

# Analysis of cross-reactive allergens from American and German cockroaches by human IgE

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Serum from atopics hypersensitive to the American cockroach were examined for their specific IgE to American and German cockroaches by the fluoroallergosorbent test (FAST). Of 44 sera tested, 86.4% (38/44) contained IgE to American and German cockroaches, and 13.6% (6/44) were found to be positive for American cockroach alone by FAST. Nine individual sera containing IgE antibodies to both cockroaches were used to analyze the cross-reacting allergens in the crude American and German cockroach extracts by FAST inhibition and immunoblotting. FAST-inhibition studies showed various degrees but similar inhibition of binding of human IgE to solid-phase American cockroach extract. Proteins from both cockroach extracts were separated on SDS-PAGE followed by immunoblotting, and the results showed considerable heterogeneity in the IgE-binding patterns with each of the cockroach extracts for the same nine individual sera. Components with apparent molecular weights of 60, 52, 49, 38, and 12 kDa from both the American and German cockroaches were able to bind IgE antibody. These results suggest the presence of cross-reactive allergens in the American and the German cockroaches.

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Cockroaches, commonly found in urban dwellings worldwide, have long been considered vectors of various infectious diseases (1) and recognized as one of the major causes of inhalant allergy and asthma (2–12). A high incidence of cockroach hypersensitivity in atopic and asthmatic populations has been reported (3, 4, 9, 10, 12). Allergens with masses ranging from 6 to 120 kDa from American cockroach (9, 13–20) and allergens in a similar range from German cockroach (14–18, 21–23) have been identified by various immunochemical techniques. Recently, Bla g 2 and Bla g 4 of German cockroach have been sequenced (24, 25), and cDNAs encoding for major allergens of American cockroach have been isolated and sequenced in our laboratory (26, 28). It has been suggested that crude extracts from German (*Blattella germanica*), American (*Periplaneta americana*), Oriental (*Blatta orientalis*), and Asian (*Blattella asahinai*) cockroaches contained common IgE-binding components (14–17, 23).

Over 60 species of cockroaches have been described in Taiwan. However, only seven species (*P. americana*,

*P. brunnea*, *P. australasiae*, *B. germanica*, *Nauphoeta cinera*, *Neostylophyga rhombifolia*, and *Pycnoscelus surinamensis*) are frequently found in homes. According to the consulted entomologists, *P. americana* is the major domiciliary cockroach found in the Taichung area. On the other hand, *B. germanica* is the predominant cockroach in the Taipei area. In this study, we analyzed the allergenic cross-reactivity of prominent components from these two species, using the serum of subjects hypersensitive to the American cockroach extract.

## Material and methods

### Cockroach extracts

Batches of live adult American cockroaches were trapped, identified, and stored in a –70°C deep freezer. Crude *P. americana* extract (CRa-A) was prepared in our laboratory with Coca's solution, as previously described (13). Commercial American cockroach (1 : 20 w/v, lot no. GB26-30B-15A) and German cockroach (1 : 20 w/v, lot no. GB46-3B-1A3)

extracts were obtained from Greer Laboratories (Lenoir, NC, USA). In order to be used as antigens for the fluoroallergosorbent test (FAST), FAST inhibition, and immunoblotting, German cockroach extract (CRa-G, lot no. GB46-3B-1A3, Greer) was extensively dialyzed against 0.02 M phosphate-buffered saline (PBS) and concentrated.

### *Subjects and allergy skin test*

Adult patients with symptomatic allergic rhinitis or asthma, from the allergy clinics at Taichung Veterans General Hospital in Taichung and Chung Gung Memorial Hospital in Taipei, Taiwan, were selected for skin test with commercial American cockroach (1 : 20 w/v, Greer Laboratories) and other common inhalant allergens. Physiologic buffered saline and histamine phosphate solution (0.1 mg/ml) were used as negative and positive controls, respectively. The prick test was performed with a 27-gauge needle, and the result was read 15 min later by measuring the diameter of flare and wheal. The interpretation was recorded according to the following criteria: (–) flare <10 mm, (1+) flare 11–20 mm, (2+) flare 21–30 mm and wheal 5–10 mm, (3+) flare 31–40 mm and wheal 5–10 mm, (4+) flare >41 mm or wheal >10 mm or with pseudopods. A total of 84 sera of atopic patients, with a range of 17–64 years, who gave over (1+) immediate skin reaction to CRa-A, was collected for specific IgE test by FAST. Sera of nine patients (21–61 years old), containing both CRa-A and CRa-G IgE antibodies, and the serum of a CRa-A- and CRa-G-negative atopic patient (49 years old) were subjected to FAST inhibition and immunoblotting studies.

### *FAST and FAST inhibition*

Optimal concentrations of crude CRa-A (4.0 µg/well) and CRa-G (4.0 µg/well, Greer Laboratories) were determined by checkerboard titration and coated onto blank microtitration wells (BioWhittaker, Walkersville, MD, USA) in our laboratory. Cockroach-specific IgE antibody of atopic subjects was determined with an enzyme immunoassay kit according to the manufacturer's instructions (IgE FAST-Plus Test, BioWhittaker). Either 50 µl of serum alone or 30 µl of serum and 30 µl of serial dilutions of inhibitor (200 µg/ml of CRa-A or CRa-G) were added to CRa-A-coated wells (4.0 µg/well) for FAST or FAST inhibition, respectively, followed by 2-h incubation at room temperature. After incubation and washing, alkaline phosphatase-labeled, monoclonal antihuman IgE conjugate was added and incubated at room temperature for another 2 h. Excess enzyme-labeled anti-IgE was

washed away, and fluorescence substrate (4-methylumbelliferyl phosphate in 2-amino-2-methyl propanol buffer, pH 9.5) was then added to the wells and incubated at room temperature for 40–60 min. After incubation, fluorescence signal units (FSU) and IgE concentration were determined by a fluorometer using calibrators containing 0.35, 0.8, 4.0, and 20.0 IU/ml of perennial ryegrass-specific IgE as standards (BioWhittaker). A class-scoring system based on specific IgE concentration was assigned according to the manufacturer's recommendation as follows: class 0, <0.35 IU/ml; class I, 0.35–0.99 IU/ml; class II, 1.0–2.99 IU/ml; class III, 3.0–6.99 IU/ml; class IV, 7.0–16.9 IU/ml; class V, 17.0–43.99 IU/ml; and class VI, >44.0 IU/ml. Inhibition was expressed as:  $[1 - (b - c) / a - c] \times 100$ , where a = FSU in the absence of inhibitor, b = FSU in the presence of inhibitor, and c = FSU in the absence of a reaginic serum.

### *SDS-PAGE and immunoblotting*

Proteins were separated by SDS-PAGE under denaturing conditions with a discontinuous buffer by Laemmli's method (29). After electrophoresis, protein bands were transferred onto a 0.45-µm nitrocellulose (NC) membrane by the method of Towbin et al. (30), as previously described (31). After blotting, NC membranes were washed with 10% methanol in Tris-buffered saline (TBS, 20 mM Tris, 0.5 M NaCl, pH 7.5) for 1 h with rocking, and then blocked for 1 h with TBS containing 3% gelatin. Blots were washed three times with TBS containing 0.05% Tween 20 (TBST) and then incubated overnight at room temperature with sera of atopic subjects (diluted 1 in 15 with 0.05% Tween 20 and 0.1% sodium azide in TBS). After incubation, NC strips were washed twice with TBST for 1 h with rocking and then incubated with <sup>125</sup>I-labeled antihuman IgE (Pharmacia, Piscataway, NJ, USA) overnight at 4°C. After extensive washing, strips were air-dried and then autoradiographed on an X-OMAT film (Eastman Kodak, Rochester, NY, USA) for 7 days at –70°C with an intensifying screen.

### *Protein determination*

Protein concentration was determined by Bradford's assay (32) with bovine serum albumin as the standard.

## **Results**

Of 84 samples tested, 52.4% (44/84) were found to have IgE antibodies against CRa-A. Sera of FAST-positive subjects were further tested for IgE specificity against CRa-G (Greer Laboratories),

86.4% (38/44) being found to contain antibodies against both CRa-A and CRa-G. The result of FAST analysis showing the distribution of CRa-G-specific IgE from CRa-A-positive subjects is summarized in Table 1. CRa-A-coated wells were used to analyze the cross-reactivity of CRa-G extract (Greer Laboratories) in FAST-inhibition experiments. CRa-A or CRa-G was used as inhibitor and tested for its inhibition of IgE binding. Sera of nine patients containing CRa-A-specific IgE (FAST class score of IV or V) and CRa-G-specific IgE (FAST class score of II–IV) (Table 2), and the serum of a CRa-A- and CRa-G-negative atopic patient was selected for FAST inhibition (Fig. 1). A range of 63–90% inhibition was found from the nine individual sera at the highest concentration (6 µg/well) of CRa-G (Fig. 1B). A control experiment using CRa-A as reference was done, and a similar result with higher degrees of inhibition (76–94%) was also obtained at the concentration of 6.0 µg/well (Fig. 1A).

The masses of the major components from CRa-A and CRa-G were estimated from SDS-PAGE under dissociating condition (Fig. 2A). Coomassie brilliant blue-stained gel revealed heterogeneity both in protein patterns and in intensity of staining among and within extracts. The IgE-binding patterns of CRa-A and CRa-G are shown in the autoradiographs from immunoblotting of the individual serum and radiolabeled antihuman IgE (Fig. 2B and C). None of the sera demonstrated identical IgE-binding patterns, while serum from the CRa-A-negative atopic control did not display any IgE binding to CRa-A or CRa-G proteins (lane j, Fig. 2B and C). In CRa-A, four proteins with apparent masses of 78, 72, 45, and 28 kDa could be identified from all the patient sera (100%), and IgE bindings also occurred mainly at 98 (66.7%), 90 (55.6%), 60 (88.9%), 52 (77.8%), 49 (44.4%), 47 (44.4%), 38 (66.7%), 32 (44.4%), 26 (66.7%), 18 (88.9%), and 12 kDa (44.4%) (Fig. 2B). On the other hand, the immunoblot of CRa-G showed five predominant proteins with apparent masses of 60, 52, 49, 38, and 12 kDa that bound 100%, 77.8%, 44.4%, 66.7%,

Table 2. Results of skin test of patients and FAST class score of sera used in FAST inhibition and immunoblot analyses

| Patients | Skin test<br>(CRa-A) | FAST class score |       |
|----------|----------------------|------------------|-------|
|          |                      | CRa-A            | CRa-G |
| A        | 2+                   | IV               | II    |
| B        | 4+                   | V                | III   |
| C        | 3+                   | IV               | III   |
| D        | 3+                   | V                | IV    |
| E        | 3+                   | IV               | III   |
| F        | 3+                   | IV               | III   |
| G        | 2+                   | IV               | II    |
| H        | 3+                   | IV               | III   |
| I        | 2+                   | IV               | II    |
| J        | –                    | 0                | 0     |

and 33.3% human IgE, respectively. Thus, it appears that these components from both the American and German cockroaches were able to bind IgE antibody. Some less frequently stained bands of >100, 94, 80, 42, and 40 kDa could also be identified.

## Discussion

*P. americana* and *B. germanica* are two major domiciliary cockroaches occurring commonly in homes worldwide. Similarity in physicochemical characteristics and cross-reaction of components from allergens of these two species were first suggested by Twarog et al. (9). In contrast, Richman et al. (23) and Helm et al. (17) demonstrated little cross-reactivity between crude extracts of American and German cockroaches by radioallergosorbent test (RAST) inhibition. By skin test analysis, Stankus & O'Neil (14) demonstrated reactivity to CRa-G alone in only two subjects and reactivity to both CRa-A and CRa-G in 52/100 atopics tested. Skin test analysis conducted by Schou et al. (15) showed that 42.1% (8/19), 21.1% (4/19), and 36.8% (7/19) of subjects were positive for CRa-G, CRa-A, and both CRa-A and CRa-G, respectively. Recently, Kang et al. (16) reported that the allergen with apparent masses of 40–45 kDa from American, German, and Oriental cockroaches was recognized by most individual sera tested by immunoblot analysis. In this study, we obtained skin testing, immunoblot analysis, RAST, and RAST inhibition, and the results suggest the existence of shared interspecies cockroach allergens.

Direct FAST analysis with CRa-A and CRa-G in 44 atopic subjects revealed only six sera containing IgE antibodies to CRa-A alone, the remaining 38 sera containing both specific CRa-A- and CRa-G-binding IgE antibodies (Table 1). In the FAST-inhibition studies, both CRa-A and CRa-G

Table 1. Distribution of CRa-G-specific IgE in CRa-A-FAST-positive subjects

| CRa-A<br>FAST class score | CRa-G FAST class score |    |    |     |    |   |
|---------------------------|------------------------|----|----|-----|----|---|
|                           | 0                      | I  | II | III | IV | V |
| I (n=12)                  | 2                      | 10 | 0  | 0   | 0  | 0 |
| II (n=13)                 | 3                      | 8  | 2  | 0   | 0  | 0 |
| III (n=8)                 | 1                      | 4  | 2  | 1   | 0  | 0 |
| IV (n=9)                  | 0                      | 2  | 3  | 4   | 0  | 0 |
| V (n=2)                   | 0                      | 0  | 0  | 1   | 1  | 0 |
| Total patients (n=44)     | 6                      | 24 | 7  | 6   | 1  | 0 |

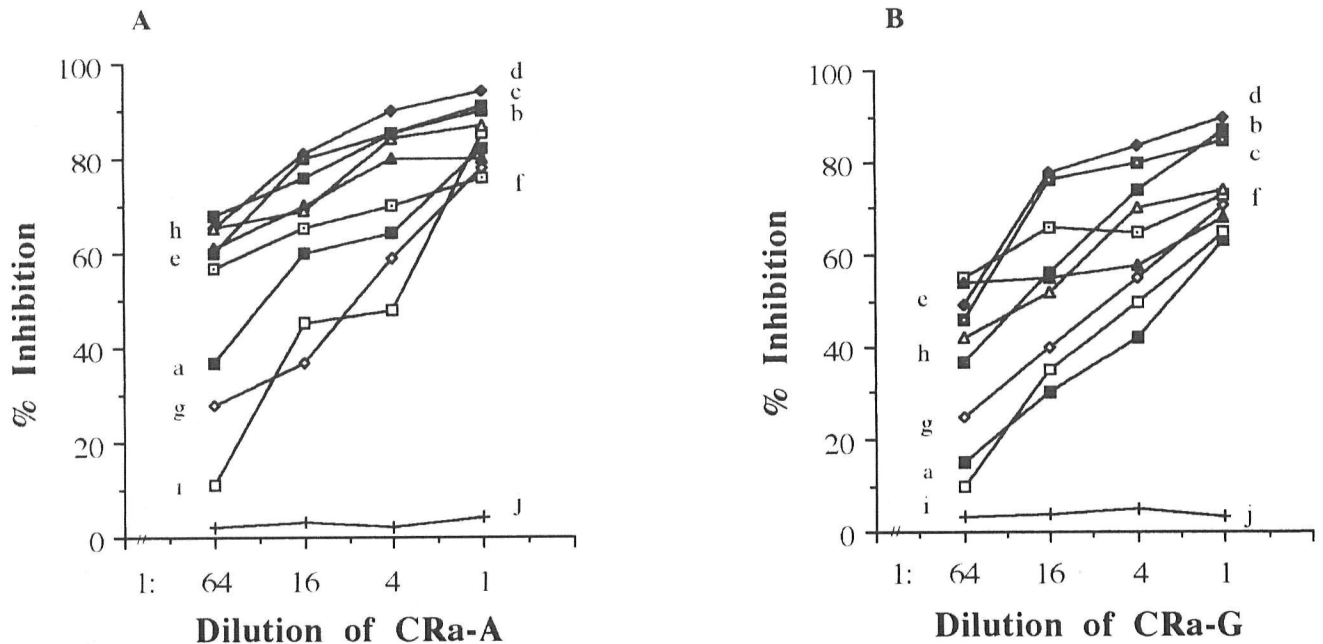


Fig. 1. FAST-inhibition results using American (A) or German (B) cockroach extract. Thirty microliters of serial dilutions of CRa-A (200  $\mu\text{g}/\text{ml}$ ) or CRa-G (200  $\mu\text{g}/\text{ml}$ ) and 30  $\mu\text{l}$  of human IgE serum (a-i, CRa-A- and CRa-G-positive atopic subjects A-I; j, CRa-A- and CRa-G-negative atopic patient J) were reacted with solid-phase CRa-A (4.0  $\mu\text{g}/\text{well}$ ). After overnight incubation at 4°C, bindings of human IgE antibodies to CRa-A were detected by FAST.

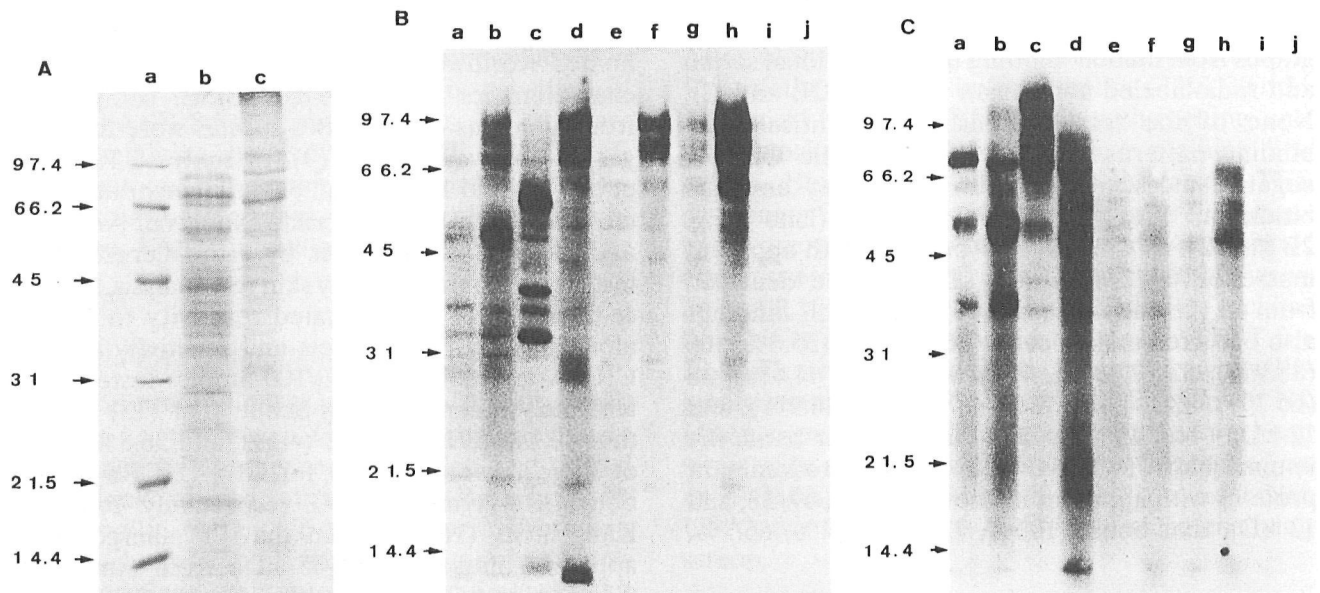


Fig. 2. SDS-PAGE patterns of CRa-A and CRa-G (A), and autographs of SDS-PAGE-separated components of CRa-A (B) and CRa-G (C) after electroblotting on NC, and followed by immunodetection with human IgE. Concentration of protein loaded for SDS-PAGE was 30  $\mu\text{g}$  of CRa-A or CRa-G. (A): lane a) protein markers; lane b) CRa-A; lane c) CRa-G; (B) and (C): lanes a-i) CRa-A-positive atopic patients A-I; lane j) CRa-A- and CRa-G-negative atopic patient J. Numbers at left indicate masses of protein markers in kDa.

demonstrated various degrees of inhibition of binding in the sera of CRa-A- and CRa-G-FAST-positive subjects tested (Fig. 1A and B). Results of FAST and FAST-inhibition studies indicated that all sera used in these assays contained specific IgE antibodies

against both CRa-A and CRa-G. Individual sera used in FAST-inhibition experiments were also used in immunoblot analysis. Both species-specific and cross-reacting allergens could be found in the heterogeneities of IgE-binding patterns of CRa-A

and CRa-G (Fig. 2B and C). These results mean that these patients were simultaneously exposed environmentally to both American and German cockroaches and/or that some allergenic components from both cockroach extracts cross-reacted. The immunoblotting revealed that allergens of apparent masses of 60, 52, 49, 38, and 12 kDa may be considered important cross-reacting allergens of American and German cockroaches on the basis of the electrophoretic mobility of protein and IgE-binding patterns (Fig. 2B and C).

Previously, proteins of 78 and 72 kDa from partially purified Per a 3 (Cr-PI) (13) and of 28 and 45 kDa from Cr-PII (20) have been identified as the major allergens of American cockroach. In this study, the 78-, 72-, 45-, and 28-kDa components of American cockroach bound to IgE with various degrees of activity from all sera tested, confirming that these components are indeed major allergens of the American cockroach. In addition, immunoblot analysis revealed that some low-mol.-wt. components of CRa-A bound IgE in high frequency with selected sera tested (Fig. 2B). Indeed, these low-mol.-wt. components of CRa-A are prominent components of previously purified Cr-PII, containing important allergens of American cockroach, and monoclonal antibodies to Cr-PII have been generated (20). Recently, two cDNAs encoding American cockroach Per a 3 allergens (79.3 and 75.5 kDa) were sequenced, and a high degree of identity (69.1%) with similar biologic functions was found between these two isoallergens (28). While no sequence similarity was found to other known allergens, including Bla g 2 and Bla g 4, these aromatic amino acid-rich allergens were found to have high sequence identities to insect hemolymph proteins (20.1–36.4%).

From our results and the results of others (14–16, 24, 25), it appears that cross-reactive allergens and species-specific allergens exist in the American and German cockroaches, although there may be some variability in assigned mol. wt. according to the immunochemical technique used. Recently, two important allergens, Bla g 2 and Bla g 4, of *B. germanica*, have been cloned and sequenced (24, 25). Bla g 2 is a 36-kDa protein sharing sequence homology with the aspartic proteases, and Bla g 4, a 21-kDa allergen, is a ligand binding protein or calycin. Both Bla g 2 and Bla g 4, like Per a 3 (28), have been found to be species-specific allergens. Cloning of Cr-PII cDNA, encoding lower mol. wt. allergens, is currently in progress in our laboratory. With more allergens being cloned and sequenced, it is essential to assess and define the relative importance of the different species-specific, cross-reactive, and major and minor allergens in cockroaches. The availability of unlimited amounts of

well-defined recombinant allergens produced *in vitro* will improve the accuracy and reliability of diagnostic tests and facilitate many studies in cockroach allergy, such as those on the definition and characterization of allergens, and epitope mapping of allergen-specific T and B cells. Moreover, structurally modified recombinant allergens can be used to develop new forms of immunotherapy.

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