Identification, quantitation, and purification of cockroach allergens using monoclonal antibodies

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A panel of murine IgG monoclonal antibodies (MAbs) was raised against German cockroach (CR) (Blattella germanica) *extract and selectively screened to identify MAb directed against allergen(s) recognized by IgE antibodies. Sera from 28 CR-allergic patients were used as sources of IgE antibodies to detect allergens "presented" by the MAb. Four clones (10A6, 3G12, 8F4, and 1D4) produced MAb to allergen(s) that bound IgE antibodies. Quantitative radioimmunoassays were used to compare levels of the MAb-defined allergens in CR extracts. MAb 10A6 reacted with a cross-reacting allergen that was detected in 9/14 CR species, including* Blattella, Periplaneta, Blatta, Leucophea, *and* Supella *spp, at concentrations of 100 to 10,000 U/ml. In contrast, MAb 3G12, 8F4, and 1D4 were* Blattella *specific. The allergen defined by MAb 8F4 was purified by MAb affinity chromatography and size-exclusion by high-performance liquid chromatography. It is a 36 kd heat-sensitive protein, isoelectric point, 5.2 to 5.4. Allergen 10A6 was partially purified by isoelectric focusing and high-performance liquid chromatography. It is a heat-stable, acidic protein (isoelectric point 3.15). Based on comparison of their properties with properties of previously described CR allergens, the allergens defined by MAb 10A6 and 8F4 have been provisionally designated* Blattella germanica *allergen I* (Bla g *I*) *and* Blattella germanica *allergen II* (Bla g *II*), *respectively. Assays of six commercial CR skin test extracts demonstrated a 200-fold difference in* Bla g *I levels (4.7 to 1085 U/ml) and only two extracts that contained detectable* Bla g *II (248 and 324 U/ml). The results demonstrate that MAb can be used to identify and define CR allergens and that the strategy of the use of MAb as a first step in allergen analysis and purification can be very effective, especially for poorly characterized allergen extracts. (J* ALLERGY CLIN IMMUNOL *1991;87:511-21.)*

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Cockroaches are an important source of house-dust allergens and appear to be a significant cause of asthma, particularly in urban areas of the United States (e.g., New York, Washington, D.C., Chicago, Detroit, Atlanta, and Boston).[1-7] A high proportion of dust-allergic patients from these areas (up to 60%) have positive immediate skin tests, positive bronchial challenge tests, and serum IgE antibodies to CR allergens.[4, 7-12] Both immediate and late bronchoconstrictive responses to CR extract have been reported, and these responses can be blocked by cromolyn sodium.[3, 5, 11] Exposure to CR allergens is also a well-recognized cause of occupational asthma in laboratory workers handling these insects.[13] Previous studies suggest that CR exposure should be evaluated as a cause of asthma in individuals who present with allergic disease and live in houses with suspected CR infestation. Our own investigation of the prevalence of

Abbreviations used

MAb:	Monoclonal antibody
RIA:	Radioimmunoassay
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
PBS-T:	Phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20
BBS:	Borate-buffered saline, pH 8.0
IEF:	Isoelectric focusing
HPLC:	High performance liquid chromatography
MW:	Molecular weight
UVA:	University of Virginia
CNBr:	Cyanogen bromide
pI:	Isoelectric point

allergen-specific IgE in adult patients presenting for emergency treatment of asthma in central Virginia, found that the prevalence of CR-specific IgE antibodies was second only to that of the dust mite. Furthermore, comparison of the patients with asthma with a random control population demonstrated that serum IgE antibody to CR was a significant risk factor for acute asthma.[14] CRs have also been reported as a cause of asthma in several other parts of the world, for example, Puerto Rico, South Africa, Thailand, Singapore, and Taiwan.[15-19]

There are >50 species of CR in the United States, of which only seven or eight are important indoors.[1] Studies of CR allergens have concentrated on two species, German CR *(Blattella germanica)* and American CR *(Periplaneta americana)*. CR allergens have previously been identified by immunochemical techniques, such as crossed radioimmunoelectrophoresis, immunoblotting, and RAST inhibition.[20-25] Partial purification of two allergens, MW ~25 kd and 69 to 70 kd, from American and German CR extracts has been reported, and low MW allergens (<10 kd) have been identified by skin testing and on immunoblotting.[4, 21, 24] Although several groups have used conventional biochemical techniques to isolate CR allergens, the antigenic and allergenic relationships between the allergens that have been reported are not clear, in part because specific antibodies to these proteins have not been available.

As an alternate approach, we raised a panel of MAbs to German CR extract that were then selectively screened to identify MAb specific for allergen(s) recognized by human IgE antibodies. The MAbs recognize two distinct allergens, a 25 kd acidic allergen, which has recently been reported as *Bla g* I,[24] and a second allergen of 36 kd, which is specific for *Blattella* species (*Bla g* II). We have developed quantitative immunoassays for each allergen and purified *Bla g* II by MAb-affinity chromatography and HPLC. The results demonstrate that MAb can be used as a first step in allergen purification and also establish the identity of two CR allergens.

MATERIAL AND METHODS

CR extracts

German CR *(Blattella germanica)* frass (CR debris containing body parts, fecal material, and egg cases) was obtained from CR-culture jars. Twenty-eight grams of frass was ground into a thick paste with a mortar and pestle and added to 140 ml of BBS, pH 8.0. The material was stirred overnight at 4° C and then centrifuged at 18,000 rpm for 1 hour. The supernatant was dialyzed extensively against BBS and ether extracted. The protein concentration of the aqueous CR extract was 5 mg/ml, as determined according to the Bradford[26] method with gammaglobulin as the standard. This extract, designated UVA 86/04, was used throughout these studies as an "in-house" reference for CR-allergen quantitation. Extracts of other CR species, *Periplaneta americana, P. brunnae, P. australasia,* and *P. fuliginosa; Blaberus cranifer, B. giganteus* and *B. discoidalis; Supella longipalpa; Leucophea maderae; Blatta orientalis; Blattella asahinae; Eublaberus posticus,* and *Gromphadorhina portentosa* were prepared by extracting 400 mg of frass in 2 ml of BBS overnight at 4° C. These species were maintained in the Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, Va.

Commercial CR extracts were obtained from six U.S. manufacturers: Allergy Laboratories, Inc. (Oklahoma City, Okla.), Allergy Laboratories of Ohio, Inc. (Columbus, Ohio), Berkeley Biologicals (Berkeley, Calif.), Center Laboratories (Port Washington, N.Y.), Greer Laboratories (Lenoir, N.C.), and Hollister-Stier Laboratories (Spokane, Wash.). These extracts were marked for skin prick testing in solution containing 50% glycerin. Unless it is otherwise stated, all other insect or arachnid extracts were commercial prick-test reagents supplied by Hollister-Stier Laboratories.

Human sera

Sera were obtained from 33 subjects observed in the University of Virginia Allergy Clinic. Twenty-eight subjects were patients with asthma who demonstrated positive skin tests and/or detectable serum IgE antibodies to CR allergens by RAST; 21 of these patients had presented to the UVA Hospital Emergency Room with acute asthma. They had been enrolled in a previous study, from which their CR RAST results were obtained.[14] Five sera were obtained from control subjects. Two subjects were patients followed in UVA allergy clinic with positive skin tests and RAST to at least one inhalant allergen, but with negative skin tests and negative RAST (<16 RAST units) for CR. The other three control subjects were nonatopic and had negative RAST to common inhalant allergens. Patients were skin tested with Hollister-Stier mixed CR extract (*B. germanica, B. orientalis,* and *P. americana* species). CR-allergic individuals were defined as individuals with a >5 × 5 mm wheal on skin prick test or a >6 × 6 mm wheal on intradermal testing

TABLE I. Binding of human IgE antibodies to CR allergen(s) presented by four different MAbs*

Serum	^{125}I-labeled anti-IgE bound to allergen presented by MAbs			
	3G12	8F4	10A6	1D4
CR allergic				
C. G.	1,027	4,846	4,402	11,022
J. N.	5,019	3,848	465	8,307
A. S.	3,776	1,652	6,150	3,304
S. W.	3,290	6,236	22,328	15,031
M. L.	1,344	18,204	348	22,149
B. S.	1,548	887	17,832	1,637
J. O.	2,820	ND	22,255	ND
T. W.	21,249	ND	30,164	ND
G. W.	14,202	ND	1,153	ND
P. J.	11,232	ND	838	ND
M. S.	8,263	ND	27,589	ND
C. S.	800	ND	8,326	ND
Serum pool	4,874	2,102	8,672	7,667
Control subjects				
C. B.	186	402	164	855
Horse serum	235	503	159	1,022

ND, Not done.

*CNBr-activated paper disks were coupled with MAb (1 μg per disk) and incubated successively with German-CR extract (UVA 86/04), patients' serum, and ^{125}I-labeled anti-IgE. Values are expressed as counts per minute of ^{125}I-labeled anti-IgE bound. (Total radioactivity added was ~100,000 cpm.)

with 1/100 dilution of CR extract. The 28 CR-allergic subjects had >200 RAST units per milliliter of CR-specific IgE (1 unit is ~0.1 ng of IgE).[14] Sera were also obtained from 59 CR-allergic patients who presented to either the Pediatric Allergy Clinic, UVA (N = 7), or to hospital emergency rooms in Atlanta, Ga. (N = 22) or Wilmington, Del. (n = 30). These sera all contained IgE antibody to CR (>40 RAST units per milliliter). A serum pool was prepared with serum from four patients with high levels of CR-specific IgE (1280 to 4000 RAST units to milliliter). This pool (UVA 86/03) comprised two sera from patients with IgE antibody to German and American CR (each contributing 30% of the pool) and two sera from patients with predominant IgE antibody to German CR (each 10% to 15% of the pool). Collection of human sera for use in these studies was approved by the Human Investigation Committee of the UVA.

Production and selection of MAbs

BALB/c mice were administered three intraperitoneal injections of 25 μg German CR extract (UVA 86/04) in complete Freund's adjuvant at 10-day intervals. Two weeks after the final injection, sera were screened for IgG anti-CR antibodies with an ELISA. A mouse with a high titer of anti-CR IgG (positive at a dilution >1/20,000) received a final intraperitoneal injection followed 2 weeks later by an intrasplenic boost with 50 μg of CR protein. Three days later, this mouse was killed, and its spleen cells were fused with Sp_2O-Ag14 myeloma cells with 37% polyethylene glycol, as described previously.[27] Two weeks after fusion, the hybrids were screened for antibody production by ELISA. Positive hybrids were tested for the ability to capture protein recognized by IgE antibody in the CR-serum pool with an indirect RAST procedure. The hybrids were cultured in RPMI 1640 medium containing 1% fetal calf serum, and supernatants from these cells were coupled to CNBr-activated paper disks (100 μl supernatant/disk) overnight at 4° C. The disks were washed twice with PBS-T, incubated for 1 hour at room temperature with 1% BSA PBS-T, washed twice with PBS-T, and dried; 100 μl of CR extract (~1 μg of CR protein) in undiluted horse serum was added to the paper disks and incubated at room temperature for 6 hours. Unbound CR proteins were washed from the disks with PBS-T, and the disks were incubated overnight with 50 μl of CR-allergic serum pool diluted in 50 μl of horse serum. The disks were washed five times in PBS-T and then incubated with 100 μl ^{125}I-labeled antihuman IgE (2 ng, ~100,000 cpm) in horse serum for 4 hours at room temperature. After five washes with PBS-T, the disks were counted in a gamma counter.

Hybrids selected on the basis of the IgE binding assay were cloned by limiting dilution. Positive clones were selectively expanded and injected intraperitoneally into pristane-primed female BALB/c mice. The resulting ascites was precipitated with 50% saturated ammonium sulfate, resuspended in BBS at a concentration of 10 mg/ml, and stored at −20° C. The isotype of each MAb was determined by immunodiffusion.[27] The use of animals in this study was

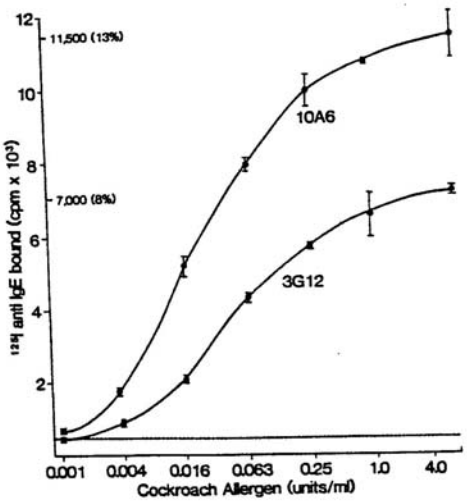

FIG. 1. Typical control curves obtained in the quantitative RIA for CR allergens with MAb 10A6 or 3G12. The *dashed horizontal line* indicates the mean level of nonspecific binding with sera from five control patients.

approved by the Animal Research Committee (UVA) under guidelines for the use and care of animals formulated by the National Council for Medical Research.

Immunoassays for CR allergens

Four MAbs, 10A6, 8F4, 3G12, and 1D4, were used to assay extracts of CR and other insects by solid-phase RIA. MAbs were bound to plastic removawells (Immulon II, Dynatech Laboratories, Alexandria, Va.) by coating overnight with 1 μg per well of a 50% saturated ammonium sulfate cut of ascites in 0.1 mol/L of bicarbonate buffer, pH 9.6, at 4° C. The wells were incubated with dilutions of CR extracts for 2 hours, washed five times with PBS-T, and incubated with 50 μl of pooled serum from CR-allergic patients. IgE antibodies bound to the CR allergen were detected by the addition of 2 ng of ^{125}I-labeled anti-IgE. CR extracts were usually assayed at doubling dilutions between 1/10 and 1/1000, with 50% horse serum as diluent. The assay was quantitated with German-CR extract (UVA 86/04) that was arbitrarily assigned a value of 5000 units of CR allergen per milliliter. A control curve was generated with serial twofold dilutions of this extract, from 0.01 to 4.0 U/ml, and results were interpolated from the linear portion of the curve.

MAb affinity purification of 8F4 allergen

MAb 8F4 (~200 mg of a 50% ammonium sulfate fraction of ascites) was coupled to 7 gm of CNBr-activated Sepharose 4B (Pharmacia, Piscataway, N.J.); 200 ml of CR extract was absorbed over the column at a flow rate of 20 ml/hr. After absorption, the column was washed with 500 ml of PBS, and the bound allergen was eluted with 0.005 mol/L of glycine in 50% ethylene glycol, pH 10.0 (2.2 ml fractions were collected into 0.8 ml of 0.2 mol/L of phosphate buffer, pH 7.0). Approximately 30 eluted fractions were collected, and fractions containing significant allergen content by RIA were pooled, dialyzed against three changes of BBS, and concentrated by ultrafiltration with an Amicon PM10 filter (Amicon Corp., Danvers, Mass.). Allergen isolated from the 8F4 MAb-affinity column was additionally purified by size exclusion HPLC with a Superose 12 HR 10/30 column (Pharmacia) equilibrated in 0.05 mol/L of phosphate buffer, pH 7.2, containing 0.15 mol/L of NaCl; 100 μl of 8F4 allergen (13,000 units) was injected onto the column and eluted at a flow rate of 0.4 ml/min (total run time, 60 minutes). The purity of the allergen preparations was assessed by CIE and SDS-PAGE, as described previously.[28] Polyclonal rabbit antisera to German CR extract were used for CIE. The antisera were prepared by immunizing two New Zealand white rabbits four times, intramuscularly, with 1 mg of German-CR extract (UVA 86/04) in complete Freund's adjuvant at 14-day intervals. Sera were collected 14 to 21 days after the final injection and stored at −20° C. For SDS-PAGE analysis, 1 to 3 μl of allergen was analyzed under nonreducing conditions on an 8% to 25% gel with silver stain (PhastSystem, Pharmacia).

Partial purification of allergen defined by MAb 10A6

The 10A6 allergen was partially purified by preparative IEF and size exclusion HPLC; 50 ml of German CR extract (containing 3000 U/ml of 10A6 allergen) was fractionated by preparative IEF with a pH 3 to 8 gradient, as described previously.[27] Fractions were analyzed for 10A6 allergen by immunoassay, and the 10A6 peak (pI 3.15 to 3.25) was pooled, precipitated with 80% ammonium sulfate, dialyzed against 5 mmol/L of ammonium bicarbonate, and lyophilized. The material was resuspended in 100 μl of PBS (90,000 U/ml of 10A6 allergen) and 10 μl was analyzed by size exclusion HPLC. Eluted fractions were assayed for 10A6 allergen by immunoassay. Allergen was also purified by MAb-affinity chromatography, as described above, with 10A6 immunosorbent.

RESULTS

Analysis of MAbs

Fusion of CR-immune spleen cells and SP_2O myeloma cells resulted in 195/960 (20%) hybrids with supernatants containing significant levels of IgG anti-CR antibody on ELISA (optical density, >0.7). Sixty-three hybrids had optical densities >1.0, and supernatants from these hybrids were coupled to CNBr-activated paper disks and screened for binding of allergen(s) recognized by human IgE antibodies. Twenty hybrids bound >5% of ^{125}I-labeled antihuman IgE in this solid-phase RIA, and the four hybrids that bound >10% of the anti-IgE were cloned. All four

TABLE II. Species specificity of anti-CR MAbs*

CR species†	Allergen (units/ml) detected with MAb			
	10A6	8F4	3G12	1D4
Blattella germanica (UVA 86/04)	5,000	5,000	5,000	5,000
B. asahinae	>10,000	>10,000	ND	>10,000
Periplaneta americana	120	0.5	2.0	0.4
P. brunnae	100	<0.1	<0.1	<0.1
P. australasia	600	0.3	<0.1	<0.1
P. fuliginosa	230	1.9	<0.1	0.6
Supella longipalpa	340	2.3	2.7	0.7
Leucophea maderae	130	<0.1	<0.1	<0.1
Blatta orientalis	320	2.7	6.3	4.2
Blaberus sp‡	<0.1	<0.1	<0.1	<0.1
Eublaberus posticus	<0.1	<0.1	<0.1	<0.1
Gromphadorhina portentosa	<0.1	<0.1	<0.1	<0.1

ND, Not done.

*Extracts of CR frass were assayed by RIA for allergen presented by each of the four MAbs. Results for each MAb are expressed as units of allergen per milliliter and were interpolated from a control curve with a *B. germanica* extract (UVA 86/04). This reference was arbitrarily assigned 5000 units of CR allergen per milliliter. (One unit corresponds to ~1 μg of protein in the UVA 86/04 extract.)

†Extracts of the following insects: ant, butterfly, caddis fly, deerfly, flea, horsefly, housefly, mayfly, moth, cricket, midge, termite, *Triatoma protracta*, and *T. rubida*, did not contain detectable allergen in any of the MAb assays. Tick, spider, storage-mite (*Acarus* sp and *Tyrophagus* sp) and dust-mite (*Dermatophagoides* sp) extracts were also negative in these assays.

‡*Blaberus giganteus*, *B. cranifer*, and *B. discoidalis*.

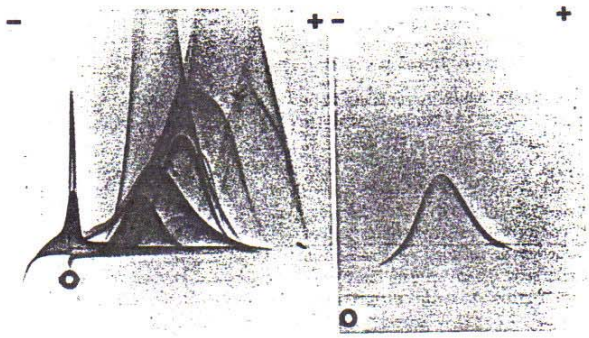

FIG. 2. Analysis of German-CR extract *(left panel)* and single-step MAb affinity-purified 8F4 allergen *(right panel)* by crossed immunoelectrophoresis with polyclonal rabbit antibodies.

MAbs, clones 10A6, 8F4, 3G12, and 1D4, were isotype IgG1. To investigate the prevalence of IgE antibodies to the MAb-defined CR antigens, the MAbs were used as capture antibodies to present CR proteins to IgE antibodies in 28 selected sera from CR-allergic patients (with >200 RAST units per milliliter). Results with 12 sera and a CR-allergic serum pool are presented in Table I. Each MAb presented a protein that was recognized by IgE antibodies in most of the sera: 89% (25/28) for 10A6; 89% (25/28) for 3G12; 88% (7/8) for 8F4; and 75% (6/8) for 1D4. Some sera demonstrated significant quantitative differences in binding to allergens presented by particular MAb; for example, serum from B. S. bound predominantly

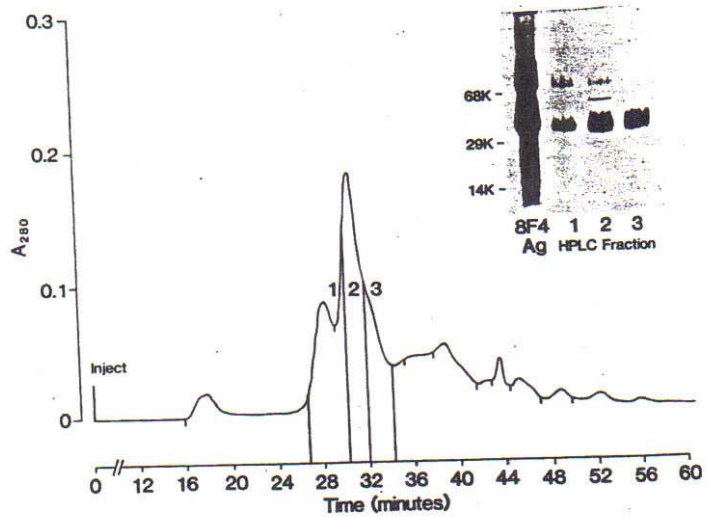

FIG. 3. Purification of 8F4 allergen by size-exclusion HPLC. MAb affinity-purified 8F4 (13,000 units) was applied to a Superose 12 HPLC column, and fractions were analyzed for protein content (A_{280}, *solid line*) and for 8F4 allergen and were pooled as indicated (fractions 1, 2, and 3). The fractions contained 592, >2000, and 1024 U/ml of 8F4, respectively, and eluted with an apparent MW of 35 to 45 kd by comparison with the elution times of MW markers (ovalbumin, 32 minutes) and (cytochrome c, 37 minutes). **Inset,** SDS-PAGE analysis of single-step MAb affinity-purified 8F4 and HPLC fractions 1 to 3.

to 10A6, serum from G. W. and P. J. bound strongly to 3G12, and serum from M. L. bound strongly to 8F4 and 1D4, but weakly to the other MAbs.

Specificity of MAb

The serologic assays suggested that these four MAbs recognized one or more allergens but did not fully distinguish whether or not they recognized the same or different allergens. To investigate these possibilities, binding of the four MAbs to extracts prepared from a panel of 14 CR species and 15 other insects was compared by two-site immunoassay. Typical control curves for these RIAs, with MAbs 10A6 and 3G12, are depicted in Fig. 1. MAb 10A6 recognized a cross-reactive allergen that was detected in most (9/14) of the CR species tested at allergen concentrations of 100 to >10,000 U/ml. In contrast, 8F4, 3G12, and 1D4 bound strongly to *Blattella* species (*B. germanica* and *B. asahinai*) but demonstrated <1% cross-reactivity with other CR extracts, suggesting that they recognized predominantly genus-specific allergen(s). None of the other common insect or arachnid extracts tested in the RIA (including spider, moth, termite, and ant), bound significantly to any of the four anti-CR MAb (Table II).

MAb-affinity purification of CR allergen

The allergen defined by MAb 8F4 was purified from German-CR extract by MAb-affinity chromatography with an 8F4 immunosorbent column and a 5 mmol/L glycine/50% ethylene glycol-eluting buffer, pH 10.0. The 8F4 allergen elicited a single immunoprecipitation line on CIE against polyclonal rabbit antibodies that identified at least 12 different antigens in German-CR extract (Fig. 2). However, on SDS-PAGE, this material was clearly not fully purified and demonstrated a major band at 36 kd as well as several other protein-stained bands, the most dense being at 70 kd (Fig. 3). The allergen was additionally purified by size-exclusion HPLC (Fig. 3). The trace demonstrated two overlapping peaks, eluting at 26 to 35 minutes, with the sharpest peak eluting at 30 to

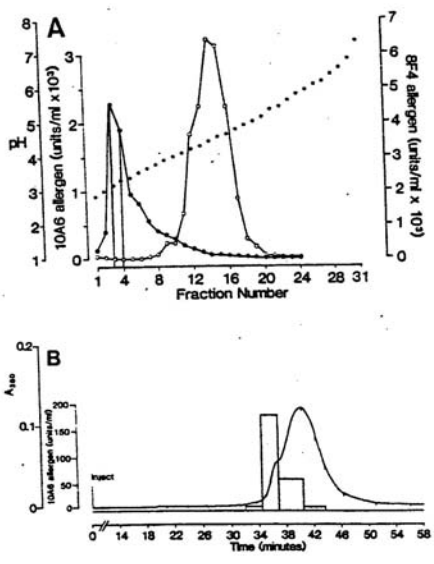

FIG. 4. Partial purification of 10A6 allergen. **A**, Preparative IEF of German-CR frass extract; pH gradient, 3 to 8 (●). Eluted fractions were assayed for 10A6 (●——●) and for 8F4 (o——o) by immunoassay. **B**, Analysis of 10A6 IEF fractions by HPLC. The 10A6 IEF peak *(shaded area, A)* was pooled, concentrated, and applied to a Superose 12 HPLC column. The protein trace (——) and the 10A6 allergen content *(columns)* of eluted fractions are illustrated. Elution times for MW markers on this Superose 12 column were BSA, 33.0 minutes; ovalbumin, 35.1 minutes; myoglobin, 38.5 minutes; and cytochrome c, 39.6 minutes.

TABLE III. Comparison of immunochemical properties of allergens defined by MAb (*Bla g* I) and 8F4 (*Bla g* II)

Analysis	Allergen	
	10A6 (*Bla g* I)	8F4 (*Bla g* II)
MW	25-35 kd*	36 kd
IEF		
Preparative	3.15	4.5
Analytical†	2.3	5.2-5.4
Heat stability	Heat stable	Heat sensitive (100° C)
pH sensitivity‡	Stable, pH 2-12	Stable, pH 2-12
Rabbit polyclonal IgG antibodies§	Nonprecipitating	Precipitating
Immunoassay		
For 8F4	—	+ + +
For 10A6	+ + +	—

*Based on data illustrated in Fig. 4, *B*, and on previous gel filtration and SDS-PAGE analyses.[4, 24] Shou et al.[24] obtained an MW of 33 to 37 kd on gel filtration (similar to our estimate of 35 kd on size-exclusion HPLC, Fig. 4, *B*).

†With HPLC-purified 8F4 allergen, values of pI 5.2 to 5.4 were obtained on silver-stained analytical IEF gels. By immunoisoelectric focusing, pI values of 2.3 and 5.85 were obtained with MAbs 10A6 and 8F4, respectively (Dr. Samuel Lehrer, Tulane University, New Orleans, personal communication).

‡10A6 allergen (450 units) or 8F4 allergen (30 units) was incubated for 2 hours at room temperature in PBS-T; 20 mmol/L of HCL, pH 2.0; 20 mmol/L of NaOH, pH 12.0; 5 mmol/L as glycine/50% ethylene glycol, pH 10.0; or 100 mmol/L of citrate phosphate buffer, pH 4.2. After neutralization, there was no significant difference between the ELISA reactivity of the pH-treated solutions or PBS-T controls for either allergen (data not presented).

§With two separate antisera raised against German-CR extract (UVA 86/04). Neither antiserum elicited precipitin lines against partially purified 10A6 allergen, even though they both demonstrated good binding to 10A6 in enzyme immunoassay.[31]

34 minutes, coincident with the 8F4 allergen as determined by immunoassay. Three fractions from this peak were pooled, concentrated, and analyzed by SDS-PAGE. All three fractions demonstrated a dense 36 kd band. The first two fractions were contaminated with trace amounts of the 70 kd protein; however, the third fraction demonstrated only a dense homogeneous band at 36 kd (Fig. 3).

Additional evidence that MAbs 8F4 and 10A6 recognize different allergens

When German-CR extract was separated by preparative IEF, the isolated fractions demonstrated two nonoverlapping peaks of 10A6 and 8F4 immunoreactivity of pI 3.15 and 4.5, respectively (Fig. 4, *A*). The pI values for 8F4 were in good agreement with values obtained on silver-stained analytical IEF gels with purified 8F4 allergen (HPLC fraction 3) and, similarly, the values for 10A6 were consistent with values obtained by immunoisoelectric focusing (data kindly provided by Dr. Samuel Lehrer, Tulane University, New Orleans, La.) (Table III). The 10A6 IEF peak (fractions 3 and 4) was further purified by size-exclusion HPLC. A broad 45 to 10 kd protein peak eluted at 34 to 46 minutes, and the 10A6 allergen eluted coincident with a shoulder on this peak at 36

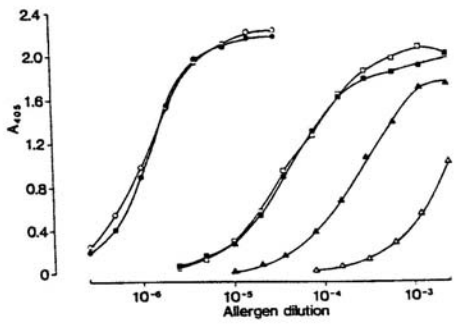

FIG. 5. Heat stability of the 10A6 and 8F4 allergens. Allergen was heated successively for 1 hour at room temperature, 22° C, 10A6 (○), or 8F4 (□); or at 56° C, 8F4 (■); or at 100° C for 5 minutes (▲) or 20 minutes, 10A6 (●), or 8F4 (△), and aliquots were withdrawn and tested for immune reactivity by ELISA.[31] Values for each curve are the mean of duplicate points.

to 37 minutes (~35 kd) (Fig. 4, *B*). Heat-stability experiments demonstrated that 10A6 allergen was heat stable (at 100° C for 20 minutes), whereas 8F4 allergen was stable at 56° C but progressively lost immune reactivity at 100° C (Fig. 5). These allergens also differed in their ability to induce precipitating polyclonal antibodies in rabbits. A summary of the properties of the allergens defined by MAbs 10A6 and 8F4 is presented in Table III. The 10A6 and 8F4 allergens have been designated *B. germanica* allergen I (*Bla g* I) and *B. germanica* allergen II (*Bla g* II), respectively.

Allergenic importance of *Bla g* I and *Bla g* II

The MAbs were selected using 28 sera with moderate to high IgE anti-CR RAST values (>200 U/ml). To assess the relative importance of *Bla g* I and *Bla g* II in causing sensitization, we compared IgE antibody responses to both allergens in the 28 sera, together with an additional 59 sera from CR-allergic patients with RAST >40 U/ml. Of these 87 sera, 27 (31%) had detectable IgE antibodies to *Bla g* I and 55 sera (63%) had IgE antibodies to *Bla g* II. The biologic activity of both allergens was confirmed by quantitative intradermal skin testing on six selected patients. Four patients had positive skin tests (>6 × 6 mm wheal) to *Bla g* I at 10^{-1} to 10^{-4} μg/ml, and five patients had positive reactions to *Bla g* II at 10^{-1} to 10^{-4} μg/ml. Four nonatopic control subjects had negative skin tests to 10 μg/ml of each allergen. There was a good correlation between skin test reactivity and serum IgE antibody responses to the two allergens; for example, patient S. W. (Table I) had IgE antibodies to *Bla g* I and *Bla g* II and had skin test responses to 10^{-3} and 10^{-2} μg/ml of each allergen, respectively. In contrast, there was a 4-log difference in the skin test reactions of patient B. S. (10^{-3} μg/ml, *Bla g* I and 1 μg/ml, *Bla g* II) who only had serum IgE antibodies to *Bla g* I.

Allergen levels in commercial CR extracts

Six commercial CR skin test reagents that are currently marketed in the United States were compared for *Bla g* I and *Bla g* II levels. All extracts contained detectable *Bla g* I, although there was a 200-fold difference between the quantity of this allergen in the extracts (from 4.7 to 1085 U/ml; Table IV). Four extracts contained no detectable *Bla g* II; however, three of those extracts were prepared from American CRs and would not be expected to contain the *Blattella*-specific allergen. Extracts B and E were prepared from different source materials but contained comparable levels of *Bla g* I and *Bla g* II at significantly higher levels than any of the other extracts.

DISCUSSION

The use of MAbs raised against unpurified allergen extracts to identify and characterize individual allergens was originally described for mite and cat allergens (*Der p* I, *Der p* II, and *Fel d* I).[29, 30] We raised a panel of MAbs to the German CR, *B. germanica*, and successfully used these MAbs to identify CR allergens. The advantage of this approach was that MAbs that define at least two CR allergens were produced from a single fusion. The results demonstrate that MAbs 10A6 and 8F4 are directed against physicochemically and antigenically distinct allergens that appear to correspond to CR allergens identified in previous studies. Immunochemical comparisons suggested that 10A6 was the same as the 25 kd acidic, cross-reacting allergen previously identified by Twarog et al.[4] and Lind et al.,[24] which has provisionally been designated as *B. germanica* allergen I (*Bla g* I).[24] An excellent correlation between ELISA assays for 10A6 and for *Bla g* I has been demonstrated (see accompanying article[31]), and we have recently confirmed that 10A6 allergen and *Bla g* I are antigenically identical by exchange of antibodies and purified allergens with Drs. Carsten Schou and Henning Lowenstein (ALK Research Laboratories, Horsholm, Denmark). The 8F4 allergen is a 36 kd *Blattella*-specific allergen that is clearly distinct from *Bla g* I. Helm et al.[25] also recently identified a 36 kd *Blattella*-specific allergen on immunoblotting. The 8F4 allergen has been purified and fulfills the assessment criteria recommended by the International Union of Immunological Societies allergen nomenclature subcom-

TABLE IV. Allergen content of six commercially available CR extracts

Extract	Species*	Manufacturer's concentration (wt/vol)	*Bla g* I† (U/ml)	*Bla g* II† (U/ml)
A	American	1:20	10.1	<0.005
B	German	1:20	1,085	248
C	American and German	1:100	6.5	<0.005
D	American	1:20	4.7	<0.005
E	American, German, and Oriental	1:10	675	324
F	American	1:10	8.2	<0.005

*Extracts A, C, and F were marketed as "cockroach" extract with no delineation of the species; however, the manufacturers revealed that extracts A and F were prepared from American CRs (*Periplaneta americana*); extract C was prepared from a mixture of American and German CRs. Extracts B, D, and E were labeled with the CR species from which they were prepared.
†Units of allergen per milliliter of extract corrected to 1:10 wt/vol.

mittee.[32] We propose that it should be designated *Blattella germanica* allergen II (*Bla g* II). Recent cross-inhibition immunoassays with ^{125}I-labeled 8F4 MAb demonstrate that MAb 1D4 is also directed against *Bla g* II (data not presented). The proposed assignment of nomenclature for *Bla g* I and *Bla g* II is based both on our data and data of other laboratories and appears to reconcile all the current data.

In terms of sensitization, *Bla g* II appears to be the more important allergen since approximately twice as many patients had IgE antibodies to *Bla g* II than to *Bla g* I. Overall, 70% (26/87) of patients with a positive CR RAST had IgE antibodies to either *Bla g* II or *Bla g* I. Several sera were also identified with strongly positive CR RAST (>800 U/ml) but with no detectable IgE antibody to either allergen. Thus, although these allergens are good markers of CR sensitization (particularly *Bla g* II), the data suggests that CRs also produce other important allergen(s).

The development of specific immunoassays for *Bla g* I and *Bla g* II with the MAb will facilitate additional studies, both on the role of CRs in causing asthma and on the source and biologic role of these allergens. These assays have been modified to ELISA format and used to assess environmental exposure to CRs in the houses of patients with asthma.[31] We also used the ELISA to compare allergen levels in commercial CR skin test extracts as part of the present study. The results demonstrate significant differences (up to 200-fold) in the levels of both *Bla g* I and *Bla g* II in commercial CR extracts and suggest that better standardization of CR extracts is necessary and that standardization could be improved with assays for these two allergens. The results also raise the possibility that sensitization to CR may be underestimated because of the limited number of German-CR extracts that are currently marketed for skin testing. The *Bla g* I assay detects allergen produced by all common indoor CR species, although the level of cross-reactivity is rather weak, and *Blattella* extracts appear to contain more *Bla g* I than extracts of other species. It is possible that the affinity of MAb 10A6 for the putative homologous allergen in other CRs (e.g., *Per a* I,[24]) is lower than for *Bla g* I, and as a result, the quantities of *Bla g* I in genera other than *Blattella* may be underestimated. This problem would best be resolved by the production of MAb to *Per a* I and comparison of their specificity and affinity with MAb 10A6. All the MAbs recognize allergens common to both species of *Blattella*. *B. germanica* is widely distributed in temperate areas of the United States, and the Asian CR, *B. asahinai*, has recently been introduced into Florida and appears to be spreading through the Southeast. *B. asahinai* was the most potent source of *Bla g* I and *Bla g* II in the extracts that we tested; however, it is not known whether or not exposure to *B. asahinai* causes significant allergic disease. *B. asahinai* is not usually found in houses, but large outdoor populations are common in Florida and could potentially exacerbate the symptoms of CR-allergic patients living in affected areas.

Previous studies with RAST analyses suggested that CR bodies were the main source of CR allergens.[16, 18] The source material for the CR extracts used in our studies was frass. This material accumulates at the bottom of CR-culture jars and consists mainly of feces, secretions, egg cases, and some body parts, but relatively few whole bodies. Many of the extracts consisted almost entirely of fecal material, which suggests that *Bla g* I and *Bla g* II are secreted or excreted. In keeping with this finding, Menon et al.[33] and Lehrer et al.[34] have recently demonstrated allergenic activity

in fecal extracts by skin testing and RAST inhibition, and we have also demonstrated that body washes of live CRs are a potent source of both *Bla g* I and *Bla g* II.[35] The precise source of these allergens is currently being investigated by comparing allergen levels in different source materials and in different parts of the CR.

Our results confirm the advantages of the use of MAbs in allergen identification, purification, and characterization.[27-30, 36] Although it is obviously preferable to make MAbs against purified or partially purified allergens, the strategy of screening MAbs against an unpurified extract with sera from allergic patients is particularly suited to poorly defined allergens and is a useful alternative to biochemical purification procedures. In addition, as a result of the present studies, we stored a panel of 30 cryopreserved hybrids that produced antibodies to CR antigens that did not bind IgE, and these antibodies may have applications in other areas of CR biology.

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