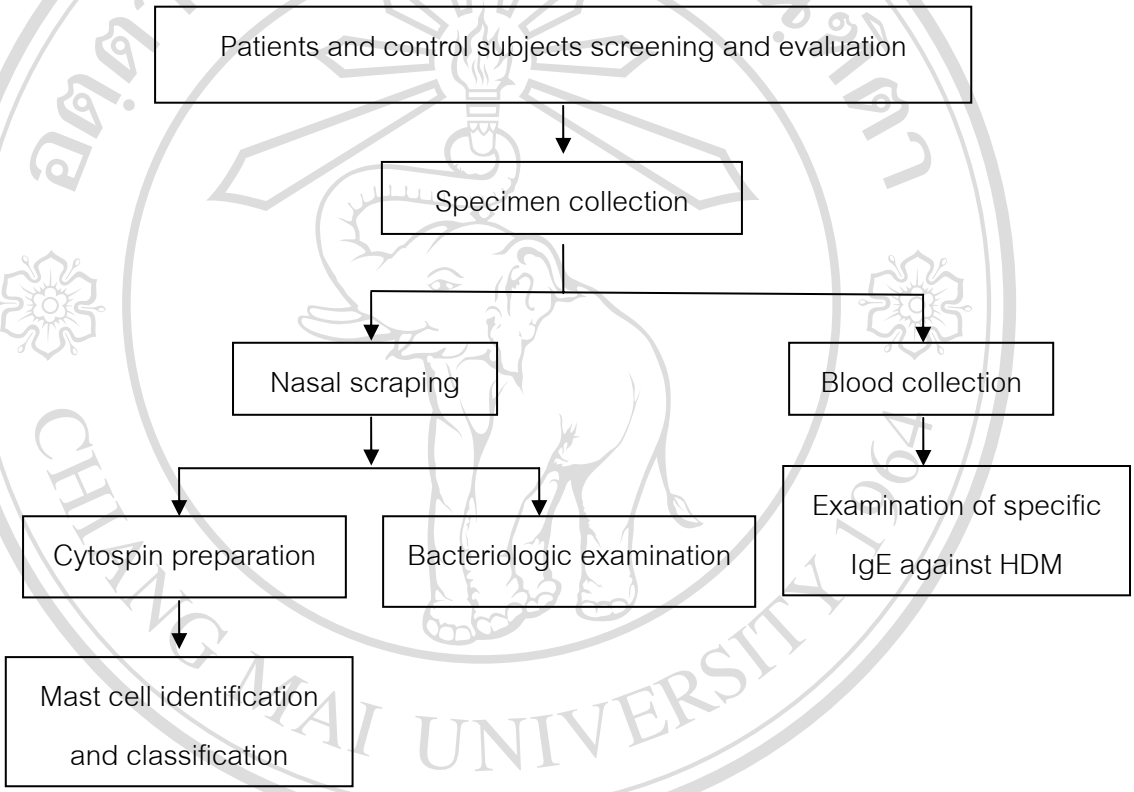
III. MATERIALS AND METHODS

A. Experimental plan

The following approaches were utilized to reach the goals of this study.



B. Patients and control subjects screening and evaluation

1. Subjects

Twenty-six nonallergic healthy subjects (control group) and 69 patients (allergic group) with clinical diagnoses of allergic rhinitis by history and physical examination and with positive skin tests to a panel of common allergens were recruited from the Allergy Clinic, Maharaj Nakorn Chiang Mai, Chiang Mai University, Chiang Mai, Thailand. The mean age of the subjects was 25.2 years (range 10-63 years; 10 men and 16 women) in the control group and 27.3 years (range 9-75 years; 40 men and 29 women) in the allergic group.

All subjects in the allergic group satisfied the following criteria:

1. Neither history of asthma nor atopic dermatitis
2. No respiratory tract infection
3. No nasal polyposis
4. No eosinophilia syndromes
5. No medical treatment, including antibiotics, antihistamines and steroids during the 2 weeks before enrollment
6. No previous immunotherapy

The nonallergic group had no history and family history of allergic rhinitis, allergic asthma and atopic dermatitis and had no sign and symptom of respiratory tract infection before sample collection. (All subjects in this study gave their informed consents for participation in the study.)

2. Clinical evaluation

The nasal symptom scores were evaluated according to the criteria of Meltzer, as described in Journal of Allergy Clinical Immunology, 1988; 82: 900-908. The concordance rate for the allergist determined nasal scores, graded the five nasal symptoms (sneezing, itching, nasal congestion, runny nose/sniffing, and postnasal drip/snorting) on a scale of 0-3 (0, absent; 1, mild; 2, moderate; 3, severe). The total score was determined and classified the severity as mild with score 0-5, moderate with score 6-10 and severe with score 11-15. In this study, the numbers of patients were classified in mild, moderate, and severe groups were 15, 24 and 30, respectively.

C. Specimen collection

1. Nasal scraping

The nasal mucosal specimen was collected by gently scraping with a disposable standard plastic Rhinoprobe scoop at the surfaces of middle thirds of inferior turbinates. Elimination of nasal discharge was done before sample collection. The probe was pressed on mucosal surface and quickly moved outwardly 2-3 mm. This step repeated once or twice if insufficient sample was obtained. The obtained nasal mucosal specimen was immediately suspended in 300 µl of sterile normal saline solution.

Scores for allergic rhinitis symptoms (*J Allergy Clin Immunol*. 1988; 82: 900-908.)

Sneezing	
0	Absent
1	Mild; 1 to 4 sneezes per day
2	Moderate; 5 to 10 sneezes per day
3	Severe; distressing spells or fits that may interfere with sleep or concentration
Itching	
0	Absent
1	Mild; occasional itching
2	Moderate; annoying itching continuous for ≥ 30 min
3	Severe; interfere sleeping or concentration
Nasal congestion	
0	Absent
1	Mild; some hindrance to breathing, not uncomfortable
2	Moderate; nostril(s) feel blocked, need to breathe through
3	mouth most of the time; annoying
4	Severe; cannot breathe comfortable through nose at all; may interfere with sleep, alter odor perception, or affect quality of voice
Runny nose/sniffing	
0	Absent
1	Mild; sniffing or tissues needed 1 to 4 times daily
2	Moderate; sniffing or tissues needed 5 to 10 times daily
3	Severe; nose runs freely despite frequent use of handkerchief or tissues
Postnasal drip/snorting	
0	Absent
1	Mild; slight awareness or ticking sensation in throat
2	Moderate; causes frequent throat clearing; annoying
3	Severe; causes gagging or frequent cough; uncomfortable

2. Blood collection

The blood samples were collected for specific IgE examination against different types of house dust mites, Der p 1, Der f 1, and Blo t 1. Fifty-five sera samples were obtained from patients, who satisfied with the criteria of the allergic rhinitis group. Twenty-nine sera samples were collected from nonallergic healthy subjects that satisfied with the criteria of the nonallergic group. All sera were kept at –20 ˚C before testing.

D. Cytospin preparation and Wright-Giemsa staining

The specimen suspensions were cytospun by using a cytocentrifuge (Cytospin 2; Shandon Southern Products Limited, Cheshire, UK). A portion (30 µl) of the specimen suspension was placed in centrifuge chamber. The cytocentrifugation was carried out at a speed of 1,200 rpm for 3 minutes. The cytocentrifuge-prepared smears were rapidly air dried, immediately fixed with 95 % ethyl alcohol for 60 seconds. The slides were stained with Wright-Giemsa solution (Appendix A) for 5 minutes then added distilled water until metallic sheen appeared, then left for 3 minutes. The slides were washed with distilled water, air dried, and mounted with Permount® (Fisher Scientific Company, USA.). Nasal cytology and differential cell count was examined under oil immersion at 1000x magnification. The differential cell count of individual cell type was expressed as a percentage.

E. Mast cell identification and classification

Mast cell identification and classification were evaluated by Toluidine blue and Alcian blue/Safranin staining (Supajatura et al., 2002). The slide was dipped in a jar containing Toluidine blue solution (Appendix A) or Alcian blue/Safranin solution (Appendix A) for 10 minutes and then rinsed with distilled water, air dried, and mounted with Permount®.

The degree of mast cell degranulation was classified into three categories (Supajatura et al., 2002);

1. Extensively degranulated (+++): 50 % of the cytoplasmic granules exhibiting fusion, staining alterations and extrusion from the cell

2. Slightly (+) to moderately degranulated (++): 10-15 % of the granules exhibiting fusion or discharge

3. Normal (N): contact granules

The cells were classified by morphology under an oil immersion of light microscope.

F. Bacteriologic examination

The specimen suspensions were diluted with sterile normal saline solution at 10-fold serial dilution, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} subsequently. One hundred microliters of each dilution was spread on blood agar (Tryptone Soya agar supplemented with 5% human blood), Mannitol salt agar, and MacConkey agar (Oxoid LTD., Basingstoke, Hampshire, England.) then aerobically incubated in 5% CO_2 at 35-37 °C. All cultures were examined at 24 to 48 hours. Identification of microorganisms was done with the standard microbiological method, including colonial pigmentation, colonial morphology, Gram staining, and biochemical tests.

G. Examination of house dust mites specific IgE by indirect ELISA

Fifty microliters of the diluted dust mite antigen (recombinant Der p 1, r Der f 1 or r Blot 1), at a final concentration of 100 μg/ml/well, was added to 96 well microtiter plates (Costar). The plates were covered and incubated overnight at 4 °C in a humidified chamber. The plates were then washed 3 times with 0.05 % PBSTween 20 (PBS-T) and leaved for 30 seconds. The plates were blocked with 100 μl of blocking solution (1 % Bovine serum albumin + PBS-T) for 1 hour at room temperature, then washed 3 times as previously described. Fifty microliter of diluted sera (1:5) was added. The plates were covered and incubated overnight at 4 °C in a humidified chamber. After washing 3 times, 50 μl of diluted mouse anti-human IgE biotin conjugate (1:1,000) was added and incubated for 1 hour at room temperature. After washing, 50 μl of diluted Streptavidin-HRP conjugated (1:2,000) was added and incubated for 1 hour at room temperature. After 3 times washing, the substrate ABTS (2,2'- azino-bis-3- ethyl benzyl thiazoline-6-sulfonic acid) in buffer containing citric acid-water and 3 % H_2O_2, 50 μl, was applied and allowed the reaction to proceed for exactly 60 minutes in the dark at room temperature. The optical density (OD) was measured by the microplate spectrophotometer at wavelength 405 nm. The mean of

OD plus 3 standard deviation (Mean+3SD) value of normal serum was defined as the cut-off value.

H. Statistical analysis

Data were analyzed using the student *t* - test. Significant differences were accepted when *P* values were less than 0.05.