Asian ladybugs (*Harmonia axyridis*): A new seasonal indoor allergen

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Background: *Harmonia axyridis*, the Asian ladybug (ALB), was repeatedly introduced between 1916 and 1990. These beetles are intolerant to cold and move indoors during the winter.
Objective: To investigate sensitization to ALB.
Methods: Proteins in ALB extracts were purified by gel filtration and ion exchange chromatography. Purified fractions were screened for IgE antibody using the streptavidin CAP technique in sera from 20 patients with allergy living in ALB-infested houses. Two proteins were fully purified. Serum antibodies were also assessed in sera from 68 adult patients with asthma.
Results: Fifteen of the 20 sera had measurable IgE antibody, 7 with high titers, > 10 IU/mL, to ALB extract. The 2 proteins, Har a 1, 10 kd, and Har a 2, 55 kd, bound IgE antibody in 65% and 75% of the sera, respectively. Sequencing revealed a novel N-terminal sequence for Har a 1. Sequencing of Har a 2 demonstrated homology to a dehydrogenase from the red flour beetle. Although sera from 18 of the patients with asthma were positive for IgE antibody to ALB, they were also positive to *Blatella germanica*. These subjects did not report exposure to *H axyridis*, and inhibition studies with *B germanica* blocked ≥ 95% of ALB IgE antibody binding.
Conclusion: Two proteins of ALB have been purified and used to demonstrate that patients exposed to this beetle can develop high-titer IgE antibody. Cross-reactivity with *B germanica* was found but was significant only among patients primarily exposed to cockroaches.
Clinical implications: Asian ladybug has become an important seasonal indoor allergen in the United States. (J Allergy Clin Immunol 2007;119:421-7.)

Key words: *Allergen, ladybugs, streptavidin CAP, insect, cross reactivity, winter indoor allergy*

Abbreviation used
ALB: Asian ladybug

After its first introduction to the United States from Asia in 1916, the ladybug *Harmonia axyridis* was released repeatedly as an ecological control for aphids by state, local, and federal entomologists until 1990.[1] It was believed that the beetle population would be controlled by the cold winters. However, rather than freezing, these ladybugs swarm and invade houses in the early fall. The first reports of persistent ladybug populations came from Louisiana in 1988.[1] Since then, the beetle has become established through large areas of the country with reports of infestation from Wisconsin, Kentucky, Missouri, West Virginia, and along the East Coast as far south as Georgia. Hundreds to thousands of beetles can be collected each week from houses during the winter months. The problem can be severe in rural and suburban areas, but is less common within cities. Reports on allergic symptoms related to the beetle have come from St Louis, Mo; Appleton, Wis; Louisville, Ky; West Virginia; and Georgia.[2-9] Patients with sensitivity to *H axyridis* have presented with a range of allergic symptoms including rhinitis, conjunctivitis, chronic cough, and asthma.[2,3]

We have recently identified 20 patients presenting with allergic symptoms and a history of exposure to Asian ladybugs (ALBs). Ten were skin-tested using an extract of *H axyridis* made specifically for this purpose. We report here the development of an *in vitro* assay for the measurement of IgE antibody to *H axyridis*, and the purification of 2 allergens derived from the beetle. In addition, we have documented cross-reactivity between *H axyridis* and *Blatella germanica*.

METHODS

Detection of *H axyridis*

Beetles found in homes were identified as *H axyridis* by using a classification scheme for ladybug and beetle infestation produced by the University of Kentucky, Department of Entomology.[10]

Extract of *H axyridis*

Live and dead *H axyridis* were collected from patients' houses and kept at –20°C. The beetles were frozen with liquid nitrogen and crushed with a mortar and pestle. Proteins were extracted from the resulting powder by incubating in borate buffered saline, pH 8.0,

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Supported by grants from National Institutes of Health (AI-20565, AI-50989).
Disclosure of potential conflict of interest: T. A. E. Platts-Mills has received grant support from Pharmacia. The rest of the authors have declared that they have no conflict of interest.
Received for publication October 16, 2006; revised November 22, 2006; accepted for publication November 22, 2006.
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0091-6749/$32.00

doi:10.1016/j.jaci.2006.11.633

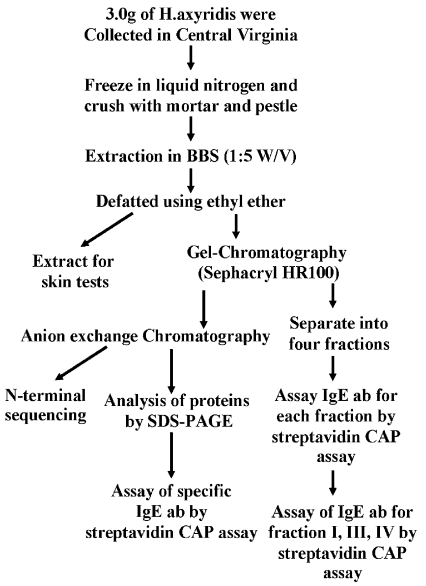

FIG 1. Outline of extraction, purification, protein characterization, and serum IgE antibody detection. *BBS*, Borate buffered saline.

overnight at 4°C. The protein solution was defatted using ethyl ether (Sigma-Aldrich, St Louis, Mo). After centrifugation at 2000 rpm for 5 minutes, the sample was separated into 3 layers. The lowest layer was collected for analysis (Fig 1).

Separation of proteins by gel filtration

Protein extract was separated with an ACTA prime plus low pressure computerized protein purification system and a Sephacryl 100, 26/60 column (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), eluted with PBS (150 mmol/L, pH 7.2, 0.02% sodium azide, 1.3 mL/min, total 320 mL) and pooled into 4 fractions, I to IV. Each fraction was biotinylated by using sulfo-succinimidyl-6-(biotin-amido)-hexanoate (Pierce, Rockford, Ill) and bound to streptavidin CAP (Phadia AB, Uppsala, Sweden) to measure serum IgE antibody.[11,12] Significant background fluorescence was associated with fraction II; therefore, an allergen complex was made with fractions I, III, and IV. The proteins in this mixture were biotinylated for streptavidin CAP assays.

Further allergen purification using ion exchange chromatography

Proteins from fractions II and IV were purified using the ACTA prime plus system and an ion exchange Resource Q or Hi trap Q column (GE Healthcare). After equilibration with 50 mmol/L TRIS, pH 8.5, 0.5 to 2 mg protein from fraction II or IV was bound to the column. The column was washed and eluted using a linear salt gradient. SDS-PAGE was performed using Phast system (GE Healthcare). A 55-kd and a 10-kd protein were isolated from fraction II and IV, respectively. N-terminal sequencing was performed with Applied Biosystem's Procise 494 protein sequencer (Foster City, Calif). Trypsin digestion and tandem mass spectrometry using Finnigan LCQ Deca XP (Thermo Electron Co, Waltham Mass)[13,14] was performed on fraction II. The nomenclature for the 2 proteins was confirmed by the International Union Immunological Societies (IUIS) nomenclature committee as Har a 1, 10 kd, and Har a 2, 55 kd.

Patient groups

Two groups of patients' sera were tested. The ladybug group consisted of outpatients who presented with allergic symptoms and reported that their home was infested with *H axyridis* (Table I). All these subjects lived in either rural or suburban areas. The emergency department group consisted of patients presenting to the emergency department with asthma (n = 68), enrolled in 1998 and 1999.[15] Patients answered a questionnaire about infestation of their homes, focusing on cockroaches, and none mentioned ladybugs. However, ALB infestation was still rare in this area at that time. Control sera were collected from subjects without allergy. Skin testing was performed on subjects using *H axyridis* extracts made specifically for clinical diagnosis. These experiments were approved by the Human Investigation Committee at the University of Virginia.

Serum IgE antibody titers

The IgE antibody in patients' sera was measured using streptavidin CAP (kindly provided by Phadia AB, Kalamazoo, Mich).[12] Biotinylated allergen was incubated with streptavidin CAPs and incubated with serum samples. Allergen-specific IgE antibodies were measured by the fluorometric enzyme assay.

Inhibition of ALB IgE antibody binding

Cross-reactivity was assessed using a modification of RAST inhibition experiments.[16] Seven sera, 2 from the emergency room study and 5 from the ALB study, were preincubated with serial dilutions of *B germanica* allergen extract (from 1:1-1:1000; Greer Laboratories, Lenoir, NC). After overnight incubation at 4°C with the allergens, the serum was tested for unbound IgE antibody to ALB extract using the streptavidin CAP assay.

Statistics

Correlation between IgE antibodies to *H axyridis* and *B germanica* was assessed using the Pearson rank correlation. Data were analyzed with SPSS for Windows (version 10.0; SPSS Inc, Chicago, Ill).

RESULTS

Separation of *H axyridis* extract using gel filtration

Proteins extracted from crushed beetles were defatted and separated into 4 fractions by using gel chromatography (Fig 1 and Fig 2, *A*). SDS PAGE analysis of the fractions is shown in Fig 2, *B*. Fractions were tested for serum IgE antibody binding by streptavidin CAP. The 8 sera selected for testing had a distinct pattern of binding with 3 features (Fig 3, *A*). High titer IgE antibody levels were found to each of the fractions with 5 sera in which titers were >10 IU/mL. Two patients, patient 6 and patient 7, had significant IgE antibody levels to fraction IV but very low levels to fraction I. Last, all the sera showed some positive binding to fractions I and II, including an asymptomatic control. This pattern was consistent in all control sera (Fig 3, *B*) with apparent IgE antibody levels of ~0.5 IU/mL and ~3.0 IU/mL to fractions I and II, respectively.

Purification of allergens using ion exchange chromatography

A 10-kd protein was purified from fraction IV by anion exchange chromatography (Fig 4, *A, track #3*). Thirteen of the 20 patients with symptoms and a history of exposure

TABLE I. Twenty patients reporting symptoms to ALB: environmental exposure and serum IgE antibody levels

	Age (y)/race/sex	Symptoms	Season	Skin test	Household infestation	ALB	IgE antibody (IU/mL)			
							CR	Mite	Cat	Other*
1	46/W/F	Rhinitis, cough	Winter	ND	+++	59	1.5	2.1	Neg	Moth
2	60/W/M	Cough, asthma	Year round	++	++	30	5	10.6	10.6	Grass, moth, Berlin beetle
3	54/W/M	Rhinitis, cough	Winter	++	+++	41	Neg	Neg	Neg	Grass
4	56/W/F	Rhinitis, cough	Winter	ND	++	14	2.3	Neg	Neg	Grass, Berlin beetle
5	57/W/F	Asthma, rhinitis	Winter	++	++	10	0.4	22	8.9	Dog, moth, grass
6	72/W/F	Asthma, rhinitis	Winter	++	++	1.6	0.6	0.4	Neg	Moth‡
7	58/W/F	Cough, conjunctivitis	Winter	++	++	4.1	Neg	Neg	Neg	Neg
8	18/W/M	Rhinitis	Spring	ND	+	Neg	1.5	Neg	Neg	Moth‡
9	47/W/F	Asthma	Fall	++	+++	26	Neg	Neg	Neg	Neg
10	59/W/F	Asthma	Year round	+	++	1.6	2.3	1.8	3.1	Dog, moth, grass, Berlin beetle
11	78/W/F	Asthma	Worse in winter	++	++	0.6	Neg	Neg	Neg	Neg
12	42/W/F	Rhinitis	Spring/fall	ND	+	Neg	Neg	1.2	Neg	Neg
13†	51/W/F	Rhinitis, conjunctivitis	Fall	ND	++	14.5	Neg	Neg	1.0	Neg
14	59/W/F	Chronic cough, rhinitis	Year round	ND	++	0.38	Neg	Neg	Neg	Neg
15	45/W/F	Asthma, rhinitis	Year round	ND	+	0.4	Neg	Neg	Neg	Grass
16	45/W/F	Asthma, hives	Fall	ND	++	Neg	Neg	Neg	Neg	Moth
17	63/W/F	Rhinitis, cough	Winter	ND	+++	3.6	Neg	Neg	Neg	Neg
18	49/W/F	Asthma	Winter	++	++	Neg	Neg	Neg	Neg	Neg
19	39/W/F	Asthma	Year round	ND	+	Neg	Neg	15.2	1.1	Dog, moth
20	34/W/F	Asthma	Year round	++	+++	0.68	0.67	19.6	0.7	Grass, moth

CR, Cockroach; *F,* female; *M,* male; *W,* white.
*Other allergens tested included rye grass, moth, Berlin beetle, and dog.
†Patient #13 reported a large local swelling of the neck after being bitten by a ladybug.
‡The IgE antibody responses to moth included 2 sera with high titer.

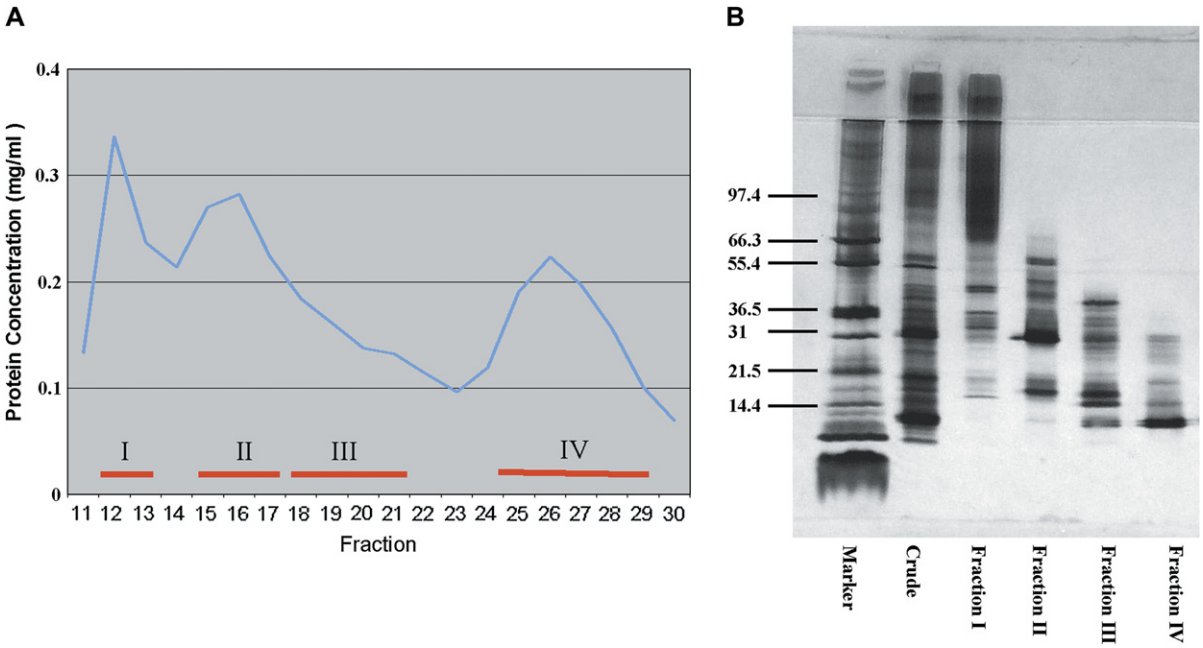

FIG 2. Identification of 4 fractions from ALB extract. Protein concentrations were determined for 5-mL aliquots eluted from a gel chromatography column. Using these concentrations, we defined 4 fractions (Fig 2, *A*). The 4 fractions and crude extract were run on an SDS-PAGE gel to identify proteins (Fig 2, *B*).

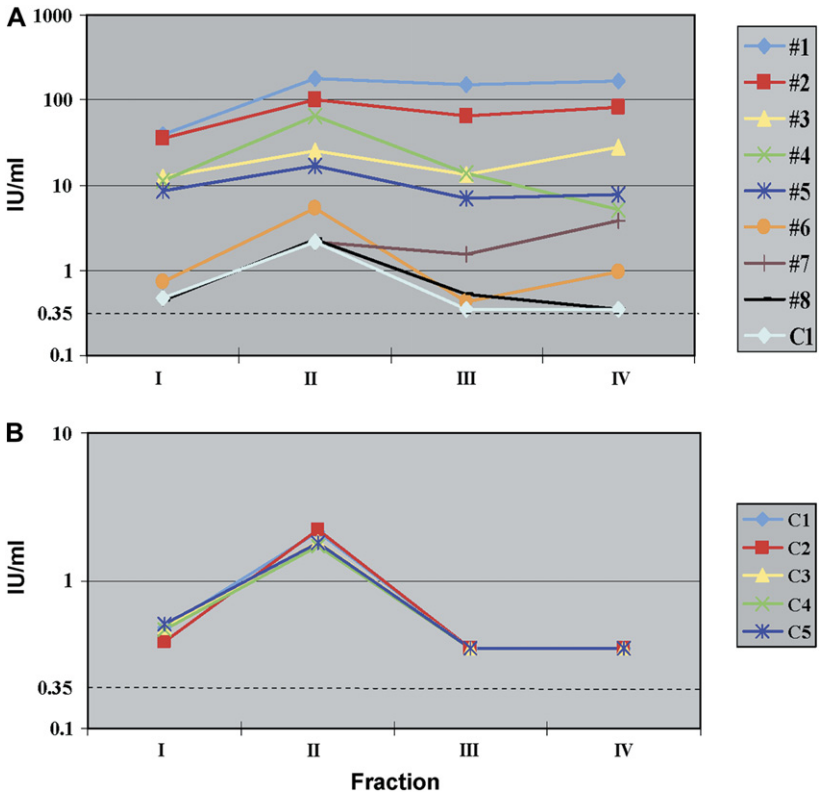

FIG 3. Serum IgE antibody titers to the 4 isolated fractions. **A,** Eight patients with symptoms and an exposure history to ALB. **B,** Five control sera show detectable IgE antibody levels to fractions I and II, indicating nonspecific activating activity by a protein in these fractions.

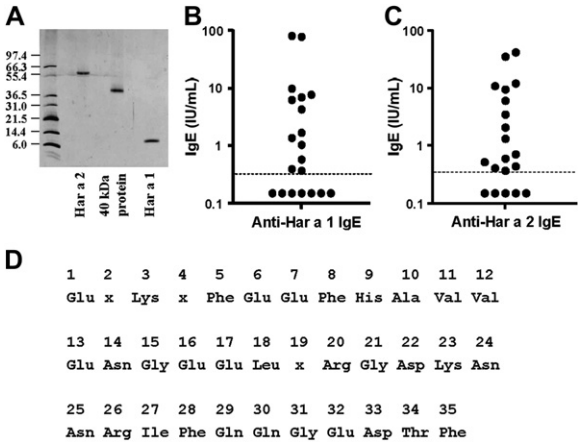

FIG 4. Characterization of Har a 1 and Har a 2, two new ALB allergens. **A,** SDS-PAGE analysis of 3 purified proteins: 10-kd Har a 1, 55-kd Har a 2, and a 40-kd protein. The 40-kd protein showed no binding to IgE antibody. **B and C,** Anti–Har a 1 *(B)* and anti–Har a 2 *(C)* IgE antibody was measured in sera from 20 patients. The *dotted line* indicates the limit of detection. **D,** N-terminal sequence of Har a 1.

had significant IgE antibody to this protein (Fig 4, *B*). The N-terminal sequence of this protein was obtained by Edman degradation (Fig 4, *D*). Database searches of Genbank, European Molecular Biology Laboratory, and DNA Data Bank of Japan revealed no significant homologies. Thus, it appears that this protein, officially named Har a 1, may represent a previously unrecognized beetle protein.

The second protein was purified from fraction II using an anion exchange chromatography. This 55-kd protein showed apparent IgE binding with all sera, including controls. Further analysis revealed this to be a result of nonspecific activation of the assay system used in CAP assays, and not dependent on the presence of human serum. Direct experiments confirmed that the protein is not autofluorescent, but does have β-galactosidase activity sufficient to activate the CAP system (data not shown). We normalized the fluorescent intensity of our positive samples by subtracting the fluorescence seen with a negative control serum. Fifteen out of 20 patients had positive results when tested for IgE antibody to the 55-kd protein, officially named Har a 2 (Fig 4, *C*). Because the purified protein could not be sequenced by Edman degradation, it was subjected to trypsin digestion followed by tandem mass spectrometry.[13,14] Search on the Genbank, DDBJ, and EMBL identified extensive homology with the gene sequence of an aldehyde dehydrogenase of red flour beetle, *Tribolium castaneum*.[17,18] Further, sequence data were obtained using the peptides as primers for PCR (Fig 5).

Assay development for IgE Abs to *H axyridis*

Because of the nonspecific activity of the proteins in fraction II (Fig 3), a pool of fractions I, III, and IV was

*Genbank sequence of Red Flour Beetle Aldehyde dehydrogenase, XP_9108431.
$Polypeptides sequenced by Tandem mass spectrometry.
#Partial sequence of Har a 2 derived from PCR of mRNA library.

FIG 5. Partial sequence data on the 55-kd allergen Har a 2. The Har a 2 sequences *(bottom)* were aligned to an aldehyde dehydrogenase from the red flour beetle *(top)*, accession #XP_969405.

biotinylated to assess sensitization to *H axyridis*. Fifteen out of 20 sera were positive for serum IgE antibody to this pool (Table I). By contrast, all 15 sera from asymptomatic subjects were negative (<0.35 IU/mL). Using this assay, we tested 68 sera from patients presenting to the emergency department of the University of Virginia with acute asthma.[15] Eighteen sera were positive for IgE antibody to the ALB protein mixture. Seventeen out of 18 sera were from African American patients, most of whom lived in the city of Charlottesville. This was surprising because the sera were collected in 1998 and 1999, before ALB infestation was common in this area. In addition, there have been very few reports of ladybug infestation in the city. Comparison with other results for these sera showed that all of these sera also had IgE antibody to the German cockroach *B germanica* (Fig 6, *A*). The strong correlation ($r = 0.83$, $P < .001$) between IgE antibody titers to cockroach extract and ladybug proteins suggested *in vitro* cross-reactivity (Fig 6, *A*). On the other hand, there was no significant correlation ($r = 0.34$, $P = .15$) between IgE antibody to German cockroach allergen and ladybug allergen among the ladybug group (Fig 6, *B*). Cross-reactivity between ALB and *B germanica* was confirmed by CAP inhibition experiments with 9 sera. In each of the ER sera, ≥95% of the binding to ALB could be blocked by preincubation with cockroach extract (Fig 6, *C*). By contrast, IgE antibody to ALB in 5 sera from patients primarily exposed to ALB showed only 5% to 10% inhibition by cockroach extract (Fig 6, *C*). Preliminary experiments suggest that the cross-reactivity relates primarily to a protein present in fraction I (molecular weight, 30 kd).

The ladybug sera were tested for IgE antibody to common indoor allergens and for 2 other insects: Berlin beetle and moth. Berlin beetle was the only beetle for which a commercial solid phase was available. The results showed only 3 sera were positive for the Berlin beetle but 8 sera were positive to moth, including 2 sera with titers > 10 IU/mL (Table I). Interestingly, in addition to identifying 7 sera with high-titer IgE antibody, we identified 5 subjects who were positive to ALB but not to any of the other allergens tested.

DISCUSSION

It is now clear that people whose houses become infested with ALBs can become sensitized to proteins derived from these insects. In addition, many of the patients recognize a strong seasonal basis for their symptoms, September through March, not a season for any of the pollens, molds, or dust mites. Thus, we have a new and apparently increasing seasonal indoor allergen. Most of the associated symptoms were not distinctive. However, we have been impressed by chronic cough, and several patients have reported conjunctivitis related to touching their eyes after catching ladybugs. One patient living in an infested home was admitted to the hospital with asthma. In addition, these beetles can bite and cause local reactions at

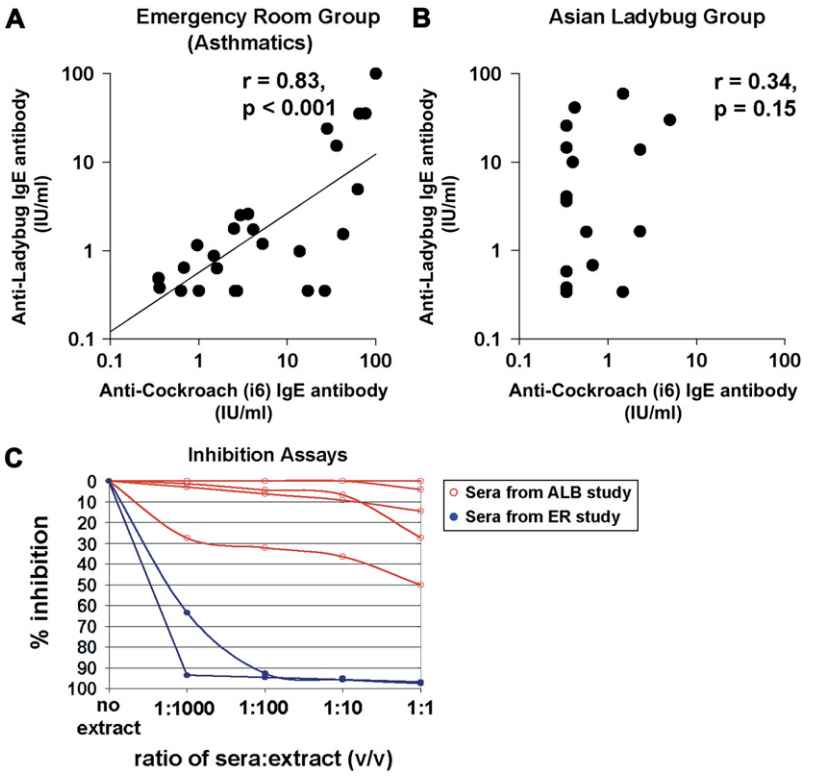

FIG 6. Evidence of cross-reactivity between antiladybug (using a mixture of fractions I, III, and IV) IgE and anticockroach IgE in the ER study sera. A strong correlation was observed in patients with asthma living within the city of Charlottesville, Va **(A)**, but not for patients with allergy living in rural areas with ALB-infested homes **(B)**. Preincubation of sera with *B germanica* extract showed extensive inhibition of ALB IgE antibody binding with ER sera, but not with ALB sera **(C)**.

the site. *H axyridis* is a different species from traditional ladybugs; they can be many different colors, but often they are slightly browner in color, they have an unpleasant smell when handled, and they can fly indoors, but usually only for small distances.

Sensitization can be identified either by skin tests or *in vitro* assays. At present, neither approach is commercially available. The lack of readily available extracts supports our view that sensitization was not common before ladybugs moved indoors. It is relatively easy to collect the bugs, and many practitioners have made extracts for use in their own clinics. Given our current understanding of the extract, it will be possible to establish a solid phase for *in vitro* testing. When we measured serum IgE antibodies to *H axyridis*, we were impressed that 7 of the 15 positives (47%) had a titer > 10 IU/mL (Fig 3, *A,* and Table I). Although we do not yet have objective measurements of exposure to these proteins, it is striking to observe high titers of IgE antibody to this insect. Recent studies have suggested that there are major differences in the titer of IgE antibody induced by different allergens. In particular, high-titer IgE antibody to cat and dog are less common than with dust mite, cockroach, or pollen.[12]

Our data show significant cross-reactivity between *H axyridis* and *B germanica*. Cross-reactivity between closely related species, such as grass pollens, mites, and some mammals, is well recognized and is not surprising given that the proteins often have > 80% homology.[19] Cross-reactivity between insects has been reported previously.[19-21] However, the nature of cross-reactivity between more distantly related species is not well understood. In addition, there is evidence of cross-reactivity between some proteins produced by dust mites and cockroaches. The truly interesting question is how much cross-reactivity is influenced by the evolutionary distance between species. Certainly, cockroaches and ladybugs have only limited separation compared with the separation between mammals and mites, plants, or molds. Our data show that the patients sensitized by exposure to cockroach allergens had extensive *in vitro* cross-reactivity to ladybug extract.

In keeping with our results on Har a 1, a recent report identified a low-molecular-weight allergen by Western blot.[22] To investigate further the significance of this allergen, we have recently radiolabeled Har a 1 with ^{125}I and performed experiments to measure both exposure and IgG antibody. Using an inhibition radioimmunoassay, Har a 1 could be measured in dust and airborne samples. In addition, we have found very high levels of Har a 1 in washings from live ladybugs. By using this radiolabeled protein, we have also assayed IgG antibody in sera from the ALB group (data not shown).

For some patients, the simple information that they are allergic to the ladybugs infesting their homes is a major relief. The next logical treatment approach is avoidance. Many techniques have been tried, including chemicals, traps, sound waves, and painting the house a dark color to discourage swarming. No method has been consistently successful, but the best results have been reported when treating the outside of the house with pyrethroids before cold weather. The problem appears to be that the beetles enter the cavities within the walls, and it is difficult to attack them once they are established. Whether the allergens are primarily derived from the brown liquid ladybugs excrete, making distinctive marks on the walls, or from the debris of dead beetles is not clear.[3] It seems likely that cleaning to reduce debris will be important. On the other hand, there is no evidence that infestation reflects an available supply of food for the beetles. Unfortunately, moving into the city or building a new very tight house may be the most successful approach to controlling exposure. Immunotherapy with extracts of *H axyridis* has been reported anecdotally, but no controlled trials have been reported. Because controlled trials are essential, it is unlikely that commercial extracts will be approved for immunotherapy for several years.

In conclusion, seasonal infestation of homes with ALB has given rise to a new source of indoor allergens. Some of the exposed individuals become highly sensitized, as judged by allergic symptoms including asthma, chronic cough, and titers of IgE antibody. It is important that allergists in areas of the country where infestation is common be aware of the problem.[22] Analysis of the proteins has already identified 2 specific allergens, Har a 1 and Har a 2. We have also identified *in vitro* cross-reactivity with *B germanica*. Our preliminary data suggest that the 10-kd protein Har a 1 is specific for ladybugs and may be suitable for measuring IgG antibodies and for assessing environmental exposure. Treatment approaches are limited because avoidance is difficult and immunotherapy can be provided only on an individual basis. However, many patients are aided by understanding that ladybugs are the cause of their symptoms.

We thank Dr John Shannon and Nicholas E. Sherman of the Biomolecular Research Facility at the University of Virginia for sequencing Har a 1 and Har a 2. We are grateful to Michael Land of Phadia Inc for supplying the streptavidin CAPs used in these studies. We also thank Dr Robert Esch at Greer Laboratories for independent confirmation of the low-molecular-weight allergen using our sera for Western blot experiments.

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