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Efficacy of Recombinant Allergens for Diagnosis of Cockroach Allergy in Patients with Asthma and/or Rhinitis

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Key Words
Tropomyosin · Cockroach allergy · Asthma · Recombinant allergens

Abstract
Background: Immunoglobulin E (IgE) reactivity to individual allergens among cockroach-allergic patients has revealed wide variability. The aim of this study was to assess the effectiveness of recombinant cockroach allergens for skin testing, and to determine sensitization profiles among cockroach-allergic patients living in Brazil. ***Methods:*** Fifty-seven cockroach-allergic patients with asthma and/or rhinitis were recruited. Skin testing with recombinant (r) allergens from *Periplaneta americana* (rPer a 1 and rPer a 7) and *Blattella germanica* (rBla g 2, rBla g 4 and rBla g 5) were performed at 10 μg/ml and 5 μg/ml (rPer a 1). IgE antibodies to rPer a 7 and rPer a 1 were quantitated by ELISA. ***Results:*** Of 57 patients tested, 3 (5.3%), 24 (42.1%), 4 (7%), 3 (5.3%) and 4 (7%) showed positive reactions to rPer a 1, rPer a 7, rBla g 2, rBla g 4 and rBla g 5, respectively. Twenty-eight patients (49.1%) had positive tests to at least one allergen. In keeping with skin test results, 31/57 patients (54.4%) and 5/55 patients (9%) had detectable IgE to rPer a 7 and rPer a 1, respectively. Levels of IgE to rPer a 7 were higher in patients with positive tests to rPer a 7 than those with negative tests (geometric mean 13.2 and 1.8 IU/ml, $p < 0.05$). There was good concordance of results of skin tests and measurements of serum IgE to rPer a 7. ***Conclusion:*** IgE reactivity to rPer a 7 (*P. americana* tropomyosin) was dominant among patients in Brazil. However, 50% of the patients did not present reactivity to any of the recombinant allergens tested.

Introduction

Cockroaches are an important source of proteins which cause immunoglobulin E (IgE)-mediated responses associated with asthma and allergic rhinitis [1]. Cockroach allergy is associated with increased asthma-related morbidity, particularly among underprivileged patients living in inner-city environments and those from nonaffluent countries [2–4]. Allergens from the two most common domiciliary cockroach species, *Blattella germanica* and *Periplaneta americana*, have been identified, with diverse structure and biological function. Cockroach allergens comprise at least 10 groups of distinct proteins, including Bla g 1 and Per a 1 (midgut microvilli protein homologues), Bla g 2 and Per a 2 (inactive aspartic prote-

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ases), Per a 3 (arylphorin/hemocyanin), Bla g 4 and Per a 4 (male pheromone transport lipocalins), Bla g 5 and Per a 5 (glutathione-S-transferases), Bla g 6 and Per a 6 (troponins), Bla g 7 and Per a 7 (tropomyosins), Bla g 8 (myosin light chain), Bla g 9 and Per a 9 (arginine kinases) and Per a 10 (serine protease) [5]. *B. germanica* enolase and vitellogenin have also been identified as novel cockroach allergens by proteomics approach [6]. In clinical practice, exclusive reactivity to either German or American cockroach extracts on skin testing is rarely observed among cockroach-allergic patients, and this is thought to be due to cross-reactivity to the homologous allergens.

Environmental exposure to cockroach results from release of allergens produced in the digestive tract and excreted with feces (groups 1 and 2, Per a 3), or secretion from the male reproductive tract during sexual activity (Bla g 4 and Per a 4) [7, 8]. Dead bodies may also be source of allergens, in particular muscle proteins (groups 6, 7 and Bla g 8) [9, 10]. Allergens become airborne in particles of 10–40 μm in diameter, which may reach the lungs and induce IgE antibody responses. It has recently been reported that the proteolytic activity of the serine protease allergen Per a 10 caused enhanced lung inflammation in a mouse model of asthma [11, 12].

Diagnosis of cockroach allergy is routinely performed by skin testing and/or measurement of specific IgE to cockroach, using crude extracts. Cockroach extracts manufactured in the USA for allergy diagnosis show variations in levels of major allergens Bla g 1 and Bla g 2 of up to 7-fold [13]. Slater et al. [14] have shown that the mean potency of 3 US German cockroach extracts was 3,300 BAU/ml, using the intradermal D50 method, whereas standardized mite, cat or grass extracts contain typically 5,000–100,000 BAU/ml. A lack of well-characterized extracts may have hampered research on immunotherapy for cockroach allergy. Few published studies on cockroach immunotherapy among patients with asthma and/or rhinitis have shown beneficial changes in immunological and clinical parameters [15–17]. Studies on subcutaneous and sublingual immunotherapy with German cockroach extract are currently being conducted in the USA.

Component-resolved diagnosis has been proposed as a strategy to improve specificity, to refine investigation of allergen cross-reactivity and to establish clinical correlations including predictions of severity of disease and of long-term outcomes [18–22]. One initial step to component-resolved diagnosis is to define the most appropriate allergen repertoire for a given allergen source, which would identify the vast majority of patients presenting clinical disease. In this study, we performed skin prick testing in a group of 57 cockroach-allergic patients living in Brazil, using a panel of 5 *P. americana* and *B. germanica* recombinant allergens, and we then examined serum IgE antibody responses to Per a 1 and Per a 7. Our results revealed that cockroach tropomyosin (Per a 7) is an immunodominant allergen in Brazil, and indicated that further studies may be necessary to establish a panel of cockroach allergens which could facilitate identification of the majority of cockroach-allergic patients by skin testing.

Subjects and Methods

Subjects and Skin Testing

A total of 57 patients, 7–54 years of age, with asthma and/or rhinitis participated in this study. Patients were selected consecutively from those attending the Allergy Clinic at the Clinical Hospital of the School of Medicine of Ribeirão Preto in Brazil. All subjects presented positive skin tests to extracts of *P. americana* and *B. germanica* (1:20 w:v, Greer Laboratories, Lenoir, N.C., USA), and underwent skin prick testing with recombinant (r) allergens of *B. germanica*, rBlag 2, rBlag 4, rBla g 5 and *P. americana*, rPer a 1 and rPer a 7 at concentrations of 10 or 5 μg/ml (rPer a 1). Previous studies by our group with recombinant mite allergens have established that concentrations of 5–10 μg/ml were safe and optimal for skin testing, and that increasing the dose to 50 μg/ml did not increase the positivity of the tests (manuscript in preparation). The concentration of 5 μg/ml for rPer a 1 was chosen due to limited availability of the allergen for skin testing. Ten nonallergic nonasthmatic subjects, who presented negative skin tests to a panel of inhalant allergens, including cockroach, were evaluated as negative controls. Recombinant allergens from *B. germanica* and Per a 1 were produced at Indoor Biotechnologies, Charlottesville, Va., USA, according to previously described methods [23–28]; the purity of these allergens was determined as >95% by silver-stained SDS-PAGE and the IgE-binding activity of these preparations has been reported [23–28]. Recombinant *P. americana* tropomyosin (Per a 7 allergen) was expressed in the *Pichia pastoris* system using the pPIC9 vector, as previously described [10].

Skin prick tests were performed on the forearm by using a prick lancetter (Allergoprick Inox, Flexor, SP, Brazil). A reaction was considered positive when a wheal with a diameter 4 × 4 mm or greater, accompanied by erythema, developed within 15 min after application of the allergen, based on the work by Peat et al. [29]; in a longitudinal study involving children older than 6 years, they showed that skin wheals of 4 mm or greater were most consistently associated with allergic illnesses. Positive (10 mg/ml histamine dihydrochloride) and negative (sterile albumin-saline with phenol) (both Hollister-Stier Laboratories, Spokane, Wash., USA) controls were applied in all tests. The study was approved by the ethics committee of our institution, and all patients or their guardians gave their informed consent to participate in the study.

Total IgE and Specific IgE to P. americana and B. germanica

Levels of total IgE and specific IgE to *P. americana and B. germanica* were determined using the ImmunoCAP system (Phadia, Brazil), and results were expressed as kU/l.

Table 1. Characteristics of cockroach-allergic patients participating in the study and skin tests and IgE antibodies to cockroach allergens

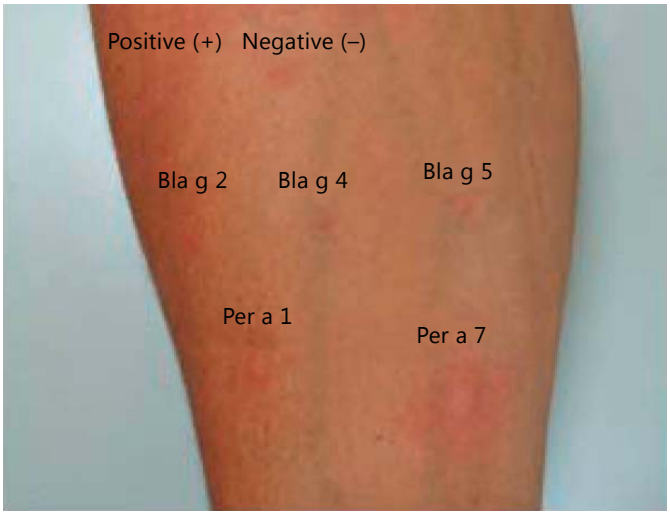
Patient characteristics	(n = 57)
Sex, female	29/57 (51)
Age in years, mean	16 [7–52]
Diagnosis	
Asthma	18 (31.6)
Asthma and rhinitis	28 (49.1)
Rhinitis	9 (15.8)
Asthma/rhinitis/atopic dermatitis	2 (3.5)
Positive skin tests to *P. americana*	57/57 (100)
Mean wheal diameter, mm	6 [5–10]
Positive skin tests to *B. germanica*	57/57 (100)
Mean wheal diameter, mm	5 [4–8]
Positive skin tests to *D. pteronyssinus*	54/57 (94.7)
Mean wheal diameter, mm	7 [5–10]
Total IgE levels, GM, kU/l	531.0 [17.0–4,727.0]
IgE to *P. americana* and/or *B. germanica*, number of positive	21/28[a] (75)
GM, kU/l	4.0 [0.7–44.3]
Positive skin tests to recombinant cockroach allergens	
At least one allergen	28/57 (49)
rPer a 1	3/57 (5.3)
rPer a 7	24/57 (42.1)
rBla g 2	4/57 (7)
rBla g 4	3/57 (5.3)
rBla g 5	4/57 (7)
IgE to rPer a 1, number of positive	5/55 (9)
GM, IU/ml	0.6 [0.5–12.5]
IgE to rPer a 7, number of positive	31/57 (54.4)
GM, IU/ml	2.4 [0.5–200]

Data are expressed with percentages in parentheses and range in brackets.

[a] Specific IgE to *P. americana* and/or *B. germanica* was available for 28/57 patients with positive skin tests to cockroach.

Chimeric ELISA for Specific IgE to Per a 1 and to Per a 7 (P. americana tropomyosin) in Sera from Cockroach-Allergic Patients

Specific IgE antibodies to Per a 1 and Per a 7 were measured using a chimeric ELISA, as previously described [30, 31]. In brief, microtiter plates were coated with 1 μg/well of monoclonal antibody 10A6 directed against Bla g 1, which is antigenically cross-reactive with Per a 1, or with mAb 1A6 directed against *D. pteronyssinus* tropomyosin, which also recognizes shrimp and cockroach tropomyosin, overnight at 4°C, in carbonate-bicarbonate buffer (pH = 9.6). After washing, plates were incubated with rPer a 1 or rPer a 7 at a concentration of 0.5 μg/ml. Subsequently, patients' sera were added at 1:10 dilution, followed by incubation with biotinylated goat anti-human IgE (1:4,000, KPL, Md., USA), and streptavidin-peroxidase (1:1,000, Sigma, St Louis, Mo., USA). The reaction was developed using 1 mmol 2,2′-azino-bis (3-ethylbenzthiazoline-6 sulphonic acid) (Sigma) and H_2O_2. The ELISA was quantitated using a chimeric mouse Fab/human Fc epsilon antibody (clone 2B12-IgE), as previously described [30, 31].



Color version available online

Fig. 1. Skin prick testing in a representative patient who participated in the study. Positive skin tests were observed for rPer a 1 and rPer a 7 and negative tests were obtained for rBlag 2, rBla g 4 and rBla g 5.

Statistical Analysis

The comparison of specific IgE levels to Per a 7 in the groups with positive and negative skin tests to rPer a 7 was carried out by the Mann-Whitney test, and a p value <0.05 was considered significant. Concordance of results of skin tests and IgE measurements to rPer a 7 was assessed by the Kappa statistics; Kappa index and its 95% confidence interval were calculated according to Fleiss [32] using the SAS version 9.2 software.

Results

In this study, a total of 57 subjects underwent skin prick testing with recombinant allergens of *B. germanica* and *P. americana*. Characteristics of patients participating in the study are shown in table 1. Of 57 patients tested, 3 (5.3%), 24 (42.1%), 4 (7%), 3 (5.3%) and 4 (7%) showed positive skin prick tests to Per a 1, Per a 7, Bla g 2, Bla g 4 and Bla g 5, respectively. Twenty-eight of these 57 patients (49.1%) had positive skin tests to at least one recombinant allergen (table 1). Ten nonallergic control subjects showed no skin reactivity to recombinant cockroach allergens (data not shown). There were no adverse reactions after skin prick testing. Figure 1 shows the results of skin prick tests in a representative patient who participated in the study, who presented positive tests to rPer a 1 and rPer a 7.

Levels of specific IgE to rPer a 7 and to rPer a 1 were determined by chimeric ELISA. Thirty-one of the 57 patients (54.4%) had detectable IgE to rPer a 7 (table 1). There was good concordance of results of skin tests with rPer a 7 and measurements of specific IgE to rPer a 7 in sera. Twenty-three of the 24 (96%) patients with positive skin tests to rPer a 7 had detectable IgE to rPer a 7; however, 8/33 (24%) with negative skin tests had detectable IgE to this allergen (table 2). Levels of IgE antibodies to Per a 7 varied from 0.5 to 200 IU/ml [geometric mean (GM) 2.4 IU/ml], and they were significantly higher in the group with positive tests to rPer a 7 than in those with negative tests (GM 13.2 and 1.8 IU/ml, respectively, $p < 0.05$; fig. 2). IgE antibodies to rPer a 1 were very low (table 1). We found no correlation of age, gender, duration or severity of disease and presence of positive skin tests and/or specific IgE to Per a 7 in this group of patients (data not shown). In addition, there were no differences in patterns of recognition of allergens between children and adults (data not shown). The complete panel of results from the 57 patients who participated in the study is presented in online supplementary table 3 (for all online suppl. material, see www.karger.com/doi/10.1159/000346318).

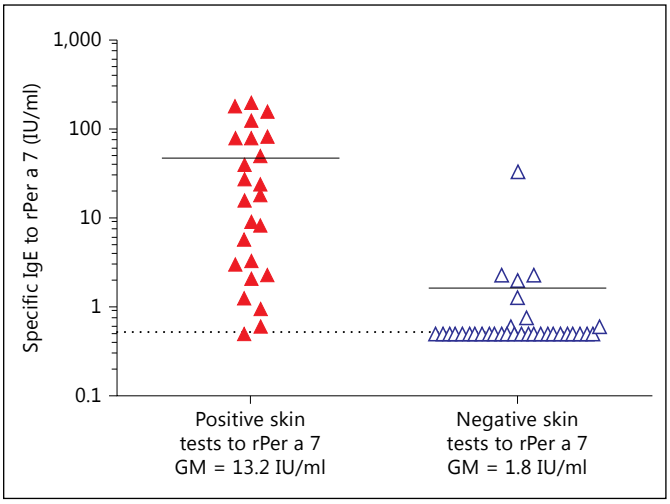


Fig. 2. Levels of specific IgE to rPer a 7 in sera from patients with positive and negative skin tests to rPer a 7. GM levels of specific IgE to rPer a 7 were significantly higher in the group with positive skin tests to rPer a 7 than in the group with negative tests (GM 13.2 IU/ml and 1.8 IU/ml, respectively, Mann-Whitney test, $p < 0.05$). Assay detection limit of 0.5 IU/ml (dotted line).

Table 2. Concordance of results of skin tests with recombinant Per a 7 and measurements of IgE to recombinant Per a 7 in sera from cockroach-allergic patients

	Detectable IgE to rPer a 7 in serum	Undetectable IgE to rPer a 7 in serum
Positive skin test to rPer a 7	23/57	1/57
Negative skin test to rPer a 7	8/57	25/57

Kappa index 0.69 (95% CI 0.51–0.87): good concordance.

Discussion

In this study, we showed that cockroach tropomysosin (Per a 7 allergen) is the dominant allergen among cockroach-allergic patients in Brazil with asthma, rhinitis or both. Skin testing using a panel of 5 recombinant allergens was performed on 57 cockroach-allergic patients, and 24 patients (42%) had positive responses to rPer a 7. The results of these skin tests paralleled those of in vitro tests for IgE to Per a 7, with a good concordance rate. The frequency of sensitization to Per a 7 of 43–54%, considering in vivo and in vitro testing, respectively, was in keeping with previous studies from our group [10].

Recombinant cockroach allergens have been used for in vitro studies, which revealed that patients present variable allergen sensitization profiles; no single major allergen appears to account for most of the IgE reactivity to cockroach. Using streptavidin CAP and a multiplex flow cytometric assay, Satinover et al. [33] demonstrated that a panel of 5 purified recombinant allergens (rBla g 1, rBla g 2, rBla g 4, rBla g 5 and rPer a 7) could identify 62% of cockroach-allergic US patients, defined by a positive ImmunoCAP to *B. germanica* extract. Prevalence of IgE antibodies was highest for rBla g 2 (54.4%) and rBla g 5 (37.4%) among the 118 sera analyzed; however, patterns of IgE antibody binding were unique to each subject. More recently, Chuang et al. [6] also showed that IgE reactivity profiles were heterogeneous, in a group of 32 cockroach-allergic patients presumably from Taiwan. Using a panel of recombinant cockroach allergens Bla g 1, Bla g 2, Bla g 4, Bla g 5, Bla g 7, the newly identified *B. germanica* enolase, arginine kinase and vitellogenin, the authors demonstrated substantial differences in the prevalence of IgE reactivity to each of the allergens. All patients reacted to at least one recombinant allergen on an IgE dot-blot immunoassay, and IgE reactivity ranged from 62.5% to Bla g 2 to 25% to enolase, as judged by relative densitometric indexes.

Surprisingly, in the study by Satinover et al. [33], the reactivity to rPer a 7 was very low; only 12.7% of sera pre-

sented IgE to this allergen. Even when analyzing sera of patients with cosensitization to house-dust mites, only 15/93 (16%) were positive to cockroach tropomyosin, suggesting that cosensitization was due to concomitant exposure to dust mites and cockroach, rather than to allergenic cross-reactivity. In keeping with this, studies carried out in Europe have revealed a low frequency of IgE reactivity to mite tropomyosin (Der p 10); among mite-allergic patients from various European countries, IgE to Der p 10 was found in 6–18% of the patients [34]. On the other hand, analysis of sensitization profiles in a large group of allergic patients in Africa revealed that 55% of patients presented IgE to mite tropomyosin [35].

It is possible that the high frequency of reactivity to cockroach tropomyosin seen in our patients may reflect cross-reactivity to mite tropomyosin, which shares 80% sequence identity to cockroach tropomyosin; however, we cannot rule out cosensitization. We hypothesize that the high frequency of sensitization to tropomyosins in Brazil and Africa may be due to cross-reactivity to tropomyosin from intestinal parasites. *Ascaris lumbricoides* is the leading cause of parasite infection among underprivileged populations in Brazil and worldwide, with estimates of 1.4 billion currently infected individuals in the world. In endemic areas, children get exposed to *A. lumbricoides* early in life, and infection may target the lungs through passage of larvae during the life-cycle of the parasite [36]. Several reports and a meta-analysis revealed that current infection with *A. lumbricoides* was associated with a significant increase in the risk of asthma [37]. Our group [38], and subsequently Acevedo et al. [39], working in Cartagena, Colombia, has cloned and produced *A. lumbricoides* tropomyosin as a recombinant protein. *A. lumbricoides* tropomyosin shows an approximately 70% amino acid sequence identity to tropomyosins from mites and cockroach, and the recombinant protein was shown to bind IgE from patients with asthma and/or rhinitis in 42% (assessed by ELISA among patients in Brazil) to 56% (assessed by skin testing in Cartagena) and 68% (evaluated by dot-blot assay in Cartagena) of patients. IgE responses to tropomyosins derived from inhalant allergens could be amplified or develop more promptly as a result of previous sensitization to *Ascaris* tropomyosin, triggering persistent lung inflammation [38]. In affluent countries, where frequency of infections with intestinal parasites is much lower, this effect would not play a significant role.

The frequency of positive responses to the other recombinant allergens was lower than 10% in our patients, raising the question of whether the recombinant allergens were equivalent to their natural counterparts. A good correlation of IgE binding to natural and recombinant Bla g 1 has been reported; Bla g 1 and Per a 1 present sequence identity of 70%, and are highly cross-reactive [28]. Likewise, an excellent correlation of IgE binding to natural and recombinant Blag 2 and Bla g 5 has been described [23, 27]. IgE reactivity to rBla g 4 has been demonstrated, both in vivo and in vitro [25, 26], and the detection of Bla g 4 protein and RNA in tissue of the adult male reproductive system has been reported [40]; however, Bla g 4 has never been purified from the natural source, hampering direct comparisons of IgE reactivity to natural and recombinant proteins. Previous data revealed that IgG and IgE binding activity of recombinant Per a 7 produced in *Escherichia coli* was somewhat lower than that of natural Per a 7 [41]. However, production of recombinant protein in a prokaryotic system may result in altered folding, leading to less biological activity. In this study, we used rPer a 7 produced in *P. pastoris*, previously shown to have good biological activity [38]. Therefore, the data available support the equivalence of the recombinant cockroach allergens to their natural counterparts, making it unlikely that the low degree of reactivity to rPer a 1, rBla g 2, rBla g 4 and rBla g 5 in our study was due to the loss of biological activity during the recombinant preparation of the allergens.

Alternative explanations may include the fact that in the USA, patients are primarily exposed to *B. germanica*, whereas in Brazil and in other parts of the world, *P. americana* is the dominant species in the home. However, most *B. germanica* allergens present homologues in *P. americana* which could account for IgE cross-reactivity [5]. Another hypothesis would be that the pattern of environmental exposure to cockroach allergens leading to sensitization may be distinct. In our region, the style of housing is peculiar to warmer climates, with homes and schools being ventilated by keeping windows open; these conditions may not be as conducive to a heavy and sustained cockroach infestation as those observed in US inner-city apartments [2, 42]. Consistent with this, levels of cockroach allergens Bla g 1 and Bla g 2 in the homes of asthmatic patients in our area were at least 10-fold lower than those previously reported in the homes of inner-city asthmatic patients in the USA [43]. Finally, it is possible that cockroach-allergic patients in Brazil show reactivity to allergens other than those evaluated in this study, although these have been reported as minor allergens, with a low prevalence of sensitization (approx. 14% for Bla g 6 and Bla g 8 [43] and 12% for Per a 3; manuscript in preparation). Allergens from groups 9 and 10 were recently

identified, and reagents are not readily available for measuring IgE antibody levels.

In conclusion, we report, for the first time, the use of recombinant allergens for the diagnosis of cockroach allergy in patients with asthma and/or rhinitis living in Brazil. Recombinant allergens induced positive skin test reactions comparable to those caused by commercial cockroach extracts, and these were safe. The results showed prominent differences of IgE reactivity profile in our patients when compared to those from the USA and Taiwan, particularly with regard to reactivity to cockroach tropomyosin, which was much higher in our population. It is possible that IgE responses to *A. lumbricoides* tropomyosin could promote/enhance the development of sensitization to tropomyosins from other sources including cockroach and mites. Our results highlight the need to consider regional differences in IgE reactivity profiles among patients with allergic diseases. Further studies including additional cockroach allergens will be necessary to establish a panel of allergens which could identify the majority of cockroach-allergic patients by skin testing.

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