T-CELL RESPONSES TO DER F 2 MITE ALLERGENS IN THAI ALLERGIC PATIENTS

By
Duangthep Thongdee

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree
MASTER OF SCIENCE
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การตอบสนองของเซลล์ T Iymphocyte ต่อสารก่อภูมิแพ้จากไรฝุ่นชนิด Der f 2 ในคนไทย

โดย

นายดวงเทพ ทองดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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DUANGTHEP THONGDEE: T-CELL RESPONSES TO DER F 2 MITE ALLERGENS IN THAI ALLERGIC PATIENTS. THESIS ADVISORS: JUNDEE RABABLERT, Ph.D., ASST. PROF. TITIYA CHITTIHUNSA, Ph.D., AND ASST. PROF. NAT MALAINUAL, Ph.D. 96 pp.

House dust mite (HDM) *Dermatophagoides farinae* causes of atopic diseases such as allergic rhinitis and asthma in tropical region. The effect of the Df extract and recombinant Der f 2.0116 on antibody and T-cell responses have not been compared. The aim of the present study was to evaluate IgE binding, transcription and translation for IL-5, IFN-γ and TGF-β induced by mite allergens. Sera from 24 HDM-allergic patients and 20 non-allergic subjects were measured for IgE reactivity by ELISA. Peripheral blood mononuclear cells (PBMCs) were cultured with mite allergens (Df, rDer f 2) and mitogen (PHA). The supernatant and cell pellet obtained were evaluated for cytokine production by ELISA and cytokine gene expression by RT-PCR, respectively. Four patients showed IgE reactivity to both allergens. Five patients showed IgE reactivity to Df. Both HDM allergens showed similar levels of IL-5 and IFN-γ transcriptions in allergic patients and non-allergic subjects. The rDer f 2 induced IL-5 protein from allergic patients higher than non-allergic subjects, while Df showed IL-5 protein from allergic patients similar to non-allergic subjects. Df induced IFN-γ protein from allergic patients higher than non-allergic subjects whereas rDer f 2 induced IFN-γ protein from allergic patients similar to non-allergic subjects. The major findings of this study are as follows. First, PBMCs from allergic patients with allergic rhinitis and non-allergic subjects had the Th2-type cytokine response to HDM allergens. Second, allergic patients did have an enhanced Th2-type cytokine response to HDM compared with non-allergic subjects. Our data demonstrated that both HDM allergens can be used for allergen immunotherapy of Thai-allergic patients.

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สารก่อภูมิแพ้ไรฝุ่น *Dermatophagoides farinae* เป็นสาเหตุของโรคภูมิแพ้ ตัวอย่างเช่น โรคจมูกอักเสบ และหอบหืด ในเขตร้อน ผลของสารสกัด Df และ Der f 2.0116 ที่มีต่อการตอบสนองของ แอนติบอดี และ ทีเซลล์ ยังไม่เคยได้รับการเปรียบเทียบ วัตถุประสงค์ในการศึกษาครั้งนี้ต้องการประเมินประสิทธิภาพของ การจับกับ IgE (IgE binding activity) การถอดรหัส (transcription) และการแปลรหัส (translation) สำหรับ IL-5, IFN-γ และ TGF-β ซึ่งถูกเหนี่ยวนำโดยสารก่อภูมิแพ้ไรฝุ่น ปริมาณ IgE จากซีรัมของผู้ป่วยภูมิแพ้ไรฝุ่น จำนวน 24 ราย และผู้ไม่มีประวัติภูมิแพ้ จำนวน 20 ราย ถูกวัดด้วยวิธี ELISA เม็ดเลือดขาว (PBMCs) จะถูกกระตุ้นด้วยสารก่อภูมิแพ้ไรฝุ่น (Df และ rDer f 2) และไมโตเจน (PHA) ปริมาณไซโตไคน์ในส่วนใสจะถูกวัดด้วยวิธี ELISA ปริมาณไซโตไคน์ในเม็ดเลือดขาวจะถูกวัดด้วยวิธี RT-PCR ผู้ป่วย 4 รายมีค่า IgE ต่อสารก่อภูมิแพ้ทั้งสองชนิด ผู้ป่วย 5 รายมีค่า IgE ต่อสารก่อภูมิแพ้ Df การแสดงออกของยีน IL-5 และ IFN-γ ในผู้ป่วยภูมิแพ้และผู้ไม่มีประวัติภูมิแพ้หลังจากกระตุ้นด้วยสารก่อภูมิแพ้ทั้งสองชนิดพบว่ามีปริมาณเท่ากัน สารก่อภูมิแพ้ rDer f 2 เหนี่ยวนำการสร้างโปรตีน IL-5 จากผู้ป่วยภูมิแพ้ มากกว่า ผู้ไม่มีประวัติภูมิแพ้ ขณะที่ สารก่อภูมิแพ้ Df สร้างโปรตีน IL-5 ในผู้ป่วยภูมิแพ้ เท่ากับผู้ไม่มีประวัติภูมิแพ้ สารก่อภูมิแพ้ Df สร้างโปรตีน IFN-γ จากผู้ป่วยภูมิแพ้เท่ากับผู้ไม่มีประวัติภูมิแพ้ สัดส่วนของการสร้าง IFN-γ ต่อ IL-5 หลังจากการกระตุ้นด้วย rDef f 2 ในผู้ไม่มีประวัติภูมิแพ้ สูงกว่าผู้ป่วยภูมิแพ้ การศึกษานี้พบว่า PBMCs จากผู้ป่วยภูมิแพ้ซึ่งมีอาการจมูกอักเสบ และผู้ไม่มีประวัติภูมิแพ้ มีการตอบสนองของ ไซโตไคน์ ชนิด Th2 ต่อสารก่อภูมิแพ้ไรฝุ่น โดยที่ ผู้ป่วยภูมิแพ้มีการส่งเสริม ไซโตไคน์ชนิด Th2 ต่อสารก่อภูมิแพ้ไรฝุ่นสูงกว่า ผู้ไม่มีประวัติภูมิแพ้ ข้อมูลเหล่านี้แสดงให้เห็นว่า สารก่อภูมิแพ้ไรฝุ่นทั้งสองชนิดเหมาะสมสำหรับนำมาใช้ในการรักษาผู้ป่วยภูมิแพ้ไรฝุ่นคนไทยได้

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Duangthep Thongdee

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Dermatophagoides farinae is a species of house dust mites (HDM) which belong to the family Pyroglyphidae. The mite has been ubiquitously found in common houses in tropical and subtropical areas (134). *D. farinae* has been recognized as important source of allergens associated with allergic diseases such as asthma, rhinitis, and atopic dermatitis (105). Group 2 allergen (Der f 2 from *D. farinae*) is products of single gene, but it shows frequent allelic variation affecting several amino acids (106). Recombinant Der f 2 (rDer f 2) is a convenient molecule for investigating polymorphic proteins. It can be readily produced as a highly allergenic recombinant protein which has been used for X-ray crystallography and other conformational analyses (43). Seven amino acid substitution differences from native Df are S57N, L58I, D59N, I63V, F75Y, V76I, and I88A. Different variants of Der f 2 have different immunoglobulin E binding activities (106). The amino acid substitutions can render T-cell epitopes active or inactive. The cytokine pattern of T-cell responses induced by different variants of rDer f 2 was also found to differ even with a single amino acid substitution (44).

Allergen specific T-cell producing Th1 and Th2 cytokines can be detected in blood of atopic adults (138, 114). The cause of this allergen-specific Th2 predominance in atopic could be related to a dysfunction of the regulatory cytokine that modulate Th1 and Th2 responses. Experimental and theoretical data support the idea that activation of Th2 cells leads to production of IL-4, IL-5 and IL-13 that are cytokines involved in the synthesis of specific IgE and eosinophylic inflammation (110). On the other hand, Th1 cells, that produce IFN-γ, inhibit the biological effects of Th2 cytokine and are involved on delay type hypersensitivity (111). In spite of this observation and of their mutual inhibitory properties, the Th1/Th2 differentiation does not explain some features such as increasing IFN-γ production by PBMCs stimulated by Der f antigens in atopic children (61) and the presence of activated Th1 cells in

asthmatic patients with bronchial inflammation and hyper-responsiveness () and in some experimental models of asthma (130). TGF-β inhibits T-cell differentiation and proliferation of both Th1 and Th2 cells and attracts macrophages, dendritic cells and other inflammatory cells to sites of antigen exposure. TGF-β inhibits the function of these cells once they are activated (76). The increased levels of regulatory cytokines TGF-β in atopic patients suggest attempts to control the inflammatory response (91).

The major diagnosis methods of allergic diseases include skin pick tests (SPT) and serum specific IgE detection. Since the SPT possesses simple, quick, inexpensive and highly sensitive characteristics, it has long been one of most essential methods for diagnosis of type I allergic diseases in spite of the rapid development of science and technology over the past century (37, 141). SPT allows the detection if IgE-mediated sensitivity with simple device and low risk of causing an allergic reaction (37, 141). Enzyme linked immunosorbent assays (ELISA) is accurate and precise measurement of IgE and cytokine production. Reverse transcriptase-polymerase chain reaction (RT-PCR) allows detection of cytokine gene expression due to it has good reproducibility and specificity (85).

The aims of the present study were to evaluate humoral and cellular immune responses to *D. farinae* in allergic patients and non-allergic subjects by determining the levels of specific IgE to Df and rDer f 2, the cytokine (IL-5, IFN-γ and TGF-β) gene expression and production by PBMCs stimulated with both allergens.

CHAPTER II

LITERATURE REVIEW

1. House dust mites

House dust mites make up a large part of house dust allergens and belong to the Pyroglyphidae family; subclass Acari, class of Arachnid, phylum of Arthropods. The most important species are *Dermatophagoides farinae*, *D. pteronyssinus* (122), *Euroglyphus maynei* (8), *Lepidoglyphus destructor* (27) and *Blomia tropicalis* particularly, but not only, in tropical and subtropical regions (7, 18). Most mite allergens are associated with enzymatic activities (133). Which were shown to have direct nonspecific action on the respiratory epithelium (47), some of which may potentiate a Th2 cell response (107).

The body of a house dust mite is just visible against a dark background in normal light. A typical house dust mite measures 420 micrometers in length and 250–320 micrometres (0.0098–0.013 in) in width. Both male and female adult house dust mites are creamy blue and have a rectangular shape. The body of the house dust mite also contains a striated cuticle. Like all acari, house dust mites have eight legs (except 3 pairs in the first instar). The average life cycle for a male house dust mite is 10 to 19 days. A mated female house dust mite can live for 70 days, laying 60 to 100 eggs in the last 5 weeks of her life. In a 10 week life span, a house dust mite will produce approximately 2000 fecal particles and an even larger number of partially digested enzyme-covered dust particles. The house dust mite survives in all climates, even at high altitude. House dust mites thrive in the indoor environment provided by homes, specifically in bedrooms and kitchens. Dust mites survive well in mattresses, carpets, furniture and bedding, with figures around 188 animals/g dusts. Even in dry climates, house dust mites survive and reproduce easily in bedding (especially in pillows), deriving moisture from the humidity generated by human breathing, perspiration, and saliva. House dust mites consume minute particles of organic matter. House dust mites have a simple gut; they have no stomach but rather *Diverticulae*, which are sacs

or pouches that divert out of hollow organs. Like many decomposer animals, they select food that has been pre-decomposed by fungi (1).

Dermatophagoides and *Euroglyphus* feed on human skin danders which are particularly abundant in mattresses, bed bases, pillows, carpets, upholstered furniture or fluffy toys (5). Their growth is maximal in hot (above 20°C) and humid conditions (80% relative humidity). When humidity is inferior to 50%, mites dry out and die (79). This is why they are practically nonexistent above 1,800 m in European mountains (23) where the air is dry, whereas they are abundant in tropical mountain areas (145,). Even though mites are present in the home all year round, there are usually peak seasons (86,). Many patients have symptoms all year round but with a recrudescence during humid periods (20).

House dust mite allergen is contained in fecal pellets. Airborne exposure occurs with the active disturbance of contaminated fabrics and settles rapidly after disturbance. Mite allergen in dust is associated with the prevalence of sensitization and control of the disease (88). The presence of 100 mites per gram of house dust (or 2 µg of Der p 1 per gram of dust) is sufficient to sensitize an infant. For around 500 mites or 10 µg of Der p 1 per gram of house dust, the sensitized patient shows a greater risk of developing asthma at a later date (70). The higher the numbers of mites in dust the earlier the first episode of wheezing (126). The prevalence of sensitization to mites in the general population is more important in humid than in dry regions.

D. pteronyssinus and *D. farinae* extracts for cross-species diagnosis and immunotherapy allergen extracts cannot be used to attribute sensitisation to different *Dermatophagoides* sp. The allergens are cross-reactive and their allergen content varies (14, 74). The Australian experience is instructive. Different studies have reported more skin test reactivity to *D. farinae* than *D. pteronyssinus* (13), the same reactivity (35), or less reactivity to *D. farinae* (140).

Cross reactivity of D. pteronyssinus and *D. farinae* allergens about 50-60% of the IgE binding activity to *D. pteronyssinus* extracts can be accounted for by the binding of the Der p 1 and Der p 2 (45), collectively most of the remaining binding is to the group 4, 5, 7 and 21 allergens (151, 152) similar conclusions have been drawn from skin prick testing (148). With few exceptions there is a good correlation between

the IgE binding to Der p 1, Der p 2 (97) and HDM extract (142). The inter-species cross reactivity of these allergens is therefore paramount. A good correlation of IgE binding to Der p 1 and Der f 1 was found in a Japanese population exposed to both species (156). The titres to Der p 1 were however over twice those to Der f 1 in a third of the subjects and the ability to absorb IgE binding to Der p 1 with Der f 1 varied from 15% to almost 100%. A good correlation was also found in Virginia, USA where the patients would be exposed to both species, biased to *D. farinae* (49). There were however 10 fold differences for some individuals. Using a small sample of sera from England and the USA it was found that *D. pteronyssinus* could absorb out over 80% of the anti-Der f 1 reactivity of subjects mainly exposed to *D. farinae* and all of the reactivity of subjects exposed to *D. pteronyssinus*. Absorption studies with a saturating dose of allergen found that about 50% of the IgE binding, of atopic dogs from Tokyo, was species specific (87). A study of sera from Belgium, where *D. pteronyssinus* predominates, used competitive inhibition. For most sera over 100 fold more Der f 1 was required for 50% inhibition (77) with the inhibitory concentrations of Der f 1 approaching those found for denatured Der p 1. Thus using an assay that might reflect affinity a large effect was found. The study in Japan showed that cross reactivity of the group 2 allergens was higher than that found for the group 1 allergens (156), with crossabsorption removing all IgE binding. A close correlation of IgE binding to Der f 2 and Der p 2 was also found in Virginia (48). Absorption was not performed but it is unlikely that the IgE binding would be with antibodies to an unrelated antigen. Jin *et al.* showed that the IgE from a pool of sera from Seoul, Korea bound recombinant Der p 2 better than a recombinant Der f 2 (53) indicating high cross reactivity since subjects in Korea are exposed primarily to Der f 2. A comparison of IgE reactivity of sera from England with natural Der p 2 and recombinant Eur m 2 from the pyroglyphid mite *Euroglyphus maynei* found less correlation than that between recombinant Der p 2 variants and natural Der p 2 (124). The sera frequently had a two-fold difference in IgE binding. Since Eur m 2 has the same sequence disparity from Der p 2 as Der f 2, 20%, this shows a larger difference than the earlier studies. Similarly the sera from atopic dogs in Tokyo showed a higher inter-species disparity of IgE binding to the group 2 allergens than the group 1

allergens (87). The cross reactivity of Der f 7 and Der p 7 can be inferred from Shen *et al.* who showed that the IgE binding to Der f 7 were about 30-40% of the titres to Der p7 (119). This concurs with the low *D. farinae* levels found in the study area of Taipei. The cross reactivity of the group 4, 5 and 21 pyroglyphid allergens has not been published. The probability of children developing asthma has been shown to double from 0.25 to 0.5 for a 100-fold change in the titer of IgE (123). Accordingly it is unlikely that species-specific differences allergens would affect any assessment of the prognosis of patients. Disease is associated with much smaller amounts of IgE than those typically produced by HDM allergic patients so if residual allergic sensitivity is left by immunotherapy with the wrong species, or if the incorrect specificity of blocking antibody is induced, then treatment could be compromised.

T-cell cross reactivity Inter-species T-cell cross reactivity of the group 1 and group 7 allergens has been measured in Western Australians who are exposed to *D. pteronyssinus*. *In vitro* responses showed equal proliferative to *D. pteronyssinus* and *D. farinae* allergens and the same Th1 and Th2 cytokine release (43). The allergen preparations had not been depleted of endotoxin so it is possible a difference could be found with ultra purified allergen. Th2 cytokine release was however not induced in cells of subjects sensitised to non-HDM allergens so allergen specificity was required. When synthetic peptides were used to induce *in vitro* proliferation, cross reactive responses were found Der p 1 and Der f 1 peptides and indeed some of the stimulatory epitopes had the same sequence (42). Fewer peptides of Der f 1 were however stimulatory showing that the species may be important especially for peptide immunotherapy. The early studies of Th2 responses in HDM allergy were conducted with Der p1 in Rome using patients that would have mostly been sensitised to *D. farinae* (103).

Allergen variants the group 1 and 2 allergens are single gene products with frequent allelic variation. Variants in different regions of allergen could exist and be required for optimal diagnosis and immunotherapy and it is conceivable that variation contributes to allergenicity, either by increasing the diversity of epitopes, or the inherent allergenicity of particular variants. If binding to toll-like receptors (TLR) is important for the allergenicity of Der p 2, and there is evidence for this (137, 143),

then variants that bind best would be more allergenic. The variants were initially found by cDNA cloning but much of the information has been derived from PCR. The calculated and observed rates of sequence error for high fidelity PCR of 300 base pairs would lead to a nucleotide error in less than 1% of the sequences (12). For a set of 50 sequences the probability an incorrect sequence would be 0.26 and for two would be 0.08. The probability of obtaining frequent errors is according very small and finding changes in the same amino acid is most improbable. The veracity of the nature of single changes however must be established. The sequences of Der p 1 cDNA clones from a commercial culture revealed frequent amino acid substitutions (24) that were then shown to present in HDM in homes from Australia (125) and Thailand (106). In all 23 variants from 44 sequences were found with substitutions found in 18 of the 221 amino acids (26). Similar diversity has been found in China (156). The histidine 50 in the first cDNA sequence, Der p 1.0101, was only found in commercial HDM with other sequences having tyrosine. The only frequent exchange was an alanine to valine at position 124 with multiple but infrequent substitutions at positions 19, 81 and 215. Since most substitutions are sporadic the core sequence defined by Der p 1.0102 and Der p 1.0105 which only differ at position 124 would the most abundant. The exchange at 124 is in a region that contains T-cell epitopes recognised by most HDM-allergic subjects (42) so it could be important. Unlike Der p 1, Der f 1 has little polymorphism as noted in Thailand (106) and China (160). The nucleotide sequences were however diverse and the Der f 2 sequences from the same mites were polymorphic. The lack of polymorphism of Der f 1 suggests that polymorphism is not important for allergenicity. The substitutions in Der p 2 and Der f 2 showed an evolutionary pattern of polymorphism (25, 94, 106, 124, 125). The variable amino acids in each species were however not in the same structural regions. The important polymorphisms of Der p 2 were in amino acids 40, 47, 111 and 114 that cluster together in and near a loop region (106, 125). Der p 2.0101 has valine, threonine, methionine and aspartate in these positions while Der p 2.0104 has leucine, serine, leucine and asparagine. Other variants have different combinations of these amino acids. The dominant allergen in Western Australia was Der p 2.0101 found in 50% of cDNA clones with most other clones showing incomplete substitution towards

the Der p 2.0104 types. About half the clones of Der p 2 from Korea were Der p 2.0104 like198 and the HDM in Bangkok showed an even stronger bias to Der p 2.0104. The canonical Der p 2.0101 was not even found. Single sequences published from Germany and Denmark (25) show Der p 0104 like sequences. A sequence reported from China is very interesting because it shows substitutions in positions 40 and 47 to charged aspartate and arginine amino acids and not the typical leucine and serine. Recombinant Der p 2.0101 binds less IgE than recombinant Der p 2.0104-like variants (44, 102) and correlates less with the IgE binding to natural Der p 2 (124) even with sera from an environment where Der p 2.0101 is common (44). The substitution in residue 114 is well known to determine the binding of monoclonal antibodies ID8 and 4G7 which require asparagines at that position (40). It is unlikely that the 50% difference in the IgE binding between Der p 2.0101 and Der p 2.0104-like sequences would affect IgE mediated allergy because the titres are still large and this has been demonstrated by basophil degranulation. Combinations of human IgE monoclonal antibodies however had a 100-fold difference in their ability to produce degranulation with different variants (25). It is therefore possible that the variants contribute to the allergenicity by increasing the diversity of the antibody responses. They could be critical if recombinant allergens were used for immunotherapy. If blocking antibodies were important, then those produced against a recombinant Der p 2.0101 might not be sufficient to block effectors responses to Der p 2.0104. To date the variants have not been isolated as natural proteins and, if they are important, this would be a research priority since structural differences between recombinant and natural Der p 2 have been demonstrated (132). Of immediate significance is that the monoclonal antibodies that are used to measure environmental Der p 2 do not bind to all variants. Der f 2 from *D. farinae* in Asia (53, 81, 106) and Europe200 fall into 2 groups. Der f 2.0101-like sequences show conservative uncharged substitutions in residues 88, 11 and 125. Another group that can be described as Der f 2.0107-like has characteristic changes in amino acids 57, 58 and 59 including substitution of the surface accessible aspirate 59. In the one letter code the Der f 2.0107-like variants change SLD to NIN in amino acids 57-59. About 30% of the sequences described from Germany were of the Der f 2.0101 type and 70% the 0107 type while the

proportion was reversed in Bangkok. Like Der p 2 the variants vary in different locations. Four sequences from Japan (94) and those from Korea (53) were of the Der f 20101-type and sequences from China showed both groups (81).

Since allergy and allergic disease has been associated with economic development (72, 128) it is anticipated that there will be an increased need to combat HDM allergy in new geographical regions. The need to diagnose allergy in new circumstances will require allergen preparation appropriate for the HDM in the regions and to take into account cross relativities that are not encountered in the regions where the allergen extracts are used today. It has already been shown in tropical Australia (44) that diagnosis with a standard extract was not suitable for a new environment. Also, like infectious disease, there would be an enormous benefit in preventing or effectively treating allergic disease with new types of vaccination and immunotherapy. There are many new innovations that could profoundly improve immunologically based treatments and as summarized elsewhere they are being developed (136). Many however will depend on new technologies with molecularly defined medicaments. Their optimal formulation with respect to the allergens is becoming apparent (137) and as described here they can be further tailored for the allergens in environment where they will be used. In the absence of further information this should include the variants of the major allergens since, as is cussed, it is plausible that variant specific responses could be an impediment to effective immunotherapy. These considerations do not just apply to new environments since as documented here it is not uncommon for investigations, therapy and diagnosis to be conducted without correct regard to or knowledge of the HDM allergens in the patient's environment.

2. Group of allergen

As shown in Table 1, HDM allergens were grouped based on their biological function. According to the International Union of Immunological Societies (IUIS) Allergen Nomenclature Subcommittee, the designation for a characterized allergen was the first 3 letters of genus, the first letter of species name and a number the other

already characterized allergen it matched in homology and molecular weight, there were 23 known mite allergens (57).

Group 1 (Major cysteine protease allergen) as first characterized for Der p 1 these allergens are cysteine proteases in the same family as papain and cathepsin L. The possible that they have serine protease activity has been refuted and this is important with respect to 1 can however bind to benzamidine columns but this binding does not necessarily have to be via the active site. Although organisms typically produce a variety of cathepsin L molecules the expressed sequence tag analysis showed that *D. pteronyssinus* produces one abundant enzyme from this family, Der p 1. A similar conclusion has been shown by the proteomic analysis of Bartard where resis and the only cysteine protease listed. The sequence homology Der p 1 with the well characterized papain and related enzyme provided immediate insights into the nature of its tertiary structure and information for modeling, the main difference from papain being the presence of two extracysteies now shown to make the disulphide bond (Cys 4 and Cys 117).

The production of enzymatically active recombinant allergens has now been achieved and the structure of recombinant polypeptide of proDer p 1 and the mature recombinant Der p 1 has been solved by X-ray crystallography (43). For the most part Der p 1 resembles papain. Amagnesium-binding site not found on other C1 type cysteine protease was found but given that Der p 1 retains its enzymatic activity in the presence of EDTA it is not required for proteolysis. More importantly the structure showed that the non-glycosylated Der p 1 existed as a dimmer in physiological condition. The Der p 1 polypeptide has a calculated molecule mass of 25kDa and its migration by SDS-PAGE is consistent with this. Its size in more physiological conditions has however not been previously determined and its migration is retarded during standaed gel filtration chromatography. Silica gel columns however showed that Der p 1 behaved as a 40 kDa dimmer in pH 8 and a monomer when arginine was added to prevent dimerisation. It stuck to the column below pH 7.5 and it was speculated that this might be due to the exposure of the dimerisation interface.

The group 1 allergens form *D. pteronyssinus, D. farinae* and *E. maynei* show 85% sequence identity. The region 80-130 that is part of flexible loop connecting the

left and righi domains. Most of these are surface exposed with the ability to contribute to the allergenic differences between the species. The recombinant polypeptides has directly demonstrated that the importance of the central regions for the antigenic difference of Der p 1 and Der f 1. Peptides found throughout the structure of the molecule, however bind IgE and several noncross-inhibitory monoclonal antibodies block IgE binding. A striking difference between Der p 1 and Der f 1 has been the paucity of polymorphism for Der f 1. Of 19 sequences described from Thailand, 15 were of the Der f 1.0101 type reported for mites from CSL-Ltd concentration in the environment is not known. Recent quantitative studies have confirmed the early results showing that the titre of IgE activity is low. They are thus not important allergens and this cast further doubt onto any adjuvant role for proteolytic activity.

Group 2 the allergenicity of group 2 mite allergens remained unexplained until recently. However, an elegant study clearly evidenced that Der p 2, by structural homology, acts as a functional homolog of MD-2 to drive airway inflammation in a TLR4-dependent manner. Airway sensitization and challenge with recombinant Der p2 (0.1 g), under conditions of very low levels of LPS exposure (0.026 pg), led to experimental allergic asthma in wildtype and MD-2-deficient, but not TLR4-deficient mice. Der p 2 consequently displays auto-adjuvant properties which are critical for the allergenicity of this mite allergen (142). It is noteworthy that the LPS binding activity of Der f 2 was also recently evidenced. Surprisingly, another study showed that recombinant Der p 2 stimulates airway smooth muscle cells in a TLR4- independent manner but triggered the MyD88 signaling pathway through TLR2. Lately, natural Der p 2 was shown to elicit the production of TNF through direct binding with DC-SIGN on DCs, whereas the unglycosylated recombinant form was ineffective in receptor activation. Recombinant Der p 2 caused nuclear factor B-dependent upregulation of pro-inflammatory cytokines in bronchial (BEAS-2B and NHBE) but not alveolar (A549) airway epithelial cells; whereas recombinant Der f 2 can directly stimulate IL-13 production in BEAS-2B cells (125).

Table 1 Molecular cloning of *Dermatophagoides* spp. allergens.

Group	Allergen	Number of amino acid residues	Deduced MW (kDa)	Potential glycosyla- tion site(s)	Biochemical identity or homology
Group 1	Der p1	222	24	1	Cysteine protease; homology with papain, actinidin, cathepsins B and H; sequence polymorphism
	Der f1	223	27	1	
Group 2	Der p2	129	15	0	NPC2 family
	Der f 2	129	15	0	
Group 3	Der p 3	232	31	0	Trypsin; homology with Der p 6 and Der f 6, mammalian and invertebrate trypsins, and other serine proteases; sequence polymorphism
	Der f 3	232	29	0	
Group 4	Der p 4	496	60	1	Amylase; homology with insect and mammalian a-amylases
Group 5	Der p 5	113	14	0	Function unknown; cleavage site 98 unknown
Group 6	Der p 6	231	25	0	Chymotrypsin; homology with Der p 3 and Der f 3, mammalian and invertebrate chymotrypsins, and other serine proteases
	Der f 6	230	25	0	
Group 7	Der p 7	198	26, 30, 31	1	Function unknown (25,30,3 1 kDa)
	Der f 7	196	30-31	1	
Group 8	Der p 8	219	27	2	GST; homology with glutathione S-transferases
Group 9	Der p 9		29		Collagenolytic serine protease
Group 10	Der p 10	284	36	0	Tropomyosin; homology with invertebrate tropomyosins
	Der f 10	284	37	0	
Group 11	Der p 11		103		Paramyosin (98 kDa); homology with invertebrate paramyosins; glycoprotein
	Der f 11		98	2	
Group 13	Der f 13				Fatty acid binding protein
Group 14	Der p 14	1650	177	0	Apolipophorin-like protein; identity with a M-l?? allergen, &E-binding Magl, Dl3961 (P39673); peptide fragments Magl and Mag3
	Der f 14		177		
Group 15	Der f 15	555	98/109	1	Chitinase (98/109 kDa, cDNA: 63 kDa)
Group 16	Der f 16	480	53	0	Gelsolin/villin, homology with gelsolin family
Group 17	Der f 17	284	53	4	EF-hand calcium-binding allergen
Group 18	Der f 18		60		Chitinase
Group 20	Der p 20				Arginine kinase
Group 21	Der p 21				
Group 22	Der f 22				
Group 23	Der p 23		14		Unknown function, homology to peritrophin-A domain (PF01607)

Group 3, 6 and 9 (Serine Protease) with collagenolytic activity. Its IgE binding activity although reportedly frequent is less than the low IgE binding Der p 3. Recent sequence for Der p 9 (AAN02511, AAP57077) show a protein with the known N-terminal of the natural allergen and a 38% amino acid sequence identity to Der p 3 trypsin and 40% identity to Der p 6 chymotrypsin. It has the catalytic triad typical of serine proteases but with an alanine at the substrate-determining region of substrate binding pocket (6 residues proceding the active serine), instead of the aspartate or serine of trypsin and chymotrypsin repectively (161). Der p or f 3, 6 and 9 represent the three HDM serine proteases which display tryptic, chymotryptic and collagenolytic activities, respectively. Similarly to mite cysteine proteases, the serine proteolytic activity of mite proteases was shown to induce an increase in epithelial permeability through cleavage of the tight junction proteins occludin and ZO-1. Purified natural Der p 3 and Der p 9 induced the release of GM-CSF, eotaxin, IL-6 and IL-8 from cultured airway epithelial cells through PAR-2 activation.

Group 4 (Amylase) the 60 kDa Der p 4 is one of the most important allergens besides Der p 1 and 2. Its sequence is typical of alpha-amylases and its structure models well the family 13 glycosyl hydrolases. This is an 8-stranded alpha/beta barrel containing the active site, interrupted from residue 99-165 by a calcium-binding domain protruding between beta strand 3 and alpha helix 3 and a carboxylterminal beta-berrel domain from residues 402-508. There is one substitution in the chlorine-binding motif but the recombinant allergen hydrolyses starch with high activity in a chlorine dependent reaction. Overall the sequences of the allergens Der p 4 and Eur m 4 are 50% identical to insect and mammalian alpha-amylases and contain the cysteines, required to from the conserved disulphide bonds. An N-glycosylation site was found for Der p 4 but this was not present in Eur m 4. The sequences of Eur m 4 and Der p 4 were 90% identical, which is greater similarity than that found for the other allergens. With respect to allergenic cross reactivity, it should be noted that the high sequence conservation is predominantly due to amylase-specific sequences conserved throughout the animal kingdom, such that while insects and mollusks show 50% identity to the allergens, humans have the same conserved sequences.

Group 5 (Unknown) Der p 5 has similar degree of IgE binding to Der p 4 and thus an important mild-range allergen. A sequence determination of natural Der p 5 showed that the aspartate at residue 2 of sequence predicted from cDNA analysis was in fact the N-terminal. Yi et al. have however shown that Blo t 5 has a ragged N-terminal usually starting with residues from 2-4 of the predicted sequence indicating the possibility that this could also occur for the pyroglyphid allergens. The C-terminal region has a sequence strongly predicted to be coiled coil region and this agrees with recent data on the similar allergen Der p 21. Here short angle X-ray scattering analysis was used to show that the allergen was a dimmer of a double coiled coil. The Der p 5 and Der p 21 however only show about 30% sequence identity so this structure cannot be assumed for Der p 5 and indeed there is less coiled coil predicted for Der p 5 (122). Interestingly the predicted regions of coiled coil structure match better for Der p 21 and Blo t 5 than Der p 5 although simple sequence compassion show both Der p 5 and 21 show 40% identity to Blo t 5.

Group 7 (Unknown) the group 7 allergens are one of the three mild-potency allergens of probable importance in half the mite-allergic subjects. Little is known about their structure. One important aspect is the high and variable glycosylation. By SDS-PAGE the recombinant polypeptide has a molecular mass of 22 kDa but allergen is found in mite extracts as molecules of 26, 30 and 31 kDa, as well as a lower molecular weight form that is presumed to be a degradation produce. The abundance of each molecular species varies from mite extract to extract. N-glycosidase treatment can reduce the molecules to 26 kDa. There are no unambiguous homologues from other species but recent genomic analyses have revealed significant sequence similarity to prenylcysteine lyase domains found in hypothetical insect proteins. The sequence similarity extends over most of the sequence of insect sequences and their size is the same as the allergens, indicating they may be homologues. The classical prenylcysteine lyases are however different in that they are large multidomain proteins. It is nevertheless interesting given that lipid binding is a very common property of major allergens. The group 7 allergens are the 44th most abundant protein made by mites comparing well to the 31st and 41st ranking of Der p 1 and 2. They are however very minor proteins of the house dust mite extracts indicating they are labile.

Allergen formulations with adequate concentrations of this specificity need to be developed.

Group 8 (Glutathione-S-Transferase) these are allergens that frequently binds IgE but with very low titre. This applies to the recombinant Der p 8 which is a μ-class glutathione-S transferase and to glutathione-S-transferase isolated from mite extracts with glutathione conjugated Sepharose and Hales et al. unpublished. Ther are many isoforms of glutathione-S-transferase so the possibility exists that one could be more allergic than that revealed to due but the 2-dimensional gel immunoblotting analysis of Huang shows that, if one exists, it is not very abundant in mite extracts. Huang also showed evidence for low level, but frequent, cross reactivity with glutathione-S-transferase from the American cockroach.

Group 10 (Tropomyosin) the group 10 allergens are tropomyosins. They show high sequence identity to the slow twitch tropomyosins of arthropods including other important allergens from prawns and cockroaches. Anti Der p 10 antibodies strongly stain the skeletal muscle of the mites suggesting that these are the allergens but, given the conserved sequence identity of all the isoforms of tropomyosin, the actual sensitizing isoform cannot be known complete certainly. Der p 10 was the 23rd most abundant protein produced by the mite.

The tissues with tropomyosins are sequence identity and cross reactivity with other species and probably relatedly, the high variability of IgE binding found in different populations. The sequence identity is 96% to the tropomyosins of glycyphagid mites, 80% to crustaceans, 75% to insect tropomyosins and 50% to human tropomyosins. The IgE antibody crosses reactivity of mite tropomyosin with cockroach and shrimp is well known and is consistent with their highly conserved sequence. IgE binding to tropomyosins is however not that common so cross reactivities are correspondingly infrequent. IgE from sera from cockroach allergic subjects could bind tropomyosin from mites and cockroach equally well it only occurred at low frequency. For the pyroglyphid mite tropomyosins, the original description from Japan showed a very high frequency and in semiquantitative assays, a high degree of binding compared to the major Der f 2 allergen (87). Subsequent studies in Europe (124), Australia (13) and Singapore found a low frequency and

amount of reactivity to Der p 10 and to glycyphagid Blo t 10 tropomyosin. IgE binding assays in Africa however showed frequent responses suggesting the presence of a cross reacting sensitization. On balance it appears that for most populations, the most important aspect of tropomyosin reactivity in mite allergy is its ability to confuse diagnosis by cross reactivity.

Group 11 (Paramyosin) studies from Taiwan and Singapore have reported a high frequency of IgE binding by paramyosin from D. farinae an D. pteronyssinus as well as B. tropicalis. The original studies repored for Der f 11 showed a very high frequency of IgE binding (80% although no quantitative was performed and there was evidence for non-specific binding of IgE. Similar results were found for Der p 11. Better controlled studies of natural Blo t 11 also showed 67% IgE binding but the assays used were not quantitative, despite being labeled as such. These authors also showed that most of natural allergen was degraded and was present in mite extracts at less than 1 μg/ml. The reactivity of paramyosin with IgE from sera from other populations has not been examined but Western blotting studies have reported prominent IgE binding bands that could be due to this allergen. Quantitative tudies need to be performed and the difficulties found in purifying undegraded natural and recombinant allergens overcome. Paramyosins from a number of parasites are prominent antigens and this could conceivably affect regional responses by cross reactivity. Paramyosin is however not as evolutionary conserved as tropomyosin. There is 95% amino acid sequence identity with B. tropicalis and the scabies mites but only 60% with insect paramyosins and 50% with those of parasites.

Group 12 (Unknown) has not been described for the pyroglyphid mites. The reported Bol t12 has a sequence with some similarity to the chitin binding domains of larger chitinases and appears to be an authentic protein because the cDNA of the 124 residue protein had a leader sequence and untranslated flanking regions. It bound IgE in half the allergic sera with strong immunostaining in a phage plaque assays suggesting it could be important. Except for a recent Genbank entry for a homologue from Lepidoglyphus destructor (AAQ55550) there are no other reports of allergens or sequences from any organism resembling this molecule. The L. destructor and B.

tropicalis sequences had a sequence identity of 93%, which is unusual because other comparisons between these species show less than 50%.

Group 13 (Fatty Acid Binding Protein) the group 13 fatty acid binding proteins are the 15th most abundant protein in mite extracts, ranking behind a series of cuticle-associated proteins and ahead of all other denominated allergens. Despite its name and abundance their lack of allergenicity is perhaps the most prominent feature. Only occasional people produce IgE antibody to this protein in both the glycyphagid mites and for Der f 13 and Der p 13 (unpublished). Immunological investigation on this protein could best concentrate on the reason why an abundant protein is not allergenic. The tertiary structure of Der f 13 has been solved by NMR and shows the typical β-barrel-helix-loop helix structure of the fatty acid binding protein family. The amino acid sequence is more conserved (70-80% identity) with respect to the glycyphagid mites than most allergens, but are quite disparate from mammalian homologues (40%).

Group 14 (Large Lipid Transfer Protein) the group 14 allergens are members of the large lipid transfer protein (LLTP) family that includes the egg storage vitellogenins and the lipid transporting apolipophorins, as well as other molecules of the haematopoietic and insect defense system. The ca 250 amino acid sequences that forms the beta barrel structure of the N-terminal lipid transfer module is highly conserved and areas of sequence similarity can be identified throughout the first 800 amino acids of its 1760 residue sequence. It was originally described as an apolipophorin-like protein because of its similarity to published insect apolipophorins but these sequences are also similar to crustacean vitellogenins. The subtilisin-like converts cleavage site (RXR/KR) conserved at about residue 725 in the crustacean vitellogenins is however not present suggesting another function. Antibodies against the Der f 14 and Der p 14 stain the haemocoels and react by immunoblot with male and female mites (unpublished). The lack of a sex difference indicates a function other than a vitellogenin. In a recent review of the LLTP family Avvare emphasized the diversity and lack of knowledge of the function of this family and the fact that the vitellogenins cannot be distinguished from the apolipophorin by sequence analysis. IgE binding to LTP antigens with high sequence identity to the group 14 allergens

have also been described for scabies mite infection in humans and sheep scab. Complete cDNA sequence are available for the allergens from *D. pteronyysinus* and E. maynei while presumably due to the technical difficulties in making large full length cDNA, only C-terminal regions determination have been made for *D. farinae*, *B. tropicalis* and *S. scabiei*. The C-terminal region of *S. scabiei* and *B. tropicalis* show an identity of 60 and 44%, with Der p 14, which is high considering the disparity of the species and that the C-terminal region of LLTPs have more diverse sequence than the N-terminal. For *D. farinae* the protein was defined as M-177 by immunoblotting with antibodies prepared against the C-terminal recombinant peptide fragments. These fragments with laboratory designations of Mag-1 and Mag-3 respectively represent the extreme C-terminal 1322-1662 and an almost immediately proximal region 903-1251. They the products of incomplete cDNA transcripts with no known or suspected significance as natural entities.

The group 14 allergens are possibly important but have received little attention. The studies of Fujikawa showed high reactivity compared to Der p 2 in immunoblotting studies with the M-177 allergen. Unpublished studies of Hales et al. using a combinant peptides 1-260 and 1322-1662 showed a possible mid range activity with IgE binding one third of the allergic subjects at similar levels 5-10 ng/ml as Der p 4, 5 and 7. Studies to determine the allergenicity of the whole allergen appear warranted. Of particular importance is that the allergens have been shown to be highly susceptible to proteolytic degradation in mite extracts, a process that may not occur in dead mite bodies where compartmentalization from the digestive enzymes could be retained.

Group 15 (Chitinase) the group 15 chitinase allergens are of particular interest because although they have not been extensively studies in humans, they are the major allergens of house-dust-mite-allergic dogs, binding IgE in almost all dogs compared to less than 50% shown by the group 1 and 2 allergens. Their sequence have a near perfect signature for the active site and chitin binding domains of the family 18 chitinases and have the characteristic PEST region of O-glycosylation. This as shown by O'Neil et al. can exist in a long and a short form, both of which can be amplified from genomic DNA indicating two genes or alleles and not alternate

splicing. The group 15 cDNAs encode polypeptides of 61 kDa but the natural allergens have molecular masses of 98 and 106 kDa almost certainly due to glycosylation of the proline, serine rich PEST region. Consistent with this McCall et al., reported the detection of glycan by a blotting technique. A semiquantitative assay of the IgE binding to a recombinant Der p 15 polypeptide has shown that it binds very frequently at 70% suggesting it could be an important allergen but this needs to be examined by quantitative assays using the natural protein. Another interesting property of the group 15 allergens is they are found in the gut of mites, but not in the faecal pellets, showing they are distributed differently to the group 1 and 2 allergens.

Group 16 And 17 Allegens (Gelsolin And EF Hand Binding Proteins) the IgE binding to Der f 16 and 17 has been detected from sdreening cDNA libraries. The binding was found in about 50% of subjects and was low compared to other allergens.

Group 18 (Chitinase-Like) the group 18 chitinase-like allergens have sequences that resemble chitin binding domains but the second catalytic domain is truncated and does not contain the critical Glu 160, as for example found in the conserved FDGLDLDWEYP sequence of Der p 15. They also do not have a PEST region. A small number of similar sequences have been described including a conceptual translation from the *Drosophila* sp. Genomes (Genbank EAL26490, AAL90100) and family of mammalian lectins related to the YMI protein. While their functions are not known that are thought to be involved in inflammation and tissue remodeling. Natural Der f 18 was shown to bind IgE in 45% of humans typically with weak binding activity compared to the signal with *D. farinae* extract and this contrasted with the stronger reactivity reported by the same laboratory for Der f 15. The reactivity with dogs was still at a high frequency 77% but was also less than Der f 15. In humans showed that the frequency IgE binding to a recombinant polypeptide produced in E. coli was, at 63%, less than Der p 15 but the binding of positive sera appeared similar to the Der p 18 binding. Note that the group 15 and 18 only has 25% sequence identity and as expected from this they have no serological cross reactivity. Like Der f 18 the Der f 15 was found in the upper digestive system but not the faeces.

Group 19 (Anti Microbial Peptide) the group 19 designations were given to an as yet unpublished and poorly allergenic homologue of an anti microbial peptide from *B. tropicalis*.

Group 20 the group 20 is the mite arginine kinase, which was investigated because its sequence is highly conserved amongst invertebrates showing 80% identity to crustaceans and 75% to insects compared with 45% for mammalian enzymes. It was shown to bind IgE in 40% of sera from mite allergic people, but with low titres. Isoforms of arginine kinases are the 29th and 30th most abundant proteins in mite extracts.

Group 21 the group 21 allergen entered into the sequence (ABC73706) and allergen databases is a protein shown to bind IgE antibodies in 30% of sera from Austria. Further IgE binding studies including quantitative assessments need to be performed before is relative importance can be assessed. It is of structural interest because its sequence has similarity to the group 5 allergens. Interestingly however, it has only 26% sequence identity to Der p 5 compared to the 38% sequence identity of Der p 5 and Blo t 5 and the 44% identity of Der p 21 and Blo t 5. The study of these proteins will provide an interesting example of divergence. The predicted coiled coil sequences of Der p 21 found across the whole molecule more closely resemble the predictions for Blo t 5 suggesting that Der p 21 and not Der p 5, could be the real evolutionary homologue of Blo t 5 although further investigation is required. Like Der p 5 however no cross reactivity was found with glycyphagid allergens.

A major requirement for the mucosalimmune system is to make harmless responses to innocuous inhaled antigens. House dust mites (HDM) are traditionally studied for their allergenicity, but they are also a well-characterized source of antigens that can be investigated for insights into the immunoregulatory processes. *Dermatophagoides pteronyssinus* is the most widespread species while D. farinae predominates in regions with low relative humidity. Mixed populations occur, for example, in some regions of the US (6, 51) and Japan (115). The allergens of the different species typically have 80-85% sequence identity and show antibody cross-reactivity (77). In general, most important sources of allergens have one or two specificities that dominate the response. Birch pollen allergy is the best example with

80% of the IgE antibody in 90% of subjects binding Bet v1 with titers of about 50 ng/mL. Major allergens from other sources bind about 50% of the IgE antibody with titers of about 50 ng/mL except for those to the cat allergen Fel d 1, which are often low (32). Groups 1 and 2 are the major HDM allergens. Combined they typically bind about 50 ng/mL of IgE (45) constituting about 50% of IgE binding to all the HDM allergens regardless of whether the total titers are high or low (46).

3. IgE-mediated reactions

D. farinae and *D. pteronyssinus* were identified in house dust samples from all over the world since 1964 (153). An evidence of exposure to the HDM allergens *D. farinae* and *D. pteronyssinus* was an important risk factor for allergic sensitisation, asthma development and asthma symptom exacerbation (133). Studies of house dust-allergic individuals around the world had shown that house dust mites caused symptoms such as perennial-type asthma, rhinitis and conjunctivitis, often with nocturnal or early morning episodes (83). In Thailand, allergic conditions have been very common among children residing in Bangkok (66, 108).

The other constituents of native *D. pteronyssinus* allergens, but not Der p 1 or Der p 2, did not significantly influence on the IgE-mediated early asthmatic response but contributed significantly to the allergen-induced late asthmatic response and bronchial hyperactivity (146). *D. pteronyssinus* had also been reported to play an important role as a trigger in patients with atopic dermatitis, including adult patients (71). However, in a crosssectional study of 1669 school beginners 6 to 7 years old in Augsburg (Bavaria, Germany), it was concluded that current eczema in these children were related to Der f 1 exposure and not to Der p 1 exposure (67). Patients who had been induced by house dust mite allergen for more than 48 hours continuously and contributed to eczematous eruptions were characterised by considerably increased levels of IgE antibodies, high activity of atopic dermatitis (56). It was possible that a number of features described to *D. pteronyssinus* would be applicable to *D. farinae* but had not specifically been investigated for this mite. The reader was referred to the entry on *D. pteronyssinus*. Various studies reported that the rate of sensitisation was higher among atopic children and that high mite infestation increased the rate of

sensitisation. The European Community Respiratory Health Survey, an international study of asthma prevalence and risk factors for asthma, collected information on IgE antibodies to common allergens in over 13,000 adults living in 37 centres in 16 countries and found a median prevalence of 20.3% (range 6.7-35.1%) for sensitisation to *D. pteronyssinus* (16). In the Linkoping area Der m 1 was the major house dust mite allergen (58%). Of the children with IgE antibodies against house dust mite, 67% reacted to all 3 mites. Mite sensitisation rates were marginally increased (7%) by the addition of IgE analysis of *D. microceras* to the routine analysis of IgE antibodies against *D. pteronyssinus* and *D. farinae*. The authors concluded that Der m 1 may in this instance be an important house dust mite allergen and should be considered when House dust mite exposure data were assessed in areas with a climate like that of Sweden (148). In tropical Singapore, a prospective evaluation was made of 175 newly diagnosed allergic rhinitis patients, of whom 39% reported a concomitant diagnosis and/or clinical complaints of bronchial asthma and 48% of atopic dermatitis; skin reactivity for *D. pteronyssinus* and *D. farinae* mix was detected in 85% of patients (and 62% for *B. tropicalis*) (60). In a study of 579 asthmatic patients in Taiwan, it was shown through measuring allergen-specific IgE antibodies that almost 59% were sensitised to *D. microceras*, compared to 59.8% to *D. pteronyssinus* and 56.8% to *D. farinae*. Sensitisation to Cockroach was found in 38.3%, to Dog dander in 26.3%, to *Candida albicans* in 13.3%, to Cat dander in 10% and to *Cladosporium herbarum* in 6.6%. The study indicates the importance of considering *D. microceras* when evaluating allergic individuals (24). Among 93 Taiwanese asthmatic children aged 3 to 15 years evaluated for sensitisation to 5 species of mites, 63 were found to have IgE antibodies to at least 1 of the following mites: *D. pteronyssinus*, *D. farinae*, *D. microceras*, *Euroglyphus maynei*, and *Blomia tropicalis*. Sensitisation to *D. pteronyssinus* was found in 87%, to *D. farinae* in 85%, to *D. microceras* in 84%, to *Euroglyphus maynei* in 77% and to *Blomia tropicalis* in 65% (73). Similarly, in a Taiwanese study of 498 atopic children aged 2 to 16 years, high prevalences of sensitisation were documented: 90.2% to *D. pteronyssinus,* 88.2% to *D. farinae*, 79.5% to *D. microceras*, and 76.7% to *Blomia tropicalis* (50).

A group of 25 atopic children under 11 years of age in Oxford in the United Kingdom was studied for skin reactivity and IgE antibodies to 4 species of House dust mites: *D. pteronyssinus*, *D. farinae*, *D. microceras* and *Euroglyphus maynei*. All of the children were sensitised to *D. pteronyssinus* and 80% of these children were also sensitised to *D. farinae* and *D. microceras.* Importantly, dust samples from various sites in the homes of the children revealed *D. pteronyssinus* in all the homes, but no *D. farinae* or *D. microceras*. A control group of 20 atopic children of similar ages who were not sensitised to HDM allergens had similar exposure to the 4 mite species. These results suggest that factors in addition to mite exposure were important in the development of specific IgE responses to house dust mites (158). Interestingly, in habitats were conditions were not favourable for mites, mites had still managed to survive and may cause sensitisation. The presence of *D. farinae* and *D. pteronyssinus* had been reported in Egypt (31).

4. Allergic disease

Allergic diseases were produced by immunological hypersensitivity reactions to otherwise harmless substances caused by proteins called allergens. These reactions were included in the term “allergy”. The world allergy was introduced by Von Pirquet in 1906 to designate “altered reactivity” as a result of previous exposure. Allergy was mostly used as general term for reaction of discomfort of unknown origin. The most common allergic diseases were associated with type 1 hypersensiticity where the allergen induced IgE antibody production and sensitized T cells to produce Th2 cytokines. The effects produced by atopic or anaphylactic reaction were the results of teophase system. The sensitization phase was initiated by antigen presenting cells uptake anigen and presented the digested fragment to naïve T cells, which led to the T-cell proliferation, differentiation, cytokine production and the subsequently induced high specific IgE production from B cells. The other phase, elicitation phase, occurs upon by re-exposure to the same allergen. Following reaction, at least 2 molecules of specific IgE which bounded to the FcεRI on the mast cells and basophils were crosslinked with allergens leads to the release biological substances including histamine, heparin and serotonin. These mediators were

transformed by other immunological cells into prostaglandin and leukotrienes which were responsible for the late phase allergic reaction. The effect of these mediators included smooth muscle contraction, increased vascular permeability, gastric nasal and lacrimal secretion. The type of reaction depended upon the dose of allergen, the route of contact allergen, the frequency of contact with allergen and the degree of sensitivity of involved individual. Allergic diseases were multifactorial diseases. Factors that were found to influence the occurrence of allergies were the host and environment. Host factors involved in the risk for allergy were heredity, sex, race and age, with heredity being by far the most important. Persons who had a family history of allergic diseases would be at a high risk of developing the diseases. Environmental factors could act as the trigger in agent such as allergens, or acted like an adjuvant such as passive smoking and pollution (11). In general, an exposure dose was determined by two factors: the exposure concentration and the exposure time. For allergens, the exposure concentration was uncertain. For house dust mite, allergen particles contaminated infested fabrics and then became airborne with disturbance. Particles were cleared by settling, but some were also absorbed onto walls, furniture, and other reservoirs. Most studies of exposure had measured allergen levels in settled dust; only rarely airborne concentrations had been assessed. Settled dust and airborne dust mite allergen concentrations were highly variable, with reported coefficients of variation of 30% or more (161).

Histamine and tryptase are the major mediators stored in the mast calls. Cytokines, which appear to orchestrate the continuity between the early and late phases of the allergic reaction, are also newly synthesized mediators. The effects of mast cell and basophile degranulation are due to the responses of target cells. The mediators stimulate glands, nerves, and blood vessels to create the typical symptoms of allergic rhinitis: clear rhino rhea, sneezing, pruritus, and nasal congestion. Peptide hormones, insect venoms, radio contrast solutions, cold, and drugs such as narcotics and muscle relaxants can mimic the clinical reactions of immediate hypersentivity.

Patients commonly experience a second increase in mediators, called the late-phase reaction, 4 to 8 hours after the nitial reaction. In allergic rhinitis, this period is characterized by increased nasal obstruction and nonspecific nasal hyper

responsiveness. The clinical significance of the late-phase reaction is the actions of the leukocytes recruited to the nasal passages. These include basophiles, eosinophils, neutrophils, and macrophages, all of which provide additional pro-inflammatory mediators and cytokines. Although the principal cells in the nasal secretions are eosinophils, lymphocytes predominate in biopsy specimens of the nasal mucosa, suggesting that secretions and mucosa are distinct compartments with unique cell and cytokine configurations.

A number of environmental factors have been reported to affect the development and severity of asthma, including outdoor air pollutants (e.g., particulates, ozone), indoor irritants, and agents such as environmental tobacco smoke. However, it can be argued that the most significant inhaled agents that modulate the development of respiratory allergy and asthma are biologics. Indeed, one important aspect of innate immunity involves the response of monocytes and macrophages, which is mediated by receptors whose primary ligands arise from various microorganisms. Furthermore, many pathogens, especially viruses, target epithelial cells, and the resulting responses of epithelial cells and surrounding monocytes greatly affect the host response to those pathogens.

It has been suggested that the primary acquired immune response to a given antigen is influenced by the nature of the innate immune system (and its associated cytokine response). Thus, products of innate responses to microbes that are more effectively cleared by IgG and Th1 inflammation might be expected to promote Th1-acquired responses. In the absence of such inflammation, Th2 responses can dominate, especially if inhaled bioaerosols contain agents that derive from multicellular organisms (which may mimic parasites). Ultimately, it is the total exposure and immune experience of an individual, coupled with genetic factors that control their innate and acquired immune responses that determine if allergy develops in the airway. Central to Th1/Th2 balance is the composition of contaminants that derive from microbes (111). Hence, in this review we examine the biology of response to allergens, viruses, and bacterial products (primarily endotoxin) in the context of development of allergy and asthma.

The question of asthma induction usually brings to mind infants who experience asthma for the first time; however, at least two other examples illustrate the importance of allergen exposure to asthma incidence in adults. The first example is occupational asthma, especially that caused by laboratory animal allergy, where 25-30% of workers who are sensitized to laboratory animal allergens develop symptoms within 1 year of beginning work (17). About 25% of symptomatic workers have asthma symptoms, thereby making laboratory animal allergens a relatively common cause of incident asthma associated with a new allergen exposure in adults (17). A second example is the report of markedly increased rates of asthma in primitive villagers from the New Guinea highlands. In the 1980s adult men in these villages developed severe asthma, and 91% were sensitive to many allergens, including house dust mites (29). Cotton blankets that had been donated by Western charities were found to be heavily contaminated with dust mites, thus suggesting that they had been presented with a new, unique exposure that led to sensitization and incident asthma (29).

Children who develop asthma typically have symptoms by the age of 4-5 years, and a significant portion of them develop persistent asthma (127). Data from birth cohort studies suggest that atopy (defined by family history, other allergic manifestations such as eczema, elevated IgE, or sensitization) is a major risk factor for the development of childhood asthma. In asthmatic children age 6 years and older, sensitization to airborne environmental allergens is very common (80-90% of cases), and the combination of sensitization and exposure is strongly associated with more severe disease (112). House dust is heavily contaminated, but removal is difficult because of the inaccessible reservoirs. Air sampling studies have shown that mite particle is 10-40 μ diameters (28).

In general, an exposure dose is determined by two factors: the exposure concentration and the exposure time. For allergens, the exposure concentration is uncertain. For house dust mite, allergen particles contaminate infested fabrics and then become airborne with disturbance. Particles are cleared by settling, but some are also absorbed onto walls, furniture, and other reservoirs. Most studies of exposure have measured allergen levels in settled dust; only rarely have airborne concentrations

been assessed. Settled dust and airborne dust mite allergen concentrations are highly variable, with reported coefficients of variation of 30% or more (159).

House dust mite (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) allergens are major causative factors of various allergic diseases such as asthma, rhinitis, and atopic dermatitis.

5. Mechanisms of Allergic Diseases

Allergy is a disease in which multiple cell types, their involved in the blood and immune system originate from pluripotent stem cells in the bone marrow. During hematopoiesis, these pluripotent cells give rise to two lineages: the lymphoid cells and the myeloid cells. The myeloid lineage of cells differentiates into all other cells, including red blood cells, platelets, basophils. eosinophils, neutrophils and monocytes. The lymphoid lineage differentiates into the three different types of lymphocytes: T lymphocytes, B lymphocytes, and natural killer cells. The allergic reaction involved mediator and pathways (68). Particular interesting pathways are the role of IgE and the pathways regulated by T lymphocytes, in particular CD4+ T cells that release cytokines and stimulate B lymphocytes to produce IgE antibody. There are two populations of CD4+ T cells: Th1 and Th2. Th1 cells secrete IL-2, IFN-γ and lymphotoxin whereas Th2 cels secrete IL-4, IL-5, IL-6 and IL-13 (92). This activation proceeds by an initial proteolytic digestion of the peptide chain of the putative antigen. This is carried out as a first step of interaction with a number of antigen presenting cell (APC) types, the most prominent among them dendritic cells, macrophages and even B-cells. The 13-amino-acid-long proteolytic fraction of the chain, called the T-cell epitope, is then bound to the MHC-II complex on the APC and presented to the T-cell receptor complex on the specific T-cell to be activated. The interaction involves additional binding of receptor pairs on the two cells. This complex interaction leads to activated T-cells that, both by exogenous effector molecules and by congnate interaction, activate the B-cell clones chosen by antigen binding to their B-cell receptor (BCR). The activated clones proliferate and differentiate into antibody-producing plasma cells. Most protein antigens activate this T-cell-linked path of activation. The thymus-independent pathway allows direct

activation of the specific B-cell clones, elimination the need for the T-cell epitope. Most bacterial sugar-based antigens belong to this class. Hundreds of aeroallergens and other kind of allergens isolated contain protein and trigger the T-cell-dependent pathway. In addition to these two classes of antigens, a third, superantigen class exists, where antigens are able to trigger a general nonspecific activation of T-cell response leading to wide antibody response. There has been some speculation about the superantigenic nature of some allergic response (Definition of an allergen).

Antibodies (immunoglobulins) are proteins produced primarity after a response elicited by the immune system. They circulate in serum and specifically bind to antigens in both the recognition phase and the effector phase of humeral immunity. There are five classes of immunoglobulins, which are distinguished by their antigenic and structural characteristics. The basic molecule of an immunoglobulin consists of four protein chains linked together by disulfide bonds in a “Y”-shape. The two arms of the “Y” form antigen-binding sites, and the base forms the cell-binding site. The use of enzymatic cleavage of the immunoglobulin the Fab portions and one Fc fragment. IgE Originally known as *regain*, IgE is found only in trace amounts in serum. It is primarily membrane bound to effector cells (i.e., mast cells and basophils) (94). It has a role in protection against parasitic infections, as well as in allergic diseases.

6. Cytokines Mechanisms

Cytokines are any of a number of substances that are secreted by specific cells of the immune system which carry signals locally between cells, and thus have an effect on other cells. They are a category of signaling molecules that are used extensively in cellular communication. They are proteins, peptides, or glycoproteins. The term cytokine encompasses a large and diverse family of polypeptide regulators that are produced widely throughout the body by cells of diverse embryological origin. Basically, the term "cytokine" has been used to refer to the immunomodulating agents (interleukins, interferons, etc.). Conflicting opinion exists about what is termed a cytokine and what is termed a hormone. Anatomic and structural distinctions between cytokines and classic hormones are fading as we learn more about each.

Classic protein hormones circulate in nanomolar (10^{-9}) concentrations that usually vary by less than one order of magnitude. In contrast, some cytokines (such as IL-6) circulate in picomolar (10^{-12}) concentrations that can increase up to 1,000-fold during trauma or infection. The widespread distribution of cellular sources for cytokines may be a feature that differentiates them from hormones. Virtually all nucleated cells, but especially endo/epithelial cells and resident macrophages (many near the interface with the external environment) are potent producers of IL-1, IL-6, and TNF-α. In contrast, classic hormones, such as insulin, are secreted from discrete glands. The current terminology refers to cytokines as immunomodulating agents. However, more research is needed in this area of defining cytokines and hormones.

Part of the difficulty with distinguishing cytokines from hormones is that some of the immunomodulating effects of cytokines are systemic rather than local. For instance, to use hormone terminology, the action of cytokines may be autocrine or paracrine in chemotaxis and endocrine as a pyrogen. Further, as molecules, cytokines are not limited to their immunomodulatory role. For instance, cytokines are also involved in several developmental processes during embryogenesis.

Interleukin 5 (IL-5) is an interleukin produced by T helper-2 cells and mast cells. Its functions are to stimulate B cell growth and increase immunoglobulin secretion. It is also a key mediator in eosinophil activation. IL-5 is a 115 amino acid (in man, 133 in the mouse) long TH2 cytokine which is part of the hematopoietic family. Unlike other members of this cytokine family (namely IL-3 and GM-CSF), this glycoprotein in its active form is a homodimer. The IL-5 gene is located on chromosome 11 (in the mouse, chromosome 5 in humans) in close proximity to the genes encoding IL-3, IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) which are often co-expressed in TH2 cells. Interleukin-5 is also expressed by eosinophils and has been observed in the mast cells of asthmatic airways by immunohistochemistry. IL-5 expression is regulated by several transcription factors including GATA-3 IL-5. IL-5 is a heavily glycosylated homodimer secreted by T cells. Human T cells make L-5 mRNA, but natural human IL-5 has not yet been purified. Both human and mouse (57) IL-5 cDNAs have been cloned. The gene for IL-5 is on human chrosome 5q23-31 and on mouse chromosome 11. In both cases it is

closely linked to IL-3, IL-4 and granulocyte and macrophage colony stimulating factor (GM-CSF). In humans these four cytokines are found within a 500 kb segment and all have the same orientation suggesting they may be derived from a common ancestral gene. High- and low-affinity Il-5 binding sites have been reported and cross-linking studies have identified a protein of Mr 46.5 K that may constitute the low-affinity receptor. A protein of Mr 114 k may be the hight-affinity binding site.

Interferon-gamma (IFN-γ) is a dimerized soluble cytokine that is the only member of the type II class of interferons. This interferon was originally called macrophage-activating factor, a term now used to describe a larger family of proteins to which IFN-γ belongs. In humans, the IFN-γ protein is encoded by the IFNG gene. IFN-γ, or type II interferon, is a cytokine that is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control. Aberrant IFN-γ expression is associated with a number of autoinflammatory and autoimmune diseases. The importance of IFN-γ in the immune system stems in part from its ability to inhibit viral replication directly, but, most important, derives from its immunostimulatory and immunomodulatory effects. IFN-γ is produced predominantly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by CD4 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops. In contrast to interferon-α and interferon-β, which can be expressed by all cells, IFN-γ is secreted by Th1 cells, Tc cells, dendritic cells and NK cells. Also known as immune interferon, IFN-γ is the only Type II interferon. It is serologically distinct from Type I interferons and it is acid-labile, while the type I variants are acid-stable.IFN-γ has antiviral, immunoregulatory, and anti-tumor properties. It alters transcription in up to 30 genes producing a variety of physiological and cellular responses. Amongst the effects are:Increase antigen presentation of macrophages., Activate and increase lysosome activity in macrophages, Suppress Th2 cell activity., Cause normal cells to increase expression of class I MHC molecules, Promotes adhesion and binding required for leukocyte migration, Promotes NK cell activity, Activates APCs and promotes Th1 differentiation by upregulating the transcription factor T-bet., Activate inducible Nitric Oxide Synthase iNOS. IFN-γ is a Mr 40-70 K aggregate of two molecular

weight species of Mr 20 and 25 K (109). These differ only in degrees of glycosylation. The 20 K species is glycosylated at residues 25 and 97. Both human (38) and mouse (39) IFN-γ have been cloned. Each is a single polypeptide with two potential N-linked glycosylation siments of tes. There are five extracellular N-linked glycosylation sites and 11 cysteine residues in the human receptor, with at least one disulphide bridge essential for function.

Transforming growth factor β (TGF- β), a powerful regulator of the tissue repair response, has been strongly implicated in the development of airway remodeling. Clinical studies have documented increased TGF-β expression on both the level of mRNA and protein in bronchial biopsies and in the bronchoalveolar lavage (BAL) of humans with asthma compared with control subjects (84) Similarly, TGF-β expression has also been shown to be increased in animal models of allergic airway disease (55, 58). In addition, the number of TGF-β expressing epithelial or sub-mucosal cells has been correlated with the basement membrane thickness in patients with asthma (147). Direct evidence in support of a role for TGF-β in the development of allergic airway remodeling comes from a study by McMillan and colleagues, who showed that TGF-β blockade using a neutralizing antibody (Ab) reduced the extent of ovalbumin (OVA)-induced remod-eling in a chronic mouse model (90). In addition, two very recent studies, one using SMAD3 knockout (KO) animals (75) and the other using blocking Abs (2), have also demonstrated that TGF-β contributes to the development of airway remodeling in similar OVA-based systems. Animal studies demonstrating a critical role for TGF-β in the pathogenesis of related diseases involving aberrant tissue repair, such as liver, kidney, and lung fibrosis, further support the notion of TGF-b involvement in allergic airway remodeling (65, 118). Importantly, it is clear that TGF-β is also one of the most potent negative regulators of inflammation (76). Indeed, TGF-β deficient mice die soon after birth due to rampant, multifocal inflammation (120). This activity may also be of particular relevance to allergic airway disease as TGF-β is believed to contribute, at least in part, to the regulation of the Th2-polarized airway inflammatory response that is characteristic of this disease. Numerous in vitro studies have collectively shown that TGF-β suppresses the activity and/or proliferation of various inflammatory cells,

including Th2 cells, B cells, macrophages, and eosinophils (33, 104), and moreover, TGF-β can promote their apoptosis (82). Three separate studies have investigated the impact of genetically interfering with TGF-β activity on allergic sensitization and airway inflammation in acute models of allergic airway disease using OVA. Their findings consistently demonstrate that abrogation of TGF-β signaling pathways substantially potentiates the immune response and exacerbates the allergic inflammatory response (116, 117). In light of these reports, we and others have proposed that TGF-β may play a dual role in asthma by coordinately regulating both the inflammatory and reparative responses; specifically, that TGF-β may dampen the inflammatory response elicited upon aeroallergen exposure in a sensitized individual while subsequently initiating tissue repair processes. In this study, we investigated the role of TGF-β in the regulation of chronic allergic airway inflammation and remodeling. TGF-β is a family of small non-glycosylated polypeptides and has multifunctional molecules.

CHAPTER III

MATERIAL AND METHODS

1. House dust mite (HDM) allergens

Total HDM extracts of *D. farinae* (Df) was obtained from mite cultures provided by Siriraj Dust Mite Center of Services and Research, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

1.1 Preparation of House dust mite allergen

Briefly, mite culture was stirred in phosphate buffered saline (PBS), pH 7.4 for overnight at 4 degree Celsius (°C). The mixture was centrifuged for 20 min at 12,000xg at 4°C. The supernatant was filtered through No.1 Whatman paper and through 0.22 μ filter. The sterile mite extract was then stored frozen at -20°C (21).

Recombinant allergen group 2 of *Dermatophagoides farinae* (rDer f 2) was donated by Assistant Professor Surapon Piboonpocanun from the Institute of Molecular Biosciences, Mahidol University, Thailand.

The positive transformants containing the integration of interested genes were selected for small-scaled expression. Briefly, positive transformants (rDer f 2) and plasmic vecter (pPICZ) of *Pichia pastoris* (*P. pastoris*) (132) were grown in 2 milliliters (ml) of yeast extract peptone dextrose (YPD) containing 100 μg/ml Zeocin™ at 30 °C with vigorous shaking (250 rpm) for approximately 2 days. The starter cultures were added into 5 ml of fresh buffered minimal glycerol complex medium (BMGY) and incubated at 30 °C until the absorbance of BMGY was 0.1 at optical density (OD) 600 nanometer (nm). After that, the starter culture with OD 0.1 were cultured and harvested until the absorbanes at of BMGY were 5.0. The BMGY cultures were centrifuged at 10,000xg for 5 min, and the cell pellets were resuspended in buffered minimal methanol complex medium (BMMY) using 1/5 volume of the original culture volume (approximately 1 ml). To induce expression of recombinant allergens, absolute methanol was added to a final concentration of 3% v/v to the BMMY culture every 24 h. The culture media were collected after 3 day induction.

The collected culture media were centrifuged at 12,000xg in a bench-top microcentrifuge for 3 min at room temperature. The supernatant was transferred to a separate tube and were stored frozen at -20 °C until used.

1.2 Determination of HDM protein

Protein concentration of HDM was determined by using the BioRad Protein Assay (dye-binding assay), (BioRad, Laboratory, CA) based on the method of Bradford. For standard protein, bovine serum albumin (Sigma, Steinheim, Germany) concentration was ranged from 0.0625 to 1 mg/ml was used. Ten microliters of each HDM protein was mixed with 90 µl of acid Comassie blue dye and incubated at room temperature. After incubation for 5 min, the mixture was measured for the OD 595 nm.

1.3 Characterization of HDM allergen

Characterize the protein components of the extracted antigen were prepared from *D. farinae* and *D. pteronyssinus* by using SDS-PAGE. The SDS-PAGE was using 4% stacking gel and 12.5 % resolving gel. The extracted protein had been reduced and denatured by mixing with 4X sample buffer and then boiled in a water bath at 95°C for 10 min before loading onto the gel. The protein content used to load the gel was approximately 2 µg per lane. Electrophoresis was carried out with a constant voltage of 100 volts (v) until the front dye reaches the bottom of the gel. After that, the gel was stained with Coomassie Brilliant Blue.

Proteins were firstly separated by SDS-PAGE followed by transferring the fractionated proteins to a nitrocellulose membrane as prescribed by the manufacturer with a wet blotting apparatus (Bio-Rad, USA). The blotted membrane was blocked to prevent non-specific binding of antiserum to the membrane by gently rocking overnight at 4 °C in blocking solution. The blot was then washed by shaking vigorously in washing buffer for 5 min. The washing step was performed in six times replica. The blot was then incubated with continuous shaking at room temperature for 120 min in a 1:2000 dilution of monoclonal anti-group 2 antibodies (mAb 1D8 clone, Indoor biotecnologies Ltd, Manchester, UK). After six-time washing, the membrane was incubated with biotinylated rabbit-anti mouse IgG solution (1:5000)

(SouthernBiotech, Birmingham, USA) for another 60 min at room temperature. After six-time washing, the membrane was incubated with streptavidin-AP solution (1: 2000) (KPL, USA) for another 30 min at room temperature. The membrane was then washed with the same condition and the band of specific proteins was visualized by adding BCIP/NBT 1 component substrate (KPL, USA) and incubating at room temperature with gently shaking until color was developed.

2. Subjects

A total of 24 patients with perennial allergic rhinitis and a positive Skin prick test (SPT) to house dust mite allergen (Dp and Df), were selected for this investigation by Dr. Tharit Muninnobpamas and Dr. Piyalarp Wasuwat of Department of Otolaryngology, Pramongkutklao Hospital, Bangkok, Thailand. As the control group, 20 healthy subjects with no history of allergic diseases and a negative SPT to all aeroallergen extracts tested were included. The study donor was approved by the Ethics committee in Human Research of the Phramongkutklao Hospital and written informed consent was obtained from all volunteers.

2.1 Skin prick test (SPT).

The allergen extract of commercial Dp (*Dermatophagoides pteronyssinus*) and Df (*D. farinae*), CR (American Cockroach), Grass (Bermuda grass pollen), Dog (Dog Epithelium), Cat (Cat pelt), Weed, Smut (*Sporobolus indicus*), M (*Penicillium notatum*, *Aspergillus niger*, Drechslera, *Cladosprium spharospermum*, Alternaria) manufactured by allertech (Table 2). Histamine dihydrochloride (10 mg/ml) was used as positive control and normal saline was used as negative control.

SPT was performed on the skin of volunteer forearm using allergen extracts as mentioned above along with positive and negative controls. Briefly, the arm was first cleaned, then a drop of Allergen extract or control solution was placed onto a marked area of skin, with a distance of 2-3 centimeter (cm) apart. A sterile No 23G needle was used to prick the skin through the allergen drop, which allowed a small amount of allergen enter the skin. Each allergen was pricked with a separate needle. Two to three minutes later, the residue fluid on the skin was carefully wiped off to avoid mixing the adjacent samples. The reaction was measured after 15 min by

copying the wheal size with transparent adhesive and kept tape onto a record sheet for later analysis. All the patients were observed for another had for any unpleasant reactions such as local itch and swelling, local rash, generalized rash or anaphylaxis.

After SPT was completed, 55 ml of venous blood was taken from each donor. 5 ml of blood was centrifuged at 400xg for 30 min. The serum was collected and stored at -20°C for measurement of specific IgE. And 50 ml of blood was used for PBMCs culture.

Table 2. List of commercial Allergens used for select the patient groups

No.	Allergens	Concentrations
1.	HDM (Dp. and Df.)	10,000 AU/ml
2.	Dust	10,000 AU/ml
3.	CR (American Cockroach)	1:20 W/V
4.	Smut (*Sporobolus indicus*)	1:10 W/V
5.	Weed	1:10 W/V
6.	Grass (Bermuda grass pollen)	10,000 BAU/ml
7.	Dog (Dog Epithelium)	15,000 PNU/ml
		1:10 W/V
8.	Cat (Cat Pelt)	10,000 BAU/ml
9.	Mold: *Penicillium notatum*	20,000 PNU/ml
	Mold: *Aspergillus niger*	1:10 W/V
	Mold: *Drechslera*	
	Mold: *Cladosprium sphaerospermum*	
	Mold: *Alternaria*	

2.2 Measurement of specific IgE

IgE reactivity to a panel of proteins from Df and rDer f 2 was determined by ELISA (41). Briefly, dilute each allergen in coating buffer (50 nM carbonate/bicarbonate buffer) at concentration of 1000 ng/ml. Two-hundred µl of each allergen was coated in each well of 96-well polystyrene microtiter wells (Nunc^{TM}, Denmark) and incubated at 4 °C overnight. The blocking buffer was added into each well and incubated at 37 °C for 60 min. The sera at dilution of 1: 10 was added into the well and incubated at 37 °C for 120 min. Biotinylated-labeled mouse anti-human IgE antibody (SouthernBiotech, Birmingham, USA) at dil 1: 1000 was added into each well and incubated at 37 °C for 60 min. During each step, the plates were washed with PBS-Tween20 for 5 times. The plates was added with streptavidin-peroxidase solution at dil 1: 1000 and incubated at 37 °C for 60 min. The color reaction was developed by adding ABTS® peroxidase substrate (KPL, USA). Absorbance of IgE antibody was measurced at OD405nm with a Wallac 1420 microplate reader (Auto DELFIA Wallac 1420; Wallac, Turku, Finland).

3. Cytokine production assays

3.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Blood was collected and diluted with an equal volume of RPMI-1640. PBMCs were isolated on a density gradient (Lymphoprep^{TM}, Norway), washed two times with RPMI-1640, and resuspended in AIM-V culture medium (Invitrogen, Australia). The cells were seeded at $1x10^6$ cells/well in 12-well plate in a volume of 1 ml.

3.2 Activation of PBMCs for cytokine production

PBMCs dissolved in AIM-V medium was into each well of 12-well plates (1×10^6 cells/ml per well). The PBMCs were stimulated with 100 µl allergen extracts (30µg of Df or rDer f 2), mitogens (PHA), or medium alone and then incubated at 37°C in 5% CO_2. The cells were harvested using centrifugation at 250 x g for 5 min 4ºC at 24, 48, 72, 96 and 120 h. post-incubation. The supernatants and the

cell pellets were used for detection of IL-5, IFN-γ and TGF-β cytokine protein by ELISA and cytokine gene expression by RT-PCR, respectively.

3.3 Cytokine gene expression measurement used RT-PCR

RNA extraction was carried out through using TRI Reagent® (Molecular research center, USA) according with the manufacturer's instructions. The PBMCs were macerated, and the resulting solution was transferred to 1.5ml tubes. In each sample, 0.1 ml of BCP Reagent was added, and after vigorous mixing, and incubated on ice for 10 minutes, followed by centrifugation at 12,000 x g per 15 minutes at 4°C. The upper layer resulting from the centrifugation was then transferred to a new 1.5ml tube and the RNA was precipitated with isopropanol (Sigma, Singapore). The RNA was then resuspended in 1 ml of 75% ethanol and centrifugation at 12,000 x g per 5 minutes at 4°C. RNA pellet was dry for 5 minutes on ice. The RNA was dissolved in 20 µl of *DNase/RNase-Free* Distilled Water. The RNA concentration was quantified in a spectrophotometer at 260nm wavelength and its integrity was assessed by denaturizing electrophoresis in 0.8% agarose gel. The cDNA was synthesized using Oligo(dT) primers and the reverse transcriptase system (iNtRON Biotechnology, Korean) according to manufacturer's instructions. PCR was carried out in a final volume of 20 µl, in the presence of 5 µl of cDNA, 2.5mM of dNTP, 20 µM of each primer, The primer sequences were as follows: ***IL-5*****,** 5'-GAG GAT GCT TCT GCA TTT GAG TTT-3' and 5'-GTC AAT GTA TTT CTT TAT TAA GGA CAA-3'; ***IFN-γ*****,** 5'-AGC TCT GCA TCG TTT GGG TTC-3' and 5'-GTT GGC CCC TGA GAT AAA GCC-3'; ***TGF-β*****,** 5'-ACC ACT GCC GCA CAA CTC CGG TGA C-3' and 5'-ATC TAT GAC AAG TTC AAG CAG AGT A-3'; ***β-Actin*****,** 5'-ATC TGG CAC ACT TCT ACA-3' and 5'-GTT TCG TGG ATG CCA CAG GAC-3', and 5 U/ µl of Platinum TaqDNA polymerase (iNtRON Biotechnology, Korean). PCR products were analyzed by electrophoresis in an 8% polyacrylamide gel dyed by ethidium bromide. The gels were photographed and the results were analyzed by densitometry using Gel Doc. (Bio-Rad. Quantity One Version 4.6.3). The semi-quantitative RT-PCR and the β-Actin (housekeeping) was considered the reference gene.

3.4 Cytokine measurement used Enzyme-Linked Immunosorbent Assay (ELISA)

Concentrations of IFN-γ and IL-5 levels in culture supernatants were determined using commercially available ELISA kits according to manufacturer instructions (R&D Systems, USA). Briefly, 100 µl of capture antibody in coating buffer was coated on Maxisorp 96 well ELISA plate (Nunc^{TM}, Denmark). The plate was sealed and incubated overnight at 4 ^{o} C. The well was aspirated and washed 3 times with >300 µl/well wash buffer. The plate was inverted and blotted on absorbent paper to remove any residual buffer. The well was blocked with 200 µl/well of 1X assay diluent and incubated at RT for 1 h. The well was aspirated and washed 3 times with >300 µl/well wash buffer as in step 2. One hundred µl/well of standard was added to the appropriate wells. Two-fold serial dilutions of the standard were assay for making the standard curve. The plate was sealed and incubated at RT for 1 h and washed 5 times. The 100 µl/well of avidin-HRP was diluted in 1X assay diluents. The plate was sealed and incubated at RT for 30 min, aspirated and washed. In this wash step, the wells were soaked in wash buffer for 1 to 2 min prior to aspiration. One hundred µl/well of substrate solution is added to each well. The plate was incubated at RT for 15 min. Fifty µl/well of stop solution was added to each well and the plate was read at 450 nm. All samples and standards were run in duplicate.

4. Stastistic analysis

Data were expressed as the means (±SEM). The statistical differences were analyzed using the Mann-Whitney U-test in the SPSS statistical software package (Version 13 Chicago, IL, USA). A significant value was defined as $p < 0.05$.

CHAPTER IV

RESULTS

1. Subjects Characteristics

The demographic and clinical characteristics of the study subjects (Table 3) were distributed into two groups according to SPT positivity to commercial HDM-total extract: 1) HDM+ group: patients with positive SPT to HDM-total; 2) HDM- group: Non-allergic subjects with negative SPT to HDM-total and aeroallergens. Rhinitis was the most frequent clinical diagnosis observed in HDM+ group but not be observed in HDM- group. The HDM+ group showed the mean wheal size to HDM-total and histamine dihydrochloride (positive control) were $\geq$ 3 mm. In contrast, all subjects did not show the mean wheal size to normal saline which was used as negative control. HDM- group showed a negative SPT to mite extracts as well as other aeroallergens tested according to the selection criteria used.

The highest SPT was observing at 37.5% from patients suffering from two HDM-totals (Df and Dp) whereas the least SPT was observing at 4.17% from patients suffering from two HDM-total and other aeroallergens.

Table 3. Demographic and clinical characteristics of the study subject

Characteristics	Groups	
	HDM+	HDM-
Number of subject (N)	24	20
Age (years, means ± SD)	35.17±12.06	30.75±5.46
Gender (male/female)	10/14	9/11
Clinical diagnosis (N, %)	24, 100%	20, 100%
Categorized by allergens (N, %)		
Df, Dp,	9, 37.50%	0
Df, Dp, CR, Dust,	6, 25.00%	0
Df, Dp, CR, Dust, Smut	2, 8.33%	0
Df, Dp, CR	2, 8.33%	0
Df, Dp, CR, Weed	1, 4.17%	0
Df, Dp, Cat, Dog	1, 4.17%	0
Df, Dp, CR, Smut	1, 4.17%	0
Df, Dp, Dust, CR, Dog	1, 4.17%	0
Df, Dp, Dust, CR, Weed, Cat, Dog	1, 4.17%	0

HDM+; HDM-allergic subjects, HDM-; Non-allergic subjects, Dust; House dust, Df; *Dermatophagoides farina*, Dp; *Dermatophagoides pteronyssinus*, CR; American Cockroach, Smut; *Sporobolus indicus*, Dog; Dog epithelium, Cat; Cat pelt

2. HDM–allergen characteristics

In the present study, HDM allergens consisted of native (Dp and Df) and recombinant (rDer f 2) allergens expressed in *P. pastoris*. For negative control, protein of pPICZ was used. The characters of HDM allergens were determined by SDS-PAGE and western blot, as shown in Figure 1. The molecular weight of native allergens (Df and Dp) were ranged from 10 to 72 kDa whereas that of rDer f 2 was 14 kDa. Molecular weight of pPICZ was not found. Additionally, molecular weight of rDer f 2 was comfirmed by western blot hybridization was 14 kDa (Figure 2).

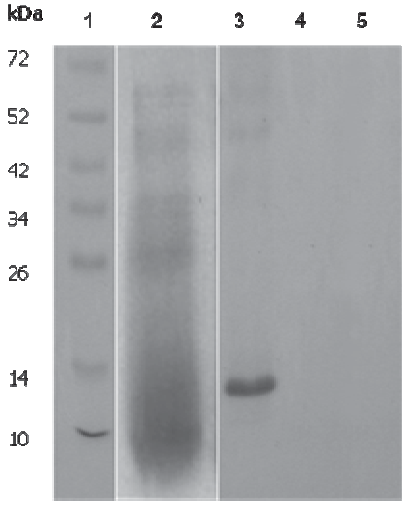

Figure 1. The protein components of HDM allergen were determined by SDS-PAGE; Lane 1: Molecular mass standards (in kilo Dalton); Lane 2: Df; Lane 3: r Der f 2; Lane 4: PBS pH7.4; Lane 5: pPICZ

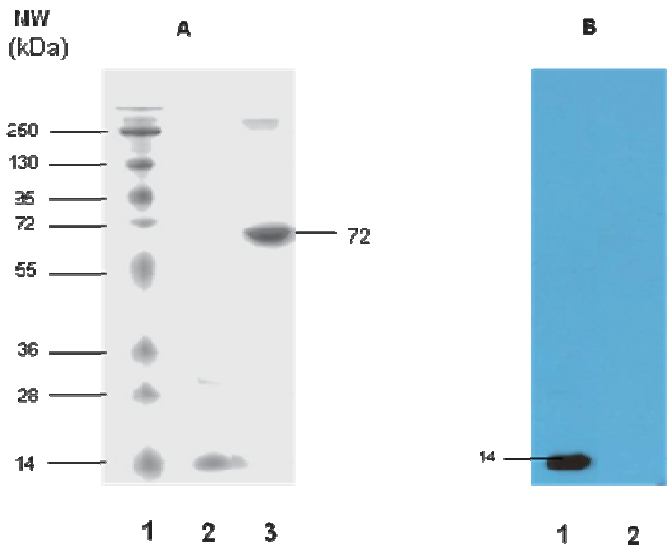

Figure 2. The protein components of HDM allergen were determined by SDS-PAGE (A); Lane 1: Molecular mass standards (in kilo Dalton); Lane 2: rDer f 2; Lane 3: BSA; and western blot (B); Lane 1: rDer f 2; Lane 2: BSA, respectively.

3. T-cell responses to Df and rDer f 2 allergens at molecular level

IL-5, IFN-γ, TGF-β cytokine genes from allergic patients and control subjects were stimulated with PHA, Df, rDer f 2, pPICZ and medium alone by RT-PCR. The results revealed that all stimulators induced *IL-5* and *IFN-γ* genes expression in both allergic patients and control subjects. In contrast, PHA, Df, rDer f 2, pPICZ and medium alone did not induce *TGF-β* gene expression in either allergic patients or control subjects (Figure 3). It was suggesting that expression of *IL-5, IFN-γ, TGF-β* genes in allergic patients were similar to those from non-allergic subjects at molecular level.

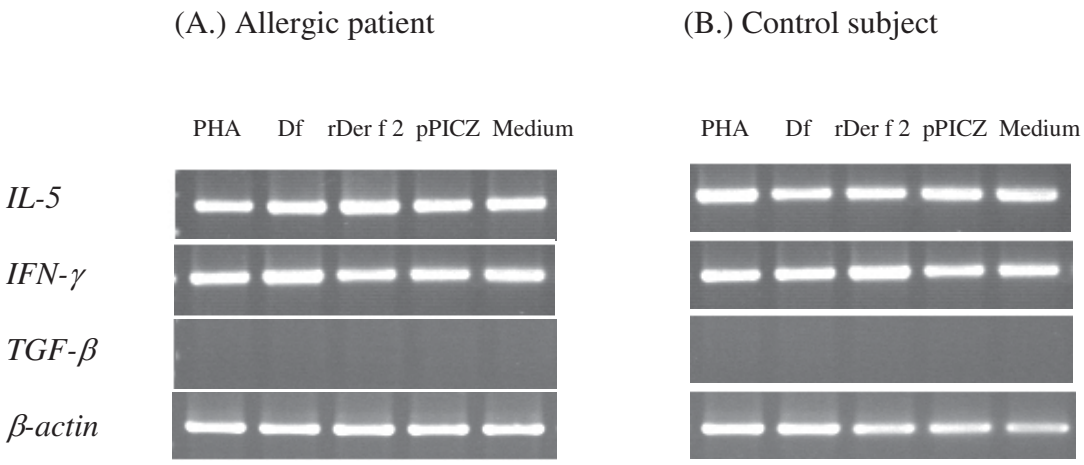

Figure.3 *IL-5, IFN-γ* and *TGF-β* gene expression response to PHA, Df, rDer f 2, pPICZ and medium compared with *β-actin* housekeeping gene in PBMCs of allergic patient (A) and control subject (B) were determined by 1% w/v Agarose gel electrophoresis, stained with ethidium bromide.

4. T cell responses to Df and rDer f 2 allergens at cellular level

IL-5, IFN-γ, TGF-β cytokine protein production from allergic patients and control subjects were stimulated with PHA, Df and rDer f 2 by ELISA. For allergic patients, PHA, Df and rDer f 2 induced IL-5 production at 217.76±27.84, 7.47±0.61 and 58.33±4.92 pg/ml, respectively. For non-allergic subjects, PHA, Df and rDer f 2 induced IL-5 production at 165.50±16.61, 4.43±0.174 and 6.31±0.85 pg/ml, respectively (Table 4). No correlation between PHA and rDer f 2 as well as PHA and Df were found in allergic patients and in non-allergic subjects (Figure 4). For allergic patients, PHA, Df and rDer f 2 induced IFN-γ production at 863.98±67.78, 70.32±6.74 and 619.62±61.43 pg/ml, respectively. For non-allergic subjects, PHA, Df and rDer f 2 induced IFN-γ production at 1142.61±78.40, 6.16±0.45 and 410.73±48.56 pg/ml, respectively. No correlation between PHA and Df as well as PHA and rDer f 2 was found in allergic patients and non-allergic subjects. For allergic patients and non-allergic subjects, PHA, Df and rDer f 2 showed level of TGF-β from PBMCs of allergic patients and non-allergic subjects at <60 pg/m (Table 4).

To analyze the effect on Th1/Th2 bias the responses the allergic patients and non-allergic subjects, the data showed that the cytokine release to PHA did not differ from allergic patients and non-allergic subjects. The Df allergen showed IFN-γ/IL-5 ratio from allergic patients (7.16±0.70) higher than non-allergic subjects (1.43±0.1), suggesting that Df allergen slightly induced Th1/Th2 bias in allergic patients, but not good enough for non-allergic patients. The reverse rDer f 2 allergen showed IFN-γ/IL-5 ratio from non-allergic subjects (53.57±9.71) higher than allergic patients (11.25±1.08), suggesting that rDer f 2 induced Th1/Th2 bias in non-allergic subjects better than allergic patients.

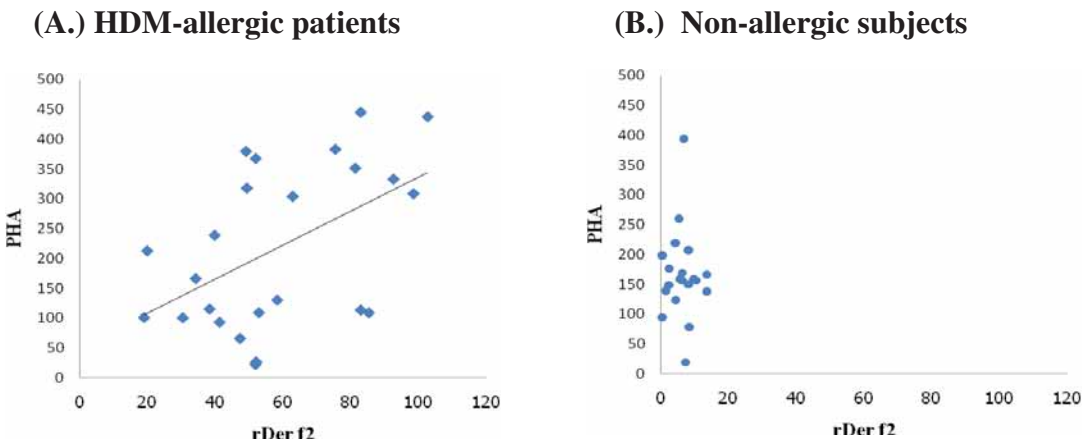

Figure 4 Correlation of IL-5 production of PBMCs from HDM-allergic patients (r = 0.253; p≤0.05) (A.) and Non-allergic subjects (B.) response to PHA and rDer f 2 allergens.

Table 4. Cytokine protein responses to the PHA and house dust mite allergens from HDM-allergic and non-allergic subjects*.

	IL-5 (pg/ml)		IFN-γ (pg/ml)		TGF-β (pg/ml)		IFN-γ/IL-5 ratio	
	Allergic	Non-allergic	Allergic	Non-allergic	Allergic	Non-allergic	Allergic	Non-allergic
PHA	217.76±27.84	165.50±16.61	863.98±67.78	1142.61±78.40	<60.00	<60.00	6.83±1.49	10.06±2.98
Df	7.47±0.61‡	4.43±0.174	70.32±6.74†‡	6.16±0.45‡	<60.00	<60.00	7.16±0.70†	1.43±0.12‡
rDer f2	58.33±4.92†	6.31±0.85	619.62±61.43	410.73±48.56	<60.00	<60.00	11.25±1.08†	53.57±9.71

*Results are presented as means (±SEM)

† Significant differences between Allergic Non-allergic groups. $p \leq 0.05$

‡ Significant differences between Df and rDer f2 allergens. $p \leq 0.05$

Cut off of IL-5= 4 pg/ml, IFN-γ = 4 pg/ml and TGF-β= 60 pg/ml

5. Specific IgE reactivity to Df and rDer f 2 allergens

Level of specific IgE from allergic patients and non-allergic subjects to Df and rDer f 2 were measured by ELISA. As shown in Table 5, of 24 allergic patients who had been SPT positivity to commercial HDM-total extract, only 4 patients (No. 3, 5, 6 and 7) showed specific IgE to both Df and rDer f 2. Only 5 patients (No. 2, 11, 14, 15 and 16) showed IgE binding to Df whereas no patient showed specific IgE to rDer f 2. Fifteen patients showed specific IgE values below 0.35 kU_A/L to Df and rDer f 2. It was suggesting that the SPT positivity to commercial HDM-total extract was not related to IgE-binding reactivity to HDM allergens in allergic patients. Of 20 non-allergic subjects, all subjects showed specific IgE values below 0.35 kU_A/L to Df and rDer f 2. It was suggesting that the SPT negatively to commercial HDM-total extract was related to specific IgE reactivity to HDM allergens in non-allergic subjects. No correlation between the level of IL-5 production and the degree of IgE-binding to the rDer f 2 from HDM-allergic patients and non-allergic subjects were observed (Figure 5).

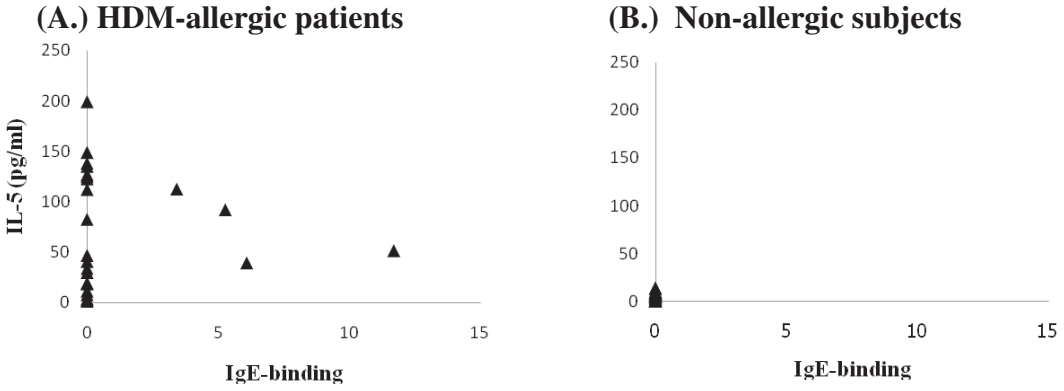

Figure 5 No correlation (r=0.01) between the level of IL-5 production and the degree of IgE-binding to the rDer f 2 from HDM-allergic patients (A.) and Non-allergic subjects (B.) were observed.

Table 5. IgE-binding to HDM allergens in HDM-allergic patients

No.	SPT	Allergic history	IgE –binding (kU_A/L)	
			Df	rDer f 2
1.	+	Df, Dp, Dust, CR, Smut	11.24	3.42
2.	+	Df, Dp	11.07	0.40
3.	+	Df, Dp, Dust, CR,	6.09	3.40
4.	+	Df, Dp, Dust, CR, Dog	5.27	3.18
5.	+	Df, Dp, CR, Smut	5.74	<0.35
6.	+	Df, Dp, Cat, Dog	5.09	<0.35
7.	+	Df, Dp, Dust, CR,	3.39	<0.35
8.	+	Df, Dp, CR, Weed	1.88	<0.35
9.	+	Df, Dp, Dust, CR,	0.99	<0.35
10.	+	Df, Dp, CR, Weed	<0.35	<0.35
11.	+	Df, Dp, Dust, CR, Smut	<0.35	<0.35
12.	+	Df, Dp, Dust, CR,	<0.35	<0.35
13.	+	Df, Dp, Dust, CR,	<0.35	<0.35
14.	+	Df, Dp, Dust, CR,	<0.35	<0.35
15.	+	Df, Dp, CR,	<0.35	<0.35
16.	+	Df, Dp, CR,	<0.35	<0.35
17.	+	Df, Dp	<0.35	<0.35
18.	+	Df, Dp	<0.35	<0.35
19.	+	Df, Dp	<0.35	<0.35
20.	+	Df, Dp	<0.35	<0.35
21.	+	Df, Dp	<0.35	<0.35
22.	+	Df, Dp	<0.35	<0.35
23.	+	Df, Dp	<0.35	<0.35
24.	+	Df, Dp	<0.35	<0.35

*SPT, skin prick tests; +, SPT wheal response diameter was more than 3 mm;

*Dust; House dust, Df; *Dermatophagoides farina*e, Dp; *Dermatophagoides pteronyssinus*, CR; American Cockroach, Smut; *Sporobolus indicus*, Dog; Dog epithelium, Cat; Cat pelt

*Cut off of IgE –binding; 0.35 kU_A/L

CHAPTER V

DISCUSSION

House dust mites (HDMs) as a source of allergens and their contribution to allergic diseases, particularly asthma and rhinitis, have been recognized for many years (4). Allergic rhinitis is an inflammatory reaction of the nasal mucosa, in consequence of an IgE mediated hypersensitive reaction to inhaling allergens, involving different mediators and cytokine cells (121). Positive skin tests and/or serum IgE antibodies to HDM have been widely demonstrated in genetically predisposed individuals. In the present study, we found only 37.5 % allergic patients presenting positive SPT to both mites (Df and Dp), while 62.5 % of allergic patients sensitized to a panel of aeroallergen. These results were similar to those of our previous studies carried out in another group of allergic patients from Police hospital in Bangkok (105), confirming that the high rate of this concurrent sensitization has certainly complicated the evaluation of the role of *D. farinae* and *D. pteronyssinus* in these patients, particularly in tropical and subtropical regions where both mites coexist. In this context, Df-total extract was fractioned by SDS-PAGE and confirmed by Western blot analysis using 1D8 monoclonal antibody. The recombinant Der f 2 allergen derived from *P. pastoris* was also fractioned by SDS-PAGE and confirmed by Western blot analysis. The recombinant Der f 2 allergens presented predominantly molecular weight at 14 kDa. Both allergens were used to detect IgE antibody in allergic patients and healthy subjects. We found levels of specific IgE antibody detected by Df extract were higher than those by recombinant Der f 2 allergen in allergic patients. These results are consistent with report of Almedida et al (3) and Pipatchaipaisan, (105). It is implied that specific IgE antibody could be related to natural allergen exposure, thus reflecting a normal consequence of immediate hypersensitivity reactions in allergic patients in vivo (3). In our data, we found that IgE are preferentially involved in this cross-reactivity in some allergic patients to both

Df extract and recombinant Der f 2 allergen. This result indicated that IgE might be recognizing same epitope in the extract allergen and recombinant allergen.

Several reports revealed that both the IgE-dependent early response and antigen-primed Th2 cells are necessary for a persistent atopic inflammation characterized by the accumulation of activated inflammatory cells (98). Previous studies have highlighted the role of IL-5 in the development of the chronic phase of atopic inflammation and in the expression of allergic diseases (59, 62, 149). The hypothesis of our study was that immunotherapy with the relevant mite allergic would induce the functional modification from Th2 to Th1 phenotype in allergic patients. To investigate the cellular immunity against HDM allergens, we evaluated intracellular IL-5, and IFN-γ gene expression as well as the cytokine release from the PBMCs of the allergic patients relative to healthy subjects. We did not find significantly difference in IL-5 or IFN-γ gene expression from PBMCs of allergic patients and healthy control subjects. This data is consistent with other investigators (121). However, we found that IL-5 production induced by Df extract was slightly positively correlated in both allergic patients and healthy subjects. On the other hand, we found that IL-5 production induced by recombinant Der f 2 allergen was significantly increased in allergic patients relative to healthy subject. This data was consistent with the evidence that activated T cells and the Th2 cytokine IL-5 production is increased in lung of atopic asthmatic subjects (69, 100, 149). We found no correlation between serum HDM-specific IgE levels and IL-5 production stimulated in either allergic patients or healthy subjects. This is agreement with data from some but not all studies (61, 138, 154).

Investigations of allergen-simulated release of IFN-γ from PBMCs have produced mixed results. Some studies found decreased IFN-γ production in subjects with atopic diseases relative to healthy control subjects (89, 91, 155), whereas other studies reported no difference (15, 62) In our study, there was no significant difference in recombinant Der f 2-stimulated IFN-γ production between allergic patients and control subjects, while we found that degree of Df-total extract-stimulated IFN-γ production was higher in allergic patients than in the control

subjects. It has been reported that IFN-γ production closely correlated with atopic dermatitis in infants (61). No absolute definition of a Th1- or Th2-type cytokine response was demonstrated. An excess of Th2 cytokines relative to the Th1 cytokine is implicated in the cause of atopy (78, 157). Studies with PBMCs have also analyzed allergen-stimulated cytokine production as the amount of IL-5, Th2 cytokine relative to IFN-γ, Th1 cytokine (89). We analyzed cytokine production induced by HDM allergens as the ratio of the IL-5 to IFN-γ production in each culture that had a positive response to the stimulators and expressed this ratio as a Th2/Th1cytokine index. We found that for allergic patients and control subjects, the Th2/Th1cytokine index was induced by both HDM allergens, although different degree of Th2/Th1cytokine index was observed, suggesting that HDM is the Th2-type stimulators that are consistent with other reports (30). Additionally, we found that there is a lower frequency of HDM allergen-responsive T cells in healthy control subjects than in allergic patients. Evidence of our data focuses research attention on mechanisms of T-cell recognition of, and activation by, HDM allergens. Both allergic patients and normal subjects had a Th2-type cytokine response to HDM allergen; our data did suggest that patients with atopic rhinitis have an enhanced Th2 cytokine response to HDM allergen relative to healthy control subjects. It is indicated that both HDM allergens increased IL-5 production and Th2/Th1cytokine index in patients with allergic rhinitis and normal subjects.

CHAPTER VI

CONCLUSION

Df extract and recombinant Der f 2 allergens are stimulators of PBMC from allergic patients relative to healthy subjects. IFN-γ released from Th1 and IL-5 released from Th2 are studied. The major findings of this study are as follows. First, PBMC from allergic patients with allergic rhinitis and healthy control subjects have the Th2-type cytokine response to HDM allergens. Second, allergic patients did have an enhanced Th2-type cytokine response to HDM compared with control subjects. These data indicated that both HDM allergens can be used for allergen immunotherapy for Thai mite-allergic patients.

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APPENDIX

APPENDIX A

Table 6. Profiles of 44 subjects responsed to panel of allergens sensitization. (A= allergic individuals, H= healthy subjects, M=male, F=Female, -=not determine)

			Antigens sensitization (wheal ≥ 3 mm)								
ID	**Sex**	**Age**	HDM	Dust	CR	Smut	Weed	Grass	Mold	Cat	Dog
A1	F	55	+	-	+	-	-	-	-	-	-
A2	F	46	+	-	-	-	-	-	-	+	+
A3	M	40	+	+	+	-	-	-	-	-	+
A4	M	50	+	+	+	-	-	-	-	-	-
A5	F	35	+	+	+	+	-	-	-	-	-
A6	M	48	+	-	-	-	-	-	-	-	-
A7	M	32	+	+	+	-	-	-	-	-	-
A8	M	36	+	-	+	-	-	-	-	-	-
A9	F	28	+	+	+	-	-	-	-	-	-
A10	F	50	+	+	+	+	-	-	-	-	-
A11	F	48	+	+	+	-	-	-	-	-	-
A12	F	38	+	-	+	+	-	-	-	-	-
A13	F	28	+	+	+	-	-	-	-	-	-
A14	F	36	+	+	+	-	-	-	-	-	-
A15	F	16	+	+	+	-	+	-	-	+	+
A16	M	22	+	-	+	-	+	-	-	-	-
A17	F	48	+	+	-	-	-	-	-	-	-
A18	M	19	+	+	-	-	-	-	-	-	-
A19	M	20	+	+	-	-	-	-	-	-	-
A20	F	19	+	+	-	-	-	-	-	-	-
A21	F	20	+	+	-	-	-	-	-	-	-
A22	M	27	+	+	-	-	-	-	-	-	-
A23	M	48	+	+	-	-	-	-	-	-	-
A24	F	35	+	+	-	-	-	-	-	-	-
H1	F	30	-	-	-	-	-	-	-	-	-
H2	F	29	-	-	+	-	+	-	-	-	-
H3	F	41	-	-	-	-	-	-	-	-	-
H4	M	38	-	-	-	-	-	-	-	-	-
H5	M	22	-	-	-	-	-	-	-	-	-

Table 6. (continue) Profiles of 44 subjects responsed to panel of allergens sensitization. (A= allergic individuals, H= healthy subjects, M=male, F=Female, -=not determine)

			Antigens sensitization (wheal ≥ 3 mm)								
ID	**Sex**	**Age**	HDM	Dust	CR	Smut	Weed	Grass	Mold	Cat	Dog
H6	F	28	-	-	-	+	-	-	-	-	-
H7	M	32	-	-	-	-	-	-	-	-	-
H8	M	31	-	-	-	-	-	-	-	-	-
H9	M	40	-	-	-	-	-	-	-	-	-
H10	M	28	-	-	+	-	-	-	-	-	-
H11	F	35	-	-	-	-	-	-	-	-	-
H12	F	24	-	-	-	-	-	-	-	-	-
H13	F	22	-	-	-	-	-	+	-	-	-
H14	F	30	-	-	-	-	-	-	-	-	-
H15	F	26	-	-	-	-	-	-	-	-	-
H16	F	28	-	-	-	-	-	-	-	-	-
H17	F	32	-	-	+	-	-	-	-	-	-
H18	M	36	-	-	-	-	-	-	-	-	-
H19	M	35	-	-	-	-	-	+	-	-	-
H20	M	28	-	-	-	-	-	-	-	-	-

Table 7. Symptom Profiles of 44 subjects. (A= allergic individuals, H= healthy subjects, M=male, F=Female, -=not determine)

ID	Sex	Age	Cases					
			Sneezing	Nasal blockage	Running nose	Cough	Asthma	itch
A1	F	55	+	+	+	-	-	-
A2	F	46	+	+	+	-	-	-
A3	M	40	+	+	+	-	-	-
A4	M	50	+	+	+	+	-	-
A5	F	35	+	+	+	+	-	+
A6	M	48	+	+	+	-	-	-
A7	M	32	-	+	+	-	-	-
A8	M	36	+	+	+	-	-	-
A9	F	28	+	+	+	-	-	+
A10	F	50	-	-	+	-	-	+
A11	F	48	+	+	+	-	-	+
A12	F	38	+	+	+	-	-	+
A13	F	28	+	+	+	-	-	+
A14	F	36	+	+	+	-	-	+
A15	F	16	+	+	+	+	-	+
A16	M	22	+	-	+	+	-	+
A17	F	48	+	+	+	-	-	-
A18	M	19	+	+	+	-	-	-
A19	M	20	+	+	+	-	-	-
A20	F	19	+	+	+	-	-	-
A21	F	20	-	+	+	-	-	-
A22	M	27	+	+	+	-	-	-
A23	M	48	+	+	+	-	-	-
A24	F	35	+	+	+	-	-	-
H1	F	30	-	-	-	-	-	-
H2	F	29	-	-	-	-	-	-
H3	F	41	-	-	-	-	-	-
H4	M	38	-	-	-	-	-	-
H5	M	22	-	-	-	-	-	-

Table 7. (continue) Symptom Profiles of 44 subjects. (A= allergic individuals, H= healthy subjects, M=male, F=Female, -=not determine)

			Cases					
ID	**Sex**	**Age**	Sneezing	Nasal blockage	Running nose	Cough	Asthma	itch
H6	F	28	-	-	-	-	-	-
H7	M	32	-	-	-	-	-	-
H8	M	31	-	-	-	-	-	-
H9	M	40	-	-	-	-	-	-
H10	M	28	-	-	-	-	-	-
H11	F	35	-	-	-	-	-	-
H12	F	24	-	-	-	-	-	-
H13	F	22	-	-	-	-	-	-
H14	F	30	-	-	-	-	-	-
H15	F	26	-	-	-	-	-	-
H16	F	28	-	-	-	-	-	-
H17	F	32	-	-	+	-	-	-
H18	M	36	-	-	-	-	-	-
H19	M	35	-	-	-	-	-	-
H20	M	28	-	-	-	-	-	-

Table 8. Cytokine Profiles of 44 subjects. (A= allergic individuals, H= healthy subjects, M=male, F=Female, -=not determine)

			IFN-γ (pg/ml)			IL-5 (pg/ml)		
ID	**Sex**	**Age**	PHA	Df	rDer f 2	PHA	Df	rDer f 2
A1	F	55	736.25	31.25	523.75	308.22	7.50	98.38
A2	F	46	424.79	66.36	452.29	100.52	8.50	30.46
A3	M	40	880.67	78.36	1244.50	332.50	16.50	92.50
A4	M	50	370.00	99.56	875.81	113.43	14.20	82.89
A5	F	35	1323.55	59.32	406.45	444.49	5.21	82.87
A6	M	48	1188.50	86.65	545.00	367.13	14.24	51.95
A7	M	32	808.09	70.23	815.11	238.68	8.26	39.83
A8	M	36	868.44	29.35	393.77	437.01	9.56	102.57
A9	F	28	1198.50	108.36	404.44	317.38	11.24	49.31
A10	F	50	1243.54	77.23	935.04	108.77	17.24	85.25
A11	F	48	360.00	91.35	645.45	382.50	10.37	75.38
A12	F	38	524.76	80.77	572.54	303.67	10.24	62.83
A13	F	28	1100.16	39.36	657.93	378.96	12.24	49.09
A14	F	36	954.13	46.32	781.51	350.91	4.00	81.23
A15	F	16	1026.36	24.36	427.86	130.19	4.00	58.26
A16	M	22	1016.00	139.36	430.36	166.11	14.12	34.26
A17	F	48	770.00	92.53	134.33	212.50	5.36	20.00
A18	M	19	415.79	115.33	439.38	65.95	8.87	47.32
A19	M	20	1204.32	61.36	659.40	115.18	9.56	38.35
A20	F	19	1202.39	92.33	1407.82	109.06	8.25	52.86
A21	F	20	533.65	78.36	84.77	100.99	14.24	19.06
A22	M	27	1386.71	60.44	583.57	93.15	12.63	41.30
A23	M	48	776.00	28.36	726.00	22.22	4.00	51.85
A24	F	35	422.84	30.88	723.82	26.75	4.00	52.04
H1	F	30	1442.67	536.25	6.70	158.82	4.50	9.65
H2	F	29	770.74	103.42	7.60	136.91	4.00	13.56
H3	F	41	1283.12	401.16	4.00	156.70	6.53	6.34
H4	M	38	1297.43	608.14	4.00	123.63	4.00	4.25
H5	M	22	847.43	524.64	9.63	176.48	4.00	2.36
H6	F	28	2251.63	212.50	4.00	394.21	4.35	6.78
H7	M	32	1257.76	228.01	4.00	19.21	4.00	7.25
H8	M	31	1131.56	550.97	8.40	149.25	5.59	2.55

Table 8. (continue) Cytokine Profiles of 44 subjects. (A= allergic individuals, H= healthy subjects, M=male, F=Female, -=not determine)

			IFN-γ (pg/ml)			IL-5 (pg/ml)		
ID	**Sex**	**Age**	PHA	Df	rDer f 2	PHA	Df	rDer f 2
H9	M	40	839.36	82.88	4.00	219.25	6.11	4.48
H10	M	28	1419.56	221.26	9.53	94.76	4.00	0.56
H11	F	35	792.35	442.72	7.54	159.24	4.13	5.86
H12	F	24	1285.35	548.28	4.00	206.37	8.35	4.00
H13	F	22	1352.24	315.92	6.50	166.33	13.52	4.00
H14	F	30	1118.24	211.33	5.58	150.13	8.35	4.00
H15	F	26	961.32	716.70	7.45	138.36	1.35	5.14
H16	F	28	898.37	855.14	7.50	197.24	0.57	4.00
H17	F	32	839.56	245.23	4.00	259.25	5.25	4.00
H18	M	36	1321.70	224.04	8.23	169.25	6.24	4.00
H19	M	35	789.52	523.01	4.00	78.35	8.59	4.25
H20	M	28	952.36	663.00	6.45	156.25	10.33	4.00

List of Abbreviations

Abbreviation	Term
A	Allergic
APCs	Antigen presenting cells
BMGY	buffered minimal glycerol complex medium
BMMY	buffered minimal methanol complex medium
Bt	*Blomia tropicalis*
BSA	bovine serum albumin
CR	Cockroach
°C	degree Celsius
cm	centimeter
cts	counts per second
DAS-TR-IFMA	Double-antigen Sandwich Time-resolved immunofluorometric assay
Df	*Dermatophagoides farinae*
Dp	*Dermatophagoides pteronyssinus*;
ELISA	Enzyme Linked Immunosorbent Assay
EU	europium
F	Female
FPA	Fluorescence polarization assay
H	Healthy
HDM	House Dust Mite
h	hour (s)
IL	interleukin
IFN	interferon
IUIS	International Union of Immunological Societies
Ig	Immunoglobulin
kDa	kilo Dalton
kU_A/l	kilo units of aprotinin-specific antibodies per liter
Abbreviation	Term
M	Male

mAb	monoclonal antibody
mg	milligram
min	minute (s)
ml	milliliter
mM	milimolar
mm	millimeter
µg	microgram
µl	microliter
ng	nanogram
nm	nanometer
OD	optical density
P. pastoris	*Pichia pastoris*
PBS	phosphate buffer saline
PHA	phytohemagglutinin
rpm	round per minute
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide gel Electrophoresis
SPT	Skin prick Test
SMM	Spent Mite Medium
TGF	Transforming growth factor
Th1	T helper cells type1
Th2	T helper cells type2
V	volt
v/v	volume / volume
w/v	weight / volume
YPD	yeast extracts peptone dextrose

APPENDIX B

General Reagents

Phosphate Buffered Saline (PBS), pH 7.4

NaCl	8.0	g
KH_2PO_4	0.24	g
Na_2HPO_4	1.44	g
KCl	0.20	g

Dissolve in deionized water, adjust to pH 7.4 with HCl and adjust to final volume of 1,000 ml with deionized water. Sterized by autoclave.

Culture Media

Yeast Extract Peptone (YP)

yeast extract	1.0	g
tryptone	2.0	g

Dissolve and adjust the volume of 90 ml with deionized water. Sterized by autoclave at 121 °C, 15 lb/square inches for 15 min.

10X Dextrose (D)

Dextrose	5.0	g

Dissolve and adjust the volume of 25 ml with deionized water. Sterized by autoclave.

Buffered minimal (BM)

yeast extract	5.0	g
tryptone	10.0	g

Dissolve and adjust the volume of 350 ml with deionized water. Sterized by autoclave.

1 M K_2HPO_4

K_2HPO_4	8.709	g

Dissolve and adjust the volume of 50 ml with deionized water.

1 M KH_2PO_4

KH_2PO_4	13.609	g

Dissolve and adjust the volume of 100 ml with deionized water.

1M Potassium Phosphate Buffer, pH 6.0

1 M K_2HPO_4	13.2	ml

1M KH_2PO_4	86.8	ml

Dissolve in deionized water, adjust to pH 6.0 and sterized by autoclave at 121 °C, 15 lb/square inches for 15 minutes.

10X 13.4% Yeast Nitrogen Base with $(NH_4)_2SO_4$ but without amino acid (YNB)

YNB	13.4	ml

Dissolve and adjust the volume of 100 ml with deionized water. Sterized by 0.22 μ filter and store at night.

10X Glycerol

Glycerol	5.0	ml

Dissolve and adjust the volume of 45 ml with deionized water. Sterized by autoclave.

10X Methanol

Methanol	3.0	ml

Dissolve and adjust the volume of 97 ml with deionized water. Sterized by autoclave.

500X Biotin

Biotin	2.0	g

Dissolve and adjust the volume of 10 ml with deionized water. Sterized by 0.22 μ filter and store at night.

Yeast extract peptone dextrose (YPD)

YP	9.0	ml
10X D	1.0	ml
Zeocin™ (100 μg/ml)	10.0	μl

Buffered minimal glycerol complex medium (BMGY)

BM	70.0	ml
1M Potassium Phosphate Buffer	10.0	ml
10X YNB	10.0	ml
10X GY	10.0	ml
500X B	100.0	μl

Buffered minimal methanol complex medium (BMMY)

BM	70.0	ml

1M Potassium Phosphate Buffer	10.0	ml
10X YNB	10.0	ml
10X M	10.0	ml
500X B	100.0	µl

Reagent for Polyacrylamine Gel Electrophoresis

Lower gel buffer

Tris base	18.2	g
Sodium dodecyl sulfate (SDS)	0.4	g

Dissolve in deionized water, adjust to pH 8.8 and adjust to final volume of 100 ml with deionized water.

Upper gel buffer

Tris base	6.0	g
SDS	0.4	g

Dissolve in deionized water, adjust to pH 6.8 and adjust to final volume of 100 ml with deionized water.

10% Ammonium persulfate (APS) (w/v)

Ammonium persulfate	1.0	g
Deionized water	10.0	ml

12.5% Resolving gel

Lower gel buffer	1.0	ml
Deionized water	1.33	ml
30% Acrylamide	1.67	ml
10% APS	20.0	µl
TEMED	8.0	µl

4% Resolving gel

Upper gel buffer	0.78	ml
Deionized water	1.83	ml
30% Acrylamind	0.39	ml
10% APS	20.0	µl
TEMED	5.0	µl

4X Sample buffer

Glycerol	4.0	ml
SDS	0.4	g
Upper gel buffer	5.0	ml
Bromophenol blue	2.0	g
2- Mercaptoethanol	1.0	ml

Dissolve and adjust the volume of 10 ml with deionized water.

10X Electrode buffer

Tris base	30.0	g
Glycine	144.0	g
SDS	10.0	g

Dissolve in deionized water, adjust to pH 8.3 and adjust to final volume of 1,000 ml with deionized water.

Working Electrode buffer

10X Electrode buffer	100	ml

Dissolve and adjust the volume of 900 ml with deionized water.

Coomassie Brilliant Blue (CBB)

CBB-G 250 stock	5	g]
Ammonium sulfate	50	g
85% O-phosphoric acid (w/w)	6	ml
Methanol	125	ml

Dissolve and adjust to final volume of 500 ml with deionized water.

5% SDS

SDS	0.25	g

Dissolve and adjust to final volume of 5 ml with deionized water.

7.5X Electroblotting buffer

Tris base	4.5375	g
Glycine	21.6	g

Dissolve and adjust to final volume of 200 ml with deionized water.

Working Electroblotting buffer

7.5X Electroblotting buffer	200	ml
Methanol	300	ml
5% SDS	3	ml

Dissolve and adjust to final volume of 1500 ml with deionized water.

0.1 % PBS-Tween 20 (PBS-T)

PBS	1,000	ml
Tween 20	1	ml

Blocking/Diluting buffer (PBST with 3% BSA)

PBS-T	100	ml
BSA	3	g

Reagent for Enzyme-Linked Immunosorbent asaay

50mM carbonate/bicarbonate buffer, pH 9.6

Na_2CO_3	1.59	g
$NaHCO_3$	2.93	g

Dissolve in deionized water, adjust pH to 9.6 and adjust to 1,000 ml final volume with deionized water. Sterized by autoclave.

0.05% PBS-Tween (PBS-T)

PBS	1,000	ml
Tween 20	0.5	ml

Blocking/Diluting buffer (PBST with 1% BSA)

PBS-T	100	ml
BSA	1	g

APPENDIX C

เอกสารชี้แจงข้อมูลแก่ผู้เข้าร่วมโครงการวิจัย

(Research Subject Information sheet)

ชื่อโครงการวิจัย

(ภาษาไทย) การตอบสนองของเซลล์ T Iymphocyte ต่อสารก่อภูมิแพ้จากไรฝุ่นชนิด Der f 2 ในคนไทย

(ภาษาอังกฤษ) T-Cell Responses to Der f 2 Mite Allergens in Thai Allergic Patients

วันที่ชี้แจง ……..

ชื่อและสถานที่ทำงานของผู้วิจัย

1. อาจารย์ ดร.จันทร์ดี ระแบบเลิศ ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศิลปากร วิทยาเขตพระราชวังสนามจันทร์ จ. นครปฐม

2. นายดวงเทพ ทองดี ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศิลปากร วิทยาเขตพระราชวังสนามจันทร์ จ. นครปฐม

ชื่อผู้วิจัยร่วม

1. ผู้ช่วยศาสตราจารย์ ดร. ณัฐ มาลัยนวล ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล กรุงเทพมหานคร

2. รองศาสตราจารย์ แพทย์หญิงสุภัทรา เตียวเจริญ ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล กรุงเทพมหานคร

3. พันเอกปิยลาภ วสุวัต กองอุบัติเหตุและห้องฉุกเฉิน โรงพยาบาลพระมงกุฎเกล้า

4. พันโทธฤต มุนินทร์นพมาศ กองโสต ศอ นาสิกกรรม โรงพยาบาลพระมงกุฎเกล้า

ท่านได้รับการเชิญชวนให้เข้าร่วมในโครงการวิจัยนี้ แต่ก่อนที่ท่านจะตกลงใจเข้าร่วมหรือไม่ โปรดอ่านข้อความในเอกสารนี้ทั้งหมด เพื่อให้ทราบว่า เหตุใดท่านจึงได้รับเชิญให้เข้าร่วมในโครงการวิจัยนี้ โครงการวิจัยนี้ทำเพื่ออะไร หากท่านเข้าร่วมโครงการวิจัยนี้ท่านจะต้องทำอะไรบ้าง รวมทั้งข้อดีและข้อเสียที่อาจจะเกิดขึ้นในระหว่างการวิจัย

ในเอกสารนี้ อาจมีข้อความที่ท่านอ่านแล้วยังไม่เข้าใจ โปรดสอบถามผู้วิจัยหรือผู้ช่วยผู้วิจัยที่ทำโครงการนี้เพื่อให้อธิบายจนกว่าท่านจะเข้าใจ ท่านจะได้รับเอกสารนี้ 1 ชุด กลับไปอ่านที่บ้านเพื่อปรึกษาหารือกับญาติพี่น้อง เพื่อน หรือแพทย์ที่ท่านรู้จัก ให้ช่วยตัดสินใจว่าควรจะเข้าร่วมโครงการวิจัยนี้หรือไม่ การเข้าร่วมในโครงการวิจัยครั้งนี้จะต้องเป็น**ความสมัครใจ**ของท่าน ไม่มีการบังคับหรือชักจูง ถึงแม้ท่านจะไม่เข้าร่วมในโครงการวิจัย ท่านก็จะได้รับการรักษาพยาบาลตามปกติ การไม่เข้าร่วมหรือถอนตัวจากโครงการวิจัยนี้ จะไม่มีผลกระทบต่อการได้รับบริการ การรักษาพยาบาลหรือผลประโยชน์ที่พึงจะได้รับของท่านแต่อย่างใด

โปรดอย่าลงลายมือชื่อของท่านในเอกสารนี้จนกว่าท่านจะแน่ใจว่ามีความประสงค์จะเข้าร่วมในโครงการวิจัยนี้ คำว่า “ท่าน” ในเอกสารนี้ หมายถึงผู้เข้าร่วมโครงการวิจัยในฐานะเป็นอาสาสมัครในโครงการวิจัยนี้ หากท่านเป็น**ผู้แทนโดยชอบธรรมตามกฎหมาย**ของผู้ที่จะเข้าร่วมในโครงการวิจัย และลงนามแทนในเอกสารนี้ โปรดเข้าใจว่า “ท่าน” ในเอกสารนี้หมายถึงผู้เข้าร่วมในโครงการวิจัยเท่านั้น

โครงการวิจัยนี้มีที่มาอย่างไร และวัตถุประสงค์ของโครงการวิจัย

ในปัจจุบันคนไทยป่วยเป็นโรคภูมิแพ้กันมากขึ้นโดยเฉพาะในเด็ก จากการสำรวจโดยนายแพทย์ ปกิต วิชยานนท์และคณะ พบว่ามีผู้ป่วยเป็นโรคหืดภูมิแพ้ร้อยละ 13 และป่วยเป็นโรคแพ้อากาศร้อยละ 40 ของประชากรเด็กไทย สาเหตุของโรคเกิดจากการที่ร่างกายมีปฏิกิริยาภูมิไวเกิน (hypersensitivity) ต่อสารก่อภูมิแพ้ต่าง ๆ ในสิ่งแวดล้อมจากการศึกษาทั่วโลก พบว่า สารก่อภูมิแพ้ที่สำคัญและพบได้บ่อยที่สุดในบ้านเรือน คือ สารก่อภูมิแพ้จากไรฝุ่นบ้านชนิด *Dermatophagoides pteronyssinus* และ *Dermatophagoides farinae* ซึ่งมีแพร่กระจายอยู่ในประเทศไทยด้วย ในบรรดาสารก่อภูมิแพ้จากไรฝุ่นบ้านทั้งหมดจำนวน 22 กลุ่มที่ถูกจำแนกตามคุณสมบัติทางชีวเคมี สารก่อภูมิแพ้กลุ่มที่ 1 และกลุ่มที่ 2 เป็นสารก่อภูมิแพ้กลุ่มที่มีความสำคัญมากที่สุด เนื่องจากพบเป็นปริมาณมากในตัวไรฝุ่นบ้าน และผู้ป่วยส่วนใหญ่มีแอนติบอดีชนิด IgE ในซีรัมที่เฉพาะเจาะจงต่อสารก่อภูมิแพ้ทั้งสองกลุ่มนี้ในระดับสูง นอกจากนั้นยังมีการศึกษาพบว่าสารก่อภูมิแพ้ทั้งสองกลุ่มนี้สามารถกระตุ้นให้เซลล์ T lymphocytes ในหลอดทดลองมีการแบ่งตัวเพิ่มจำนวนอีกด้วย ซึ่งเป็นที่

ทราบกันดีว่าเซลล์ T lymphocytes มีบทบาทสำคัญของกลไกในการเกิดปฏิกิริยาภูมิไวเกินต่อสารก่อภูมิแพ้

วัตถุประสงค์ของโครงการวิจัย เพื่อศึกษาปริมาณของ ไซโตไคน์ IL-5 และ IFN-γ จากผู้ป่วยโรคภูมิแพ้ หลังจากถูกกระตุ้นด้วยสารสกัดโปรตีนจากไรฝุ่น หรือ โปรตีนสังเคราะห์ Der f 2 จากยีสต์ เปรียบเทียบกับปริมาณของ ไซโตไคน์ IL-5 และ IFN-γ จากผู้ที่มีสุขภาพแข็งแรง ซึ่งสามารถใช้เป็นข้อมูลในการพัฒนาการตรวจ และรักษาโรคภูมิแพ้ที่เกิดจากสารก่อภูมิแพ้จากไรฝุ่นบ้านได้

ท่านได้รับเชิญให้เข้าร่วมโครงการวิจัยนี้เพราะคุณสมบัติที่เหมาะสมดังต่อไปนี้

1. เป็นผู้ป่วยโรคภูมิแพ้ต่อไรฝุ่น เพศ ชาย หรือ หญิง อายุระหว่าง 18 ถึง 50 ปี มีประวัติเป็นภูมิแพ้โดยมีอาการของโรคจมูกอักเสบจากภูมิแพ้ (Allergic rhinitis) และผู้ป่วยสามารถตอบสนองต่อสารก่อภูมิแพ้จากไรฝุ่น (*D. farinae)* ด้วยวิธีทดสอบ skin prick test

2. เป็นผู้ไม่มีประวัติเป็นภูมิแพ้ต่อไรฝุ่น เพศ ชาย หรือ หญิง อายุระหว่าง 18 ถึง 50 ปี

ท่านไม่สามารถเข้าร่วมโครงการวิจัยได้หากท่านมีคุณสมบัติดังต่อไปนี้

1. เป็นผู้ป่วยโรคภูมิแพ้ต่อไรฝุ่น เพศ ชาย หรือ หญิง อายุต่ำกว่า 18 หรือเกินกว่า 50 ปี มีประวัติเป็นภูมิแพ้โดยมีอาการของโรคจมูกอักเสบจากภูมิแพ้ (Allergic rhinitis) และผู้ป่วยสามารถตอบสนองต่อสารก่อภูมิแพ้จากไรฝุ่น (*D. farinae)* ด้วยวิธีทดสอบ skin prick test

2. เป็นผู้ไม่มีประวัติเป็นภูมิแพ้ต่อไรฝุ่น เพศ ชาย หรือ หญิง อายุต่ำกว่า 18 หรือเกินกว่า 50 ปี

จะมีการทำโครงการวิจัยนี้ที่ใด และมีจำนวนผู้เข้าร่วมโครงการวิจัยทั้งสิ้นเท่าไร

1. คัดกรองผู้ป่วยและเก็บตัวอย่างเลือด จำนวน 60 คน จากกองโสต ศอ นาสิกกรรม โรงพยาบาลพระมงกุฎเกล้า

2. ดำเนินการวิจัย ที่ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

ระยะเวลาที่ท่านจะต้องร่วมโครงการวิจัยและจำนวนครั้งที่นัด

1-2 ครั้ง

หากท่านเข้าร่วมโครงการวิจัยครั้งนี้ ท่านจะต้องปฏิบัติตามขั้นตอน หรือได้รับการปฏิบัติอย่างไรบ้าง

ผู้เข้าร่วมโครงการวิจัยจะถูกเก็บตัวอย่างเลือดจากโดยการเจาะเส้นเลือดดำบริเวณข้อพับแขน เป็นปริมาณ 50 มิลลิลิตร

ความไม่สุขสบาย หรือความเสี่ยงต่ออันตรายที่อาจจะได้รับจากกรรมวิธีการวิจัยมีอะไรบ้าง และวิธีการป้องกัน/แก้ไขที่ผู้วิจัยเตรียมไว้หากมีเหตุการณ์ดังกล่าวเกิดขึ้น

ไม่มี

ประโยชน์ที่คาดว่าจะได้รับจากโครงการวิจัย

สามารถใช้ข้อมูลจากการวิจัยในการพัฒนาวัคซีนป้องกันโรคภูมิแพ้ไรฝุ่น

ค่าใช้จ่ายที่ผู้เข้าร่วมในโครงการวิจัยจะต้องรับผิดชอบ (ถ้ามี)

ผู้เข้าร่วมโครงการวิจัยไม่ต้องเสียค่าใช้จ่ายใดๆ ทั้งสิ้นในการวิจัยนี้

ค่าตอบแทนที่จะได้รับเมื่อเข้าร่วมโครงการวิจัย

ไม่มี

หากท่านไม่เข้าร่วมโครงการวิจัยนี้ ท่านมีทางเลือกอื่นอย่างไรบ้าง

การเข้าร่วมโครงการวิจัยครั้งนี้ เป็นไปตามความสมัครใจของผู้เข้าร่วมโครงการวิจัย หากท่านไม่ต้องการเข้าร่วมโครงการวิจัยนี้สามารถปฏิเสธหรือยกเลิกการเข้าร่วมโครงการวิจัยได้ทันทีโดยไม่มีเงื่อนไข

หากเกิดอันตรายที่เกี่ยวข้องกับโครงการวิจัยนี้ จะติดต่อกับใคร และจะได้รับการปฏิบัติอย่างไร

พันเอกปิยลาภ วสุวัต กองอุบัติเหตุและห้องฉุกเฉิน โรงพยาบาลพระมงกุฎเกล้า

พันโทธฤต มุนินทร์นพมาศ กองโสต ศอ นาสิกกรรม โรงพยาบาลพระมงกุฎเกล้า

หากท่านมีคำถามที่เกี่ยวข้องกับโครงการวิจัย จะถามใคร ระบุชื่อผู้วิจัยหรือผู้วิจัยร่วม

อาจารย์ ดร.จันทร์ดี ระแบบเลิศ ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศิลปากร วิทยาเขตพระราชวังสนามจันทร์ จ. นครปฐม เบอร์โทร 084-7102575

ผู้ช่วยศาสตราจารย์ ดร. ณัฐ มาลัยนวล ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล กรุงเทพฯ เบอร์โทร 086-7833324

นายดวงเทพ ทองดี ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศิลปากร วิทยาเขตพระราชวังสนามจันทร์ จ. นครปฐม เบอร์โทร 089-8150779

หากท่านรู้สึกว่าได้รับการปฏิบัติอย่างไม่เป็นธรรมในระหว่างโครงการวิจัยนี้ ท่านอาจแจ้งเรื่องได้ที่

สำนักงานพิจารณาโครงการวิจัย กรมแพทย์ทหารบก ชั้น 5 อาคารพระมงกุฎเกล้าเวชวิทยา เบอร์โทร 02-3547600-28 ต่อ 94270

ข้อมูลส่วนตัวของท่านที่ได้จากโครงการวิจัยครั้งนี้จะถูกนำไปใช้ดังต่อไปนี้

ข้อมูลส่วนตัวของท่านจะถูกเก็บรักษาข้อมูลส่วนตัวและข้อมูลที่ได้จากโครงการวิจัยในคอมพิวเตอร์ เป็นเวลา 2 ปี โดยระบุรหัสแทนตัวบุคคล จะไม่เปิดเผยชื่อนามสกุล ที่อยู่ของผู้เข้าร่วมในโครงการวิจัยเป็นรายบุคคล การนำเสนอจะแสดงข้อมูลตามรหัสที่ระบุ

ท่านจะถอนตัวออกจากโครงการวิจัยหลังจากได้ลงนามเข้าร่วมโครงการวิจัยแล้วได้หรือไม่

การเข้าร่วมโครงการวิจัยครั้งนี้ เป็นไปตามความสมัครใจของผู้เข้าร่วมโครงการวิจัย หากท่านไม่ต้องการเข้าร่วมโครงการวิจัยนี้สามารถปฏิเสธหรือยกเลิกการเข้าร่วมโครงการวิจัยได้ทันทีโดยไม่มีเงื่อนไข

หากมีข้อมูลใหม่ที่เกี่ยวข้องกับโครงการวิจัย ท่านจะได้รับแจ้งข้อมูลนั้นโดยผู้วิจัยหรือผู้วิจัยร่วมนั้นทันที (ในกรณีที่เป็นการวิจัยเกี่ยวข้องกับการรักษาโดยเฉพาะการใช้ยา)

หนังสือแสดงเจตนายินยอมเข้าร่วมการวิจัย (Informed Consent)
รับรองโดยคณะอนุกรรมการพิจารณาโครงการวิจัย พบ.

ชื่อโครงการวิจัย

(ภาษาไทย) การตอบสนองของเซลล์ T Iymphocyte ต่อสารก่อภูมิแพ้จากไรฝุ่นชนิด Der f 2 ในคนไทย

(ภาษาอังกฤษ) T-Cell Responses to Der f 2 Mite Allergens in Thai Allergic Patients

วันที่ลงนาม .....

ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้ ข้าพเจ้าได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีการวิจัย อันตราย หรืออาการที่อาจเกิดขึ้นจากการวิจัย หรือจากยาที่ใช้ รวมทั้งประโยชน์ที่คาดว่าจะเกิดขึ้นจากการวิจัยอย่างละเอียด และมีความเข้าใจดีแล้ว

ผู้วิจัยรับรองว่าจะตอบคำถามที่ข้าพเจ้าสงสัยด้วยความเต็มใจ และไม่ปิดบังซ่อนเร้น จนข้าพเจ้าพอใจ

ข้าพเจ้าเข้าร่วมในโครงการวิจัยนี้ด้วยความสมัครใจ โดยปราศจากการบังคับหรือชักจูง

ข้าพเจ้ามีสิทธิที่จะบอกเลิกการเข้าร่วมในโครงการวิจัยเมื่อใดก็ได้ และการบอกเลิกนี้จะไม่มีผลต่อการรักษาพยาบาลที่ข้าพเจ้าจะพึงได้รับในปัจจุบันและในอนาคต

ผู้วิจัยรับรองว่าจะเก็บข้อมูลเกี่ยวกับตัวข้าพเจ้าเป็นความลับ และจะเปิดเผยเฉพาะในรูปของสรุปผลการวิจัยโดยไม่มีการระบุชื่อนามสกุลของข้าพเจ้า การเปิดเผยข้อมูลเกี่ยวกับตัวข้าพเจ้าต่อหน่วยงานต่างๆ ที่เกี่ยวข้อง จะกระทำด้วยเหตุผลทางวิชาการเท่านั้น

ผู้วิจัยรับรองว่าหากเกิดอันตรายใดๆ จากการวิจัย ข้าพเจ้าจะได้รับการรักษาพยาบาลตามที่ระบุในเอกสารชี้แจงข้อมูลแก่ผู้เข้าร่วมโครงการวิจัย

ข้าพเจ้าจะได้รับเอกสารชี้แจงและหนังสือยินยอมที่มีข้อความเดียวกันกับที่ผู้วิจัยเก็บไว้ เป็นส่วนตัวข้าพเจ้าเอง 1 ชุด

ข้าพเจ้าได้รับทราบข้อความข้างต้นแล้ว มีความเข้าใจดีทุกประการ และลงนามในใบยินยอมด้วยความเต็มใจ

ลงชื่อ…….....ผู้เข้าร่วมโครงการวิจัย

(…….....ชื่อ-นามสกุล ตัวบรรจง)

ลงชื่อ ……..ผู้ดำเนินโครงการวิจัย

(อาจารย์ ดร.จันทร์ดี ระแบบเลิศ)

ลงชื่อ ……..ผู้ร่วมดำเนินโครงการวิจัย

(พันเอกปิยลาภ วสุวัต)

ลงชื่อ ……..ผู้ร่วมดำเนินโครงการวิจัย

(พันโทธฤต มุนินทร์นพมาศ)

ลงชื่อ.....พยาน

(.....ชื่อ -นามสกุล ตัวบรรจง)

ลงชื่อ.....พยาน

(.....ชื่อ -นามสกุล ตัวบรรจง)

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