidity if the children living there were also sensitized to cockroach allergen. The presence of cockroach allergen in bedrooms was a risk factor for sensitization. Because sensitization appears to

occur with relatively low bedroom exposure [2], preventing sensitization by reducing allergen exposure may be difficult to achieve [3]. In one allergen abatement study, reductions of mite and cockroach allergens on the bedroom floor were correlated with fewer symptoms days, unscheduled clinic visits, and hospitalizations due to asthma [4]. While such interventions may be cost-effective in the long run, they require a substantial long-term commitment of resources that may be impractical. Because of this, allergen immunotherapy could offer the best hope for a long-term reduction in airway hyperresponsiveness due to cockroach allergens.

Although some highly successful immunotherapy studies have been performed using allergen extracts that have been poorly characterized, the best approach is to use extracts for which the biological potency has been determined. Effective allergen immunotherapy with other allergens is highly dose dependent [5], and the therapeutic index is low, especially in patients with asthma [6]. Thus, to diagnose and treat cockroach allergy with allergen extracts, the extracts need to be potent and standardized, and appropriate potency tests need to be established. We measured allergen levels and overall allergenicity of three cockroach extracts, and determined *in vivo* potency by quantitative serial intradermal testing.

## Methods

### Extracts

Three licensed US commercial glycerinated German cockroach extracts were purchased. Although there were subtle manufacturing differences among the extracts, each had been produced by aqueous extraction using cockroaches that had been frozen, acetone-defatted and pulverized before extraction. Extract B was prepared using a 1 : 10 (w/v) ratio of roach to extracting solution; for extracts A and C, the ratio was 1 : 20. For stability, glycerol was added to a final concentration of 50% (v/v) either before (extract B) or after (extracts A and C) extraction, and all contained phenol 0.4% (w/v).

### Subjects

Subjects were enrolled by the Inner City Asthma Consortium [7] in Baltimore, Chicago, Denver, and Washington, DC. To be eligible, otherwise healthy individuals between the ages of 18 and 65 years had self-reported symptoms compatible with allergic rhinitis or asthma. Individuals with unstable asthma, forced expiratory volume in 1 s < 75% predicted, skin colouring or conditions (such as dermatographism) that would preclude the measurement of erythema responses, a history of immunotherapy to cockroach allergen extracts, current or recent use of oral or parenteral glucocorticosteroids, or current use of

medications that might interfere with testing were excluded. Before entry, subjects were screened by a prick test with Extract C, which was found to be of intermediate potency in preliminary *in vitro* assessments. If the orthogonal sum of erythema skin test response ( $\Sigma E$ ) was  $\geq 30$  mm at 15 min, the individual was offered enrollment in the study.

Subjects were enrolled only after providing informed consent. This study – including the proficiency testing, pre-screening, and ID<sub>50</sub>EAL (quantitative intradermal skin testing method for establishing biological allergen potency; Intradermal Dilution for 50 mm Sum of Erythema Determines Bioequivalent Allergy Units) – was reviewed and approved by the Institution Review Boards of each member site, supervised by an independent Data and Safety Monitoring Board of the Inner City Asthma Consortium at NIAID, and performed under IND protocol 11319.

### ID<sub>50</sub>EAL method

Enrolled subjects were skin tested with the three German cockroach allergen extracts using the ID<sub>50</sub>EAL protocol originally described by Turkeltaub *et al.* [8]. In this test, the biological potency of an allergen extract is estimated by determining the extract dilution at which the  $\Sigma E$  is 50 mm.  $\Sigma E$  is determined 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 dilution at which  $\Sigma E$  is 50 mm, called the D50 is expressed as the  $-\log_3$  of the test dilution.

Extracts that produce similar D50s can be considered to be bioequivalent and are assigned equal bioequivalent allergy units (BAU). By convention, an extract with a D50 of 14 is assigned a potency of  $10^5$  BAU/mL [9]. Other BAU assignments may be made using the formula

$$\text{BAU/mL} = 10^5 \times 3^{(\text{D50}-14)}$$

**Skin test protocol.** All testers underwent training and proficiency testing using histamine injections in normal volunteers.

Seventeen serial three-fold dilutions of each extract were prepared by research pharmacists at each institution, using sterile saline (0.9%) containing human albumin (0.03%) and phenol (0.4%) (w/v; Hollister–Stier). The dilutions were stored at 4 °C until use for up to 1 week.

All injections were 0.05 mL, performed intradermally on the back, using a 27 g short-bevelled needle;  $\Sigma E$  was determined at exactly 15 min. Subjects were injected initially with histamine control (0.1 mg/mL base; ALK Abello) and a diluent, along with the  $3^{-17}$  dilutions of each allergen extract. If the  $\Sigma E$ s for histamine and the diluent control were  $\geq 35$  and  $< 5$  mm, respectively,

testing was continued. For serial testing using the cockroach allergen extracts, subjects were advanced three three-fold dilutions if there was no response to the more dilute extract injection. On the other hand, for positive ( $\geq 5$  mm)  $\Sigma$ E responses, the subjects were advanced one three-fold dilution. The objective was to establish a dose-response curve for each extract in each subject, with  $\Sigma$ E responses from 0 to 125 mm, containing at least four valid data points that bracketed 50 mm.

*D50 determinations, bioequivalence, and data analyses.* The D50s were calculated by linear interpolation, in which the serial three-fold dilutions immediately before and after the 50-mm  $\Sigma$ E threshold was reached are determined. Then, through linear interpolation, the three-fold dilution required to achieve the 50mm  $\Sigma$ E threshold is estimated [10].

The three extracts were considered to be bioequivalent if their D50s differed by  $< 20\%$ . The testing of bioequivalence was based on pair-wise 90% confidence intervals of the differences among the three cockroach extracts using estimates from a mixed-models analysis of variance comparing the three extracts. The mixed-models analysis contained fixed effects for extract and serial dilution number and a random effect for subject. The mixed-effects model was also used to test for significant differences between the mean D50s of the three extracts. Because there is no true reference extract, the mean of the two extracts being compared is used as the reference mean in determining the  $\pm 20\%$  boundaries. A pair of extracts was considered to be bioequivalent if the 90% confidence interval of the difference between the two D50s was contained within the  $\pm 20\%$  boundaries. The potency in BAU/mL was calculated from the mean D50 for each extract [11, 12].

Bioequivalence among the extracts was based upon the D50s rather than BAUs. Because the BAU/mL potency is an exponential function of the D50, extracts that are nominally bioequivalent in terms of their skin test responses may have potencies that are widely and even significantly divergent.

#### *In vitro testing of the allergen extracts*

Bla g 1, Bla g 2, and Bla g 5 levels of the three extracts were determined by two-site ELISA (Indoor Biotechnologies, Charlottesville, VA, USA). Overall potency was determined by competition ELISA [13] using pooled human anti-cockroach serum (S1Cr; CBER/FDA) and hyperimmune rabbit antisera. For each competition ELISA, the serum and other reagents were specifically optimized. The relative potency (RP) was determined by comparison with the German cockroach extract E2-Cg, for which the assigned RP value is 1.0 [14]. The RP for each extract was then evaluated for equivalency to the E2-Cg reference

extract. Based on previous work, an allergenic extract is considered to be equivalent to a reference extract in the competition ELISA if the mean RP of three replicates falls within the range 0.699–1.431 [15]. S1Cr was developed from the sera from 16 cockroach-allergic individuals, and had been used in a previous study from this laboratory of German and American cockroach allergen extracts [14].

In addition, two hyperimmune rabbit sera were developed. The first was produced by injecting a New Zealand white rabbit with three doses (200  $\mu$ L/dose) of whole German cockroach aqueous extract, subcutaneously, at 21-day intervals. The first dose was given with complete Freund's adjuvant, and the second two with incomplete Freund's adjuvant. The rabbit was then anaesthetized and exsanguinated to produce the 'polyclonal anti-CR' serum.

The second hyperimmune serum was produced by injecting three rabbits with rBla g 1, rBla g 2, or rBla g 5, respectively, according to the same schedule. The three sera thus produced were pooled to produce the 'pooled antigen-specific' serum.

#### *Serum collection and testing*

Before testing, 60 mL of blood was collected by venipuncture; serum was separated and stored at  $-20^\circ\text{C}$  at a central facility.

Sera were tested for IgE antibodies directed against *Blattella germanica* extract by a UniCAP assay (Phadia, AB; Uppsala, Sweden). Each serum with detectable anti-cockroach IgE was tested for specific IgE antibodies to rBla g 1, rBla g 2, rBla g 4, and rBla g 5 (Indoor Biotechnologies) by a streptavidin CAP assay [16, 17]. The data were tested for correlations between each subject's calculated D50 and the levels of IgE against each of the specific cockroach antigens.

## **Results**

#### *Subject selection and demographics*

In the four testing centres, 337 potential study subjects were identified, of whom 265 were eligible for screening. Of these, 251 gave consent for screening, and – on the basis of the screening prick skin test – 70 subjects were eligible for intradermal testing, and 62 completed testing; 15 or 16 subjects were enrolled at each centre. Based on statistical considerations, 61 subjects were to be enrolled. However, due to a protocol deviation at one of the sites, one individual – a 24-year-old African-American female – who should not have been eligible was allowed to proceed with intradermal testing. Although included in Table 1, this participant's data were excluded from all other analyses. The demographics of the enrolled subjects are shown in Table 1. All enrolled subjects completed testing.

### Adverse events

There were no serious adverse events. Forty-two adverse events – including local swelling (nine subjects), allergy symptoms (21), decrease in peak flow >10% but <25% (four), coughing or dry mouth (three), dizziness (four) and headache (one) – occurred in 27 subjects. All events were self-limited; none led to withdrawal from the study.

### Data analysis

**D50s.** Not all of the skin testing data could be used. One subject was excluded for protocol violations. In some instances, in spite of a positive screening prick test, intradermal testing failed to elicit measurable erythema responses. In others, responses were obtained but did not follow a consistent dose–response. For these reasons, out of 61 subjects, the D50s for 13, eight, and six of the remaining subjects could not be estimated for Extracts A, B and C, respectively (Table 2). Pearson's correlations testing for linear associations among the D50s of the

cockroach extracts were all significant ( $P < 0.0001$ ) with  $r$ -values (95% CL) of 0.73 (0.5, 0.88), 0.76 (0.58, 0.89), and 0.90 (0.82, 0.95) for extracts A vs. B, A vs. C and B vs. C, respectively. These correlation coefficients should be interpreted with caution. Although they are likely a result of differences in allergic responses between the extracts, they may also reflect within-subject variability due to differences in test locations on the back.

### Bioequivalence and potency

All three extracts were found to be bioequivalent by the pre-established criterion ( $\delta_{D50} < 20\%$ ) (Table 3); potencies were calculated from the D50s (Table 2). Although bioequivalent by pre-established criteria, extract B was the most potent, and extract A the least, with the potency of B being 4.9-fold greater than the potency of A (in BAU/mL,  $P < 0.05$ ). Extract B was also found to be significantly more potent than extract C (3.4-fold,  $P < 0.05$ ). The potency differences between extracts A and C were not statistically significant.

Table 1. Demographic data

|               |             |
|---------------|-------------|
| Age range     | 18–59 years |
| Males/females | 15/47       |
| Ethnicity     |             |
| Black         | 35          |
| Hispanic      | 14          |
| White         | 5           |
| Asian         | 1           |
| Other*        | 7           |

\*Self-identified as black/white (2), black/Asian, black/native American, white/Asian, Hispanic/native American or German.

### *In vitro* potency estimates of extracts A, B, and C

Quantitative skin testing is an estimate of the overall potency of an allergen mixture, based upon the recognition of multiple specific allergens by polyclonal IgE antibodies in the test population. Likewise, overall potency may be estimated using pooled allergic sera (IgE responses), as well as sera from hyper-immunized animals (IgG responses). Using three different sera (two rabbit, one pooled human), the potencies of all three extracts were found to be below that of reference

Table 2. Summary statistics of the D50s calculated for each of the three German cockroach allergen extracts

| Extract | <i>n</i> | D50  |                    |                | BAU/mL |                |
|---------|----------|------|--------------------|----------------|--------|----------------|
|         |          | Mean | Standard deviation | Standard error | Mean   | 95% CL         |
| A       | 48       | 10.3 | 2.66               | 0.38           | 1738   | (743, 4063)    |
| B       | 53       | 11.8 | 2.71               | 0.37           | 8570   | (3776, 19 448) |
| C       | 55       | 10.6 | 2.54               | 0.34           | 2495   | (1173, 5306)   |

D50s were determined for each subject for each of the three German cockroach allergen extracts. Of the 61 subjects, valid data (see text) were obtained from the number of subjects indicated in the *n* column. BAU/mL data were calculated from the mean D50 data.

BAU, bioequivalent allergy units.

Table 3. Pairwise D50 comparisons and bioequivalence between the cockroach extracts

| Endpoint | Extract comparison | Difference $\pm$ standard error | 90% CL of difference | $\pm 20\%$ of mean <sup>†</sup> | Bioequivalence (20% of mean criterion) |
|----------|--------------------|---------------------------------|----------------------|---------------------------------|----------------------------------------|
| D50      | A vs. B            | $-1.57 \pm 0.249^*$             | ( $-1.99, -1.16$ )   | $\pm 2.21$                      | Yes                                    |
|          | A vs. C            | $-0.41 \pm 0.246$               | ( $-0.82, -0.0062$ ) | $\pm 2.10$                      | Yes                                    |
|          | B vs. C            | $1.16 \pm 0.237^*$              | ( $0.77, 1.55$ )     | $\pm 2.24$                      | Yes                                    |

\*Difference is statistically significant at  $\alpha = 0.05$ .

<sup>†</sup>20% of (D50 extract 1 + D50 extract 2)/2.

**Table 4.** Relative potency (RP) of cockroach extracts by competition ELISA using rabbit and human sera, and specific allergen content of the cockroach extracts

| Lot # | Overall potency (competition ELISA) |              |                    |              |        |              | Specific allergen levels (two-site ELISA) |         |         |
|-------|-------------------------------------|--------------|--------------------|--------------|--------|--------------|-------------------------------------------|---------|---------|
|       | Rabbit                              |              |                    |              |        |              |                                           |         |         |
|       | Pooled monospecific                 |              | Polyclonal anti-CR |              | Human  |              |                                           |         |         |
|       | RP                                  | 95% CL       | RP                 | 95% CL       | RP     | 95% CL       | Bla g 1                                   | Bla g 2 | Bla g 5 |
| A     | 0.15                                | (0.10, 0.21) | 0.07               | (0.05, 0.10) | 0.01   | (0.01, 0.01) | 450                                       | 7.9     | 0.0     |
| B     | 0.24                                | (0.17, 0.35) | 0.29               | (0.20, 0.41) | 0.44   | (0.31, 0.63) | 585                                       | 28.4    | 25.5    |
| C     | 0.11                                | (0.08, 0.16) | 0.15               | (0.10, 0.21) | 0.11   | (0.07, 0.15) | 262                                       | 9.0     | 0.2     |
| E2Cg  | (1.00)                              |              | (1.00)             |              | (1.00) |              | 2000                                      | 149.3   | 34.0    |

RP is the unitless ratio of the overall potency of the test extract to that of E2Cg, a provisional FDA reference standard, which has a nominal RP of 1.00. RP values listed are the geometric mean RPs of the replicate samples. Extract potencies are considered to be equivalent to the reference extract if the mean RP of three replicates is within the interval 0.699–1.431 [15]. Bla g 1 is reported in U/mL; Bla g 2 and Bla g 5 are reported in µg/mL. Detection limits: Bla g 1, 0.008 U/mL; Bla g 2, 0.0015 µg/mL; Bla g 5, 0.002 µg/mL.

standard E2-Cg (Table 4). These RPs parallel the ID<sub>50</sub>EAL data (Table 2), with the observed RP of extract B found to be substantially larger than the RPs of extracts A and C.

The Bla g 1, Bla g 2, and Bla g 5 contents of extracts A, B, and C were determined by two-site ELISA. Extract B contained more of all three specific allergens than the other two extracts. All three extracts contained measurable Bla g 1 and Bla g 2, but the Bla g 5 content of extracts A and C was close to or below the limits of detection (Table 4). Once again, these data paralleled the ID<sub>50</sub>EAL data (Table 2).

#### *Specific IgE determinations in the subjects' sera*

Because immediate skin test responses result from the interactions of injected allergens with specific IgE bound to skin mast cells, we would expect ID<sub>50</sub>EAL data to reflect both the sum of the allergens within the test extracts and the specific IgE levels of the individual study subjects to the various cockroach allergens in those extracts. Thus, we determined the IgE levels of each subject to four known cockroach allergens, and compared these levels with the D50 determinations for each subject. Sera from 34 of the 55 subjects from whom D50s were obtained had detectable anti-cockroach IgE and could be evaluated in this manner (Fig. 1). Of these subjects, only three had measurable anti-rBla g 1 (9%); 12 had anti-rBla g 2 (35%); seven had anti-rBla g 4 (21%); and 14 had anti-rBla g 5 (41%). Spearman's rank correlations found the presence of anti-rBla g 5 to be positively correlated with the D50s of extracts A and C ( $P < 0.05$ ), but not B. Other specific IgE levels did not correlate significantly with the D50 responses.

#### **Discussion**

In this study, we estimated the biological potency of three commercially manufactured German cockroach extracts

using the ID<sub>50</sub>EAL protocol. In addition, we attempted to correlate the responsiveness to these extracts of the individual study subjects with their IgE-antibody specificity to four known cockroach allergens.

Our data support our previous observations [14] that US manufactured German cockroach allergen extracts are of low and variable potency. Our previous estimate was that glycerinated German cockroach extracts had potencies of <1000 BAU/mL, and this was consistent, in turn, with a previous estimate [18]. The extracts obtained for use in this study were more potent: the geometric mean potency of the three extracts tested in this study was 3300 BAU/mL. (By comparison, commonly used standardized allergen extracts contain between 5000 and 100 000 BAU/mL.) While this likely represents a normal variation in the potency of non-standardized allergen extracts, it suggests that the generation of moderately stronger German cockroach extracts is achievable by manufacturers using existing technology. Although still low in potency, it should be possible to attain therapeutic immunotherapy doses (100–2500 BAU) using any of these extracts [19].

Another study objective was to identify an *in vitro* method that might serve as a surrogate for ID<sub>50</sub>EAL potency determinations. For conceptual and practical reasons, the preferred potency assay for a standardized allergen extract is the measurement of a single major allergen. This approach has been accepted in the US for short ragweed and cat allergen extracts, because studies confirmed that, for both these extracts, overall potency in the allergic population parallels the levels of the major allergens (Amb a 1 and Fel d 1, respectively). For dust mite and grass pollen extracts, however, no single allergen dominates the immune response, and potency is determined using a competition ELISA with pooled human allergic sera [20].

In the current study, there was no evidence that any one cockroach allergen was immunodominant. Only 34

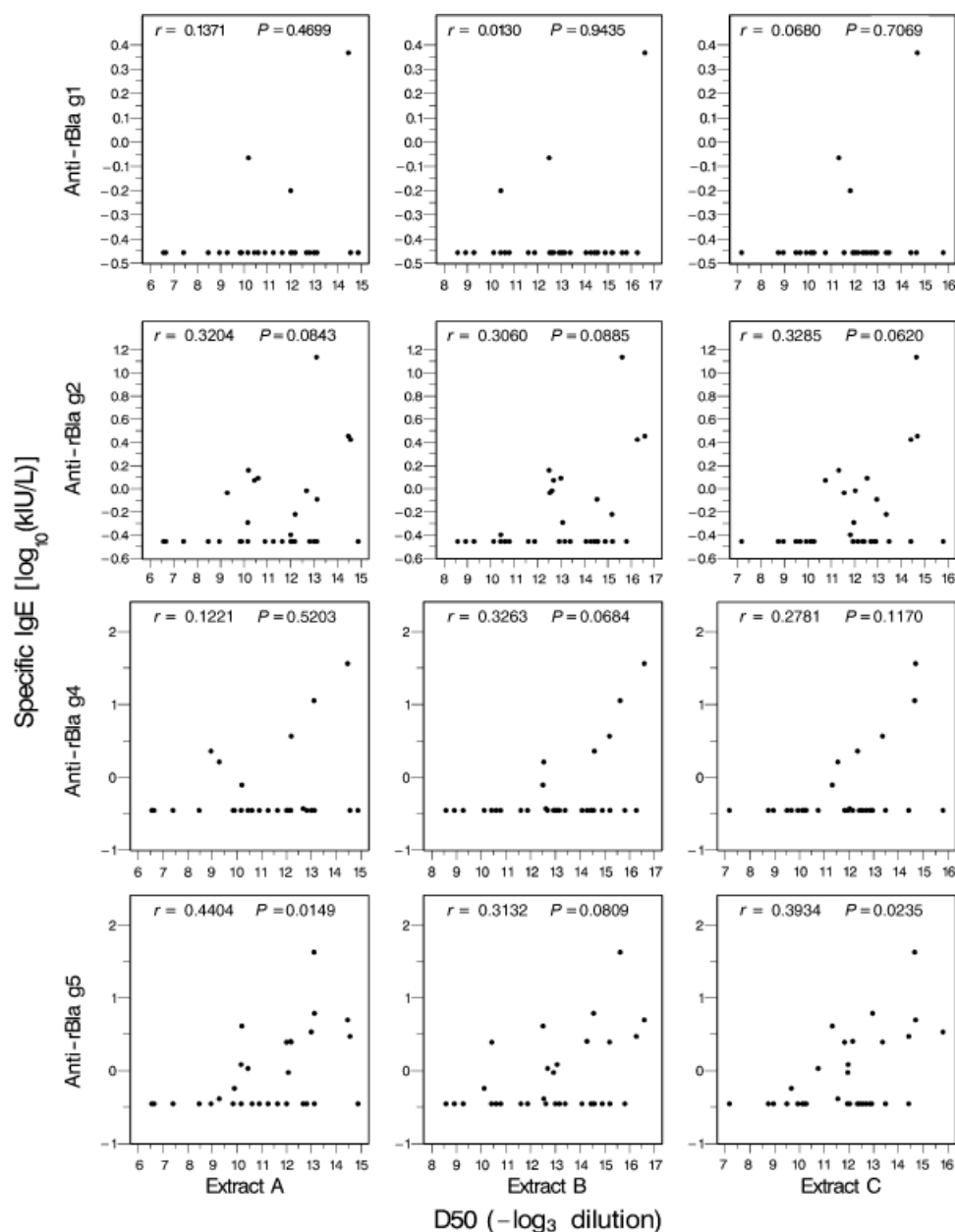


Fig. 1. Scatter plot matrix showing individual scatter plots of serum-specific IgE levels against each of the four cockroach allergens vs. the D50s of extracts A–C. Each point represents an individual study subject. Shown in each scatter plot are Spearman's rank correlation coefficient,  $r$  (a measure of association between each extract's D50 and the specific IgE concentrations), and their corresponding  $P$ -values testing whether the  $r$  differs significantly from 0.

of the 61 subjects whose sera we analysed had detectable IgE to cockroach extract, a result to be expected when all available cockroach extracts – including, perhaps, the extracts used to manufacture the UniCAP solid phase – contain relatively little allergen. Among the 17 subjects with measurable responses to at least one specific cockroach allergen, the response patterns were highly variable (data not shown). This pattern of IgE is consistent with a previous study in an inner city population in Atlanta, Wilmington, and Charlottesville [17], and

suggests that, in this important target group, no single allergen assay will provide an adequate measure of overall potency, and that allergens other than those tested may be significant. Notably, while the individual D50s of extracts A and C could be correlated to the study subjects' anti-Bla g 5 levels (Fig. 1), this finding is of questionable significance because these two extracts were virtually devoid of measurable Bla g 5 (Table 4). Thus, the surrogate potency test will have to take this uncertainty into account.

Cockroach allergy is important in the pathogenesis of asthma in the US inner city, and adequate avoidance is unlikely. We have successfully determined the biological potency of three commercially manufactured German cockroach extracts, and have begun the process of establishing an appropriate surrogate *in vitro* potency assay. This will facilitate the initiation of well-designed and scientifically valid human immunotherapy trials.

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