

Localization of Per a 3 allergen in the gut and faecal pellets of the American cockroach (*Periplaneta americana*)

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Summary

Cockroach-derived materials are known to be a major source of potent aeroallergens, causing allergic respiratory diseases such as asthma and allergic rhinitis. The aim of this study was to determine the localization of the major allergen, Per a 3 (Cr-PI), within the American cockroach (*Periplaneta americana*), which might reveal the relative importance of excreted materials and non-excreted cockroach components as allergen sources.

American cockroaches (*P. americana*) and their faecal pellets were embedded in paraffin, and serial sections were cut and collected on glass slides. After being stained with mouse polyclonal antiserum against Per a 3, the sections were incubated sequentially with biotin-labelled sheep antimouse immunoglobulin G (IgG) and a preformed fluorescent isothiocyanate (FITC)-avidin complex. Finally, the sections were mounted and examined under a fluorescent microscope.

Examination of Per a 3 immunoreactivity on the sections of the American cockroaches (*P. americana*) revealed that the midgut mucosa, gut contents and faecal pellets were all strongly labelled. Per a 3 immunoreactive products were not detected in any other internal organs of the American cockroaches. These results suggest that Per a 3 allergen might be synthesized in and secreted from the epithelia of the midgut mucosa and excreted from the body in the faecal pellets.

Introduction

Cockroaches produce a number of allergens with molecular masses ranging from 6 to 120 kDa. These allergens are one of the leading causes of allergic diseases such as asthma and allergic rhinitis (Wu & Lan, 1988; Stankus *et al.*, 1990). During the past few decades, allergens from the American cockroach (*Periplaneta americana*) and the

German cockroach (*Blattella germanica*), which are the two principal domiciliary cockroach species, have been identified and characterized by various methods at both the protein level and the DNA level (Kang *et al.*, 1991; Anna *et al.*, 1998; Wu *et al.*, 2000). However, much less attention has been paid to the localization of specific cockroach allergens *in situ*. Per a 3 (Cr-PI) is a major allergen produced by *P. americana* and it is responsible for 73% of skin reactions in cockroach-allergic individuals (Wu *et al.*, 1996). Thus, the synthesis and secretion sites of Per a 3 allergen in the bodies of the American cockroach need to be thoroughly investigated, in order to fully understand its biological function. In the current study, we demonstrated the localization of the Per a 3 allergen in the bodies and faecal pellets of American cockroaches (*P. americana*) by immunohistochemical staining.

Materials and methods

Synthesis of recombinant Per a 3

The complementary DNA (cDNA) clone encoding Per a 3 was obtained from a cDNA library of *P. americana* nymphae through immunoscreening with human sera containing cockroach-specific immunoglobulin E (IgE). Using the pGEX-5X-1 expression plasmid (Pharmacia, Uppsala, Sweden), rPer a 3 was expressed in *Escherichia coli* BL21 as a glutathione S-transferase (GST) fusion protein, which was purified by affinity chromatography with glutathione sepharose 4B (Pharmacia). After being subjected to Xa cleavage, the rPer a 3 allergen was finally purified by electroelution.

Generation of mouse antibodies to rPer a 3

For the production of polyclonal antibodies against rPer a 3, each female Balb/c mouse was injected intraperitoneally with 30 µg of purified rPer a 3 in 0.2 mL phosphate-buffered saline (PBS; 0.9% NaCl in 0.01 M sodium phosphate buffer, pH 7.4) emulsified with an equal volume of complete Freund's adjuvant. Subsequent injections were given on days 21, 34 and 51 with the same amount of antigen in incomplete Freund's adjuvant. The production of specific antibodies was monitored by Western blot analysis (data not shown).

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Received 26 April 2007; revised 26 April 2007; accepted 17 May 2007

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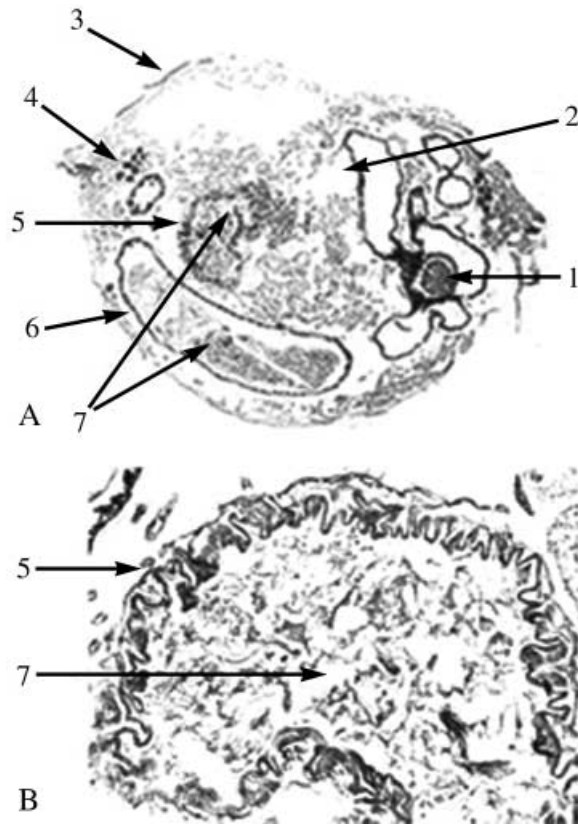


Figure 1. Micrographs of the morphological structure of body cavity of the American cockroach (*Periplaneta americana*). (a) Low-power micrograph of a transverse section through the abdomen of the American cockroach. The digestive tract occupies a large part of the body cavity. The arrows labelled with the numbers indicate the gizzard (1), crop (2), chitin crust (3), reproductive tissue (4), midgut (5), hindgut (6), and gut contents (7), respectively. (b) High-power micrograph of a cross-section of the midgut shows the gut wall (5) and dense gut contents in the lumen (7). All sections were stained with haematoxylin and eosin. Magnifications: a: $\times 100$ and b: $\times 400$.

Preparation of sections

Five male and five female American cockroaches (*P. americana*) were used in this study. Faecal pellets were carefully collected with a brush from the container within which the insects were kept. The cockroaches were killed by CO₂ inhalation. Then, the cockroaches and the faecal pellets were fixed in 4% paraformaldehyde for 16 h at room temperature, washed in PBS three times (10 min each) to remove the fixative, dehydrated through graded alcohols, and followed by two xylene washes to remove the alcohol. The specimens were then embedded in paraffin (melting point, 54 °C) and allowed to harden overnight at room temperature. Six-micrometre serial sections were cut in a transverse plane of the cockroach body and the faecal pellets. All sections were collected serially on glass slides which were coated with poly-L-lysine. Consecutive sections (3–5) were collected on each glass slide and

processed for morphological and immunohistochemical staining.

Morphological study and Per a 3 immunohistochemistry

The sections of the *P. americana* on every third slide of the serial sections were dewaxed, rinsed in tap water and stained with haematoxylin and eosin (H&E) for general morphological study of the cockroach body cavity. For immunohistochemistry, slides selected randomly from the serial sections were processed to identify Per a 3 antigen in the cockroach bodies and in the faecal pellets. The sections were dewaxed, rinsed in tap water and placed in PBS. The sections were incubated in PBS containing 5% sheep serum for 30 min at room temperature to block unspecific binding sites on the tissue sections. Then, the sections were incubated in 1 : 75 mouse polyclonal antisera against Per a 3 in 5% normal sheep serum in PBS at 4 °C overnight. After being washed in PBS three times (10 min each), the sections were incubated with 1 : 100 biotin-labelled sheep antimouse IgG (Sino-American Biotechnology Co., Henan, China) and diluted 1 : 100 in PBS with 5% sheep serum for 60 min at room temperature. The sections were washed in PBS (10 min $\times$ 3) and incubated in PBS with 1 : 100 fluorescent isothiocyanate (FITC)-avidin complex (Sigma-Aldrich, St Louis, MO, USA) for 60 min. After being washed in PBS, the sections were counterstained with 0.1% Evans blue (Fluka, Milwaukee, WI, USA) for 5 min at room temperature. Finally, the sections were mounted in buffered glycerol. Per a 3 immunoreactivity in the bodies and faecal pellets of the American cockroach was examined under a fluorescent microscope (Olympus, Tokyo, Japan). On the sections representing the negative control, normal mouse serum was used to replace the primary antiserum during the incubation. All incubations of the immunohistochemical staining were carried out in a humid chamber.

Results

General morphological study

Examination of transverse sections of the American cockroach stained with H&E revealed the general morphological structures of the body cavity (Fig. 1a). The external structure, chitin crust, and the internal structures, such as the crop, gizzard, midgut, hindgut, and reproductive tract, were all clearly demonstrated. The digestive tract occupied a large part of the body cavity. The gut contents were present in the lumen of both the midgut and the hindgut (Fig. 1a). The midgut was located close to the central part of the body cavity, and the gut contents were more densely present in the lumen of the midgut than in the hindgut (Fig. 1a,b).

Distribution of Per a 3 in the American cockroach

Under a fluorescent microscope, the Per a 3 immunoreactive products detected by the FITC secondary antibody

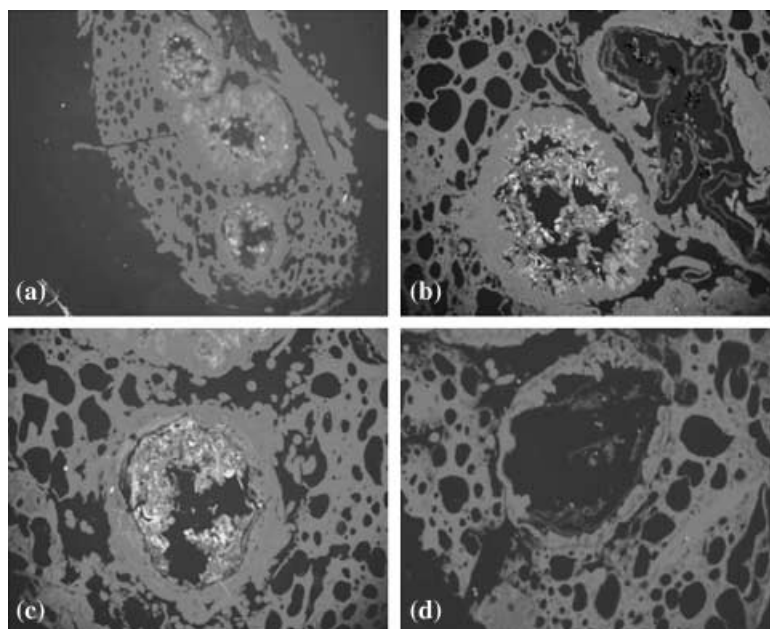


Figure 2. Localization of Per a 3 immunoreactivity in the gut of the American cockroach (*Periplaneta americana*). (a) A low-power immunofluorescent micrograph of a cross-section through the midgut, showing that Per a 3 immunoreactive products (yellow colour) are strongly located on the mucosal layer of the midgut and in the gut contents of the lumen. The morphological structure of the gut is demonstrated with Evans Blue staining (red colour). (b and c) High-power immunofluorescent micrographs show that Per a 3 immunoreactivity is restrained in the mucosal layer of the midgut and gut contents of the lumen, but not in any other structures of the American cockroach (red colour, Evans Blue staining). (d) Micrograph of a control section of the midgut in which normal mouse sera were used to replace the primary antisera in the incubation. No Per a 3 immunoreactivity is present on this section. Magnifications: a: $\times 200$, b, c and d: $\times 400$.

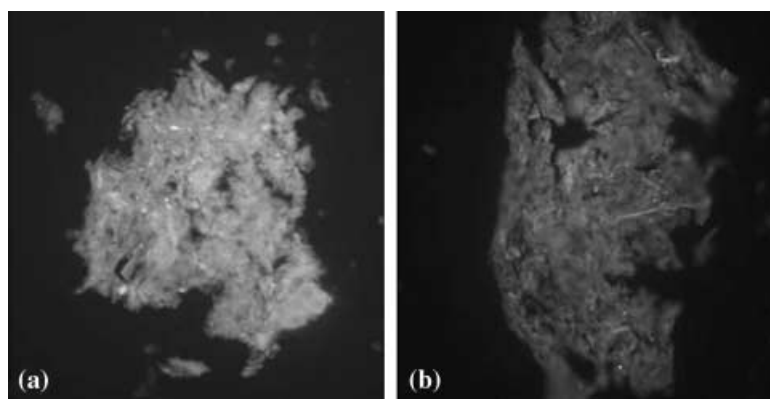


Figure 3. Per a 3 immunoreactivity is present in the faecal pellets of the American cockroach (*Periplaneta americana*). (a) Micrograph of a section of the faecal pellets shows that Per a 3 immunoreactive products are strongly located in the faecal pellets. (b) Micrograph of a control section of the faecal pellets which was incubated with normal mouse sera instead of primary antisera. No Per a 3 immunoreactivity is present on this section. $\times 1000$.

should have been green in colour. However, the Per a 3 immunoreactive products were yellow in the current study. This was due to the Evans Blue counterstaining. The Evans Blue stained all structures of the American cockroach on our paraffin sections red. The combination of green (FITC) and red (Evans Blue) made the Per a 3 immunoreactive products turn yellow. Examination of transverse sections of the American cockroach processed by Per a 3 immunohistochemistry showed that the immunoreactive products were localized to the digestive tract (Fig. 2). No other internal organs were positively labelled by the mouse Per a 3 antibodies. Under both low-power (Fig. 2a) and high-power objectives (Fig. 2b,c), Per a 3 immunoreactive products were observed in the mucosa of the midgut and in the gut contents of the lumen. In contrast, no Per a 3 immunoreactivity was observed in any of the sections of the American cockroach on which the primary antiserum was replaced with normal mouse serum during the incubation (Fig. 2d). In terms of the

staining pattern and staining intensity of Per a 3 immunoreaction, no obvious differences were observed between the sections of male and female cockroaches.

Examination of sections of the faecal pellets of the American cockroach (*P. americana*) processed by Per a 3 immunohistochemistry showed that the immunoreactivity was strongly present in the faecal pellets (Fig. 3a). However, no Per a 3 immunoreactivity was observed on any control sections of the faecal pellets on which the primary antibodies were replaced with normal mouse sera during the incubation (Fig. 3b).

Discussion

Cockroaches have long been recognized as being vectors of various pathogens and, perhaps more importantly, as one of the major causes of allergic respiratory diseases (Zhong, 1996; Leung *et al.*, 1997; Arruda *et al.*, 2001). To date, three *P. americana* allergens (Per a 1, Per a 3 and

Per a 7) and seven *B. germanica* allergens (Bla g 1, Bla g 2, Bla g 4, Bla g 5, Bla g Bd 90K, Bla g 6 and Bla g 7) have been cloned (Arruda *et al.*, 1995a,b; Wang *et al.*, 1999; Asturias *et al.*, 1999; Pomes *et al.*, 2002; Jeong *et al.*, 2003). Among these, only Per a 1 was reported to be cross-reactive with Bla g 1, and the sequence homology between the two was 52–71%. Per a 3, the major cockroach allergen, comprises a family of isoallergens. The clone obtained in our laboratory shared 98% sequence identity with Per a 3 (clone 12). Per a 3 allergens were found to be homologous to insect storage proteins (20.1–33.9%), insect juvenile hormone-suppressible proteins (30.9–36.4%) and arthropod haemocyanins (29.7–34.6%). It has been suggested that these allergens are ancestrally related to insect haemocyanin and they represent a new group of proteins within the haemocyanin super family (Wu *et al.*, 1997).

During the past few decades, the prevalence of allergic diseases has increased to an epidemic level. However, the way in which susceptible individuals become sensitized to allergens remains unknown (Meza & Gershwin, 1997). The localization of specific allergens in the bodies of the cockroach might contribute to the understanding of these sensitizing processes, and could help to elucidate the biological functions of individual allergens. Cryostat sections of the house dust mite (*Dermatophagoides pteronyssinus*) have previously been immunoprobed both with anti-Der p 1 rabbit polyclonal antibodies and mouse monoclonal antibodies, and with sera from mite-allergic individuals, respectively. When human serum containing mite-specific IgE was used to probe mite sections, the most prominently labelled areas were the anterior midgut and the faecal pellets (Tovey & Baldo, 1990). Rabbit polyclonal antibodies and monoclonal antibodies against Der p 1 recognized similar regions (that is, the anterior midgut, gut contents and posterior midgut), but labelling with monoclonal antibodies appeared to be sharper (Park *et al.*, 2000). It has also been demonstrated that group II allergens of the house dust mite (Der p 2 and Der f 2) are localized in the digestive tract and concentrated in the faecal pellets (Park *et al.*, 2000; Jeong *et al.*, 2002).

In the present study, both the midgut mucosa and the faecal pellets of *P. americana* were strongly labelled by the mouse antiserum specific for Per a 3. These findings suggest that Per a 3, like the group I and group II allergens of house dust mites, is secreted from the digestive tract and subsequently becomes incorporated into the faecal pellets. Upon disturbance, these allergens might easily become airborne and act as sources of sensitization. Interestingly, when sections of *B. germanica* were probed with the same anti-Per a 3 antibodies, no labelling was detected. This finding strongly indicates that Per a 3 is a species-specific allergen, and is consistent with the results of a previous study in which monoclonal antibodies to Per a 3 (Cr-PI) could not recognize antigens from the German cockroach (Wu *et al.*, 1996).

In conclusion, this study demonstrates that Per a 3 immunoreactivity is localized in the midgut mucosa, gut contents and faecal pellets of the American cockroach

(*P. americana*), suggesting that Per a 3 allergen might be synthesized in and secreted from the epithelia of the midgut mucosa and excreted from the body in faecal pellets.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (No. 30571625 and 39860071) and from the Science Foundation of Guangdong Province (No. 2003A3080502).

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