

Characterization and comparison of commercially available German and American cockroach allergen extracts¹

M. L. Patterson and J. E. Slater

Laboratory of Immunobiochemistry, Division of Bacterial, Parasitic and Allergenic Products, Office of Vaccines Regulation and Research, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, USA

Summary

Background In this study we examine the variability among unstandardized cockroach allergen extracts.

Methods We obtained 24 aqueous and glycerinated cockroach allergen extracts from nine manufacturers. We used previously characterized cockroach extracts, E2-Cg and E2-Ca, as references. The modified ninhydrin assay was used to determine protein concentration of each extract. Relative potencies of extracts were determined by competition ELISA, using a human allergic serum pool. Bla g 1 and Bla g 2 levels of glycerinated German cockroach extracts were determined by ELISA using monoclonal antibodies. Extracts were also analysed by SDS-PAGE.

Results Commercial cockroach allergen extracts had highly variable protein contents that were lower than the protein contents of the references. Electrophoretic data confirmed the presence of a variable number and intensity of protein bands in extracts among manufacturers. The relative potencies of the commercial extracts were between 10 and 782 BAU/mL for German cockroach and 10–250 BAU/mL for American cockroach. The mean Bla g 1 content of the commercial extracts was significantly lower than that of the reference ($P=0.001$). The mean Bla g 2 content of the commercial extracts was higher than that of the E2-Cg reference but the Bla g 2 levels were more variable compared to Bla g 1. In glycerinated German cockroach extracts, protein concentrations, relative potencies and specific allergen levels were significantly correlated ($P<0.001$).

Conclusion Our tests indicate that commercially available cockroach allergen extracts are variable in protein content, electrophoretic banding patterns, relative potency and Bla g 2 levels. In glycerinated German cockroach extracts, protein concentrations, relative potencies and specific allergen levels were significantly correlated.

Keywords cockroach, Bla g 1, Bla g 2, relative potency, total protein content, reference, German cockroach, American cockroach, allergen standardization, allergen extract

Submitted 20 August 2001; revised 30 October 2001; accepted 24 January 2002

Introduction

Allergen extracts are the most common immunotherapeutic agents in clinical use, with at least 30 million doses given each year in the US alone [1]. Unstandardized allergen extracts, which constitute the majority of products available in the US, are labelled either with the weight to volume dilutions used during manufacturing or with protein nitrogen units. These measurements have little relationship to allergen potency [2]. There are currently 19 standardized allergen extracts available in the US. The Laboratory of Immunobiochemistry at the Center for Biological Evaluation and Research (CBER) is responsible for developing and maintaining the references used

for standardized allergen extracts. An allergen extract is standardized when, as a condition of release to the public, the allergen content of each manufactured lot of the product is compared to a national reference standard. The labelled unitage on the product informs the physician of the results of that comparison. CBER has taken the position that the labelled unitage for standardized allergen extracts should predict the likelihood that a product will elicit an immediate allergic response in highly allergic individuals. This unitage is termed the overall allergen potency. The standard for overall allergen potency is an intradermal skin test titration protocol called ID₅₀EAL, in which the dilution at which a 50-mm skin test response (D50) to the allergen in question is determined in the target population [3,4]. Once the 'D50' dilution for a given extract is assigned, the relative potency (RP) of other preparations of the same allergen mixture may be determined by a related *in vivo* method, the parallel line bioassay [5,6]. These methods, though reliable, are cumbersome. *In vitro* techniques for potency determination have been developed for each standardized allergen extract [7,8].

Correspondence: Melissa L. Patterson, Laboratory of Immunobiochemistry, FDA/CBER/DBPAP, 1401 Rockville Pike (HFM-422), Rockville, MD 20852, USA. E-mail: pattersonm@cber.fda.gov

¹The views expressed in this article are the personal opinions of the authors and are not the official opinion of the U.S. Food and Drug Administration or the Department of Health and Human Services.

At the February 2000 Allergenic Products Advisory Committee Meeting, CBER announced its intention to standardize cockroach (CR) allergen extracts in the US [9]. German and American CR allergens were chosen as the next targets for standardization because cockroaches are ubiquitous, difficult to control, and frequently associated with allergy and asthma [10–15]. In this study, commercial American and German CR extracts were purchased from each allergen manufacturer that markets products in the US. The protein content and the electrophoretic banding pattern of each extract was determined and the specific allergen content of the German CR extracts was measured. In addition, the RP of each extract was estimated in comparison to lyophilized reference standards for which limited quantitative skin test data were available. Using these data, we determined the consistency of the available CR products and estimated the potency of the products in current use in the US.

Materials and methods

CR allergen extracts

A total of 24 American and German CR allergen extracts were purchased from nine different allergen extract manufacturers that are licensed to market allergen extracts in the US. These CR allergen extracts were received between March and May 2000 and stored during testing according to the manufacturer's specifications at 2–8 °C. Ten of these were German CR extracts and the remaining 14 were American CR extracts. Among these were six aqueous extracts, four American and two German, at dilutions of 1:10 or 1:1000. The remaining 18 extracts were obtained as 50% glycerin solutions at dilutions of 1:10 and 1:20.

We used two previously developed CR extracts as references in the testing. CBER references E2-Cg, a German CR extract, and E2-Ca, an American CR extract, were produced and lyophilized for CBER by Bayer (Spokane, WA, USA) in 1995. These were reconstituted in 50% glycerin/dH₂O as needed, and stored at –20 °C. These extracts were used as provisional references because they had been partially characterized through *in vitro* and *in vivo* tests. Furthermore, they were lyophilized and are available in large quantities. Both of the E2 extracts were previously used in ID₅₀EAL skin testing in 11 study subjects, eight of whom were African-American, with CR allergy (data not shown). The mean D50 for E2-Cg was 11.2, and for E2-Ca it was 10.3. Therefore, the estimated potencies of these extracts ($100\,000 \times 3^{-(14-D_{50})}$) were 4400 BAU/mL and 1700 BAU/mL, respectively [5,6].

Total protein content

The protein concentrations of the extracts were estimated by the modified ninhydrin method [16] (performed at Biopol Laboratory, Inc., Spokane, WA, USA). This method of determining the protein concentration has been used by CBER and manufacturers for standardized allergen extracts [2].

To make direct comparisons of the protein concentrations among different extracts, differences in manufacturing dilutions had to be taken into account. Modified ninhydrin protein concentrations were scaled to a 1:10 (w/v) dilution by the following equation:

$$[(\text{ninhydrin protein concentration}) \times (\text{extract dilution})]/10$$

Competition ELISA and sandwich ELISA

The RPs of the commercial CR extracts were determined by an optimized [4,17] and recently revalidated [2] competition ELISA (Laboratory of Immunobiochemistry-Standard Operating Procedure-7, revised November 1998) using a human allergic serum pool from 16 individuals (S1-Cr), and the reference extracts E2-Ca and E2-Cg. Plates were coated with E2-Cg or E2-Ca (2.5 µg/well) and blocked with 1% BSA in PBS containing 0.05% Tween-20. The wells were then incubated in sequence with a mixture of the competing antigen and a fixed dilution of S1-Cr, peroxidase-labelled goat antihuman IgE (ε-specific, KPL, Gaithersburg, MD, USA), TMB Peroxidase Substrate (KPL), and 1 M phosphoric acid. The absorbances of the micro-well solutions were read at 450 nm.

Specific Bla g 1 and Bla g 2 levels in the glycerinated German CR extracts were determined with monoclonal antibodies in an ELISA [18]. These assays were performed at Indoor Biotechnologies, Inc. (Charlottesville, VA, USA). Bla g 1 levels are reported in arbitrary units; Bla g 2 concentrations are reported in µg/mL.

The modified ninhydrin determined protein content of the reference extracts, E2-Cg and E2-Ca, was higher than the protein content of the commercial extracts. To ensure comparisons independent of these differences in protein content, values for RP and specific allergen levels were scaled using the following equation:

$$[(\text{sample RP, Bla g 1 or Bla g 2 content}) \times (\text{E2-Cg or E2-Ca protein content})]/(\text{sample protein content})$$

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

CR extract proteins were separated by electrophoresis in 4–20% polyacrylamide gels (Novex, San Diego, CA, USA) in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing 5% β-mercaptoethanol (Sigma, St Louis, MO, USA). Gels were loaded with equal amounts (25 µg) of each protein mixture. The E2 reference extracts were also analysed in a 12% SDS-PAGE gel containing 4 M urea after incubation in sample buffer that contained 8 M urea, 0.0625 M Tris-HCl (pH 6.8), 2% SDS, and 5% β-mercaptoethanol. Since the urea appeared to have a significant effect on the migration distances of the CR extract proteins, recombinant German cockroach allergens were run as controls for comparison. Recombinant German cockroach allergens, rBla g 2, rBla g 4 and rBla g 5 were obtained from Indoor Biotechnologies Inc. [19–22]. After separation, proteins were detected with Coomassie Brilliant Blue.

Statistical analyses

Bla g 1 and Bla g 2 levels of all commercial extracts were compared with the specific allergen levels of E2-Cg using Student's *t*-test. Protein contents, RPs and specific allergen levels among the extracts were analysed for correlation using Pearson's correlation coefficient.

Results

Total protein content

The protein content of commercial CR allergen extracts was highly variable. The mean protein concentration of the aqueous

sample extracts was 6.8 ± 9.7 mg/mL with a range of 0.1–24.8 mg/mL. The 18 glycerinated sample extracts had a mean protein content of 7.1 ± 5.9 mg/mL with a range of 2.4–22.9 mg/mL. When we scaled the protein concentrations to account for differences in dilutions, the content was 12.6 ± 6.7 mg/mL for the aqueous extracts and 9.6 ± 6.0 mg/mL for the glycerinated extracts. Unadjusted protein levels were used to scale the RP, Bla g 1 and Bla g 2 results in the remainder of the study.

The protein concentrations of the commercial CR extracts were lower than the E2 references. E2-Ca had a protein concentration of 30 mg/mL; E2-Cg, 25 mg/mL. Therefore, in comparing commercial extracts to the reference extracts, we scaled the potency and specific allergen data to account for these protein differences. In the correlation analyses for RP and specific allergen levels, we used the unadjusted data.

SDS-PAGE

Each of the CR allergen extracts was analysed by SDS-PAGE. The separated proteins of the aqueous extracts were at or below the detection limits of staining with Coomassie Brilliant Blue (not shown). Typical stained gels for the glycerinated extracts are shown in Fig. 1. While the protein migration patterns of different lots from a single manufacturer were fairly consistent, there were major differences in protein migration patterns among the different manufacturers. In other words, band intensities for specific bands varied among extracts from different companies. In addition, there were significant differences between the commercial extracts and the reference extracts.

Natural allergen extracts are complex mixtures of proteins but, with the exception of Per a 3, the identified allergens of German and American CRs have apparent molecular masses

less than 37 kDa [23]. The appearance of multiple bands with apparent molecular masses greater than 50 kDa in our SDS-PAGE analyses raised the possibility that some CR proteins form aggregates that resist SDS disaggregation, or that some CR proteins fail to denature completely in 1% SDS. When our reference extracts were incubated overnight in the presence of 8 M urea and 1% SDS prior to electrophoretic analysis, there was a dramatic migration shift. Nearly all of the bands migrated more rapidly than the 35 kDa molecular standard (Fig. 2).

We also examined the effect of urea denaturation on the migration of the pure recombinant German CR allergens, Bla g 2, Bla g 4 and Bla g 5 (Fig. 3). rBla g 2 and rBla g 5 appeared to be only slightly affected by the urea treatment. However, the electrophoretic migration of rBla g 4 was dramatically altered by urea denaturation, with the appearance of two new bands that may be subunits of the original single band.

Relative potency

The RPs of the commercial CR allergen extracts were determined by comparison to the E2 reference extracts using an established [4,17] and recently revalidated [2] competition ELISA technique. In this assay, the reference standard was assigned a RP value of 1.0 and the competitive binding of the test extract to pooled allergic serum IgE is compared to that of the reference standard.

The results of the competition ELISA are shown in Table 1. None of the aqueous CR extracts contained measurable IgE-binding activity even though they were tested undiluted and were scaled up to compensate for lower protein concentrations. Furthermore, none of the glycerinated CR extracts had RPs greater than 0.67, and most were substantially lower. In extensive prior testing, the standard deviation (σ) of the competition

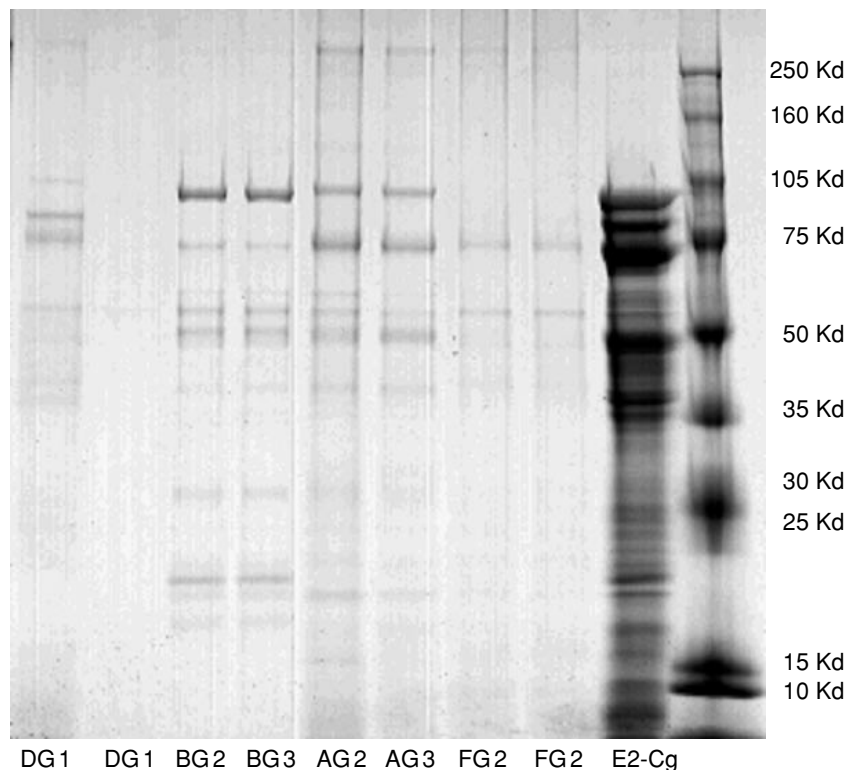


Fig. 1. SDS-PAGE of the glycerinated German CR allergen extracts. Each lane represents a different lot. Lots with the same first letter designation are from the same manufacturer.

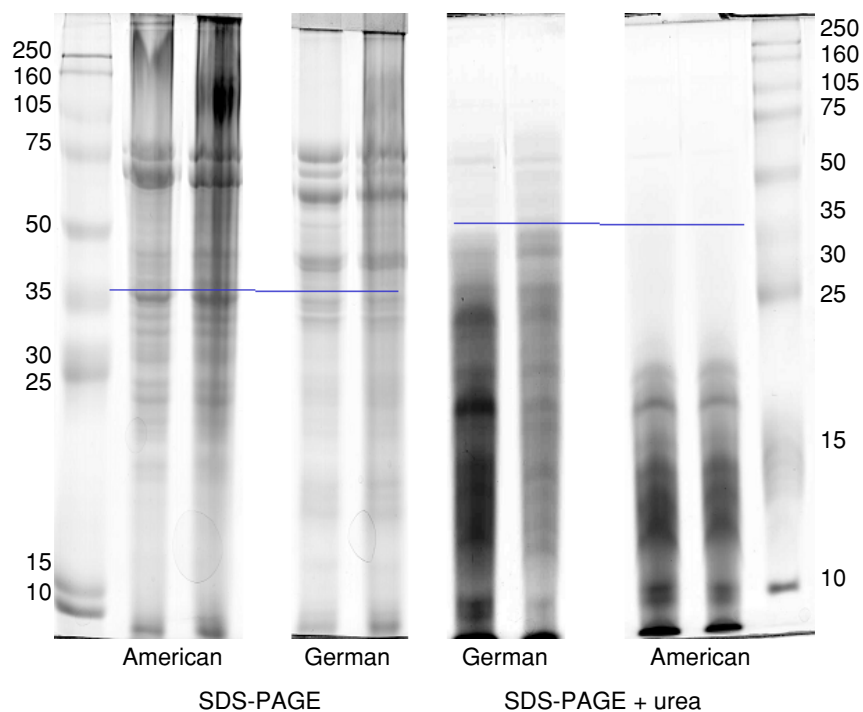


Fig. 2. The effect of denaturation with urea on the migration pattern of the E2 reference CR allergen extracts in 12% SDS polyacrylamide gels. Horizontal bars indicate the migration of the 35 kDa standards.

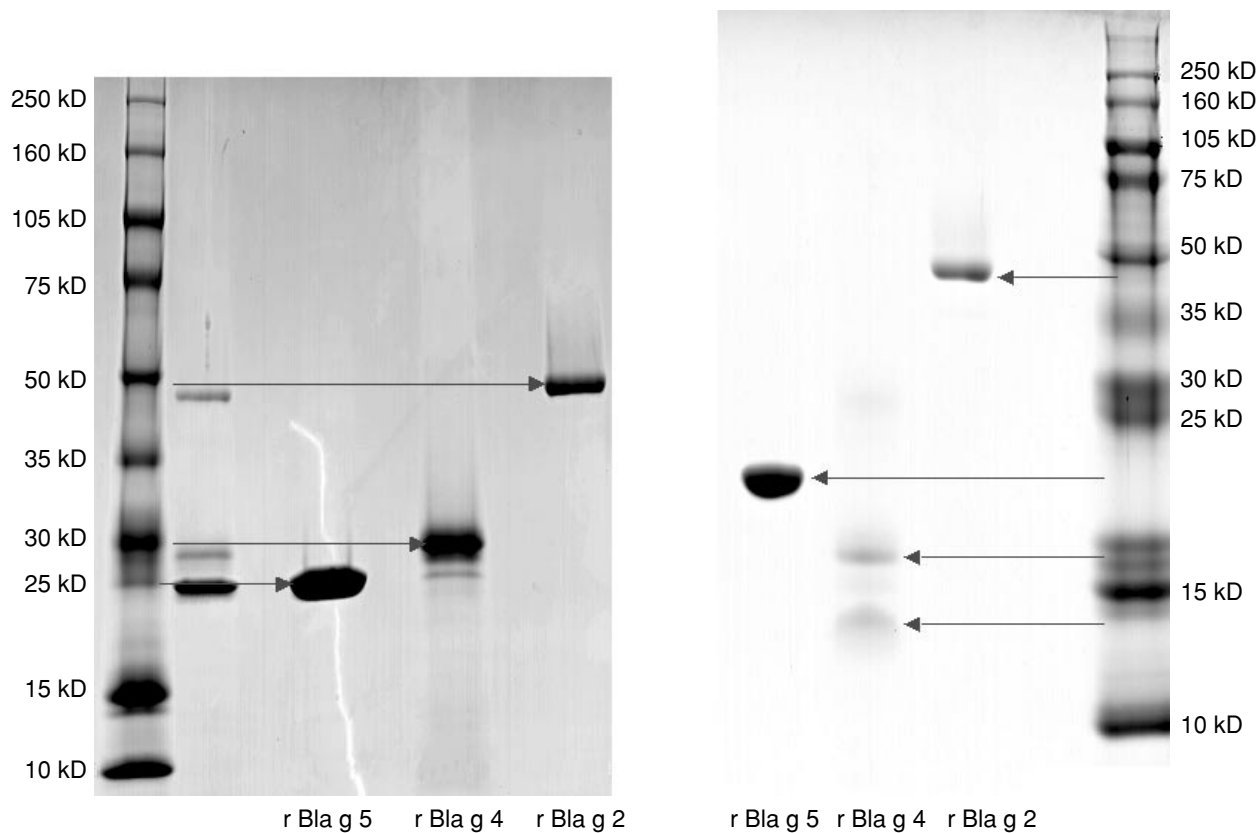


Fig. 3. 12% SDS polyacrylamide gels of recombinant CR allergens in the absence (left) and presence (right) of urea.

Table 1. Relative potencies of commercial cockroach extracts compared to E2 reference cockroach extracts determined by competition ELISA

		RP	RP*
American glycerin	Mean	0.0695	0.2916
	SD	0.0439	0.2314
	Range	0.0058 to 0.1472	0.0356 to 0.6690
German glycerin	Mean	0.0542	0.2614
	SD	0.0589	0.1819
	Range	0.0022 to 0.1777	0.023 to 0.4625
Aqueous	Mean	< 0.001	< 0.001
	SD	< 0.001	< 0.001

RP* represents potencies scaled to reflect differences in protein concentrations measured by the modified ninhydrin method.

Table 2. Scaled specific allergen levels of commercial glycerinated German CR allergen extracts and E2-Cg reference extract

	Bla g 1, U/mL	Bla g 2, µg/mL
Mean	3503	44
SD	1094	22
Range	2218–4854	8–66
E2-Cg	13829	8

ELISA for a single replicate was found to be 0.1375 in log RP. The 95% confidence interval for three replicates is given by $10 \pm 1.96\sigma/\sqrt{3}$. Consequently, a lot whose RP falls in the range 0.699–1.431 is statistically indistinguishable from the reference within a 95% confidence interval. Therefore, none of the tested extracts was equipotent with the reference material.

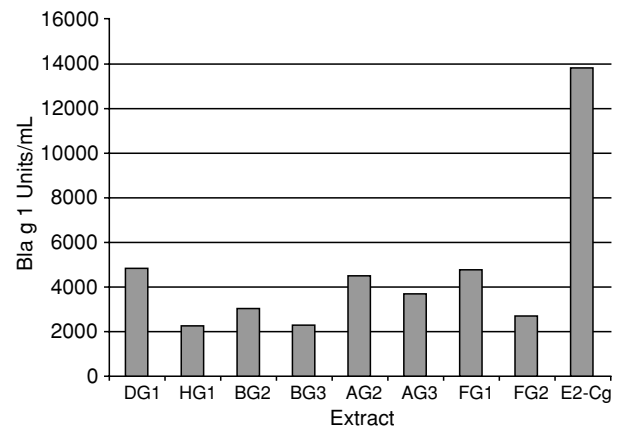
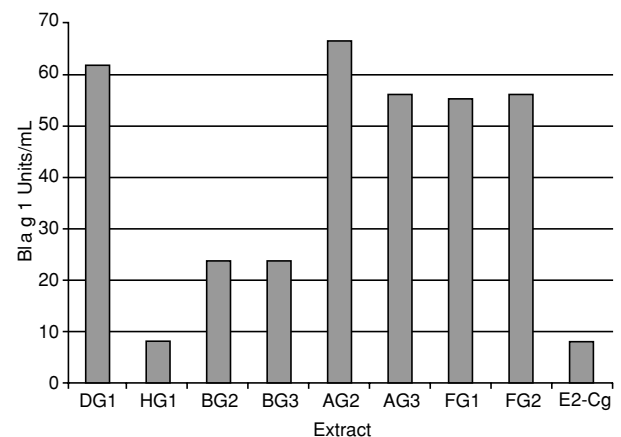
Specific allergen content

Table 2 contains the specific allergen determinations for the glycerinated German CR extracts. The Bla g 1 level in the reference extract was 3–6 times higher than the average Bla g 1 levels in the commercial extracts ($P=0.001$). Conversely, the Bla g 2 concentration in the reference extract was lower than the Bla g 2 concentrations in the commercial extracts (ns). Variation in Bla g 2 concentrations appeared to be much greater than the variation in Bla g 1 levels (Figs 4 and 5).

Comparison of the ninhydrin protein results, the RP's and the specific allergen levels for the glycerinated German CR allergen extracts indicated strong correlations among all of these parameters (Fig. 6). There was no correlation between protein concentrations and RP for the American CR extracts ($r^2=0.001$).

Discussion

In these experiments, we subjected 24 commercially available CR extracts to several different testing methods to characterize and compare them. We found that US CR allergen extracts have highly variable protein and allergen content, and the potencies of these products when compared with an arbitrary

**Fig. 4.** Bla g 1 levels in glycerinated German CR allergen extracts and the E2-Cg reference extract. Levels were scaled to account for differences in protein concentration (see Methods).**Fig. 5.** Bla g 2 concentrations (µg/mL) in the glycerinated German CR allergen extracts and the E2-Cg reference extract. Concentrations were scaled to reflect differences in protein concentrations.

reference is generally low. The reasons for this variability are unclear, but may include inaccurate species identification, inconstant growth conditions, variable harvest timing or methods, and differences in extraction techniques and storage.

The potency estimates were based on comparisons to reference standards prepared for CBER by one US allergen manufacturer in 1995. These reference standards had been subjected to limited ID₅₀EAL evaluations and, based on this testing, were estimated to have biological potencies of 4400 BAU/mL (for E2-Cg) and 1700 BAU/mL (for E2-Ca). Competition ELISA testing of the current commercial extracts suggests potencies of 10–782 BAU/mL for German CR, and 10–250 BAU/mL for American CR. These potency estimates must now be confirmed by *in vivo* testing, but for the German CR extracts, they appear to be consistent with the total protein assessments and the results of the Bla g 1 allergen assays (Fig. 6).

The threshold diagnostic and therapeutic doses for CR allergens have not been established. However, for other allergens target therapeutic doses of 5–20 µg [24–26] and 100–2500 BAU [3] have been estimated. If our BAU estimates for the CR allergens in this study were valid, then seven of the 18

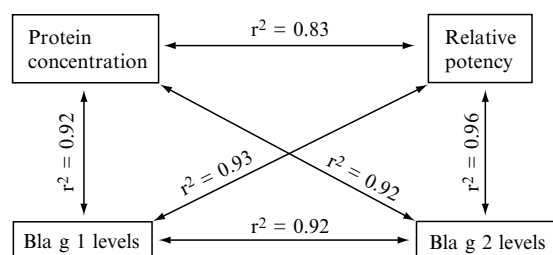


Fig. 6. Correlation of protein concentrations and ELISA results (for RP and specific allergen levels) for the glycerinated German CR extracts.

glycerinated CR allergen extracts that we examined would be subpotent for immunotherapy. For diagnosis in the general population, concentrations of approximately 100–1000 BAU/mL [27] are used. Eleven of the 18 of the glycerinated extracts that were purchased would meet this threshold. Among the eight glycerinated German CR allergen extracts that we examined, only five had Bla g 2 concentrations high enough to deliver a minimum of 5 µg/mL in a 1-mL dose. Furthermore, the variability that was measured in extract potencies and Bla g 2 concentrations suggests that the current practice of 4-fold to 10-fold dosage reductions with the introduction of new extract bottles may be insufficient to prevent adverse reactions in highly sensitive individuals.

We were not surprised to observe that aqueous CR extracts contained no detectable allergen by competition ELISA and many contained little protein. Prior studies have demonstrated that glycerin dramatically enhances allergen and protein stability [28]. Furthermore, glycerin may inhibit the action of proteases in allergen extracts [29]. Unlike standardized allergen extracts, for which expiry is based upon real-time allergen stability studies, FDA regulations permit manufacturers to set the expiry of aqueous allergen extracts between 18 and 36 months after manufacture, depending upon the time of shipment (21 CFR 610.53).

The successful diagnostic and therapeutic use of allergen extracts depends on an accurate assessment of allergen dose. The current unitage of CR allergen extracts fails to provide this basic information to prescribing physicians. Furthermore, the lack of an accurate and relevant unitage for an allergen extract precludes the performance of scientific studies with those extracts that generate meaningful and transferable data. Recent epidemiologic and skin testing data (using one glycerinated US product) have implicated CR exposure and allergy is on the rise in inner city asthma [14]. Although not specifically determined in that study, it is possible that the extract chosen was of adequate potency for that study's design, but the results could easily have been different had another product been used. In future studies using non-standardized CR extracts, investigations should ensure that a potent extract is used.

Tests for specific allergens, as opposed to overall allergen potency, have gained acceptance among many investigators and allergists [24,30]. On the evidence of clinical studies, CBER has adopted specific allergen tests as the standard potency tests for cat and ragweed allergen extracts, but has continued to use overall potency tests for standardized grasses and mites. Our preliminary testing suggests that both Bla g 1 and

Bla g 2 levels may correlate well with the RP of glycerinated German CR extracts, in spite of the low Bla g 2 content of our reference German CR extract. A prior study suggested that, among CR allergic individuals with asthma, 30% and 58% were seropositive to Bla g 1 and Bla g 2, respectively [21]. However, seroprevalence may not reflect the relative importance of each allergen in causing the disease. In addition, other specific allergens may be pathogenic, such as Bla g 4 and 5, for which 60% to 70% of CR-allergic individuals may have specific IgE antibody [15]. The safety and efficacy of extracts standardized by Bla g 1 and Bla g 2 levels alone will need to be verified before these tests can be adopted as the standards. Skin testing will be essential for developing an appropriate reference for the future and *in vitro* methods for testing commercial extracts.

For the hymenoptera venom allergens, CBER has adopted mass determinations of venom or venom protein as the standard unitage. The advantage of protein mass unitage is that it is technologically transparent, and does not require unique or expensive detection reagents. However, experience has shown that for standardized grass and dust mite extracts total protein and allergenicity correlate poorly [2]. Our limited preliminary data indicate that protein mass unitage may correlate well with the allergenicity of German CR allergen extracts, although it appears unlikely that this will be the case for American CR allergen extracts.

CBER has proposed that allergens be selected for standardization based on predefined impact criteria [9]. Criteria favouring selection of an allergen extract for standardization include the widespread use of a specific allergen as a diagnostic or therapeutic reagent in the US, and the public health impact of correct diagnosis and adequate treatment with the allergen. American and German CR allergen extracts were selected for this preliminary study based on these criteria and the concurrence of the Allergenic Products Advisory Committee. Conversely, a criterion not favouring selection of an allergen extract for standardization is consistency of currently marketed products. Standardization of an allergen that is already available on the market in a potent and consistent form would have less public health impact than the standardization of an allergen of variable and inadequate potency. On the basis of the studies described here, we conclude that currently available American and German CR allergen extracts sold in the US are characterized by inconsistent potency. Standardizing the allergens could improve the diagnosis and immunotherapy of CR-associated allergic disease.

References

- 1 Reid MJ, Lockey RF, Turkeltaub PC, Platts-Mills TA. Survey of fatalities from skin testing and immunotherapy 1985–89. *J Allergy Clin Immunol* 1993; 92:6–15.
- 2 Slater JE, Gam AA, Solanki MD, Burk SH, Pastor RW. Statistical considerations in the establishment of release criteria for allergen vaccines. *Arb. Paul. Ehrlich Institute Bundesamt Sera Impfstoffe Frankf. A. M.* 2000; 47–56.
- 3 Turkeltaub PC. Allergen Vaccine Unitage Based on Biological Standardization. *Clinical Significance*. In: Lockey R, Bukantz SC, eds. *Allergens and Allergen Immunotherapy*. 2nd Edn. New York: Marcel Dekker Inc. 1999; 321–40.
- 4 Turkeltaub PC. Biological standardization of allergenic extracts. *Allergol Immunopathol (Madr)* 1989; 17:53–65.

- 5 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; 70:343-52.
- 6 Turkeltaub PC. *In vivo* methods of standardization. *Clin Rev Allergy* 1986; 4:371-87.
- 7 Yunginger JW, Adolphson CR. Standardization of Allergens. In: Rose, NR de Macario, EC Fahey, JL Friedman, H Penn, GM, eds. *Manual of clinical laboratory immunology*. 4th Edn. Washington DC: American Society for Microbiology, 1992:678-84.
- 8 Platts-Mills TAE, Rawle F, Chapman MD. Problems in Allergen Standardization. *Clin Rev Allergy* 1985; 3:271-90.
- 9 Slater JE, Pastor RW. Allergenic Products Advisory Committee Meeting. 2000; <http://www.fda.gov/ohrms/dockets/ac/cber00.htm>
- 10 Lehrer SB, Horner WE, Menon PK, Oliver J, Hauck P. Cockroach allergenic activity: analysis of commercial cockroach and dust extracts. *J Allergy Clin Immunol* 1991; 88:895-901.
- 11 Lehrer SB, Horner WE, Menon P, Stankus RP. Comparison of cockroach allergenic activity in whole body and fecal extracts. *J Allergy Clin Immunol* 1991; 87:574-80.
- 12 Chapman MD, Pollart SM, Luczynska CM, Platts-Mills TA. Hidden allergic factors in the etiology of asthma. *Chest* 1988; 94:185-90.
- 13 Williams LW, Reinfried P, Brenner RJ. Cockroach extermination does not rapidly reduce allergen in settled dust. *J Allergy Clin Immunol* 1999; 104:702-3.
- 14 Rosenstreich DL, Eggleston P, Kattan M et al. The role of cockroach allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma. *N Engl J Medical* 1997; 336:1356-63.
- 15 Arruda LK, Vailes LD, Ferriani VP, Santos AB, Pomes A, Chapman MD. Cockroach allergens and asthma. *J Allergy Clin Immunol* 2001; 107:419-28.
- 16 Richman PG, Cissel DS. A procedure for total protein determination with special application to allergenic extract standardization. *J Biol Stand* 1988; 16:225-38.
- 17 Lin Y, Miller CA. Standardization of allergenic extracts. an update on CBER's standardization program. Arb Paul. Ehrlich Institute Bundesamt Sera Impfstoffe Frankf A M 1997, 127-30.
- 18 Pollart SM, Mullins DE, Vailes LD et al. Identification, quantitation, and purification of cockroach allergens using monoclonal antibodies. *J Allergy Clin Immunol* 1991; 87:511-21.
- 19 Arruda LK, Vailes LD, Hayden ML, Benjamin DC, Chapman MD. Cloning of cockroach allergen, Bla g 4, identifies ligand binding proteins (or calycins) as a cause of IgE antibody responses. *J Biol Chem* 1995; 270:31196-201.
- 20 Vailes LD, Kinter MT, Arruda LK, Chapman MD. High-level expression of cockroach allergen, Bla g 4, in *Pichia pastoris*. *J Allergy Clin Immunol* 1998; 101:274-80.
- 21 Arruda LK, Vailes LD, Mann BJ et al. Molecular cloning of a major cockroach (*Blattella germanica*) allergen, Bla g 2. Sequence homology to the aspartic proteases. *J Biol Chem* 1995; 270:19563-8.
- 22 Arruda LK, Vailes LD, Platts-Mills TA, Hayden ML, Chapman MD. Induction of IgE antibody responses by glutathione S-transferase from the German cockroach (*Blattella germanica*) *J Biol Chem* 1997; 272:20907-12.
- 23 Helm RM. Cockroach and other inhalant insect allergens. In: Lockey, RF Bukantz, SC, eds. *Allergens and Allergen Immunotherapy*, 2nd edn. New York: Marcel Dekker Inc. 1999: 203-23.
- 24 Bousquet J, Lockey RF, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. Geneva: January 27-29, 1997. *Allergy* 1998; 53:1-42.
- 25 Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol* 1998; 102:558-62.
- 26 Nelson HS. Preparing and mixing allergen vaccines. In: Lockey, RF Bukantz, SC, eds. *Allergens and Allergen Immunotherapy*. 2 Edn. New York: Marcel Dekker Inc. 1999; 401-22.
- 27 Bernstein IL, Storms WW. Practice parameters for allergy diagnostic testing. *Ann Allergy Asthma Immunol* 1995; 75:553-625.
- 28 Bousquet J, Djoukadar F, Hewitt B, Guerin B, Michel FB. Comparison of the stability of a mite and a pollen extract stored in normal conditions of use. *Clin Allergy* 1985; 15:29-35.
- 29 Esch RE. Role of proteases on the stability of allergenic extracts. Arb Paul. Ehrlich Institute Bundesamt Sera Impfstoffe Frankf A M 1992; 171-7.
- 30 Chapman MD, Smith AM, Vailes LD, Arruda LK, Dhanaraj V, Pomes A. Recombinant allergens for diagnosis and therapy of allergic disease. *J Allergy Clin Immunol* 2000; 106:409-18.