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A Murine Model of Allergy Caused by American Cockroach (CR), *Periplaneta americana*

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SUMMARY An animal model resembling the human immuno-pathological features of CR allergy is needed for CR allergy research, *e.g.,* measuring allergenicity of novel allergens, testing immunotherapeutic efficacies of drugs and vaccines. In this study we develop a murine model of American CR, *P. americana* allergy. BALB/c mice, 6 weeks old, were individually intraperitoneally injected with three doses (days 0, 7 and 14) of alum adjuvanted-crude extract of *P. americana*. On days 21 and 23, they were given crude CR extract in PBS intranasally (10 μl) and aerosolically (10 ml) *via* an air-pressure nebulizer, respectively. Mice received alum alone and PBS instead of the CR extract served as non-allergenic controls. All mice were bled twenty four hours after the nebulization and sacrificed. Their serum samples, broncho-alveolar lavage fluids (BALF), and lung tissues were collected. BALF of all allergen-treated mice had marked cellular infiltration notably neutrophils, eosinophils and lymphocytes. The average total cell count in BALF of the allergenic mice was 1.9 x 10^5cells/ml which out-numbered those of the non-allergenic controls (8 x 10^4 cells/ml). The eosinophil infiltration was pronounced in lungs of the allergen-treated mice. Specific serum IgE to the CR extract elevated in serum samples of all allergen treated mice and nil in the sera of the controls. None of the mice showed detectable level of IgG2a to the CR extract. RT-PCR revealed that all allergen-treated mice had marked increase of IL-13, IL-4 and TNF-α gene expressions, slight increase of IL-5 gene expression, and absence of detectable IFN-γ gene expression in comparison to the non-allergenic controls. None of the allergen-treated mice and 50% of the non-allergenic controls had IL-12 gene expression as detected by RT- PCR. One allergen treated-mouse (25%) had subpar level of the IL-18 gene expression compared to the controls. Results of the quantitative real-time PCR conformed to those of the RT-PCR. A murine model of *P. americana* resembling human allergic manifestations was successfully developed.

Cockroach (CR) is an important source of indoor allergen worldwide. In Thailand, the prevalence of CR allergy among the Thais with allergic rhinitis and/or bronchial asthma was 44-77% as determined by skin test using crude CR extract.[1-3] Several domiciliary CR species are found in the country but among them the American CR, *Periplaneta americana*, predominates and is the most important indoor allergens second only to house dust mites.[4-5] Allergic asthmatic attack caused by CR allergens among the CR allergic patients may be manifested all the year round with the more severe and prolonged clinical course than the disease caused by other type of allergens.[6] Current mainstay for treatment of the CR allergy is non-specific and based primarily on the

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clinical manifestations, *i.e.* symptomatic. Immunotherapy using small increasing intradermal dosages of crude CR extract has been limitedly practiced for minimum success in some clinical settings.[7] The crude CR extract used are varied in allergic components depending upon the inhabitants of the captured CR. The use of a more refined major CR allergen or a suitable cocktail of major and minor allergenic CR proteins would be a more appropriate immunotherapeutics. Presently, several *P. americana* allergens have been identified and characterized. These are: Per a 1,[8,9] Per a 3 or aryl phosphorin-hemocyanin,[10] Per a 6 or troponin-C [FT Chew, unpublished data], Per a 7 or tropomyosin,[11] Per a 9 (arginine kinase)[12] and Per a 10 or serine protease.[13] Among them, Per a 1 variant, *i.e*. Per a 1.0105, and Per a 9 are the major *P. americana* allergens that bound to 100% of IgE in sera of the CR allergic Thais.[9,12] These allergens in the purified native forms or their respective recombinant alternatives are not only having diagnostic potential, and for screening and monitoring the patients' allergic status, but also for evaluating as therapeutic vaccine candidates which should cause either immune tolerance, immune regulation and/or immune deviation from T_H2 (IgE) response to a non-pathogenic T_H1 response. An animal model of the American CR allergy that resembles the human disease warrants development as a biological tool for CR allergy basic and clinical research.

MATERIALS AND METHODS

Preparation of *P. americana* crude CR extract

Adult cockroaches were caught from houses in Bangkok. They were entomologically identified and only *P. americana* adults were used. The insects were frozen at -70°C before being ground to fine pieces in liquid nitrogen. Appropriate volume of 0.01 M phosphate buffered saline pH 7.4 (PBS) was added and the preparation was sonicated using a sonicator (Model VC750, Vibra Cell™ Sonics & Materials Inc., Newtown, CT) at 30% amplitude, 2 seconds pulse-on, 2.5 seconds pulse-off, for a total of 5 minutes. The preparation was then centrifuged at 12,000 x *g*, 4°C, for 20 minutes. The supernatant was collected, dialyzed against distilled water at 4°C overnight. Protein content of the crude CR extract was determined.[14]

Mice, allergen sensitization and sample collection

All animal experiments were performed following the ethical guideline of the National Research Council of Thailand. The work was approved by the Ethical Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand.

Female BALB/c mice, 6 weeks old, were purchased from the National Laboratory Animal Center, Mahidol University, Salaya Campus, Nakhon Pathom Province, Thailand. Individual mice were injected intraperitoneally with three doses of 150 μg crude *P. americana* extract adsorbed on aluminium hydroxide (alum) adjuvant (final volume of each inoculum was 200 μl) on days 0, 7 and 14. Each mouse was intranasally challenged with 10 μl of 1% crude CR extract in PBS on day 21 and aerosolized 1% crude CR extract in PBS (10 ml) using a locally made air-pressure nebulizer on day 23 (Fig. 1). Control mice received three injections of alum alone, intranasal- and aerosolized-PBS instead of the CR extract, using similar schedule and routes as for the tested mice.

Twenty four hours after the aerozolization, mice were bled from eye plexus and serum samples were individually collected. Each mouse was then humanly euthanized by cervical dislocation. Trachea was surgically exposed. A 25 gauge-needle connected to a tuberculin syringe containing 500 μl of sterile PBS was inserted into the trachea. Bronchial fluid was harvested by lavaging the mouse respiratory tract with the PBS contained in the syringe.

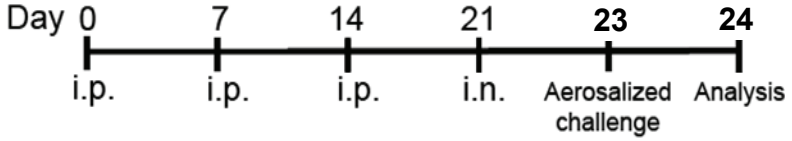

Fig. 1 Time line of sensitization and challenge of BALB/c mice with American cockroach extract. Control mice received intraperitoneal injections (i.p.) with alum, intranasal (i.n.) PBS, and aerosolized PBS instead of the CR extract.

Total cell count in each BALF was determined by using a hemocytometer.

Lung tissue of each sacrificed mouse was fixed in 10% buffered formalin overnight. The tissues were individually embedded in paraffin. Five μm sections were prepared using a microtome and the sections were stained with hematoxylin-eosin dye before light microscopy. Average number of eosinophils in lung tissue of each mouse from a total of 50 microscopic fields (100x magnifications) was determined.

Detection of antibodies to CR extract

Levels of IgE and IgG2a in sera of the allergen treated- and control mice were determined by an indirect ELISA. Crude CR extract (20 μg/ml carbonate-bicarbonate buffer pH 9.6) was added to each well (100 μl/well) in a microtiter plate (Costar, NY, USA). The plate was incubated at 37°C until dried. All antigen coated wells were washed with PBS containing 0.5% Tween-20 (PBST), added individually with 200 μl of a blocking solution (1% bovine serum albumin in PBS), and the plate was incubated at 37°C for one hour. After washing thoroughly with PBST, serial two fold dilutions of individual sera were added to appropriate wells, and the plate was incubated for three hours. Wells added with only the serum diluent served as blank. After all wells in the ELISA plate were washed as above, 100 μl of rat anti-mouse IgE-biotin conjugate or rat anti-mouse IgG2a-biotin conjugate (Southern Biotech Associates Inc, USA; diluted 1:1,000 in PBST) was added appropriately. The plate was incubated for two hours, washed with PBST, and each well was added with 100 μl of streptavidin-horseradish peroxidase conjugate (DakoCytomation) diluted 1:1,000 with PBST. After further incubating at 37°C for 30 minutes, the plate was washed as above and freshly prepared substrate solution (*o*-phenylenediamine; Zymed Laboratory) in citrate-phosphate buffer pH 5.0 containing 0.03% hydrogen peroxide was added to each well (100 μl/well). The plate was kept in the dark for 25°C for 30 minutes, then the enzymatic reaction was stopped by adding 50 μl of 4 N H_2SO_4 into each well. Optical density (OD) of the content in each well was determined at absorbance (*A*) 492 nm (A_{492nm}) using an ELISA reader (Multiscan *EX*, Labsystem, Helsinki, Finland) against the blank.

RNA isolation, RT-PCR and real-time PCR

Total RNA was prepared from lung tissue of each mouse: the tissue kept in liquid nitrogen was ground to fine powder by a tissue grinder and total RNA was isolated from the preparation by using a TRIZol reagent (Invitrogen, CA, USA). The extract was treated with RNase-free DNase 1 (Invitrogen) prior to converting to cDNA by using ReverseAid H minus (Fermentas). The cDNA was used in the reverse transcription (RT) and quantitative real-time polymerase chain reactions (PCRs) for quantification of cytokine gene expressions including *IL-13, IL-5, IL-4, IL-12, IFN-γ, IL-18* and *TNF-α.* The mouse β-actin housekeeping gene was included for normalization.

Nucleotide primers and probe sequences (Applied Biosystems) for the RT and real-time PCRs are shown in Table 1.[15-17]

RT-PCR mixture consisted of: 1 μl of cDNA, 1 μl containing 10 μM each the forward and reverse primers, 2.5 μl of 10x buffer, 3 μl of 25 mM $MgCl_2$, 2 μl of 2.5 mM dNTP, 0.2 μl of 5 units/ μl *Taq* DNA polymerase (Fermentas, Lithuania), and ultrapure distilled water (UDW). After 4 minutes of an initial denaturation at 94°C, 30 cycles of PCR reaction were performed. Each cycle was 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds, followed by final extension at 72°C for 7 minutes. The PCR product was visualized after 3% agarose gel electrophoresis and ethidium bromide staining using a UV transilluminator (UVP, CA, USA).

Real-time-PCR was performed using 1 μl cDNA and 300 and 150 nM of each PCR primers and PCR probe, respectively in a *Taq*Man Universal PCR Master Mix (Applied Biosystems). Reactions were run using Applied Biosystems 7500 Real-Time PCR System for 40 cycles. The data were analyzed using 7500 System SDS software. The level of expression of each target gene was normalized to β-actin gene.

RESULTS

Total numbers of cells in BALF of the allergen treated-mice (average $1.9x10^5$ cells/ml) were significantly higher than those of the PBS treated

Table 1 The oligonucleotide primers and probes for PCRs.[15-17]

Gene encoding	Primer	Probe	Size of PCR product (bp)
IL-13	F: 5'-agaccagactcccctgtgca-3'	5'-cgggttctgtgtagccctggattcc-3'	123
	R:5'-tgggtcctgtagatggcattg-3'		
IL-5	F: 5'-agcacagtggtgaaagagacctt-3'	5'-ctgttgacaagcaatgagacgatgagg-3'	116
	R: 5'-tccaatgcatagctggtgattt-3'		
IL-4	F: 5'-acaggagaagggacgccat-3'	5'-tcctcacagcaacgaagaacaccaca-3'	94
	R: 5'-gaagccctacagacgagctca-3'		
IL-12	F: 5'-ggaagcacggcagcagaata-3'	5'-catcatcaaaccagacccgcccaa-3'	179
	R: 5'-aacttgagggagaagtaggaatgg-3'		
IFN-γ	F: 5'-tcaagtggcatagatgtggaagaa-3'	5'-tcaccatccttttgccagttcctccag-3'	91
	R: 5'-tggctctgcaggattttcatg-3'		
IL-18	F: 5'-agacaactttggccgacttca-3'	5'-acagcctgtgttcgaggatatgactgatattga-3'	110
	R: 5'-ttcacagagggtcacagcc-3'		
TNF-α	F: 5'-catcttctcaaaattcgagtgacaa-3'	5'-cacgtcgtagcaaaccaccaagtgga-3'	174
	R: 5'-tgggagtagacaaggtacaaccc-3'		
β-actin	F: 5'-ggccaaccgtgaaaagatga-3'	5'-tttgagaccttcaacaccccagcca-3'	250
	R: 5'-cacgctcggtcaggatcttc-3'		

controls ($8x10^4$ cells/ml). Fig. 2 illustrates the histological appearances of the lung tissues of a representative mouse sensitized and challenged with the *P. americana* extract and a control mouse. While the lung tissue of the control mouse appeared normal with intact alveolar space and negligible infiltrated cells, narrowing of alveolar space and interstitial infiltration of inflammatory and mononuclear cells, *i.e.,* neutrophils, eosinophils, and lymphocytes, were markedly observed in the lung of the allergenic mouse. Numbers of infiltrated eosinophils in the peribronchial areas of allergenic mouse were significantly higher than the numbers in the control (Fig. 3).

Fig. 4 shows scattered plots of specific IgE levels in sera of the allergen treated mice and the controls. The serum IgE that bound to components of the crude *P. americana* extract was significantly elevated in sera of the CR extract treated-mice compared to the undetectable level in the sera of the negative control mice. None of the mice of both groups had serum specific IgG2a to the crude CR extract (data not shown).

Semi-quantitative RT-PCR results (Fig. 5) for detecting cytokine gene expression in lungs of the experimental mice normalized by using β-actin gene revealed markedly increased expressions of the genes encoding IL-13, IL-4 and TNF-α and marked reduction of expression of gene encoding IFN-γ in allergen treated mice as compared to the controls. None of the mice of the allergen treated group had detectable IL-12 gene expression while the expression was detected in lungs of two out of four (50%) control mice (C3 and C4) that were tested. One mouse each of the allergen treated group showed slightly increased IL-5 (T4) and marked reduction of IL-18 gene expression (T1) as compared to the controls.

Fig. 6 shows arbitary values of mRNA expression of genes encoding IL-13, IL-5, IL-4, IL-12, IL-18, IFN-γ and TNF-α relative to β-actin gene. The overall results of the quantitative real-time PCR are conformed to those detected by the RT-PCR.

DISCUSSION

An appropriate animal model of allergy with comparative immunologic responses and immunopathologic features to those of human manifestations is needed for studying basic mechanisms of the aberrant immunity and immunopathology in allergy as well as therapeutic efficacy of drugs and vaccine. Several animal models of allergies have been

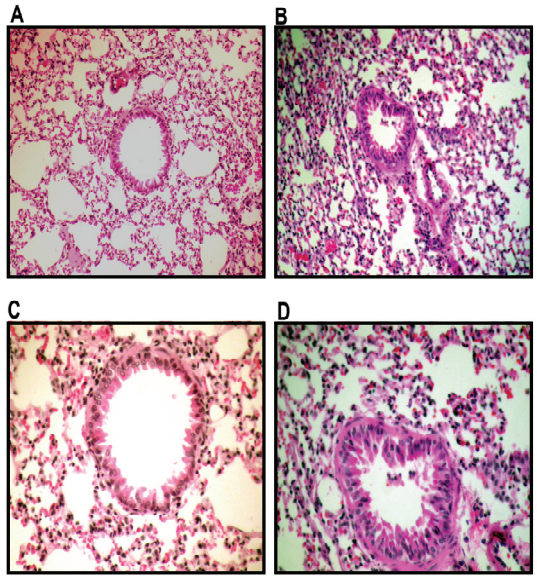

Fig. 2 Lung histology of non-allergenic control and allergenic mice stained with hematoxylin-Eosin dye. A and C, lung tissue of control mouse shows a normal appearance of alveoli and bronchiole and no inflammatory cell infiltration at low (20x) and high (40x) magnifications, respectively. B and D, lung tissue of allergenic mouse shows narrowing bronchiole with markedly cellular infiltration into alveoli and interstitial areas at low (20x) and high (40x) magnifications, respectively.

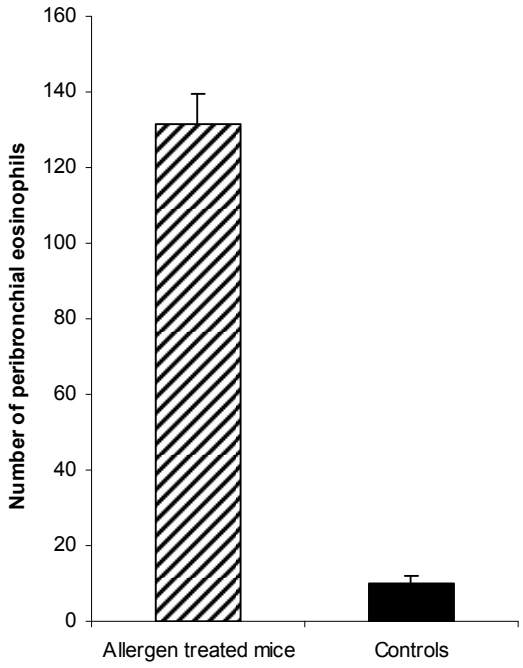

Fig. 3 Average numbers and standard deviations of eosinophils (cells/ml) in peribronchial areas of allergenic (▨) and control (■) mice.

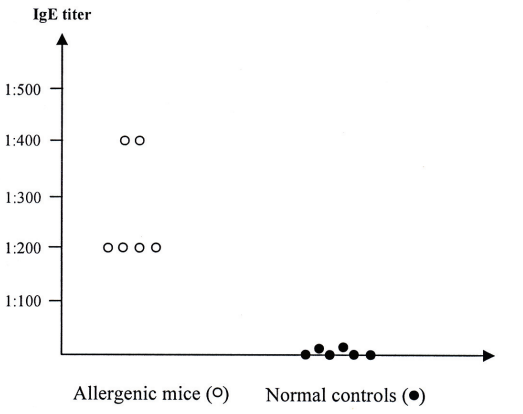

Fig. 4 Scattered plots of serum IgE levels of the CR allergen treated mice compared to the control mice.

developed, *e.g.*, atopic dog models for food allergy[18,19] and allergic asthma,[20] mouse models for lung allergy caused by fungi[21] and house dust mite,[22] mouse model of milk allergy[23] and latex glove allergy.[24] For CR allergy, Campbell *et al*.[25]developed a murine model of CR allergen-induced airway disease using C57BL/6 mice. Fourteen days after the initial sensitization of the mice with CR allergen in the incomplete Freund's adjuvant, the mice were intranasally challenged with the allergen. However, they found little cellular infiltration into the lungs of the allergen treated-mice upon histological examination. Thus, the mice were intratracheally re-challenged 6 days later with the allergen in order to induce significant inflammatory responses. After the second challenge, the allergen treated mice revealed histological, cellular and chemokine responses resembled those of human manifestations. Unfortunately, the information on the species of CR from which the allergen was prepared for their experiments was not given. In this study, therefore, we developed a mouse model of the American CR, *P. americana,* allergy using BALB/c mice which were more readily and economically available in Thailand and easier to handle than the C57BL/6 mice. After the initial sensitization by intraperitoneal injections of the mice

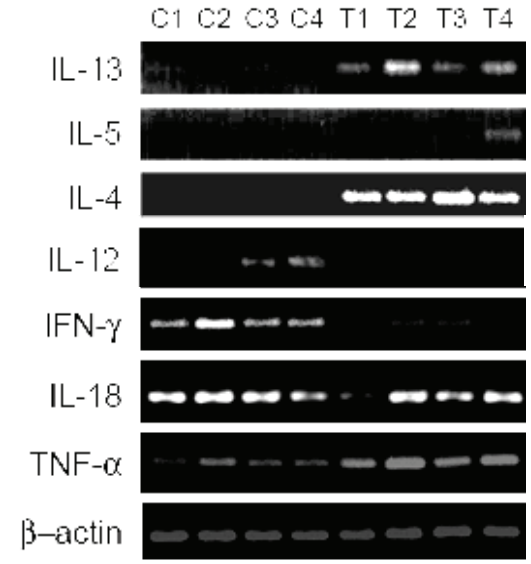

Fig. 5 Results of a semiquantitative RT-PCR for the determination of cytokine gene expression in four CR allergen treated mice (T1-T4) and four control mice (C1-C4).

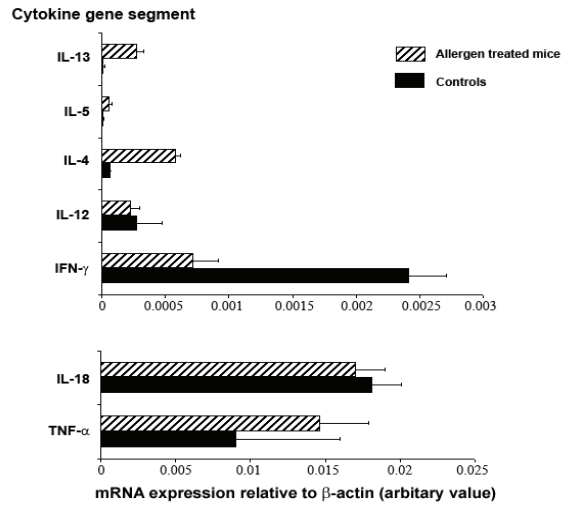

Fig. 6 Results of the real-time PCRs for the determination of cytokine gene expression in allergen treated mice and controls.

with alum-adjuvanted CR crude extract, an intranasal exposure and a nebulization of the sensitized mice with aerosolized CR allergen were performed such that the route of the allergen reexposure simulates the natural route. Alum was used because not only it has been used as human adjuvant with acceptable inocuity but it is also an adjuvant for T_H2 response.[26] All mice sensitized with the CR allergens developed not only the allergic lung inflammation with neutrophil, eosinophil, and lymphocyte infiltration but also a strong T_H2 response in terms of serum specific IgE to the allergen. They showed markedly increased expression of genes encoded IL-4 and IL-13 which are predominant cytokines of the T_H2 response and are key mediators of allergic pathogenesis.[27] The expression of genes encoding T_H1 cytokines, especially the IFN-γ were reduced significantly indicating a bias of the response towards T_H2 type in the CR allergen treated mice. Increase of TNF-α was observed which conformed to the notion that this cytokine is produced in the immediate type of allergic response and contributes to the damage of respiratory epithelium by inducing epithelial apoptosis *via* CD95L expression. All of the features developed in the mice treated with the allergen were similar to the clinical manifestation of CR allergy in human.[28] The so-developed murine model of *P. americana* allergy should be a useful tool for CR allergy research, *e.g.*, for testing the efficacies of CR allergy vaccine, and new therapeutic drugs and measures.

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