Cockroach Allergen per a 7 Down-Regulates Expression of Toll-Like Receptor 9 and IL-12 Release from P815 Cells Through PI3K and MAPK Signaling Pathways

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Key Words
American cockroach • Per a 7 • Toll-like receptor9 • IL-12 • Mast cell • ERK signaling pathway • PI3K signaling pathway

Abstract
Background: As a major source of indoor allergens, cockroach causes perennial rhinitis and asthma. Recently, cockroach feces were reported to contain TLR2 agonist, which could directly activate neutrophils to release cytokines. CpG oligodeoxynucleotide (ODN), a Toll-like receptor (TLR)9 activator was also found to induce proinflammatory cytokine release from mast cells. However, influence of specific cockroach allergen on Th1 cytokine release and expression of TLR9 in mast cells remains uninvestigated. Methods: To investigate effects of Per a 7 on TLR expression and cytokine release from mast cells and their cell signaling mechanisms, P815 cells were challenged by recombinant Per a 7 (rPer a 7), and expression of TLR9 mRNA and protein was assessed mainly by real time PCR and flow cytometry analysis. Detection of phosphorylation of cell signaling components was performed with Western blotting technique. Results: The results showed that rPer a 7 induced up to 72 and 46% down-regulation of expression of TLR9 mRNA and protein, respectively following 16 h incubation period. It induced also approximately 41.1% reduction of IL-12 release. When PD98059, U0126 and LY294002 were pre-incubated with the cells for 30 min they diminished rPer a 7 induced reduction of TLR9 expression and IL-12 release, indicating these events are via activation of ERK and PI3K/Akt signaling pathways. Conclusion: Reduction of IL-12 production and expression of TLR9 in P815 mastocytoma cells by Per a 7 suggests that this major cockroach allergen might contribute to the development of cockroach allergy.

Introduction
Cockroach allergens have been identified as major indoor allergens inducing IgE mediated allergic respiratory illness such as perennial rhinitis and asthma [1]. Most cockroach allergy was caused by the two most common domiciliary species: American cockroaches (Periplaneta
to release IL-8, IL-6 and TNF [3] and regulate TLR2 agonist, which could directly activate neutrophils [2]. Recently, cockroach feces were reported to contain namely Per a 1, Per a 3, Per a 4, Per a 7 and Per a 10 cockroaches. Five different types of major allergens americana) and German (Blattella germanica) cockroaches. Five different types of major allergens have been identified from the American cockroach, namely Per a 1, Per a 3, Per a 4, Per a 7 and Per a 10 [2]. Recently, cockroach feces were reported to contain TLR2 agonist, which could directly activate neutrophils [2].

TLRs are a group of single membrane-spanning non-catalytic receptors that recognize structurally conserved pathogen-associated molecular patterns derived from microbes, and activate immune cell responses [5]. Recently, it has been found that airway sensitization and challenge with mite major allergen Der p 2 led to experimental allergic asthma through direct interactions with TLR4 complex [6], suggesting the importance of TLRs in allergy. Moreover, accumulated evidence imply that mast cell, one of the principle effector cells in allergy can regulate adaptive immunity through TLRs [7]. For example, CpG oligodeoxynucleotide (ODN), a TLR9 activator was able to induce proinflammatory cytokines (TNF and IL-6) and chemokines (RANTES, MIP-1-alpha, and MIP-2) release from murine fetal skin-derived mast cells [8], implying that CpG-ODN may contribute to formation of inflammation. On the other hand, CpG ODNs induce Th1-type cytokine release, which can suppress the Th2-type responses that cause many of the manifestations of allergic disease, suggesting that the TLR9 activator may be useful in preventing or reversing atopic asthma [9]. Since allergens are major causative factors of allergic inflammation, and little is known of specific allergens on expression of TLRs in mast cells, we investigated influence of cockroach major allergen Per a 7 on TLR9 expression on mast cells in the present study.

We formerly found that Per a 7, a tropomyosin which is a highly cross-reactive pan-allergen between foods and inhalant allergens originating from animals [10-12], could up-regulate expression of protease activated receptor (PAR)s and activate the Th2 cytokine IL-4 and IL-13 secretion from P815 mast cell line [13]. Since little is known of effect of Per a 7 on IL-12 and IL-10 release from mast cells, and CpG ODN can act as an adjuvant of allergens [14, 15], we anticipated that Per a 7 may affect expression and function of TLR9 in mast cells, and consequently alter cytokine release ability of mast cells. The aim of the study is to investigate influence of Per a 7 on expression of TLR9 and production of IL-12 and IL-10 in mast cells. It was found that rPer a 7 down-regulated expression of TLR9 and release of IL-12 in P815 cells.

Materials and Methods

Reagents

TRIZol Reagent and SYBR Green I Stain were purchased from Invitrogen (Carlsbad, CA, USA). ExScript™ RT reagent kit and SYBR® Premix Ex Taq™ (perfect real time) were obtained from TaKaRa (DaLian, China). FITC-conjugated rat monoclonal antibody against mouse TLR9 and FITC-conjugated rat isotype control were obtained from eBioScience (Los Angeles, CA, USA). Enhanced chemiluminescence reagents, mouse IL-12p70 ELISA kits (the lowest level of detection is 5 pg/ml) were purchased from Pierce Biotechnology Inc. (Rockford, IL, USA). Tissue culture reagents including Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). LPS and CpG ODN-1826 were obtained from the Invivogen (San Diego, CA, USA). Cellular activation of signaling kits for Akt, ERK, p3 MAPK, tyrostatin (AG490), 2-(2-Diamo)-3-methoxyphenyl-4H-1-benzopyran-4-one (PD98059), 1,4-diamo-2,3-dicyano-4-bis(2-aminoarnathyio)butadiene (U0126), 1,4-diamo-2,3-dicyano-1,4-bis(methylthio) butadiene (U0124) and 2-(4-morpholinyl)- 8-phenyl-4H-1-benzopyran-4-one (LY294002) were purchased from Cell Signaling Technology (Beverly, MA). The mouse mastocytoma cell line P815 was obtained from the American Type Culture Collection (Manassas, VA, USA). Paraformaldehyde, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl) -5-(4-pyridyl)-1H-imidazole (SB203580), calcium ionophore A23187 (CI) and most of other reagents such as salt and buffer components were analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant Per a 7 (rPer a 7) was prepared as described previously [13].

P815 cell culture and challenge

P815 cells were cultured with ATCC complete growth medium including DMEM with 4 mM of L-glutamine, 1.5 mg/ml sodium bicarbonate, 4.5 mg/ml glucose, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in 75-cm² tissue culture flasks (Falcon) at 37°C in a 5% (v/v) CO₂, water-saturated atmosphere. P815 cells at a density of 1 x 10⁶ cells/ml were incubated with the serum-free basal medium for 6 h and washed twice before challenge. For challenge experiments, cells were exposed to various concentrations (0.001 – 1.0 µg/ml) of rPer a 7 with or without its specific antibody (10, 30 µg/ml), or with rPer a 1.01 (0.001 – 1.0 µg/ml), heat-treated rPer a 7 at 1.0 µg/ml, and CI at 100 ng/ml. At 2, 6 or 16 h following incubation, the culture plates were centrifuged at 450 g for 10 min at 25°C. After the supernatant (5 ml) being collected and stored at -80°C, the cell pellet containing approximately 5 x 10⁶ cells were collected for immunofluorescence and real-time PCR analysis. The purified polyclonal antibody against Per a 7 was incubated with 1.0 µg/ml of rPer a 7 on ice for 60 min before being added to P815 cell.
For certain experiments, cells were preincubated with 0.01 and 0.1 μg/ml of rPer a 7 for 1 h before adding 0.5 and 5.0 μM of CpG ODN. At 16 h following incubation, the culture plates were centrifuged at 450 g for 10 min at 25°C. The culture supernatants were collected and stored at -80°C for further use.

For cell signaling experiments, cultured cells at a density of 1.0 x 10^6 cells/ml were washed twice with the serum-free basal medium and then treated with the inhibitors of cell signaling pathways including PD98059 (50 μM), U0126 (5 μM), U0124 (5 μM), SB203580 (20 μM), LY294002 (20 μM) and AG490 (40 μM) for 30 min before being challenged with rPer a 7 (0.1 and 1.0 μg/ml) for 30 min, 2 or 6 h. The culture supernatants were then collected and stored at -80°C, and cells were collected for immunofluorescence analysis and cell signal analysis. The concentrations of inhibitors were chosen according to our previous study [16].

**Flow cytometry analysis and immunofluorescence cell staining**

P815 cells were pelleted by centrifugation at 450 g for 10 min, and then fixed and permeabilized by using a cell fixation/permeabilization kit (BD Pharmingen). Briefly, thoroughly resuspended cells were added in 100 μl of BD Cytofix/Cytopertem solution and incubated for 20 min at 4°C. Cells were then incubated with FITC-conjugated rat anti-mouse TLR9 monoclonal antibody or isotype control, respectively (at a final concentration 4 μg/ml) at 4°C for 30 min. After washing, cells were analyzed on a Fluorescence-activated cell sorting (FACS) Aria flow cytometer with CellDevia software (BD Biosciences, USA) or on a Zeiss 5 LIVE confocal laser scanning microscope (Zeiss, German).

**Western blot analysis of cell signal transduction pathways**

P815 cells were preincubated with 20 μM of LY294002, 5 μM of U0126, 50 μM of PD98059, 20 μM of SB203580, 40 μM of AG490 or medium alone for 30 min before adding 0.1 or 1.0 μg/ml rPer a 7 (or medium alone) for 30 min, 2 or 6 h. The cells were lysed in a buffer containing 20 mM of Tris–HCl (pH 7.4), 137 mM of NaCl, 10% glycerol, 1% Triton X-100, 2 mM of EDTA, 25 mM of β-glycerophosphate, 2 mM of sodium pyrophosphate and 0.5 mM of dithiothreitol at 4°C for 30 min. Cell debris was removed by centrifugation of the lysate at 12,000 x g for 10 min. The supernatants were mixed with equal volumes of 2 x sodium dodecyl sulphate sample buffer and heated to 100°C for 10 min. An equal volume of sample was fractionated by SDS-PAGE on a 10 % acrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Billerica, MA) with a Bio-Rad transfer system, according to the manufacturer’s instructions. After blocking non-specific binding sites with 5% BSA in TBST (50 mM of Tris, 0.15 M of NaCl, 0.1% Tween 20, pH 7.6) for 1 h, membranes were probed with primary antibodies at 4°C overnight, followed by incubated with HRP-conjugated secondary antibodies. Immunoreactive bands were visualized by using enhanced chemiluminescence reagents according to the manufacturer’s protocol. Densitometry analysis of immunoblots was carried out using Quantity One software (Bio-Rad, USA). The relative levels of phospho-ERK1/2 and PI3K were expressed as the ratio to β-actin, an internal control.

**Determination of levels of cytokines**

IL-10 and IL-12 were measured by using ELISA kits according to the manufacturer’s instruction.

Mast Cell TLR and Cockroach Allergen per a 7
Preparation and purification of polyclonal antibody against rPer a 7

The rPer a 7 protein was employed as immunogen to generate polyclonal antibody in New Zealand white rabbits. Briefly, rabbits were immunized subcutaneously with purified rPer a 7 (1 mg per rabbit) emulsified with an equal volume of Freund’s complete adjuvant for the first injection. After 1 week, the rabbits were boosted subsequently 3 times. One week after the last injection, antisera of the rabbits were collected and purified by Protein G Sepharose affinity chromatography (Amersham Biosciences, Piscataway, NJ, USA) following manufacturer’s instructions. The efficacy of the antibody against rPer a7 was also examined by Western-blot analysis. The antibody (20, or 60 µg/ml) was able to substantially precipitated rPer a 7 (2.0 µg/ml) following incubation for 60 min on ice (Fig. 1).

Statistics

Data are expressed as mean ± SEM for the indicated number of experiments performed in duplicate. Statistical significance between means was analyzed by one-way analysis of variance or the Student’s t test utilizing the SPSS 13.0 version. 

P < 0.05 was taken as statistically significant.
Results

Expression of TLR9 in mast cells

In order to ensure that P815 cells express TLR9, RT-PCR, FACS analysis and immunofluorescent cell staining were employed to examine expression of TLR9 in cells. The result showed that P815 cells do express TLR9 mRNA (Fig. 2A) and protein (Figs. 2B, C).

Influence of rPer a 7 on expression of TLR9 on mast cells

It was found that rPer a 7 at the concentrations of 0.01, 0.1 and 1.0 µg/ml induced a dose-dependent down-regulation of TLR9 mRNA expression following a 16 h incubation period. The maximum reduced expression of TLR9 mRNA was 72% over baseline control. Once being added at the same time with rPer a 7, anti-Per a 7 antibody was able to diminish rPer a 7 induced TLR9 mRNA down-regulation (Fig. 3A). The time course study showed that down-regulated TLR9 mRNA expression was observed at 6 h and reached almost baseline level at 16 h following incubation (Fig. 3A). Per a 1.01 and showed no effect on TLR9 mRNA expression in P815 cells.

Flow cytometry analysis showed that rPer a 7 at 0.1 and 1.0 µg/ml provoked down-regulation of expression of TLR9 in P815 cells following 16 h incubation period. Approximately up to 46% down-regulated expression of TLR9 was observed when cells were incubated with 1.0 µg/ml of rPer a 7 for 16 h. Anti-Per a 7 antibody was able to diminish rPer a 7 induced down-regulation of expression of TLR9 (Fig. 3B). The time course study showed that significant down-regulation of expression of TLR9 by rPer a 7 was first observed at 6 h, and lasted at least to 16 h following incubation (Fig. 3B). In the parallel experiments, immunofluorescent analysis showed similar pattern of decreased expression of TLR9 in P815 cells following 6 and 16 h incubation periods (data not shown). rPer a 1.01 and heat-treated rPer a 7 (data not shown) showed no effect on the TLR9 protein expression in P815 cells.

rPer a 7 induced reduction of L-12 release

It has been found that rPer a 7 can provoke Th2 cytokine IL-4 and IL-13 release from P815 cells [13]. We then anticipated that this American cockroach major allergen may reduce Th1 cytokine production in mast cells. As expected, we found that rPer a 7 at 0.1 and 1.0 µg/ml induced a concentration dependent reduction of IL-12 release from P815 cells following 16 h incubation period.

Approximately up to 41.1% reduction of IL-12 release was achieved when cells were incubated with 1.0 µg/ml of rPer a 7 (Fig. 4A). Once being added at the same time with rPer a 7, anti-Per a 7 antibody was able to diminish rPer a 7 reduced IL-12 secretion (Fig. 4A). CI induced 92.1 ± 9.8 pg/ml of IL-12 release from P815 cells (n = 4) following 16 h incubation period. rPer a 1.01 and heat-
treated rPer a 7 (data not shown) showed little effect on IL-12 release from P815 cell. rPer a 7 at the concentrations examined had little effect on IL-10 secretion from P815 cells (data not shown).

New evidence suggests that mast cells drive the development of Th2 response to allergens, particularly when allergen exposure occurs concomitantly with exposure to pathogen products present in the environment.
In order to determine if rPer a 7 affects CpG ODN induced IL-12 release, P815 cells were preincubated with lower doses of rPer a 7 (non-effective doses) for 1 h before adding CpG ODN for 16 h. The results showed that rPer a 7 at 0.1 µg/ml blocked completely CpG ODN induced IL-12 release from P815 cells (Fig. 4B).

**Effect of cell signaling inhibitors on rPer a 7 induced release of IL-12 and expression of TLR9**

We employed LY294002, a PI3K/Akt inhibitor; U0126, a selective inhibitor of MEK1/2 and thus a MAPK pathway inhibitor; PD98059, a MAPK pathway inhibitor; SB203580, a selective inhibitor of p38 MAPK; and AG490, a Janus kinase (JAK)/STAT3 pathway inhibitor to investigate the potential Per a 7 signaling pathways in P815 cells.

LY294002, U0126 and PD98059 diminished rPer a 7 induced reduction of IL-12 release by up to 71.8%, respectively when they were pre-incubated with the cells for 30 min (Fig. 5A), indicating that reduction of IL-12 release by rPer a 7 is through activation of PI3K/Akt and MAPK signaling pathways. In contrast, SB203580, U0126 a structural analogue negative control of U0126 and AG490 had little influence on rPer a 7 induced reduction of IL-12 release (data not shown). All inhibitors tested did not significantly affect basal IL-12 release. Similarly, LY294002, U0126 and PD98059 also eliminated rPer a 7 induced down-regulation of TLR9 expression by up to 69.1%, respectively (Fig. 5B). All inhibitors tested did not significantly affect basal TLR9 expression in P815 cells.

**Inhibition of rPer a 7 induced phosphorylation of ERK and PI3K by signaling inhibitors**

PD98059 and U0126 inhibited approximately up to 50 and 46.7% of rPer a 7 induced phosphorylation of ERK, respectively (Fig. 6A). LY294002 diminished rPer a 7 induced phosphorylation of PI3K by approximately 76.2% (Fig. 6B) in P815 cells following 30 min pre-incubation period. PD98059, U0126 and LY294002 inhibited also basal phosphorylation of ERK (Fig. 6A) and PI3K (Fig. 6B) at 2 and 6 h following incubation.

**Discussion**

It is interesting to learn for the first time that Per a 7 is capable of down-regulating expression of TLR9 and IL-12 release from mast cells. The results seem quite different from our previous findings that Per a 7 can up-
regulate of expression of PARs and induce IL-4 and IL-13 secretion from P815 mast cells [10]. However, since IL-12 belongs to Th1 cytokine category, which acts as an inhibitory factor for allergic inflammation, whereas IL-4 and IL-13 are Th2 cytokines, which contribute greatly to the formation of allergic inflammation, it seems likely that Per a 7 plays a causative role in the development of cockroach allergy through modulation of cytokine release from mast cells. The finding that mast cells are a crucial source of functional IL-12 [17] may support our observation that P815 mast cells can release IL-12 in the present study.

Induction of Th2 cytokine release from inflammatory cells by allergens has been widely reported. Thus, Der p 1 has been found to stimulate IL-13 release from cord blood mononuclear cells [18], and IL-4 release from a cord blood T-cell phenotype with atopic dermatitis [19]. Der f 2 was observed to induce IL-13 release from human bronchial epithelial cells [20]. On the other hand, fungal allergens has been found to diminish release of IL-12 from human dendritic cells, which directs the differentiation of human CD4+ T cells toward a Th2 cytokine profile [21]. These reports not only support our current observation that cockroach allergen Per a 7 can eliminate IL-12 release from mast cells, but also implicate that Th1 and Th2 cytokine imbalance could play a crucial role in allergen-induced inflammation.

Per a 7 is one kind of tropomyosins, which have a functional role in contraction of muscle cells and participate in regulation of cell morphology and motility in nonmuscle cells [22, 23]. In invertebrates, tropomyosin has been recognized as an important cause of IgE antibody response. It is also a highly cross-reactive pan-allergen recognized as an important cause of IgE antibody response originating from animals [10-12]. Down-regulation of expression of TLR9 by Per a 7 in mast cells suggests a novel linking between TLR and allergens, which may implicate that an allergen can affect receptor expression on mast cells to a different extent, such as down-regulation of expression of TLR9, and up-regulation of expression of PARs by Per a 7 [13].

Per a 1 is a group of major allergens consisting of 5 members, Per a 1.0101, Per a 1.0102, Per a 1.0103, Per a 1.0104, Per a 1.0105 and Per a 1.02, known as isoallergens [24]. Per a 1.0101 sequence showed 31% identity to a mosquito precursor protein, ANG12, which may be involved in digestion. Per a 1 represented a major allergen group found in American cockroaches [25], which has been shown also to be able to induce secretion of Th2 cytokines IL-4 and IL-13 from P815 cells [26]. They are neither a mutant nor a truncated molecule of Per a 7, showing no identity with Per a 7 by sequence alignment. Failure to down-regulate expression of TLR9 and IL-12 release from mast cells indicates that these two major allergens of American cockroach have different effects on mast cells.

Reduction of CpG ODN induced IL-12 release from P815 cells by rPer a 7 was quite unexpected as CpG ODN is a synthetic activator for TLR9, whereas Per a 7 is a large protein molecule, which should be more difficult to compete with CpG ODN for activation of TLR9. However, down-regulation of expression of TLR9 by Per a 7 may partially explain the inhibitory action of Per a 7 on CpG ODN induced IL-12 release. As a synthetic immunostimulatory TLR9 activator, CpG ODN could induce an innate response dominated by cytokines such as IL-10, IL-12 and IFNs, which could inhibit the allergic phenotype [27]. Once CpG was chemically linked to Amb a 1, it caused a marked increase in IFN-γ level and a shift in the antibody profile from a Th2-directed IgG1 response to a Th1-directed IgG2a response in a murine model of asthma [15]. Similarly, treatment of mite-sensitized mice with CpG ODN/Der f conjugate caused a marked increase in IFN-γ levels and decrease of IL-5 level in nasal lavage fluid [28]. Since IFN-γ and IL-12 are both typical Th1 cytokines, reduction of CpG ODN-induced IL-12 release from mast cells by Per a 7 seems disagreed with the above findings. The discrimination between them may be due to the different experimental system being used. Reduction of IL-12 release by Per a 7 may lead to Th2-polarized immune responses [29], which can help to explain a former observation that early exposure to cockroaches, but not to mites or cats, was associated with recurrent wheezing and asthma independent of specific IgE antibody [30, 31].

Per a 7 induced reduction of IL-12 release and TLR9 expression appears dependent on the intact molecular structure of Per a 7 as heat-treatment completely abolished the action of rPer a 7 on mast cells. The actions of rPer a 7 on mast cells depended also on activation of ERK and PI3K cell signaling pathways. While little information on IL-12 secretion signaling pathways in mast cells is available, the reports that CpG ODN and LPS could down-regulate IL-12 production by activating ERK1/2 in macrophages [32] and dendritic cells [33] may help to understand our current observation that activation of ERK is involved in the Per a 7 induced reduction of IL-12 release from mast cells. The former studies showed that German and American cockroach extracts could activate ERK1/2, but not p38 MAPK in A549 cells [34], and that mycobacterium tuberculosis...
potently inhibits the production of IL-12 by human monocytes by PI3K/Akt signaling pathway [35] may also support our observation above.

In conclusion, it was found in the present study that rPer a 7 can diminish IL-12 production and expression of TLR9 via the ERK and PI3K/Akt cell signaling pathways in murine mast cells. Therefore, it is likely to contribute to the development of cockroach-related allergic diseases in the body. All experiments were performed with a mastocytoma cell line where the signalling pathways are likely to be altered, and further studies are needed using normal wild-type mast cells.

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